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VOLUME 21 • NUMBER 1 • 1988

HU ISSN 0017-6559

HEMATOLOGIA

International Quarterly of Haematology

21
1988

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Haematologia is an international journal publishing original papers on all clinical and theoretical aspects of haematology. In addition to regular papers, book reviews and abstracts of selected papers from other periodicals also are a special feature of the journal.

Haematologia, published at quarterly intervals, provides the reader with complex and up-to-date information.

Manuscripts should be sent to the Editor-in-Chief:

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National Institute of Haematology and Blood Transfusion
Daróczi út 24.
H-1113 Budapest
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VSP
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Subscription price for volume 21:

DM 312,— incl. surface mail
DM 330,— incl. air mail

Haematologia is covered in *Current Contents/Clinical Practice*, *ISI/Biomed* and the *SCI*.

VOLUME 21 • NUMBER 1

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Replicate Tests for the Detection and Correction of Errors in Anti-Human Globulin (AHG) Tests: Optimum Conditions and Quality Control

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(Received 23 January, 1987; accepted 6 March, 1987)

Replicate blind AHG tests with weak IgG anti-D sensitised red cells revealed that 32% of workers caused 5 per cent or more false negative errors by using excessive agitation in reading techniques. The common quality control procedure of adding strongly sensitised cells to all negative AHG tests cannot reveal this type of error. Furthermore, strongly sensitised control cells may create an illusion of safety because AHG giving a false negative test with weak antibody in a serum sample may still show a reassuringly strong positive in the control test. "In-house" assessment of all staff and automatic cell-washers by blind replicate tests is recommended as an effective way of improving AHG test performance, thus reducing many of the errors involving false negative AHG tests seen year after year in External Proficiency Trials.

Keywords: anti-human globulin tests, cell washers, centrifuges, errors, quality control, optimum conditions, replicate tests, spin tests

Introduction

A high incidence of false negative anti-human globulin (AHG) tests has been reported by blind External Proficiency Test Surveys in the USA [1, 2, 3], Canada [4], UK [5] and Australia [6]. In addition to the failure to detect many weak antibodies (Table 1), it was also noted in one USA trial [2] that a sample of strongly sensitised (3+/4+) red cells was recorded by 13.7 per cent of participants as giving only weak 1+/2+ reactions.

It is possible that many of these failures were due to excessive agitation at the reading stage of tests. Hence, the common quality control procedure of adding red cells strongly sensitised with IgG anti-D to all negative AHG tests could not reveal the false negatives found in these surveys. However, partial neutralisation of AHG as a cause of some false negatives cannot be excluded, because strongly

Table 1
False negative errors with AHG tests in External Proficiency Test Surveys

Antibodies	Failure rates
Weak 1+	7–30%
Moderate to strong ++ to +++	1–4%
Strong sensitised RBC +++/++++ Anti-D	0–1% negative tests 13.7% obtained only 1+/2+

sensitised (3+/4+) red cells may still react with partially neutralised AHG reagents that would be unable to agglutinate more weakly sensitised red cells [7]. The common practice of using strongly sensitised red cells is a poor control system, as it may create an illusion of safety when the AHG reagent is partially neutralised for some reason, or when the error is the result of physical disruption of agglutination.

The Development of Blind Replicate Tests (7)

A Joint Working Party of the ICSH/ISBT realised that External Proficiency Tests assess only one or two workers in each laboratory, and that these studies have failed to identify the causes of error shown year after year. Blind replicate tests were therefore carried out with red cells weakly sensitised with IgG anti-D as a means to establish the incidence and cause of failures among a large number of technical personnel. The system was simple and proved useful for training pur-

Table 2
AHG test failures due to over-agitation at the reading stage

Failures	Manually washed (×4) 36 replicate +/+ + anti-D-tests*	
	Reading Method	
	Pipette transfer to slide	Agitation/shake
	Number of staff	Number of staff
Nil	31	37
Up to 5%	1	29
6–20%	0	16
21–50%	0	15
Total	32	Total 97

* Anti-D concentration 0.2–0.27 IU/mL

poses. There was a high rate of correlation between failure to detect antibody (false negatives) and unacceptable reading techniques. The examination of test results recorded by 129 staff members in 19 laboratories revealed that 97 per cent of staff using direct pipette transfer to a slide had no failures, compared with 38 per cent who used a tube-agitation reading method; details are shown in Table 2.

Assessment of Technical Proficiency and Correction of Errors (7)

We recommend that all personnel doing antibody screening and compatibility testing should be evaluated by means of blind replicate tests to assess their competence to perform AHG tests. A standard antiserum containing 0.8 to 1.0 IU/mL

Table 3

Comparison of different reading methods for the spin-tube AHG tests* to show the inferior sensitivity of the shake-tube method

Anti-D × R _{1r} Dilution (IU/mL)	Reading method**		
	Pipette transfer to slide	"Agitation or Tip and Roll"	"Shake"
<i>S/2257 low avidity</i>			
Neat (0.8)	+++	+++	+ +/+ + + +
3 (0.3)	++	++	+
4 (0.2)	+	+	±
<i>S/Pot 1 high avidity</i>			
128 (0.5)	C	C	C
256 (0.25)	+++	+++	++
1024 (0.12)	+ / + +	+ / + +	±
5000 (0.015)	+ sensitivity limit	±	-

* All tests manually washed (× 4), mixed with 2 vol AHG, spun 30s at 200 RCF.

** For explanation of terms see Appendix B.

anti-D should be used. The details of this procedure are given in Appendix A. It is advisable to give immediate tuition to any workers who are shown to have faulty technique in washing and/or reading. Such training must be followed by further blind replicate trials to verify improvement in the procedure and to restore the confidence of the person concerned.

Details of various reading methods are given in Appendix B. It is not the purpose of this report to recommend a particular reading method, but we do suggest that fewer problems of interpretation will be encountered if the first method listed is used. (See Appendix B and Table 3.)

Assessment of the Efficiency of Cell Washing Machines (7)

The Joint Working Party of the ICSH/ISBT also assessed the efficacy of 139 cell-washing centrifuges in 35 laboratories. Fifty examples (36 per cent) of false negatives were found in replicate tests using red cells weakly sensitised with IgG anti-D. However, the association of false negative AHG tests with poor reading technique made detailed assessment impossible, because all laboratories that showed greater than 10 per cent automated-centrifuge errors used reading techniques that included faulty agitation or shaking to examine the cell button.

We do, however, have sufficient information with Sorvall, Dade, and Diacent machines to provide a protocol (Appendix C) that provides reasonable assurance of reliable AHG test performance. We stress that these tests can only be done successfully by personnel whose reading technique is acceptably sensitive.

Optimum Conditions and Quality Control of AHG Tests

Replicate tests with weakly sensitised cells are very useful for the demonstration of false negatives, whether due to inadequate washing or to excessive shaking of tests. However, reliable test performance also depends on the correct procedure being used at all stages of the test. We have therefore included recommendations for the quality control of each stage of the spin-tube AHG test.

Quality Control of the Anti-Human Globulin Test

The AHG test has five main variables:

1. Sensitisation of the red cells by incubation with serum.
2. Washing the cells to remove unattached serum IgG that will neutralise the AHG reagent.
3. Selection of a high quality polyspecific AHG reagent and use of an appropriate volume and AHG quality control.
4. Use of optimum time and speed for a given centrifuge and a careful reading procedure to interpret the results.
5. Quality control of the complete test by adding red cells weakly sensitised with IgG anti-D [8a] to all negative tests and not strongly sensitised cells as commonly used [9, 10].

1. Sensitisation of the Red Cells [11, 12a, 13, 14, 15, 16]

Adequate sensitisation of red cells is achieved by using a 40 : 1 ratio of serum to packed red cells [13, 15]. It has been established [13] that this is sufficient for reliable detection of weak antibodies, including most weak examples of anti-K, by low ionic strength saline solution (LISS) tests. Small variations in red cell con-

centration are difficult to control in routine work and to ensure safe practices we recommend a higher serum to packed red cell ratio of 60 : 1 [16]. This is achieved:

- i) for normal ionic strength saline solution (NISS) by using 4 vol of serum if possible [17], but not less than 2 vol of serum to 1 vol of 3 per cent washed red cells suspended in saline or phosphate buffered saline (PBS);
- ii) for low ionic strength saline solution (LISS) by using 2 vol of serum to 2 vol of 1.5 per cent red cells suspended in 0.03 mol/l LISS [18].
- iii) for low ionic additive solutions used in accordance with the manufacturers' instructions.

The tubes should be shaken to mix the reactants, then incubated at 37 °C, preferably in a waterbath, for minimum incubation times of 15 min for low ionic strength tests and 45 min for tests at normal ionic strength.

2. Washing the Cells

Failure to wash adequately will leave small amounts of serum with the red cells during the final stage of the test. This can cause complete or partial neutralisation of the AHG reagent. Adequate washing can be achieved either manually or with cell washing machines. The latter, however, must be correctly installed and maintained [19, 20].

Although in theory three washes are more than adequate to remove unbound serum protein, we recommend four washes because of the greatly increased dilution of serum thus achieved. The most important points for efficient washing action are:

- i) thorough mixing of the red cells to resuspend the cell button,
- ii) vigorous injection of the saline wash to mix the red cells and serum thoroughly throughout the tube,
- iii) removal of as much of the supernatant fluid as possible at the end of each washing cycle.

The possibility that many false negative AHG test results are due to inadequate washing of tests by automated cell-washers was first reported in 1980 [19]. It is useful to realise that a low level of serum contamination (around 1 in 3,000) can be overcome by the use of potent AHG reagents, especially by the use of 2 vol of AHG per test [16]. This was demonstrated in a recent Canadian trial [22]: 14 Dade and nine Sorvall machines, including CW1s, were evaluated using replicate tests with IgG anti-D sensitised (1+) cells in 17 centres; 11 of which obtained better results with 2 vol compared to 1 vol of AHG per test.

An additional source of false negative antiglobulin tests has been reported by Bruce et al. [21], who showed that plasticiser(s) played a significant part in failures due to the use of saline stored at low pH in certain plastic containers.

3. The Use of High Quality Polyspecific Antiglobulin Reagents and Their Quality Control

Spin-tube AHG reagents should contain:

- i) Potent anti-IgG with levels at least twice that of the minimum potency FDA reference preparation. Tube tests performed by immediate-spin methods are practically free of the prozone problems that limit the anti-IgG activity of tile test reagents [16]. Tile tests are less sensitive than spin-tube tests and are not recommended for antibody screening or compatibility tests. These reagents, used with two volumes (70–100 μ l) per test, are very resistant to serum neutralisation (see Table 4) and will help safeguard against failure.
- ii) Adequate anti-complement activity. In conventional polyspecific reagents this will be primarily anti-C3c, but anti-C3d is also needed to detect red cells coated with complement *in vivo*. The serological activity of anti-C3d must, however, be severely restricted in order to prevent "false positive" reactions

Table 4

Anti-IgG sensitivity in the presence of partial neutralisation is related to the potency and quantity of AHG per test

Sensitised R ₁ r cells added to negative AHG tests with non-sensitised R ₁ r cells	Partial neutralisation 1 vol. 1/3000 serum/test			
	AHG	RIII	ISBT	AHG FDA Lot 16
	*potency titre	1024		potency titre 256
	1 vol	2 vol	1 vol	2 vol
Anti-D IU/ml				
0.75	++/+++	+++	+	++
0.38	++	++/+++	W	+ / ++
0.15	++	++/+++	W	+
0.038	+	+ / ++	—	VW

1 vol. = 35 μ l

GRADES OF AGGLUTINATION

C	+++	++	+	W	VW
one clump	several clumps	smaller clumps	small granules	microscopic cells/clump	(8–12) (4/6)

* The anti-IgG potency titre of an AHG is the highest dilution that gives a macroscopic + reaction with a pool of R₁r red cells strongly sensitised with IgG anti-D (75 IU/mL) (16).

due to the presence of C3d on normal red cells [16]. We recommend that all batches of AHG should be tested to show freedom from false positive reactions with red cells from CPD-A1 donor pack segments (over five days old) incubated with fresh serum, as in compatibility tests. These tests should not show macroscopic reactions, although a small proportion of weak microscopic reactions is unavoidable with AHG reagents containing a desirable level of anti-C3d.

The routine quality control of the AHG involves:

- i) The addition of a coloured dye (usually green) to the AHG reagent.

This is a very economical procedure which enables the operator to observe that AHG reagent has been added to each test [23].

- ii) The routine quality control of the potency of the anti-human globulin reagent, which could be performed by separate control tests (8b, 9, 24) using an anti-D diluted to give +/+ reactions with a pool of red cells from four donors of the phenotype R_{1r} [7].

The use of red cells weakly sensitised with anti-D is intended to demonstrate whether the washed cells are still contaminated with human immunoglobulin, for whatever reason. This weak indicator system is more sensitive to partial neutralisation of the AHG than cells strongly coated with IgG.

4. Spin Phase and Reading of the Tests

The last stage begins after the red cells have been sensitised and thoroughly washed. AHG is added to each tube, the tubes centrifuged, and the tests read by a careful reading technique (see Appendix B).

Volume of AHG Per Test

We recommend the use of two drops (70 μ l – 100 μ l) per test as being better able than one drop to overcome partial neutralisation due to inadequate washing of any test.

Final Spin Phase with AHG Reagent

The tube should be centrifuged within 15 – 30 s of adding the AHG. The centrifugal force used should be just enough to create a button of cells with clearly defined edges, but not so much as to make the button difficult to dislodge. Many combinations of RCF and time give similar results. (See Table 5).

Actual centrifuge speeds and automatic timers should be checked at monthly intervals as part of routine quality control. We recommend the use of red cells weakly sensitised with IgG anti-D and a negative control to determine the optimum spin time and force (RCF) to obtain the best results with the AHG test. Note that

Table 5

Combination of spin force (RCF) and time (seconds) satisfactory for spin-tube tests

RCF	Spin time (seconds)
110	60
200–220	25–30
500	15
1000	8–10

General guideline — doubling the RCF halves the spin time.

earlier models of machines from the same manufacturers may not have the same spin force as later models, e.g. the Sorvall CW2 has an optimum final spin phase of 30 s compared to 25 s of the earlier Sorvall CW1 machine.

5. *Quality Control by the Addition of Sensitised Red Cells to All Negative AHG Tests* [8b, 24]

The purpose of these tests is two-fold:

- i) To verify that the red cell washing procedure has removed all but insignificant traces of serum, and
- ii) to demonstrate that the AHG reagent has been added and is reactive.

Many laboratories use strongly sensitised cells for their control procedure in the mistaken belief that their procedure is satisfactory only if they see strong agglutination with the control cells. Unfortunately this practise gives a false sense of security since strongly sensitised control cells may react strongly with AHG that is sufficiently neutralised as to be unable to agglutinate weakly sensitised cells that would give 1+ / 2+ agglutination with fully active AHG [20].

Selection of the Level of Sensitisation of Control Red Cells

Table 6 illustrates an additional problem. The degree of agglutination produced by sensitised red cells and AHG alone will be reduced when they are added to non-sensitised red cells; the extent of this reduction will depend upon the Rh phenotype of the non-sensitised red cells for two reasons:

- i) Antibody transfer from the sensitised cells to the non-sensitised cells which have the corresponding antigen. The effect is greatest with non-sensitised cells that have a high antigen density [7], e.g. R_2R_2 cells cause a greater reduction than R_1r cells.
- ii) Physical interference reducing agglutination of sensitised cells by non-sensitised cells which lack the antigen to the sensitising antibody e.g. RhD negative cells reduce the agglutination of anti-D sensitised cells.

Table 6

Effect of Rh phenotype of non-sensitised red cells in reducing the anti-IgG reaction with sensitised red cells added to negative AHG tests

Phenotype of non-sensitised test cells	Range of reaction strengths in eight replicate tests with R ₁ r control red cells sensitised with anti-D		
	+++	++/+++	+/++
rr	++ to +++	++ to +++	+ to ++
R ₁ r	++ to +++	++ to +++	+ to ++
R ₂ R ₂	++ to +++	+ to ++	GW to +
	Too strong	Correct	Too weak

Problems in the Provision of IgG Sensitised Control Cells

In addition to the above effect, one must also bear in mind that the volume of anti-D serum needed to produce the desired (2+/3+) degree of agglutination is a function not only of the 'avidity' and concentration of the antibody, but also the strength of the D antigen on the red cells to be sensitised. A balance must be found so that when the control cells are added to a negative AHG test, a distinctly weaker reaction is seen in the presence of trace amounts of serum. Thus, the data in Table 7 indicates that R₁r red cells sensitised with neat serum containing 0.8 IU/mL anti-D show partial neutralisation by serum dilutions of 1/2000 or higher levels of serum contamination, whereas R₂r red cells sensitised by the same anti-D concentration detect serum contamination only at dilutions much less than 1/1000. With this particular anti-D, only 0.27 IU/mL were needed to provide a sensitive control with R₂r red cells.

Table 7

Strongly sensitised red cells added to partially neutralised negative AHG tests do not demonstrate a reduction in anti-IgG potency

Red cells sensitised	Dilution of anti-D used (IU/mL)	Sensitised red cells added to negative AHG tests in the presence of serum dilutions:*				
		Control (No serum)	1/3000	1/2000	1/1000	1/50
R ₁ r	Neat (0.8)	++	++	+	W	—
R ₂ r	Neat (0.8)	+++	+++	++	++	—
R ₂ r	1 in 3 (0.27)	+++	++	+	W	—

* 1 vol (35 µl) of the serum dilution added to each washed (4×) test

The routine production of sensitised cells for daily use during 2–3 weeks' storage at 4 °C involves the problem of some antibody dissociation from the red cells, which is a function of diluent, temperature, storage conditions and the antibody concentration and affinity. This in turn means that strongly sensitised control cells are likely to be provided to allow for deterioration during storage.

Thus the preparation of IgG anti-D sensitised cells is a complicated procedure which is difficult to resolve for satisfactory bulk production.

In conclusion, we recommend the use of replicate tests with weakly sensitised red cells for the evaluation of personnel and cell-washing centrifuges. Replicate tests offer a new way to improve AHG test performance, because they enable staff with a poor reading technique to be identified and re-trained. When combined with the optimum conditions and quality control of the AHG tests described, this type of "in-house" assessment of staff is the only effective way of reducing many of the false negative errors seen year after year in External Proficiency Trials.

APPENDIX A

Protocol for the Evaluation of AHG Reading Technique by the Use of Blind Replicate Tests

1. Titrate a suitable, not-hyperimmune IgG anti-D, as used for the control of the AHG test, against R₁r red cells to find the dilution of anti-D that gave 1+ sensitised red cells (most workers used around 0.2–0.3 IU/mL*).

2. Prepare a batch of sensitised cells by incubating 16 mL of the selected anti-D dilution with 8 mL of 3 per cent washed R₁r red cells.

3. Have another person label 12 tubes for blind tests. Place 1 vol of 3 per cent 1+ sensitised cells and 2 vol of group AB inert serum in 9 random tubes; place 1 vol of unsensitised cells + 2 vol of group AB inert serum in the remaining tubes. Record the position of the various tubes.

4. Wash the tests thoroughly four times, add AHG, spin the tests and read.

5. Record the number of false negative (and false positive) results for each worker and analyse in conjunction with the particular reading technique used.

6. Workers with false negative errors should receive immediate tuition in correct procedure (see Appendix B) and washing technique.

7. Give failed workers repeat replicate tests to verify improved competence.

* Conversion of IU/mL to ug/mL (25), divide by 5, e.g. 0.3 IU/mL = 0.06 ug/mL or 60 ng/mL.

APPENDIX B

Reading Techniques

i) Pipette transfer of the cell button to microscope slides

The pipette should have a wider than usual bore of 1.5–2 mm ID so that the column of cells will be short and agglutinates will have a better chance of reaching the slide unharmed. The tip must not be chipped or irregular. Do not agitate the tube at any stage.

Draw the cell button with the minimum possible suction into the stem of the pipette. Then gently eject the contents onto a slide while moving the pipette along it for about 1.5–2 cm.

Surface tension holds the red cells in fluid around the pipette surface and the movement distributes the cells over an area approximately 4 cm². The angle of the pipette above the horizontal controls the width of the distribution. Read the test macroscopically then microscopically if necessary.

ii) Tip and roll

Hold the tube almost horizontally (70–80°) between the thumb and first two fingers and slowly rotate and move back and forth, without shaking or agitation, until the cell button is dislodged from the tube. The free cell button/agglutinate(s) should be allowed to move no more than 1–2 cm down the tube. The test can then be read macroscopically, with the tube held horizontally over an illuminated light source. A ×5 or ×6 magnifying mirror, or a ×6 hand lens can be used for observing 1+ or stronger agglutinates. Microscopic readings can then be obtained by:

a) examination of the tube placed horizontally on the stage of an inverted microscope (e.g. Olympus, model CK, magnification ×60)

or b) by transferring the tube contents to a microscope slide either by pipette or by touching the lip of the tube to the slide and moving the tube slowly along it.

iii) Gentle agitation

Hold the tube almost vertically between the thumb and first two fingers and then gently agitate. Use a fine tremor or gentle quiver i.e. a trembling or vibrating movement, but never a coarser shaking action. The test can then be read as described above under 'tip and roll'!

i) Shake techniques

This type of procedure has unfortunately been developed by some workers from the gentle agitation procedure just mentioned. Over-vigorous action may reduce the expected degree of agglutination and we believe this technique is responsible for many false negative tests in blood transfusion work. The tests are read as described above.

APPENDIX C

*Procedure for Routine Quality Control of Cell Washing Centrifuges**New Equipment*

Machines should only be accepted after they have been installed and adjusted by the agent's representative and their washing efficiency proved by three machine loads of replicate tests with 1+ sensitised cells. The machines should conform to applicable safety standards for centrifuges e.g. BS 4402 (UK, 1982) and Electrical Safety Codes for Hospital Laboratory Equipment e.g. ESCHLE (UK).

Procedure With All Machines

- i) Use for wash cycles with 3 mL (or more) of saline or phosphate buffered saline pH 7.0–7.2 (PBS).
- ii) Use 2 vol of potent spin-tube AHG.
- iii) Determine the final spin time and RCF with AHG to give optimum reactions without false positives due to over-spin techniques.
- iv) Tubes must conform to manufacturer's recommendations. We recommend glass tubes, because cell buttons are far more difficult to dislodge from plastic tubes. 75 mm length of tube is optimal; longer tubes have been found to cause inadequate washing in several makes of machines because the tube lip makes contact with the saline wash port and prevents the correct volume of saline being dispensed into the tube. Tubes of 12 or 10 mm diameter are satisfactory.
- v) Clean the machine daily, to avoid a build-up of serum contamination that may cause back-contamination of tests from aerosol droplets created by the turbulence of the moving components of the machine.
- vi) At least once a week routinely control the washing efficiency by at least one machine load of replicate tests with 1+ sensitised cells.
Prepare the sensitised cells as in Appendix A.
- a) Label 36 tubes (3 sets) and add 1 vol of the 3 per cent sensitised (1+) cells and 2 vol of group AB inert serum to each tube.
 - b) Wash each machine load four times, add AHG, spin the tests and read.
 - c) Record and analyse the number of failures for each machine. A failure is a test that did not give the expected 1+ result i.e. a "w" or negative reading.

Action on Faulty Cell Washing Centrifuges

- i) The incidence of failures should be reported to the Head of Department. The appropriate National Organisation and manufacturer should be notified to assist in information being circulated to all users.
- ii) The machine should be taken out of routine use until the faults have been corrected.

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Advances in Therapy of Chronic Lymphocytic Leukaemia*

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(Received 4 March, 1987; accepted 11 March, 1987)

Chronic lymphocytic leukaemia (CLL) is a lymphoproliferative disorder characterized by the progressive accumulation of clonal small mature-looking lymphocytes, usually of B cell origin. In addition to a better understanding of many biological features, during the last 10–15 years a great progress has been made in the prognostic characterization of the disease. The therapeutic achievements, however, have been less impressive. The possibility to identify patients with different prognosis has renewed interest in the treatment of this disorder. A considerable number of controlled trials have been performed or are in course, and new perspectives for CLL treatment are emerging.

Basically four different kinds of measures are used: 1) chemotherapy, 2) radiotherapy, 3) adjuvant measures and 4) new modalities.

Keywords: chronic lymphocytic leukaemia, chemotherapy, new agents, treatment

Chemotherapy

The most important tool is still chemotherapy, either single or combined. A great number of agents are active in CLL. Due to its efficacy and tolerance, chlorambucyl has been selected as the drug of choice in this disease and it can be given on daily or intermittent basis, usually combined with corticosteroids. It has been suggested that the intermittent administration might be preferable to the daily one and to prednisone alone, but these facts have not yet been definitively proven in randomized trials. The usual schedule employed in our institution and shared by many groups consists of chlorambucyl 0.4 mg/kg/p.o. days 1 and 2 combined with prednisone 0.1 mg/kg/p.o. days 1 to 5. The treatment schedule is maintained for at least 6 months before evaluating the patient. The response rates are wide in range (35–90% of remissions with 8–40% of complete responses). It appears that the survival is more prolonged if the treatment is applied to previously untreated patients, and if it is given in earlier clinical stages.

* Presented as part of Plenary Session contribution at the 4th International Symposium on Therapy of Acute Leukaemias. February 7–12, 1987, Rome (Italy).

A great number of combined chemotherapy regimens have been attempted in CLL, although most of them in uncontrolled studies. Thus, this discussion will be restricted only to a few randomized trials.

There are at least 3 known studies comparing chlorambucyl to cyclophosphamide, vincristine plus prednisone (COP regimen). In one [1], the Spanish Cooperative Group PETHEMA compared intermittent chlorambucyl plus prednisone vs COP in CLL patients with advanced disease (stage C). The overall response rate was higher in chlorambucyl plus prednisone group, but this was not reflected in the survival. On the other hand, previously untreated patients survived better than those previously treated. Another randomized trial comparing intermittent chlorambucyl plus prednisone with COP administered during 9 months in stages III, IV and progressive II was carried out by the Eastern Cooperative Oncology Group [2] and again, no differences were found in median survivals and complete remission rates. Finally, the French Cooperative Group, comparing daily chlorambucyl with COP regimen in stage B [3], also failed to demonstrate any difference in survival among both groups.

The Spanish Cooperative Group PETHEMA has activated a study in which untreated patients in stages B and C are randomized to receive intermittent chlorambucyl plus prednisone or a combination therapy consisting of cyclophosphamide (160 mg/sq.m.), mephalan (6 mg/sq.m.) and prednisone (60 mg/sq.m.) for 4 days every two weeks. Both therapeutic arms are maintained for 10 months. A preliminary analysis [4] performed recently has not shown a significantly better survival with the first treatment.

Recently, the French Cooperative Group [3] compared the standard COP regimen vs modified CHOP (with a relatively small dose of adriamycin, 25 mg/sq.m.) in previously untreated patients in stage C. A longer survival was observed in the latter group with a plateau at approximately 75%. Since the median follow-up of these studies is still relatively short, the significance of these "plateaus" must be interpreted with caution.

The Southeastern Oncology Group has analyzed the value of cycle-active consolidation therapy [5]. In previously untreated patients receiving chlorambucyl plus prednisone during 6 months, a response rate of 78% with 22% of complete remissions were observed. Thereafter responders were randomized to receive 3 months therapy with the same schedule or cycle-active therapy with Ara-C (25 mg/sq.m. every 12 hours \times 8, subcutaneously) plus cyclophosphamide (25 mg/sq.m. every 12 hours \times 8 orally) every 3 weeks. Although the number of complete remissions increased in both arms of the protocol, there was no difference in the survival between the two groups the second arm being associated with a greater toxicity.

In summary, it appears that in advanced and/or progressive stages of CLL, the standard therapy schedule (chlorambucyl plus prednisone) is still the treatment of choice. Encouraging results have been obtained with the modified CHOP regimen which deserves further trials.

A completely different problem is that of patients with CLL in early stages. Some trials have been performed in this type of disease, too. In the afore-mentioned

French trial [3] stage A patients were randomized to abstinence or chlorambucyl. More than 200 patients entered each arm. The rate of progression to stages B or C was higher in the abstinence group, but in contrast the mortality was higher in those receiving chlorambucyl. The overall survival, however, was not different statistically.

A similar approach was followed by the Acute Leukemia Group B [6], comparing in 49 patients with stable CLL in stages I and II low doses of chlorambucyl (0.5 mg/kg every 28 days) versus abstinence. Although clinical progression was retarded by treatment (median of 3.8 years versus 1 year), the survival was not influenced.

Radiotherapy

Several types of radiotherapy, such as total body irradiation, total nodal irradiation, thymic irradiation, extracorporeal blood irradiation and low-dose splenic irradiation have been claimed to produce good results in CLL. There is no general agreement on the efficacy of such modalities. The present place of radiotherapy is its use as a palliative measure in order to alleviate symptoms caused by massive splenomegaly. Perhaps, it would be worth-while to further evaluate the low dose splenic irradiation [7] in comparison with other therapeutic regimens. This should be done in prospective randomized trials, some of them already in progress.

Adjuvant measures

Splenectomy can also alleviate clinical symptoms due to a huge splenomegaly and partially correct peripheral blood cytopenias. Despite clinical improvement, splenectomized patients apparently do not survive longer than nonsplenectomized patients [8], although no controlled studies are available. Extremely high lymphocyte levels can be brought down by cytoapheresis. Minor improvements of peripheral cytopenias can be achieved with androgens and/or lithium. The value of IgG administration as prophylactic measure against increased incidence of infections, typical of CLL patients, is not known. An international randomized double-blind trial addressing this issue is in progress [9], and a preliminary analysis has shown a protective efficacy of IgG administration [10].

New modalities

New therapeutic modalities, such as bone marrow transplantation, monoclonal antibodies, differentiation promoting agents, interferon and others have been tried in CLL.

There are at least 6 known cases of CLL who underwent bone marrow transplantation [11, 12, 13]. The Grenoble group [11] performed in April 1984 an allogeneic BMT in a 43 year-old female patient in stage IV, obtaining a stable complete remission of nearly 3 years duration. A Spanish team from Barcelona [12] trans-

planted in April 1986 a 40 year-old male patient in stage III, with apparently good result. The afore-mentioned French team [13] recently reported four additional allogeneic transplants with 3 deaths and only one survivor. It is interesting to note that apparently the malignant clone can be eradicated by intensive chemoradiotherapy, but for obvious reasons this approach can be only used in a reduced number of patients.

As far as monoclonal antibodies are concerned, only transient and incomplete responses have been achieved in most cases treated with T101 [14, 15]. In the future, it might be interesting to use non-modulating monoclonal antibodies, monoclonals conjugated with different cytotoxic agents (radionuclides, toxins, cytotoxic drugs), and anti-idiotypic monoclonal antibodies in early stages.

Since it has been possible to promote differentiation of B-cells *in vitro* with phorbol esters [16, 17] and different interferons [18], this approach is now being explored *in vivo*. The retinoic acid showed some efficacy in 2 cases of prolymphocytic leukemia, but no effect in 9 patients with non-Hodgkin's lymphoma or CLL [19]. It might be of interest to carry out further trials in early stages or perhaps with other agents.

With recombinant alpha-interferon less than 20% of patients achieve only a partial and transient response [20, 21, 22]. The general opinion today is that alpha-interferon is not useful in CLL. However, it seems that response rates may be higher in previously untreated patients. Thus, in future it might be interesting to explore such a therapy in early stages or try other interferons. Taking into account these facts, recently we treated 8 untreated patients with CLL in early stages, and in all of them a clear-cut reduction in lymphocyte count was obtained [23].

Future research

From the previous discussion, it clearly appears that after many years of stagnation, some progress is being achieved in CLL therapy. Many trends for future research in this field are emerging.

One of the important problems is to investigate whether CLL can be cured or not. From some anecdotal observations on bone marrow transplantation it can be assumed that intensive radiochemotherapy is probably capable of eradicating the malignant clone. But it is probable that this kind of approach could never be applied widely. On the other hand, not exceedingly rare cases of spontaneous complete remission in CLL can be observed [24], in association with different – mostly infectious – clinical events. Some subtle biological modification occurring during these episodes, must be responsible for the disappearance of the abnormal leukaemic clone. The discovery of such mechanisms might lead to the therapeutical application of biological response modifiers, a great challenge for the future. For the moment, in this context, the research should be done chiefly on the characterization of complete remission of CLL, as well as on the impact of complete remission on survival. In most clinical complete remissions (disap-

pearance of all symptomatology and correction of peripheral blood values) the abnormal clone still persists. It would be of utmost importance to characterize all clinical remissions from a clonal point of view. In this sense, at least the proportion of kappa/lambda chain-bearing lymphocytes should be normalised, though ideally the clonal remission should be characterized by DNA rearrangement technology. Another interesting topic is the study of bone marrow histology in cases of CLL in complete remission. It is presently accepted that bone marrow patterns represent a very good prognostic parameter [25] and that this pathological stage can progress during the natural course of the disease or regress under therapy [26]. The significance of residual bone marrow lymphoid nodules and of accompanying T-cell population during complete remission deserves further research. Although in some studies the achievement of complete remission apparently does not prolong the survival [5], this question should be further investigated with better definition of complete remissions.

Another crucial point for future research concerns the treatment of CLL in early stages. Since the usual clinical approach is to not treat these kinds of patients, only a few trials have been reported on the efficacy of different therapeutic modalities in early stages. From these studies it appears that chemotherapy is able to delay the progression to more advanced clinical stages, but has no benefit on survival [3, 6]. There is even some indication that it might be harmful [3]. On the other hand, it also appears clear that different therapeutic modalities are more active in terms of response in earlier than in late clinical stages [5, 23]. The key question remains unanswered: Is the therapy in early stages really needed? The primary aim of any medical treatment is the cure of the disease. When this is not possible, we try to improve the quality of patients' life and prolong their survival. CLL patients in early stages are usually symptomless. So, the chief aim of the therapy would be to prolong their survival. But, is it actually shortened?

Our preliminary data on this issue [27] have been reanalyzed recently. The survival of a series of 329 patients collected from 17 Spanish institutions was clearly shorter than that of the age- and sex-matched Spanish population. The median survivals were 5.3 years and 14.3 years, respectively ($p < 0.001$). Thus, CLL as a whole clearly shortens the survival. But is it also true for the early stages of the disease? According to the French authors [28], the survival of stage A patients does not seem different from that of the French population of the same age- and sex-distribution. In our recent analysis, the median survival of 142 stage A patients was 8.5 years in comparison to 14 years for the age- and sex-matched general population, the difference between the survival curves being statistically significant ($p < 0.01$). So, from our experience it seems that the therapy might be necessary for survival prolongation even in early stages of CLL. But, since the benefit would be relatively minor, the undertaking risky treatment modalities does not seem to be justified. Instead, trials with biological response modifiers should be favoured in these early stages of the disease.

Acknowledgement

This study was supported by grants No. 2124-83 CO2-01 and no. PA85-0234, Comisión Asesora de Investigación Científica y Técnica (CAICYT), Ministerio de Educación y Ciencia.

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Lymphocyte Proliferative Responses in Haemophiliac Patients: Relations to Clinical and Immunological Findings

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(Received 18 December, 1986; accepted 6 February, 1987)

Blood lymphocyte proliferative responses to mitogens were studied in 65 patients with haemophilia (haemophilia A: 54 patients, haemophilia B: 11 patients) in parallel with 39 male control subjects. As a group, patients with haemophilia did not demonstrate abnormal proliferative responses to phytohaemagglutinin (PHA), Concanavalin A (ConA) and pokeweed mitogen (PWM) when compared with healthy controls. When the patients were analysed according to their seropositivity for antibody to human immunodeficiency virus (HIV), those who were positive had significantly decreased PHA, ConA and PWM responses. Haemophiliac patients with T4+/T8+ ratios less than 1 had reduced proliferative responses to PHA, ConA and PWM when compared to patients with ratios greater than 1. No significant difference in mitogen responses were found when the patients were analysed according to the presence or absence of palpable lymphadenopathy. Those patients with haemophilia A who had received more than 5×10^4 units of factor VIII during the two years preceding the study showed no significant difference in PHA, ConA and PWM responses when compared to patients receiving less.

Keywords: haemophilia, T-lymphocytes, lymphocyte transformation, acquired immunodeficiency syndrome

Introduction

The first cases of acquired immune deficiency syndrome (AIDS) in haemophilia were reported in 1982 [1]. Since then several studies have demonstrated that haemophiliac patients may develop marked abnormalities of the immune functions [2–7], presumably as a consequence of infection with the human immunodeficiency virus (HIV), also known as the lymphadenopathy-associated virus (LAV) or human T-cell lymphotropic virus type III (HTLV-III), transmitted by infused blood coagulation factors [8–12]. Such abnormalities of cellular and humoral immunity primarily occur through the preferential infection of the helper-inducer (T4+ or CD4+) lymphocyte subset [13].

In the present study we compared haemophiliac patients and healthy controls for “in vitro” lymphocyte proliferative responses to various mitogens. We secondly

tried to relate any abnormal proliferative response in the patients to various clinical or biological parameters such as HIV serology, T4+/T8+ blood cells ratio, palpable lymphadenopathy and amount of transfused factor VIII.

Materials and Methods

Patients and healthy controls

The study included 54 patients with factor VIII deficiency (haemophilia A), 11 patients with factor IX deficiency (haemophilia B) and 39 healthy male controls (volunteer blood donors and healthy, non-infected children admitted to the Department of Pediatrics for minor surgery or bone marrow donation). The age distribution of patients did not differ from that of controls and is summarized in Table 1. None of the 65 patients had AIDS according to the CDC (Center for Disease Control) definition [14]; 17 of them, however, had persistent palpable lymphadenopathy (haemophilia A : 16 patients; haemophilia B : 1 patient) (Table 2). None of the patients and controls had a known history of drug abuse, homosexuality, travel to the West Indies or Central Africa, sexual relations with people from these countries. All clotting factor preparations used by the patients were obtained from healthy volunteers in metropolitan France.

Table 1

Age distribution of patients and controls

	Age (years)	
	Mean \pm 1 SD	Range
Controls (n = 39)	27 \pm 15	2-56
Haemophiliacs (n = 65)	22 \pm 13	2-57
Haemophilia A (n = 54)	22 \pm 14	2-57
Haemophilia B (n = 11)	25 \pm 9	10-41

Table 2

Patients with palpable lymphadenopathy and HIV serology

	HIV seropositive	HIV seronegative
Patients with palpable lymphadenopathy (n = 17)	11	6
Haemophilia A (n = 16)	10	6
Haemophilia B (n = 1)	1	0

Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on Ficoll Hypaque (Lymphoprep, Nyegaard, Oslo).

"In vitro" lymphocyte proliferative responses to mitogens

"In vitro" lymphocyte proliferative responses to the following mitogens were evaluated using conventional methods: phytohaemagglutinin (PHA) (Wellcome Research Laboratories, Beckenham, England), concanavalin A (ConA) (IBF Laboratories, Villeneuve La Garenne, France) and pokeweed mitogen (PWM) (IBF Laboratories, Villeneuve La Garenne, France). PBMC were resuspended at 1×10^9 cells/l in RPMI 1640 (Gibco) containing 10% foetal calf serum (Seromed, Biochrom AG, Berlin, Western Germany) and antibiotics (penicillin: 300 IU/ml, streptomycin: 200 μ g/ml). PBMC were incubated in flat-bottom wells (Microtest II Tissue Culture Plate, Falcon) at 1×10^5 cells/well. PHA, ConA and PWM were used at final concentrations of 2 μ g/ml, 5 μ g/ml and 0.1 μ g/ml respectively. Incubation was continued for 4 days in a fully humidified atmosphere of 5% CO₂ in air with addition of ³H-thymidine 18 hours before harvesting for liquid scintillation counting (specific activity: 2 μ Ci/well). All tests were performed in triplicate. Results were quantified as counts per minute (CPM).

Lymphocyte surface marker studies

T helper/inducer (T4+ or CD4+) and T suppressor/cytotoxic (T8+ or CD8+) cells were identified among PBMC using murine monoclonal antibodies (Coulter Clone T4 and T8, Coulter Electronics, Hialeah, Florida, USA) and a fluorescein-conjugated goat anti-mouse immunoglobulin serum (Coulter Clone GAM-FITC, Coulter Electronics). The percentages of fluorescein-labelled cells were determined with fluorescence microscopy (Laborlux 11, Leitz, Wetzlar, West Germany). Absolute values were calculated from peripheral blood lymphocyte and monocyte counts.

HIV serology

Haemophiliacs and controls were tested for antibodies to HIV by enzyme-linked immunosorbent assay (ELAVIA, Pasteur Institute, Paris, France) [15] and western blot as a confirmatory test in seropositive haemophiliacs.

Statistical analysis

The results were expressed as mean \pm standard deviation (1 SD). Intergroup comparisons were performed with the Wilcoxon rank sum test. For correlations between pairs of variables within each group the Spearman's rank correlation coefficient (r_s) was calculated. In all cases, the level of significance was $p < 0.05$.

Results

Lymphocyte responses "in vitro": comparisons between haemophiliacs and controls

Lymphocyte proliferative responses to PHA, Con A and PWM are shown in Table 3. They did not differ in haemophiliacs and haemophilia A and haemophilia B subgroups as compared to the control group. Comparison between haemophilia A and haemophilia B subgroups as well showed no significant difference.

Lymphocyte responses "in vitro" and HIV serology

Antibodies to HIV were found in 18 of the 65 haemophiliacs (27.7%), including 16 patients with haemophilia A (29.6%) and 2 patients with haemophilia B (18.2%). All healthy controls were seronegative. Seropositive patients had sig-

Table 3
Lymphocyte proliferative responses in patients and controls

	Control● (no mitogen added)	PHA●	ConA●	PWN●
Controls (n = 39)	0.86 ± 0.47	186.74 ± 74.85	121.09 ± 69.13	58.76 ± 30.25
Haemophiliacs (n = 65)	1.23 ± 1.11	197.83 ± 87.69	124.68 ± 80.47	52.64 ± 32.52
· Haemophilia A (n = 54)	1.17 ± 1.12	201.06 ± 83.18	131.20 ± 77.78	52.04 ± 32.45
· Haemophilia B (n = 11)	1.52 ± 1.10	181.96 ± 110.50	92.66 ± 89.54	55.60 ± 34.30
· Seronegative patients (n = 47)	1.39 ± 1.23	217.56 ± 82.81	138.12 ± 80.63	60.74 ± 30.53
· Seropositive patients (n = 18)	0.82 ± 0.59	146.32 ± 80.70*	89.59 ± 70.56*	31.47 ± 28.33*
· T4+/T8+ ≥ 1 (n = 47)	1.45 ± 1.22	216.73 ± 79.95	139.88 ± 80.25	60.84 ± 30.96
· T4+/T8+ < 1 (n = 18)	0.67 ± 0.42	148.47 ± 89.93*	85 ± 68.14*	31.23 ± 26.78*
· No lymphadenopathy (n = 48)	1.35 ± 1.22	210.21 ± 88.28	129.69 ± 77.65	57.11 ± 38.80
· Lymphadenopathy (n = 17)	0.89 ± 0.64	162.88 ± 78.18	110.55 ± 88.87	40.01 ± 32.08
· Factor VIII < 5 × 10 ⁴ U ⁺ (n = 41)	1.24 ± 1.23	211.78 ± 78.56	136.63 ± 77.68	54.02 ± 30.84
· Factor VIII ≥ 5 × 10 ⁴ U ⁺ (n = 13)	0.95 ± 0.60	167.25 ± 91.41	114.09 ± 78.65	45.78 ± 37.73

● Responses are expressed as CPM × 10⁻³ (Mean ± 1 SD)

+ Patients with haemophilia A: amount of transfused Factor VIII during the 2 years preceding the study

* p < 0.05 (Wilcoxon rank sum test)

nificantly depressed PHA and PWM responses as compared to seronegative patients (Table 3). The ConA response in seropositive patients was lower than those observed in seronegative patients and controls: the differences, however, did not reach statistically significant level ($p > 0.05$).

Lymphocyte responses "in vitro" and T4+/T8+ ratio

Eighteen of the 65 haemophiliacs (27.7%), including 15 patients with haemophilia A (27.8%) and 3 patients with haemophilia B (27.3%), had an inverted blood T4+/T8+ ratio (< 1). PHA, Con-A and PWM responses in patients with inverted T4+/T8+ ratio were significantly lower in comparison with patients showing T4+/T8+ ratio ≥ 1 (Table 3). Lymphocyte proliferative responses to mitogens were directly correlated to the T4+/T8+ ratio (PHA: $r_s = 0.40$, $p < 0.002$; ConA: $r_s = 0.38$, $p < 0.003$; PWM: $r_s = 0.39$, $p < 0.002$).

Lymphocyte responses "in vitro" and palpable lymphadenopathy

Seventeen of the 65 patients (26.2%) had palpable lymphadenopathy. PHA, Con-A and PWM responses in these patients did not significantly differ from those of patients without lymphadenopathy (Table 3). HIV seropositivity, however, was significantly more frequent among patients with palpable lymphadenopathy (11/17: 64.7%) as compared to those without lymphadenopathy (6/48: 12.5%) (chi-square test: $p < 10^{-4}$). All but one patient with palpable lymphadenopathy had haemophilia A (Table 2).

Lymphocyte responses "in vitro" and amount of transfused factor VIII

Fifty-four patients had factor VIII deficiency (haemophilia A). When the amounts of transfused factor VIII during the 2 years preceding the study were analysed, those patients who had received more than 5×10^4 units of factor VIII during the 2 years preceding the study did not show significantly depressed responses to PHA, ConA and PWM as compared with those receiving less (Table 3). The lymphocyte response to PHA and PWM was inversely correlated to the amount of transfused factor VIII ($r_s = -0.35$, $p < 0.05$) and $r_s = -0.27$, $p < 0.05$ respectively) whereas a marginally significant inverse correlation was found with ConA response ($r_s = -0.26$, $p = 0.057$).

Discussion

The mitogens PHA and ConA chiefly stimulate the T lymphocyte subset whereas PWM is an activator of both B and T lymphocytes [16]. Recent studies have demonstrated that asymptomatic haemophiliacs have abnormal "in vitro" responses to PHA [17–19], PWM (17) or ConA [18–20].

The present study shows that "in vitro" lymphocyte proliferative responses to PHA, ConA and PWM do not significantly differ in haemophiliac patients as compared to those from healthy controls. Within the hemophilic population, however, the results of our study indicate marked abnormalities of the lymphocyte response in different subgroups. Patients with T4+/T8+ ratios less than 1 have significantly reduced proliferative responses to PHA, ConA and PWM: this is in accordance with the findings of other investigators (17). Our study also demonstrates an association between HIV seropositivity and reduced proliferative response to PHA and PWM. As a subgroup, the 17 patients with palpable lymphadenopathy did not exhibit significantly decreased mitogen responses as compared to the other patients. They had a mean T4+/T8+ ratio lower than patients without palpable lymphadenopathy: the difference, however, was not statistically significant (1.27 ± 0.85 versus 1.71 ± 0.89 ; $p > 0.05$).

The 18 seropositive patients had a mean period of clinical follow-up of 15 months (range: 0 to 29 months): among them, only one patient developed full-blown AIDS and died of disseminated candidiasis and brain toxoplasmosis 22 months after his initial study which displayed a low T4+/T8+ ratio (0.32) and decreased responses to PHA, ConA and PWM (23.3×10^3 CPM, 14.4×10^3 CPM and 3.8×10^3 CPM respectively). So, we suggest that concomitant reductions of T4+/T8+ ratio and mitogen responses may have a predictive value for the development of AIDS in HIV seropositive haemophiliac patients. Longitudinal clinical studies of these patients should help to resolve this point.

Acknowledgement

We thank G. Corby, M. Chartier and C. Gourdet for technical assistance and C. Bouquet for secretarial help.

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Volume Regulatory Mechanisms of Human Platelets

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(Received 23 February, 1987; accepted 27 February, 1987)

Platelets were found to defend themselves against hypoosmotic swelling by a regulatory volume decrease mechanism, similar to that of T-lymphocytes. An oligomycin C sensitive Cl^- -channel and a Ca^{2+} -dependent, quinine- and trifluoperazine-sensitive K^+ -channel play a significant role in the process: platelets lose K^+ and Cl^- (and consequently water) through these. Activation of platelets accelerates the volume regulatory mechanism in spite of the fact that the Na^+/H^+ exchange process, activated simultaneously, works against volume decrease. The most rapid volume regulation was observed in activated platelets with inhibited Na^+/H^+ exchange process.

Keywords: platelet, swelling, shrinking, regulatory volume decrease, Ca^{2+} -dependent K^+ -channel, Cl^- -channel, Na^+/H^+ exchange

Introduction

Human T-lymphocytes suspended in hypoosmotic (Na)-based media were shown to swell transiently and then to restore their original cell volume by a regulatory volume decrease (RVD) process [1, 2, 3]. Detailed studies suggested that at a critical osmotic volume increase a Ca^{2+} -sensitive K^+ -channel and a Cl^- -channel open through which K^+ and Cl^- flow out from the cells followed by an osmotically coupled water efflux. In agreement with this assumption, in high- K^+ hypoosmotic media osmotic swelling is followed by a secondary volume increase as K^+ and Cl^- flow into the cells according to their concentration gradient, and water follows them. The findings showing that this secondary volume increase could be enhanced by the addition of the K^+ -ionophore gramicidin and abolished by the inhibitors of the Cl^- -channel mean further support of the above interpretation [4]. Unlike T-lymphocytes, human B-lymphocytes and granulocytes do not restore their volume after osmotic swelling. With this in mind we decided to examine whether human platelets are able to regulate their volume under anisoosmotic conditions.

Materials and Methods

The applied chemicals: quinine, propionic acid, gramicidin, thrombin, trifluoperazine, indomethacin (SIGMA Chemical Company, St. Louis, MO, USA) A23187, forskolin (Calbiochem, La Jolla, CA), EGTA, oligomycin C (Serva Feinbiochemica, Heidelberg), amiloride (Merck, Sharp and Dohme, Rahway, N. J.), chlorpromazine (EGIS, Budapest) were of reagent grade. Nigericine was a kind gift of Professor I. Horváth, methoxyverapamil (D-600) was a present of Professor Valerie Kecskeméti (Semmelweis University Medical School, Budapest).

Platelets were prepared from freshly drawn human ACD-blood by centrifugation. 20 μ l platelet-rich plasma (PRP) was diluted in 1 ml reaction-buffer. Platelet count of this suspension was: $1-2 \cdot 10^{10}/l$, the reaction buffer contained: 137 mM NaCl, 27 mM KCl, 0.3 mM Na_2HPO_4 , 12 mM NaHCO_3 , 2 mM CaCl_2 , 1 mM MgCl_2 , 5.5 mM glucose, and 5 mM HEPES (N-2-hydroxyethylpiperazine ethane sulfonic acid) in distilled water. 20 μ l of this platelet suspension was diluted in 5 ml of 0.16 M NaCl, 0.16 M KCl, or 0.16 M choline chloride, and the mean volume of platelets was measured. After the first volume measurement, drugs were added to the platelets as specified, and the suspension was kept at room temperature for 2 min.

To induce hypoosmotic shock 5 ml distilled water was added to 5 ml of platelet suspension. Volume measurements were done 30 seconds, 1, 2, 3, 4, 5, 10 and 15 minutes after the shock.

The mean volume of platelets was determined electronically by a Coulter-type PSL-1 Laborscale + PSA-1 Analyser (Medicor, Budapest) apparatus. A capillary of 70 μ m diameter was used for the measurements: the apparatus was calibrated with latex beads and mean platelet volume (MPV) values were registered.

For the detection of the Na^+/H^+ exchange process the swelling of platelets was followed in an isoosmotic Na propionate medium as described by Sarkadi et al. [5]. The isoosmotic Na propionate medium contained: 140 mM Na propionate, 1 mM CaCl_2 , 2 mM MgCl_2 , 1 mM glucose, 20 mM tris hydroxymethylaminomethane (pH 6.8). 20 μ l of the platelet suspension prepared in the reaction buffer was diluted in 5 ml Na propionate medium, MPV was measured and the suspension kept at room temperature for 2 min without any addition or with the addition of the drugs to be examined. Serial volume measurements were done at 30 seconds, 1, 2, 3, 4, 5, 10 and 15 minutes.

Results

Under our experimental conditions (capillary diameter, platelet count) the shape change accompanying the activation of platelets in isoosmotic media did not influence the values of volume measurements, and aggregation did not occur. Swelling in hypoosmotic medium and the consequent volume changes could be well detected; for the detection of the shrinkage caused by a hyperosmotic medium, however, the apparatus was not suitable.

As shown in Fig. 1., platelets swelled in hypoosmotic media and in low- K^+ media, this was followed by a rapid regulatory volume decrease (RVD) almost complete within 10 min. This process was unaffected by the increase of extracellular Ca^{2+} concentration, but it was impeded by the presence of a Ca^{2+} -chelator or Ca^{2+} -antagonist. 1 mM methoxy-verapamil (D-600) inhibited RVD completely, whereas 1 mM EGTA and 100 μ M indomethacin resp. caused a partial inhibition (Fig. 1). Lower concentrations of D-600 inhibited RVD partially.

Regulatory volume decrease was not influenced by ouabain, whereas it was inhibited completely by 100–250 μ M quinine, 1.5–3 μ M trifluoperazine (TFP)

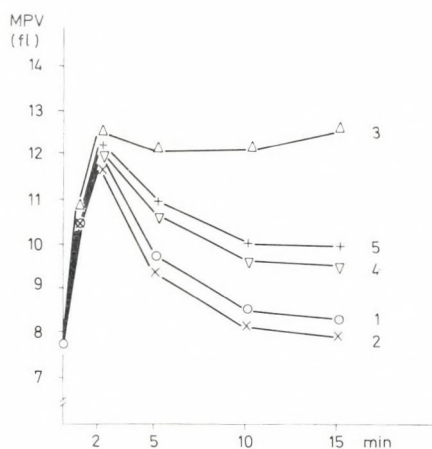


Fig. 1. The role of Ca^{2+} in the RVD of platelets in hypoosmotic NaCl media. 1. No addition, 2. 1 mM $CaCl_2$, 3. 1 mM D-600, 4. 1 mM EGTA, 5. 100 μ M indomethacin. One of 12 similar experiments

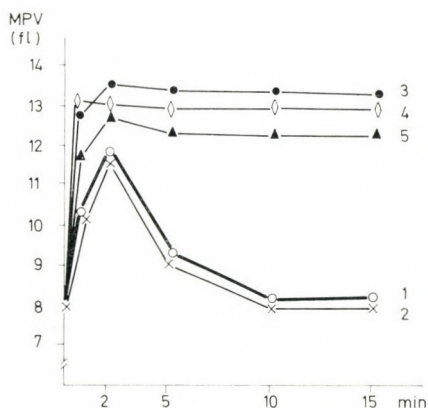


Fig. 2. Effect of drugs on the RVD of platelets in hypoosmotic NaCl media. 1. No addition, 2. 1 mM ouabain, 3. 1.5 μ M TFP, 4. 0.25 mM quinine, 5. 1 μ g/ml oligomycin C. One of 12 similar experiments

and almost completely by 1 $\mu\text{g}/\text{ml}$ oligomycin C (Fig. 2). Other anti-calmodulin drugs (10 μM chlorpromazine, 1 μM R 24571) acted like TFP, 100 μM dipyrindamole like oligomycin C.

When hypoosmotic shock was induced in a high- K^+ medium rapid swelling was not followed by a volume decrease, but by a secondary volume increase that could be inhibited by 1.5--3 μM TFP. Secondary volume increase could be enhanced and TFP-inhibition suspended by the K^+ -ionophore gramicidin. Oligomycin C in 1 $\mu\text{g}/\text{ml}$ concentration inhibited secondary swelling, irrespective of the presence or absence of gramicidin (Fig. 3). High (250--500 μM) concentrations of quinine were needed to inhibit secondary volume changes as effectively as 3 μM TFP.

Rothstein and coworkers [6] as well as our team [7] reported the existence of regulatory volume decrease in platelets at the Second European Congress of Cell Biology, Budapest. In the experiments of the Canadian group amiloride -- an inhibitor of the Na^+/H^+ exchange process -- accelerated regulatory volume decrease. Under our experimental conditions inactivation of Na^+/H^+ exchange (by substituting Na^+ with choline in the medium, or by adding 100 μM amiloride) caused no significant change in the rate of regulatory volume decrease.

On the other hand, based on the principle described by Sarkadi et al. [5], by the measurement of the swelling of platelets in isoosmotic Na propionate me-

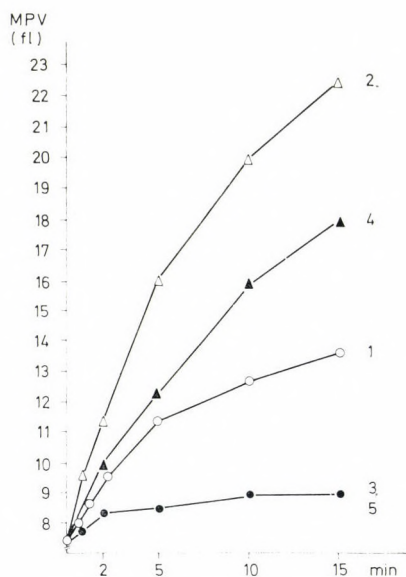


Fig. 3. Secondary volume increase of platelets in hypoosmotic KCl media. 1. No addition, 2. 0.5 μM gramicidin, 3. 3 μM TFP, 4. 0.5 μM gramicidin + 3 μM TFP, 5. 1 $\mu\text{g}/\text{ml}$ oligomycin C (\pm 0.5 μM gramicidin) One of 12 similar experiments

dium we could detect the Na^+/H^+ exchange process in our platelet preparations. Swelling was completely inhibited by $100 \mu\text{M}$ amiloride, but reappeared if the ionophore nigericin transporting Na^+ and H^+ ions was added to the system (Fig. 4).

The Na^+/H^+ exchange detected by the swelling of platelets in Na propionate medium could be reduced by $100 \mu\text{M}$ indomethacin and accelerated by thrombin (the thrombin effect being insensitive to indomethacin) (Fig. 5). Swelling was slightly reduced by $1-2 \mu\text{M}$ forskolin, an activator of the adenylate cyclase enzyme.

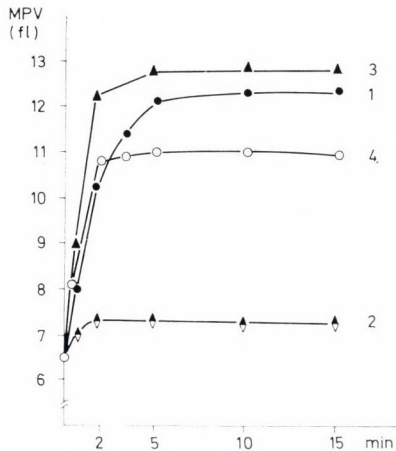


Fig. 4. Effect of amiloride + nigericin on the swelling of platelets in isoosmotic propionate. 1. No addition, 2. $100 \mu\text{M}$ amiloride, 3. $0.5 \mu\text{M}$ nigericin, 4. $0.5 \mu\text{M}$ nigericin + $100 \mu\text{M}$ amiloride. One of 8 similar experiments

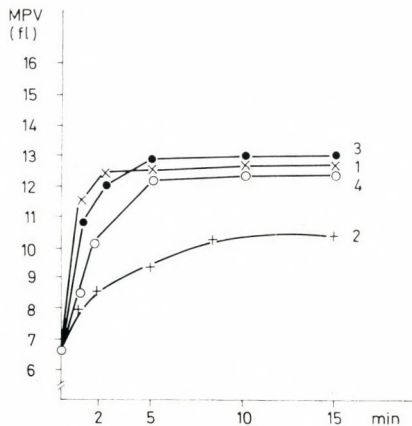


Fig. 5. Effect of thrombin and indomethacin on the swelling of platelets in isoosmotic propionate media. 1. No addition, 2. $100 \mu\text{M}$ indomethacin, 3. 0.05 U/ml thrombin (\pm indomethacin). One of 6 similar experiments

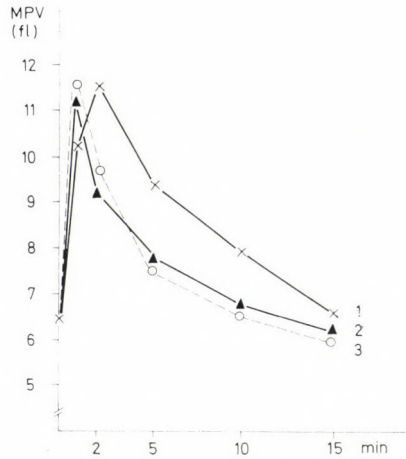


Fig. 6. Effect of thrombin and A23187 on the RVD of platelets in hypoosmotic NaCl media. 1. No addition, 2. 0.05 U/ml thrombin, 3. 10 μ M ADP. One of 6 similar experiments

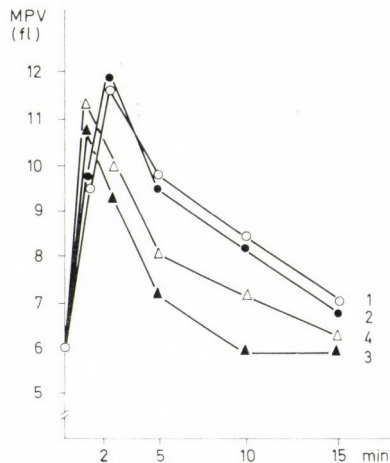


Fig. 7. Effect of thrombin + amiloride on the RVD of platelets in hypoosmotic NaCl media. 1. No addition, 2. 100 μ M amiloride, 3. 0.05 U/ml thrombin, 4. 0.05 U/ml thrombin + 100 μ M amiloride. One of 4 similar experiments

In Na^+ -based medium regulatory volume decrease following a hypoosmotic shock was fast and complete if platelet activators (thrombin, ADP) were added to the system (Fig. 6). In this case, when platelets were activated, RVD could be further accelerated by the addition of amiloride (Fig. 7).*

* Note: When the freshly prepared platelets were used immediately, speed and amiloride sensitivity of the RVD process, even without activators, approached the results shown by lines 3 and 4 of Fig. 8.

Discussion

The present investigations indicate that human platelets have an osmotic volume regulatory mechanism. The Ca^{2+} -dependence of the process (cf. Fig. 1) and its drug – (quinine, calmodulin-antagonist) – sensitivity (cf. Fig. 2) suggest that the Ca^{2+} -sensitive K^+ -pathway, the so-called Gárdos-channel is involved in the phenomenon. The oligomycin C and dipyridamol inhibition refer to the participation of a conductive Cl^- -channel. Most probably – like in T-lymphocytes – at a critical point of osmotic swelling K^+ and Cl^- channels open, and through them platelets lose KCl (and osmotically coupled water). Secondary volume increase of platelets demonstrated in hypoosmotic high- K^+ media is in agreement with this explanation. In K^+ -medium, due to the known K^+ -quinine antagonism [8], a specific quinine inhibition of the K^+ channel cannot be expected. Calmodulin antagonists, however, inhibited K^+ -transport preferentially, as indicated by the restoration of the secondary volume increase if the system contained gramicidin which forms an artificial K^+ -channel through the membrane. D-600 was an effective inhibitor of secondary volume increase, too. Since D-600 and gramicidin together caused the disruption of some platelets, in this set-up the selective inhibition of the K^+ -channel by D-600 could not be proven. Oligomycin C inhibition persisted even when the K^+ -ionophore gramicidin was added, confirming the assumption that the Cl^- -transport – the activity of which is essential for regulatory volume changes – was specifically inhibited by oligomycin C.

The above discussed transport processes seem to be basically identical with those participating in the RVD mechanism of T-lymphocytes. In platelets, however, one has to consider the eventual activation of the amiloride sensitive Na^+/H^+ exchange by Ca^{2+} [9], when Na^+ uptake accompanying swelling may reduce regulatory volume decrease. Data concerning platelet activation upon the addition of distilled water were reported [10], our osmotic shock procedure, however, did not seem to activate Na^+/H^+ exchange. Due to the propionate technique, we were able to detect the operation of Na^+/H^+ exchange in our platelet preparations. The basis of the detection is that from an isoosmotic solution of Na propionate the not dissociated, permeable propionic acid enters the platelets and decreases $[\text{pH}]_i$. For H^+ -ion equilibration H^+/Na^+ exchange is activated and the entering Na^+ forms Na propionate with the propionic acid that penetrated the cells. To achieve equilibrium distribution further propionic acid enters the platelets, this induces further H^+/Na^+ exchange with the above described consequences. As a final result, if H^+/Na^+ exchange functions, Na propionate (plus water) enters the platelets and causes a marked swelling. If the Na^+/H^+ exchange mechanism of platelets is inhibited by amiloride, swelling can be restored by supplementing the system with an artificial Na^+/H^+ carrier, like nigericin (cf. Fig. 4). The reduced swelling observed in propionate medium in the presence of indomethacin and forskolin resp., can be explained by their well-known $[\text{Ca}]_i$ reducing effects, and the Ca-sensitivity of the Na^+/H^+ exchange process. The ineffectiveness of indomethacin in the presence of thrombin, the most potent physiological activator of

platelets, is in good agreement with the efficient $[Ca]_i$ increment evoked by thrombin, even if cyclooxygenase is inhibited [11]. If regulatory volume decrease was accelerated by platelet activators, amiloride caused a further acceleration (cf. Fig. 7), indicating that under these circumstances Na^+/H^+ exchange was activated and reduced the speed of the volume regulatory system. It can be presumed that like in other cells [12] the activation of the Na^+/H^+ exchange mechanism enables platelets to regulate their volume in hyperosmotic media. Our present instrumentation, however, was not sufficient for the experimental confirmation of this possibility.

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Use of Ferrokinetics in the Follow-up of Patients with Polycythaemia Vera

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(Received 1 December, 1986; accepted 3 March, 1987)

Periodical follow-up evaluations at intervals from 3 to 6 months of haematocrit, blood volume and ferrokinetic, during a 2.5 year period were performed in 18 polycythaemia vera (PV) patients to find the most characteristic parameters for the determination of the erythropoietic system functional activity. Our results suggest that in PV it is advisable to carry out further determination of red cell volume to determine exactly the degree of erythrocytosis.

We found that plasma iron turnover (PIT) followed by $^{59}\text{Fe } T_{\frac{1}{2}}$ are the first parameters to return to normal and the first to alter after a prolonged period of a normal ferrokinetic status. We suggest these may be used as the most reliable indicators for the haematological control of PV.

Keywords: haematocrit, polycythaemia vera, plasma ^{59}Fe clearance, plasma iron turnover, red cell volume

Introduction

Ferrokinetic studies are of great importance in the characterization and investigation of erythropoiesis disorders. The erythroid tissues activity can be measured with ^{59}Fe bound to plasma transferrin [2]. Although haemoglobin and/or haematocrit levels are used for measuring red blood cell concentration, these do not always indicate the degree of anaemia or polycythaemia. Only the determination of the blood volume presents more precise data related to the anaemia or erythrocytosis in patients [12]. On the basis of blood volume changes polycythaemia is classified as absolute or relative. An essential criterion of absolute polycythaemia is the existence of an increased circulating red cell mass which can be determined by a direct red cell volume measurement with ^{51}Cr [1].

Polycythaemia vera (PV) is a disease characterized by a marked increase in erythropoiesis, which can be either accompanied or not by an increase in leukocyte and platelet production. The increased circulating red cell mass causes an increased blood viscosity with a tendency to thrombosis and haemorrhage. The frequency of these complications in PV necessitates the regular follow-up of these patients by investigations which can detect early signs of increase in erythropoietic proliferation.

The aim of this paper is to present the periodical follow-up of this 18 PV patients by measuring haematocrit, blood volume and the results of ferrokinetic studies in order to find the most sensitive parameters for the detection of increase in erythropoietic activity. This can enable us to apply therapeutic measures well before the occurrence of the characteristic symptoms of increased blood volume.

Material and Methods

Eighteen PV patients, nine males and nine females were studied. The mean age was 67.6 years with a range of 48 to 87 years. The diagnosis was established in agreement with the Polycythaemia Vera Study Group [1]. Duplicate microhaematocrits, serum iron, blood volume determination with ^{51}Cr , and ferrokinetic study with ^{59}Fe were performed in each patient [3–5, 9, 10].

The ferrokinetic study was always performed 24 hours after the blood volume measurement at 9 a.m., with the patient in a fasting state. These investigations were conducted on 18 patients who had not received any kind of previous treatment, after performing phlebotomies and giving them 5 mCi of ^{32}P . The studies were repeated periodically at intervals from 3 to 6 months during a 2.5 year follow-up. The number of patients studied in each stage ranged from 17 to 18. The time of the first study was considered as time zero (T0). One case was excluded from the study after twelve months of follow-up because this patient did not comply to the time schedule of the control investigation.

The parameters analyzed during the 30 months follow-up were: haematocrit, blood volume and circulating red cell mass in ml/kg wt, respectively, serum iron, plasma ^{59}Fe clearance $\left(^{59}\text{Fe T} \frac{1}{2} \right)$, and plasma iron turnover (PIT) in mg/24 h and mg/kg wt.

In the statistical analysis, the variables were estimated in each time period with analysis of variance I (one way) [13]. The correlations were made by the Fischer's r [13]. The significance level used was 5%. The statistical study was conducted in a NEC-PC 980 1-F computer.

Results

The results of the variables studied in each period are shown in Table 1. The haematocrit was found very high in T0 (61 ± 5.8), with a highly significant statistical difference ($P < 0.001$) as compared to haematocrits from the different periods. After the first six months this variable returned to normal and remained within the normal range in the follow-up period. Blood volume was very high in T0 with highly significant differences ($P < 0.001$) compared to the blood volume found in the other periods. This variable was also found moderately high at 6, 12 and 24 months, but the differences were not significant as compared to the first 6 months. In T0 the red cell volume was considerably high, during the first six months

Table 1

Time in months	Haematocrit	Blood volume ml/kg wt	Red cell volume ml/kg wt	Serum iron ug/%	T _{1/2} ⁵⁹ Fe* min	Plasma iron turnover	
						mg/24 h	mg/kg wt
0 (18)	61 ± 0.58 × × ×	94 ± 19.8 × × ×	87 ± 15.5 × × ×	65 ± 23 × × ×	23	53	1.00
1-6 (18)	49 ± 0.71	74 ± 16.9	36 ± 11.9 ×	85 ± 43 × ×	40	33	0.60
7-12 (18)	47 ± 0.78	70 ± 16.9	32 ± 11.1	102 ± 47	72	35	0.60
13-18 (17)	45 ± 0.58	63 ± 13.8	28 ± 7.6	86 ± 34 × ×	65	32	0.50
19-24 (17)	45 ± 0.79	70 ± 19.5	32 ± 13.7	113 ± 49	61	34	0.50
25-30 (17)	47 ± 0.72	63 ± 17.2	29 ± 10.3	113 ± 49	50	50	0.70
Normal values	40-50	66 ± 15.3	26 ± 2.1	60-180	60-120	20-40	0.6-0.8

× × × P < 0.001
 × × P < 0.01
 × P < 0.05
 ± = SD
 () = n
 * = X₅₀

it also increased but to a lesser degree. The statistical significance was P < 0.001 and P < 0.05, respectively. Within 12 months, this variable remained slightly above the normal value. The same alteration was found two years later.

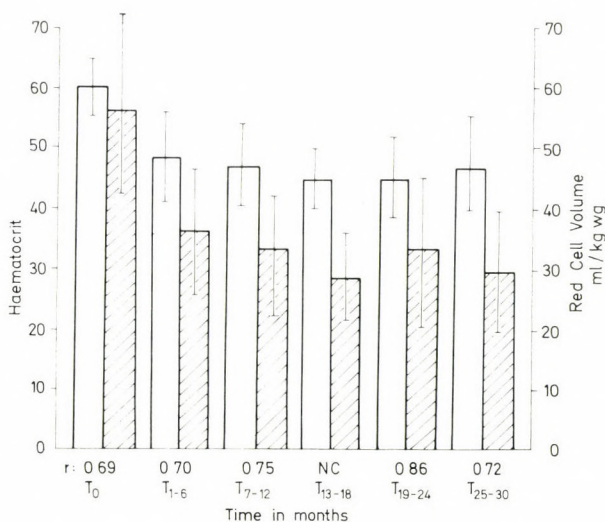


Fig. 1

Although within the normal range, the lowest serum iron value was found at the initial evaluation (T0). This parameter remained stable in the other periods.

Plasma ^{59}Fe clearance $\left(T_{\frac{1}{2}}\right)$ was very fast (23 min) in T0. In the first 6 months it was still accelerated, but slower than in T0. It became normal between 12 months and 2 years. Between 25–30 months it accelerated again.

In T0, a marked increase was observed in PIT both in mg/24 h and mg/kg wt. These values were 53 and 1.0 mg, respectively. Later, it returned to normal, but it was increased again between 25–30 months. A significant positive correlation was found between haematocrit and red cell volume in each period with the exception of the period from 13 to 18 months ($P < 0.001$) (Fig. 1).

Discussion

It has been found that there is an abnormal population of erythroid colony forming units (CFU_e) with the capacity of proliferation in the absence of erythropoietin in the bone marrow of PV patients [11, 14]. Some authors have considered that this abnormal population is more of a quantitative than a qualitative nature [6].

It is likely that the degree of erythroid production in PV patients is related to the amount of abnormal CFU_e present in the bone marrow. In addition, it has been observed that not all patients have the same degree of erythroid proliferation.

The follow-up of these patients in clinical practice in order to evaluate the disease severity is usually based on haematocrit measurements. There are, however, observations suggesting that the haematocrit value is not a good indicator of total red cell volume [7], although both variables are frequently correlated [1]. It has been observed that in cases when the haematocrit value is 60% or higher the red cell volume is also increased [1, 8]. In our work the haematocrit showed a correlation with red cell volume in each period except between 13–18 months. At the onset of the disease, when the haematocrit was over 60%, the red cell volume was also very high. In the follow-up, however, the haematocrit was found to be normal, but the red cell volumes were still high. Our results suggest that in PV under adequate haematological control it is advisable to carry out, in addition to the regular determination of red cell volume, measurements indicative of the degree of erythrocytosis.

It has been observed that there is no relationship between ^{59}Fe $T_{\frac{1}{2}}$ and marrow iron turnover (MIT), the latter being an indicator of total erythropoiesis. However, under extreme conditions it is possible to find a linear relationship between both parameters [12].

In active PV there is erythroid hyperplasia and plasma ^{59}Fe clearance is very fast. In our investigation, the lowest ^{59}Fe $T_{\frac{1}{2}}$ values were observed in T0 when

the patients had not received any kind of treatment. This fast plasma ^{59}Fe clearance does not seem to have been influenced by hyposideremia, since in this period serum iron values, although the lowest observed throughout the study, were within normal range. These observations suggest that $^{59}\text{Fe } T_{\frac{1}{2}}$ could be a good indicator of erythroid hyperplasia in PV. Another aspect of interest was that this parameter was the second one to become normal within a period of 12 months following treatment. Between 25–30 months a new reduction was observed, even when the rest of the parameters studied at that time were normal, with the exception of PIT. This behaviour of $^{59}\text{Fe } T_{\frac{1}{2}}$ in PV may be useful in determining when the erythropoietic activity starts to become normal and when the increase just starts again.

It has been demonstrated that PIT and MIT correlate significantly, although the former is usually higher [2]. With MIT increased levels as in PV, this difference is relatively stable; hence PIT in PV could be useful to evaluate total erythropoiesis. We found that PIT, besides being the first variable to return to normal, displayed alterations very similar to $^{59}\text{Fe } T_{\frac{1}{2}}$ but inversely. Thus, the highest value of PIT was found prior to the treatment. Later, it was restored to normal and at 2.5 years it was increased again. From results obtained it could be stated that PIT followed by $^{59}\text{Fe } T_{\frac{1}{2}}$ are the first parameters to become normal and the first to alter after a prolonged period of normal status of ferrokinetics. Considering that precisely these two parameters, under erythroid hyperplasia conditions as in PV, could measure total erythropoiesis indirectly, they might be used as the most reliable indicators for the haematological control of this disease. This would enable one to apply therapeutic measures before the recurrence of symptoms and signs causing increased blood volume so these patients could maintain their normal activities.

Acknowledgements

We are indebted to Dr. Maura Wade for statistical consultation. This work was supported in part by the International Atomic Energy Agency, contract No. 3304.

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Evaluation of Preoperative Blood Tests for Predicting Deep Vein Thrombosis after Total Hip Replacement

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(Received July 7, 1986; accepted November 27, 1986)

Determinations of the total calcium content and aggregability of the platelets as well as tests of coagulation and fibrinolysis were carried out on 93 patients before undergoing total hip replacement. All patients received low dose heparin, solely or combined with dihydroergotamine. Twenty-six patients developed deep vein thrombosis (DVT) detected by the labelled fibrinogen uptake test and confirmed by ascending phlebography. Only a few tests, among them the total calcium content of platelets, showed a statistical difference between the patients who subsequently developed DVT and those who did not. Combination of several tests by a multivariate statistical analysis programme proved to have more predictive value than the analysis of single tests.

Keywords: blood coagulation tests, calcium, fibrinolysis, hip prothesis, platelet function tests, venous thrombosis

Introduction

Deep vein thrombosis (DVT) occurs frequently after total hip replacement. It would be helpful in treating the condition if a means of identifying individuals at risk of developing DVT could be found. Some clinical studies have been made to establish relationships between preoperatively measured variables of the haemostatic system and the incidence of postoperative DVT [1–13; for review of papers till 1979 see 14]. Although some variables were found to differ significantly between the groups of patients who did and did not develop DVT, it is still impossible to identify the individuals at higher risk by means of a single test. Prognostic indices based on clinical and/or laboratory data produced a more reliable prediction of DVT after gynaecological [1, 15, 16] or major abdominal [6, 7, 9, 13] operations. As far as we know such a prognostic index has not yet been derived for patients undergoing hip replacement.

According to our studies some preoperative coagulation and platelet parameters, for example the total calcium content of platelets, differ significantly in patients who develop DVT following hip replacement as compared to those who do not. Further on, a multivariate discriminant function gives an improved discrimination which is comparable to earlier published prognostic indices for postoperative DVT.

Patients and Methods

The patients (34 males, 59 females) were cases for elective total hip replacement aged 40 to 80 years (mean age 61.6 years). The drugs taken by the patients were recorded. Patients who had taken acetylsalicylic acid were excluded from the study. Besides routine physiotherapy and walking exercises, all the patients received low dose heparin (5000 units twice per day) subcutaneously, solely or together with dihydroergotamine (0.5 mg), starting 2 hours before the operation.

The 125-I-fibrinogen uptake test was used to screen for DVT. The legs were scanned daily for a period of 8 to 10 days after surgery. The details of the procedure were described earlier [17]. An ascending phlebography was done in patients with positive leg scans or when thrombosis was suspected clinically. According to the phlebographic diagnosis two patients were classified into the DVT group despite a negative fibrinogen uptake test, and one patient was classified into the control group despite a positive leg scan.

Laboratory tests

Blood samples were taken 2 to 4 days before the operation and anticoagulated with citrate (0.11 M, 9 : 1 v/v). The microthrombus index (ratio of circulating platelet aggregates, MTI) according to Wu and Hoak [18] was measured immediately. All other platelet tests were performed using platelet rich plasma (PRP) prepared by centrifugation for 10 min at 200g.

Platelet aggregation was measured turbidimetrically according to Born using a non-commercial equipment. The platelet concentration was adjusted to approximately $10^5/\mu\text{l}$ using autologous platelet poor plasma as the diluent. The aggregation protocol was rigorously standardized and the agents used included ADP (Reanal, Budapest; 1.5 and 15 μM) and epinephrine (Jenapharm, GDR; 30 μM) as inducers of aggregation and diamide (azodicarboxylic acid bis-dimethylamide, Sigma; 225 μM) as an inhibitor of irreversible aggregation. The aggregation curves were analysed as follows:

- ΔT maximal change of light transmission (reversible aggregation) or change of light transmission after a fixed time interval (1.5 μM ADP: 5 min; 15 μM ADP and 30 μM epinephrine: 13 min), expressed as per cent of the difference in light transmission between PRP and platelet poor plasma.
- ΔT_{rel} ΔT as per cent of the maximal aggregation response obtained using 15 μM ADP.
- R_D ratio of the maximal change of light transmission in the presence of diamide (added simultaneously with the inducer) to ΔT of the response to the same inducer alone, as per cent of ΔT .

The total calcium content of platelets was determined as described previously [19]. 2 ml of PRP was preincubated with acetylsalicylic acid (100 μM) and then centrifuged for 10 min at 1400g. The platelet pellet was washed twice with a calcium-free buffer, washed ($\text{HNO}_3/\text{H}_2\text{O}_2$), dissolved (HCl , LaCl_3), and the cal-

cium content was determined by atomic absorption spectrophotometry. The results are given as nmol Ca per 10^9 platelets. Platelets were counted by phase contrast microscopy.

The activated partial thromboplastin time (APTT), the one-stage prothrombin time (Quick), and the bleeding time were determined by standard methods [20]. Quantitations of antithrombin III, α_2 -macroglobulin and α_1 -antitrypsin antigenic concentrations were performed by the "rocket" technique according to Laurell [21] using commercial antisera (Behringwerke, FRG). The "rocket" electroimmunoassay (EID) was carried out in agarose gel (Indubiose A37, Industrie Biologique Française, Gennevilliers, France).

Fibrin lysis was determined according to Halse [22] in a separate blood sample taken immediately after venous occlusion with a tourniquet (80 Torr, 10 min).

Statistical methods

Wilcoxon's rank test and the chi square test were used to calculate statistical significances. Furthermore, the data were processed by a computer programme to do a multivariate statistical analysis. The multivariate technique yields a linear combination of the observations — called a discriminant function — for the classification of an individual into one of two populations. It also yields a measure (Mahalanobis distance) reflecting the statistical "distance" between the two patient groups. A more theoretical approach based on the Bayes theorem allows to obtain two probabilities (one for each group) — called a-posteriori probabilities — that an individual belongs to these groups.

Results

Of the 93 patients investigated, 26 developed DVT in the postoperative period. With respect to the incidence of DVT there was no significant difference between the groups treated with heparin solely or in combination with dihydroergotamine.

Table 1 shows the mean values of some tests for the groups of patients who did and who did not develop DVT. For practical reasons it was not possible to do all the tests on all the patients. This applies especially to induced aggregations and the calcium content of platelets because these tests are time consuming and had to be done without delay after venipuncture. Bleeding time ($n = 90$), Quick test ($n = 90$), antithrombin III ($n = 88$), α_2 -macroglobulin ($n = 88$), α_1 -antitrypsin ($n = 89$), fibrin lysis ($n = 89$) and the epinephrine induced platelet aggregation ($n = 43$) did not provide statistically significant differences between the groups with and without DVT.

Some parameters were analysed by the multivariate technique. Using four variables a (microthrombus index, MTI), b (calcium content of platelets), c (APTT), and d (age), the discriminant function

$$V = 0.5 a - 0.035 b - 0.228 c + 0.120 d \quad [1]$$

was derived. The values of V calculated by this equation for the 42 patients of whom a , b , and c were measured preoperatively are shown in Fig. 1. With a cut-off point of -2.7 the discriminant function correctly identifies 90% of the DVT patients (sensitivity 90%) and wrongly allocates 12.5% of the patients without DVT into the DVT group (specificity 87.5%).

Table 1
Mean values of selected preoperative tests

Test	DVT		Controls		2P
Microthrombus index	0.92 ± 0.08	(24)	1.03 ± 0.12	(50)	a
Plt. calcium content (nmol/ 10^9 cells)	86 ± 25	(13)	129 ± 37	(44)	b
APTT (sec)	31.1 ± 5.6	(24)	33.4 ± 4.8	(63)	e
<i>Aggregation (inducer, mode of analysis)</i>					
1.5 μ M ADP, ΔT (%)	14.4 ± 8.8	(18)	10.6 ± 7.5	(26)	e
1.5 μ M ADP, ΔT_{rel} (%)	22.0 ± 9.3	(18)	16.6 ± 9.1	(26)	c
1.5 μ M ADP, R_D (%)	66 ± 31	(18)	114 ± 110	(26)	c
15 μ M ADP, ΔT (%)	63.8 ± 13.1	(18)	60.6 ± 12.5	(26)	f
15 μ M ADP, R_D (%)	23 ± 11	(18)	34 ± 18	(26)	d

Number of patients in parenthesis. Plt. = Platelet

a: $P < 0.0005$ (one tailed test); b: $2P < 0.001$; c: $2P < 0.05$; d: $2P < 0.1$; e: $2P < 0.2$; f: not significant

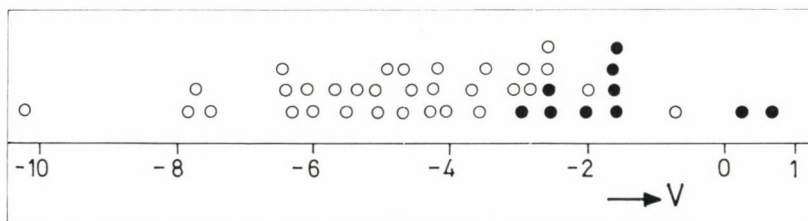


Fig. 1. Scatter diagram of values of the discriminant function V . Calculated for 42 patients on the basis of equation [1]. Solid and hollow circles represent patients who developed DVT and those who did not, respectively

Fig. 2A shows the a-posteriori probabilities (based on the Bayes theorem) of the individuals belonging to the thrombosis group. The Mahalanobis distance (D) between the two groups is also given. Fig. 2B shows the analogous results based on three variables only. The microthrombus index (MTI) was omitted first because this reduced D least. In the following steps APTT and age were omitted (Figs 2C and 2D, respectively). Regarding the statistical distance between the two

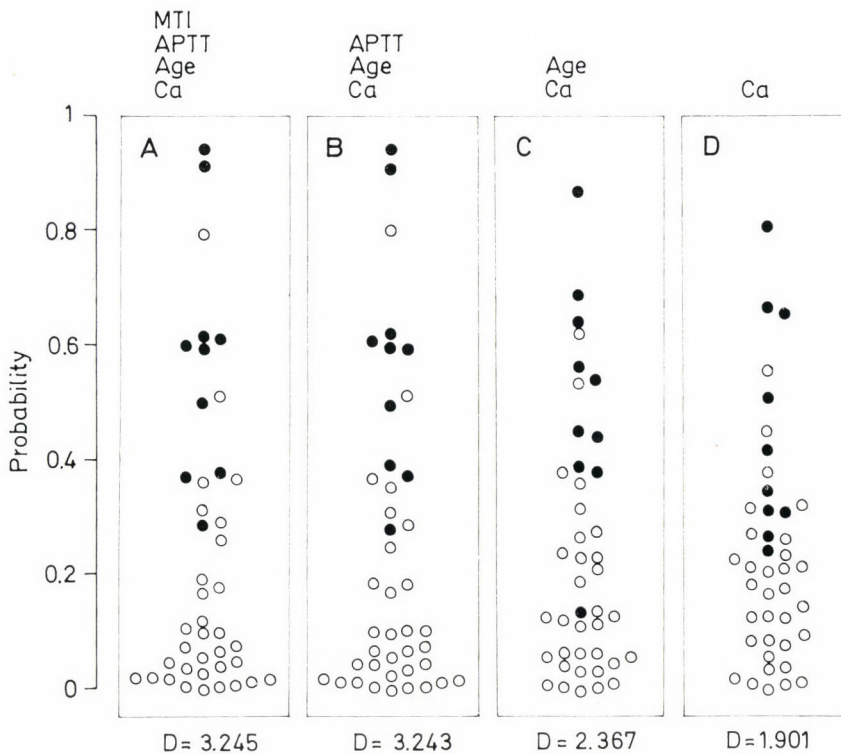


Fig. 2. Scatter diagram of the posterior probabilities (according to the theorem of Bayes) to belong to the thrombosis group, based on the indicated variables. Solid and hollow circles represent patients who developed DVT and those who did not, respectively

groups of patients, the combination of platelet calcium content with APTT and age is more effective than the combination of calcium content with age. Furthermore, this combination of variables is more effective than the evaluation of a single variable.

Although platelet aggregation induced by $1.5 \mu\text{M}$ ADP together with $225 \mu\text{M}$ diamide also yielded a significant ($2P < 0.05$) difference between the groups of patients it was not included in a multivariate analysis together with the platelet calcium content and the microthrombus index in order to avoid a further drop-out of patients on whom not all of these tests were performed. But other combinations of variables including varicosis and smoking habits were also analysed by the multivariate technique. In most cases the combination of two or more variables resulted in an improvement, but in no case was there a distinct separation of the patients of both groups.

Discussion

In the present study besides a number of established laboratory tests some recently developed tests were also used. To our knowledge this is the first evaluation of the platelet calcium content in connection with a thrombotic tendency. It appears that a predisposition to thrombosis may be associated with a continuous stimulation of some circulating platelets to undergo the release reaction thereby changing into so-called exhausted platelets [23]. During the release reaction platelets lose 60–70% of their total calcium content which is localized in dense granules [24]. Reimers et al. [25] found that when in-vitro exhausted platelets were returned to the rabbit they continued to circulate with a normal half-life. Thus the occurrence of exhausted platelets may result in a decreased average calcium content of the whole platelet population.

Were this concept true, a decreased total platelet calcium content in patients should correlate with an increased plasma level of β -thromboglobulin (β -TG). Preliminary experiments in normal subjects failed to reveal this correlation (unpublished results), probably because β -TG is localized in α -granules whereas calcium is mainly stored in dense bodies [24]. It is known that secretions from these two granule types are separate processes [26].

The mean microthrombus index of 0.92 in the DVT group reveals a weak tendency towards spontaneous formation of reversible platelet aggregates. This is in accordance with a greater extent of induced aggregation ($1.5 \mu\text{M ADP}$, ΔT_{rel}) in this group. At low concentrations of ADP the aggregation is not accompanied by a release of granule constituents [26]. Therefore, an increased response to $1.5 \mu\text{M ADP}$ is not contradictory to a decreased total calcium content of platelets.

Diamide, which was used in some aggregation tests as a modulator, is a thiol-oxidizing agent [27]. In platelets it alters proteins of the cell membrane [28] and the cytoskeleton [29], and changes the cellular thiol-disulfide status by a decrease of the reduced glutathione (GSH) level [for review see 30]. The thiol-disulfide status strongly influences the arachidonic acid metabolism [29–31] and this connection may be responsible for an altered aggregation response under pathological conditions [31].

Our intention was to check whether the susceptibility of platelets to a perturbation of the thiol-disulfide status could be discriminating with respect to a thrombotic tendency. We found that in platelets of the DVT group diamide partially inhibits the aggregation induced by ADP (resulting in $R_D < 100$). This effect is more pronounced at $15 \mu\text{M ADP}$. A better differentiation is found at $1.5 \mu\text{M ADP}$ between the patient groups with and without DVT. At the concentration of $1.5 \mu\text{M}$ the platelets of the group without DVT showed a slightly larger extent of aggregation in the presence of diamide ($R_D > 100$). These results indicate that platelets of DVT patients on the average are more sensitive to a perturbation of the cellular mechanisms underlying the aggregation response than are the platelets of patients without DVT.

Despite the fact that a hereditary deficiency of antithrombin III (AT III) is strongly associated with thromboembolism [32] there are contradicting results in the literature regarding the predictive value of a lowered AT III level for postoperative DVT. Several studies did not reveal a statistically significant difference between the preoperative AT III level of patients with and without DVT after total hip replacement [8, 10] or after other operations [12, 33]. This applies both to patients who received specific prophylaxis [8, 12] and to patients who did not [10, 33]. This is in accordance with our results, but in contrast to some other reports [4, 13, 34]. In studies on patients undergoing major abdominal surgery without specific prophylaxis [13] or total hip replacement with low dose heparin [4, 34] the authors found a significantly lower preoperative AT III level in those patients who subsequently developed DVT.

No difference was found in the plasma proteases α_1 -antitrypsin and α_2 -macroglobulin levels of the two groups of patients. The former protease is possibly the major plasma inhibitor of factor Xa [35, 36] and the latter one contributes about 25% to the overall plasma antithrombin activity [37]. The study of Lowe et al. [9] also failed to show a difference between preoperative α_2 -macroglobulin levels of the patient groups with and without postoperative DVT.

To conclude, in our study beside the APTT only some tests of platelet function and chemistry showed a statistical difference between the patients who did and those who did not develop DVT. Although the multivariate analysis of two or more of these variables marginally improved the distinct separation of the groups, the values for sensitivity (90%) and specificity (87.5%) of the discriminant function derived in the present study are similar to those found by other workers in gynaecological or general surgical patients.

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Comparison of Some Haematological Indices of Africans and Caucasians Resident in the Same Nigerian Environment

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(Received 25 August 1986; accepted 20 November, 1986)

Haemoglobin levels, total with blood cell counts and differential white blood cell counts were estimated for 512 Africans and 196 Caucasians who had been living in the same cosmopolitan Nigerian town for at least a year. Haemoglobin levels for African females differed significantly from those of Caucasians and many of the African females had values which indicated anaemia. Total white blood cell counts were similar, but an increased lymphocyte count was found in Africans and a higher neutrophil count in Caucasians. Eosinophil count was higher in Africans than in the Caucasians.

Keywords: Africans, anaemia, Caucasians, eosinophilia, haemoglobin

Introduction

In Nigeria, normal haematological values for Africans are still based on literary data established elsewhere in Caucasian subjects [1]. Several authors [2, 3, 4], however, have found values for Africans that persistently deviate from these. In the recent past several investigators [2, 5, 6, 7] have compared normal values between Africans and Caucasians. Except for the work of Ezeilo [5] most of these studies have been carried out on Africans in their African environment, without comparing the haematological values of Caucasians in the same environment. Data from such a concurrent comparative study similar to a recently published report [8] comparing biochemical values would be more convincing in the establishment of differences between Africans and Caucasians. Such data is scanty.

In this paper we report the values obtained from studies carried out concurrently on Africans represented mainly by Nigerians, and Caucasians resident in the cosmopolitan town of Port Harcourt. This town has a considerable population of Caucasian workers especially in the numerous oil industries located in it. This investigation was conducted to obtain more data necessary for establishing the real haematological reference values for Africans. Such information is essential for the better understanding of clinical conditions in which some of these haematological indices may be important in the management of patients.

Materials and Methods

Blood samples were collected over a 9 month period at the University of Port Harcourt Teaching Hospital and at the several Industrial Clinics at Port Harcourt from subjects who attended clinics for routine checks or for medical examinations before proceeding for overseas vacation. All subjects were healthy and symptom-free for at least four weeks before the study, and all were completely normal on examination. Women who were menstruating at the time or who just finished it (less than a week) were excluded from the studies. 512 Africans and 196 Caucasians aged 16 to 45 years were included in our study. Venous blood (about 2.5 ml) was collected from each subject into a sequestrene (EDTA) bottle with a plastic syringe and shaken gently to mix with the anticoagulant. Haemoglobin (Hb) concentration and total white blood cell (WBC) counts were estimated on Coultronics Model S7 Coulter Counter within 5 hours of collection of the specimen. White cell differential counts were done using Coultronics C4 cells standard.

The examined Caucasians were predominantly Europeans. Asians, Americans and other whites, however, were also included. Caucasians who had not lived in Port Harcourt for at least a year were excluded from the studies.

The mean of the values of each group were calculated and the \pm S. E. values indicated were appropriate. The significance of the results were calculated using Students' *t* test.

Results

Table 1 shows the mean haemoglobin values and ranges for both Africans and Caucasians. A significantly lower mean value ($P < 0.001$) and range was observed for female Africans. The values for male Africans are not statistically different from those of Caucasians.

A histogram of the percentage distribution of the female subjects to various ranges of the haemoglobin values can be seen in Fig. 1. This figure indicates that

Table 1
Haemoglobin values (gm l)

Subjects	Mean	Range
Africans (male)	13.6 \pm 0.9	12.9–16.0
Caucasians (male)	14.1 \pm 1.1	13.8–17.0
Africans (females)	12.3 \pm 0.7	10.5–14.8
Caucasians (females)	13.7 \pm 0.8	12.4–16.6

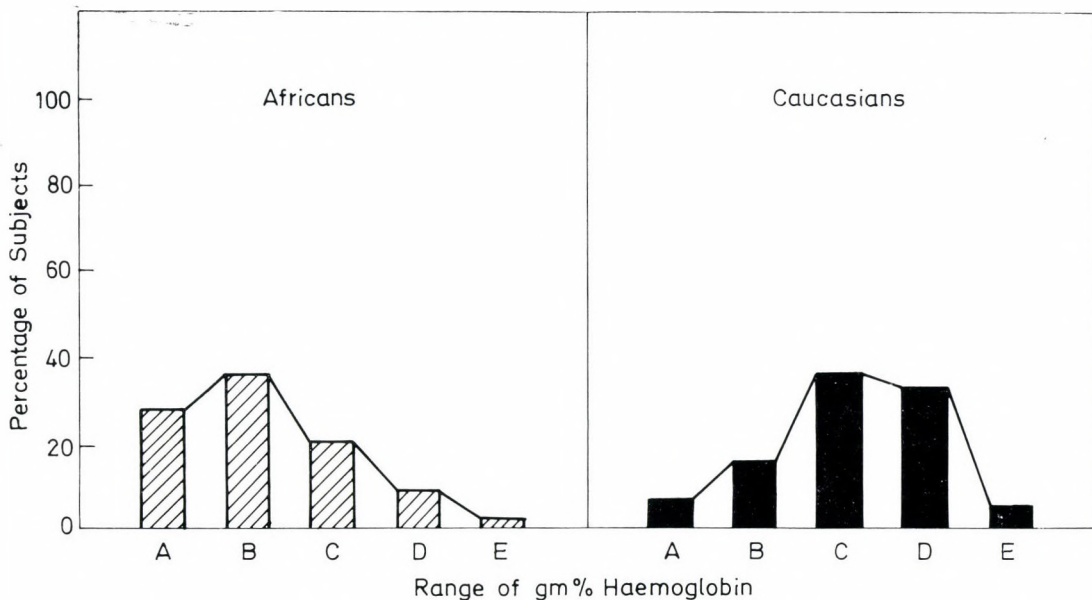


Fig. 1. Histogram of percentage of subjects against the various Hb ranges. Range of gm/l haemoglobin: A — 10.5–11.5; B — 11.6–12.5; C — 12.6–13.5; D — 13.6–14.5; E — 14.6 and above

values for many African females fall mainly in the 10.5–11.5 gm/l (29%) and the 11.6–12.4 gm/l (37%) ranges. On the other hand, that of Caucasian females fall mainly in the 12.6–13.5 gm/l (37%) and 13.6–14.5 gm/l (34%) ranges.

Table 2 shows the total WBC and differential counts for both Africans and

Table 2

Total WBC and differentials for Africans and Caucasians

WBC for Africans:	Mean total: $6.1 \pm 1.0 \times 10^9/l$	
	Range: $2.6 - 9.4 \times 10^9/l$	
WBC for Caucasians:	Mean total: $6.6 \pm 1.1 \times 10^9/l$	
	Range: $3.4 - 9.5 \times 10^9/l$	
	Mean (%)	
Differentials	Africans	Caucasians
Neutrophil	39.5 ± 5.0	54.0 ± 7.1
Lymphocyte	48.8 ± 6.3	35.5 ± 4.2
Eosinophil	9.2 ± 1.4	4.1 ± 0.5
Monocyte	2.0 ± 0.3	5.4 ± 0.7
Basophil	Less than 1%	1.0 ± 0.1

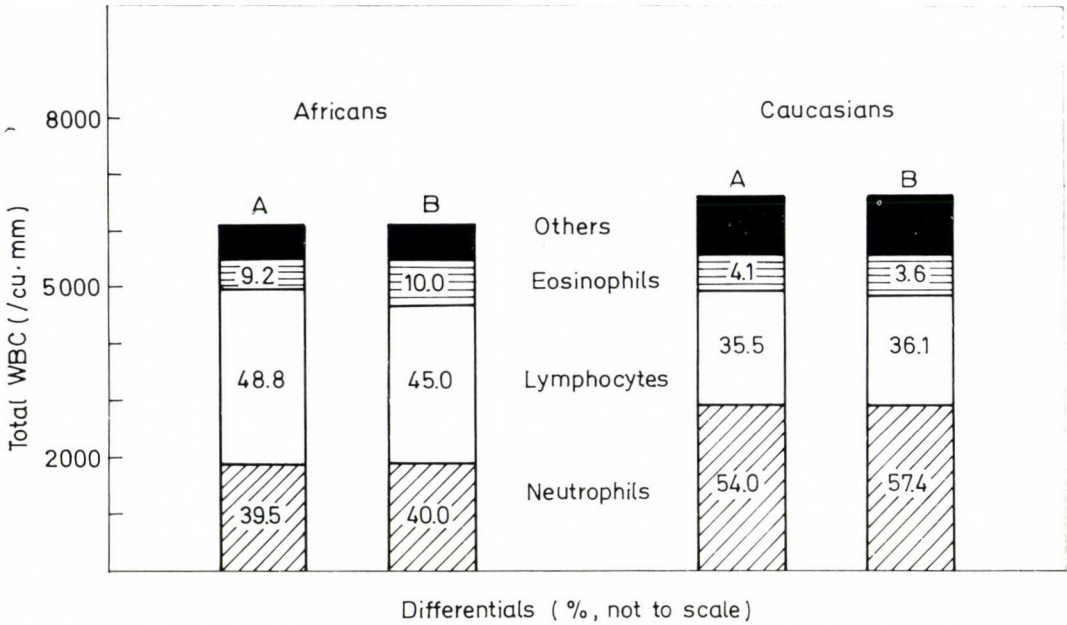


Fig. 2. Comparative histogram of total WBC and differentials obtained from this study against mean of values obtained from other studies: A: values from this study; B: mean values from other studies

Caucasians. The difference in the mean total WBC counts and range for the two groups is not statistically significant at the 5% level. There are, however, differences (statistically significant at the $P < 0.05$ level) in the differential counts of the two groups. The table indicates a predominance of lymphocytes in Africans and a predominance of neutrophils in Caucasians. The eosinophil level in Africans reaches a value that would usually be regarded in haematological literature as "eosinophilia". A histogram (Fig. 2) of the mean total WBC and differentials for both groups of subjects as obtained from this study is compared to a working set of "normal values" obtained from a review of similar studies by previous authors [9]. Our study is in agreement with the reported predominance of neutrophils in Caucasians and of lymphocytes in Africans; further on the high eosinophil count for Africans.

Discussion

Results from this study show marked differences in the haemoglobin levels of Africans and Caucasians, the Caucasians having higher mean levels. This difference in the haemoglobin level is statistically significant only in the female subjects. The mean Hb values and ranges of African females were much lower

than those reported in literature. These frequently observed low values were often associated with anaemia. The majority of the studied subjects (66%) fall within these lower ranges. These subjects, however, did not look anaemic and had no signs or symptoms indicating anaemia. Therefore their Hb values were well entitled to be considered "normal". An implication of this condition may become obvious during surgery when the anaesthetist insists that the Hb level of the patient be brought up to a certain level before the patient is anaesthetized. An anaesthetist would normally be reluctant to anaesthetize a patient with Hb value lower than 11 gm/l. This study has shown that a large proportion of African female subjects (29%) have Hb values ranging from 10.5–11.5 gm/l. In fact, of these 29%, 50 subjects, have been monitored several times, over a 4 month period and found to have Hb values consistently less than 11 gm/l (result not included). It is interesting to note that in this same environment, the Hb values found in Caucasian females are similar to values reported in literature.

The total WBC counts for Africans differ only slightly from those of Caucasians in this study. In spite of the fact that white blood cell counts are subject to certain physiological variations including pregnancy and menstrual cycle, this parameter has such a wide range of normality that we do not consider this difference significant. Also, no sex differences were apparent. The mean values and range obtained for Africans in this study are higher than a recently suggested "working set of normal values" for Africans (mean $5.5 \times 10^9/l$; range $2-9 \times 10^9/l$) [9].

The differential WBC counts obtained in our study are consistent with the well documented differences between the Africans and Caucasians [2, 5, 9] especially regarding the predominance of lymphocytes in Africans in contrast to neutrophils which predominate in Caucasians. However, the neutrophil value for Caucasians in this study is 54% which is nearer to the 52% suggested by Ezeilo [9] than the 60–70% reported in some papers [9]. In this study like in that of Ezeilo, over 300 samples were examined and because of the wide range of normality of WBC counts a sample size of at least 250 was calculated necessary for obtaining reproducible results. Apparently racial factors rather than sex differences were responsible for the differences observed in this parameter. Inter and/or intra sex differences were not obvious.

A higher eosinophil count in Africans is observed. However, many factors – including environmental ones – can cause a rise in the eosinophil level. Our results are significant because they are based on studies of two groups living in the same tropical environment. Whatever the cause of high eosinophil count, it is obvious from this and earlier studies that a definite diagnosis of "eosinophilia" for an eosinophil count up to 10% in an African could be misleading. This is because the level of this white cell in Africans is consistently 9% (see review) [9]. There have been suggestions [10] as to the role of eosinophils in health and disease. It is noteworthy that the healthy Caucasians examined in this study, living in the tropics, with greater exposure to parasitic helminth infections did not necessarily acquire eosinophil levels higher than that of their kins not living in the

tropics. It is also noteworthy that sex differences were not observed for eosinophil counts, the counts for females being similar to those of males of the two groups. The reason for the lower incidence of eosinophilia among Caucasian residents could be hereditary or may be due to a lower incidence of infestation. This, however, cannot be established from the available data. The high eosinophil counts in Africans, which may be due to frequent parasitic helminth infestation, could result in disturbances of iron absorption. In spite of the no-sex-differences observed, the low "anaemic" haemoglobin values found in the female Africans may be a consequence of iron deficiency. Females are, of course, more susceptible to iron deficiency anaemia. It is noteworthy that parity did not influence the distribution of the subjects to various Hb ranges in our study.

Data from comparative studies of haematological values in Africans and Caucasians living in the same African locality is scanty. Previous comparisons of these values have been based on the values established in the African environment against those reported in haematological literature [2, 6, 7]. The comparisons by Ezeilo [5] of these values in Africans, Europeans and Asians presumably in the same environment were performed on cord blood. This study compared values for the adult Africans and Caucasians living in the same environment.

Although there are genetic and cultural differences between Africans and Caucasians, differences in their haematological parameters due to dietary and environmental conditions as postulated by some investigators [9] are reduced to the minimum by the exclusion from our study of those Caucasians who had not lived in Nigeria for at least a year. The results obtained in this study showing differences in some of these parameters for the two groups of people are more direct and therefore correspond better to the real differences of haematological indices. We suggest that clinicians in Africa should recognize the differences and contribute to the work of compiling the reliable "African haematological reference values" to be used in the future instead of the reference values obtained from the investigation of Caucasian populations.

Acknowledgements

We thank our colleagues especially the Clinical Pathology Technologists for technical assistance, Dr. K. Chrostowski for numerous discussions on the presentation of the data. We also wish to thank the management of Shell (PD) Industrial Clinic, Michelin Tyres Industrial Clinic and Agip Oil Company for allowing us to have their employees participate in the study.

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Abstracts

Effects of inherited membrane abnormalities on the viscoelastic properties of erythrocyte membrane. R. E. Waugh (Department of Biophysics, University of Rochester Medical Center, Rochester, N. Y., 14642, USA). *Biophys. J.* 51, 363–369 (1987).

Several workers have identified molecular abnormalities associated with inherited blood disorders. This work examines how these alterations in molecular structure affect the viscoelastic properties of the red blood cell membrane. Changes in the membrane shear modulus, the membrane viscosity, and the apparent membrane bending stiffness were observed in cells of eight patients having a variety of disorders: Two had reductions in the number of high-affinity ankyrin binding sites, two had abnormalities associated with the protein band 4.1, and six were known to be deficient in spectrin. The data suggest that the membrane shear modulus is proportional to the density of spectrin on the membrane and support the view that spectrin is primarily responsible for membrane shear elasticity. Although membranes having abnormalities associated with the function of ankyrin or band 4.1 exhibited reduced elasticity, the degree of mechanical disfunction was quantitatively inconsistent with the extent of the molecular abnormality. This indicates that these skeletal components do not play a primary role in determining membrane shear elasticity. The membrane viscosity was reduced in seven of the eight patients studied. The reduction in viscosity was usually greater than the reduction in shear modulus, but the degree of reduction in viscosity was variable and did not correlate well with the degree of molecular abnormality.

B. Sarkadi

The effect of denbufylline on the viscosity of rat whole blood and on the deformability (filterability) of rat blood cell suspensions. J. J. Jukna, C. D. Nicholson (Beecham-Wülfling Research Laboratories, D-3212 Gronau, Leine, FRG). *Naunyn-Schmiedeberg's Arch. Pharmacol.* 335, 445–448 (1987).

The novel alkylxanthine, denbufylline [1,3-di-n-butyl-7-(2-oxopropyl)-xanthine] has been examined, *in vitro*, for effects on the viscosity of rat whole blood and on the filterability of rat blood cell suspensions. For comparison, pentoxifylline was also examined for rheological activity. Denbufylline reduced the viscosity of whole blood at all shear rates utilised, up to 128.5 s^{-1} . The effect was, however, more pronounced at a low (0.7 s^{-1}) than at a high (94.5 s^{-1}) shear rate indicating that the compound reduces blood cell deformability. Denbufylline also increased the filterability of blood cell suspensions. This is further evidence that the compound increases the deformability of blood cells. Denbufylline elevated the filterability of both pure erythrocyte and mixed erythrocyte/leucocyte suspensions, the effect being greatest with the latter. This suggests that denbufylline may influence the deformability of both red and white blood cells. However, the effect on white blood cells, under the experimental conditions employed, is apparently more marked. Pentoxifylline also reduced the viscosity of rat whole blood and increased the filterability of rat blood cell suspensions. However, denbufylline was 10–100-fold more potent in these tests than pentoxifylline.

B. Sarkadi

Difference between human red blood cell $\text{Na}^+ - \text{Li}^+$ countertransport and renal $\text{Na}^+ - \text{H}^+$ exchange. A. M. Kahn (Department of Medicine, University of Texas Medical School, Houston, Texas 77025, USA). *Hypertension* 9, 7-12 (1987).

Several laboratories have reported that the activities of sodium-lithium countertransport are increased in red blood cells from patients with essential hypertension. Based on the many similarities between this transport system and the renal sodium-proton exchanger, a hypothesis has been put forth in the literature that increased red blood cell sodium-lithium countertransport activity may be a marker for increased sodium-proton exchange activity in the renal proximal tubule. The present studies were designed to test the hypothesis that sodium-lithium countertransport in red blood cells from humans or rabbits is mediated by the same transport mechanism that mediates sodium-proton exchange in the renal brush border from those

species. Similar to what has been reported for the rabbit, the present studies show that an amiloride-sensitive sodium-proton exchanger is present in human renal brush border vesicles. However, $\text{Na}^+ - \text{Li}^+$ countertransport in human and rabbit red blood cells, assayed under several different conditions, was not inhibited by amiloride. In agreement with what has been reported for humans, the present studies show that extracellular proton-stimulated sodium efflux is inhibited by amiloride in rabbit red blood cells. These data demonstrate a difference (amiloride sensitivity) between the red blood cell sodium-lithium countertransporter and the renal brush border sodium-proton exchanger in humans and rabbits. These experiments detract from the hypothesis that increased red blood cell sodium-lithium countertransport activity in patients with essential hypertension is a marker for increased sodium-proton exchange activity in the renal brush border.

B. Sarkadi

Book Review

Joseph G. Sinkovics: Medical Oncology. An Advanced Course. A Self-Assessment Guide for Subspecialty Board Examinations and Practice. Second Edition, Revised and Expanded, Marcel Dekker Inc., New York and Basel, Two volumes, 2062 pages.

J. G. Sinkovics is Director of the Community Cancer Center of Saint Joseph's Hospital and holds a clinical professionship in medicine at the University of South Florida College of Medicine in Tampa, Florida. His recent publication entitled *Medical Oncology* is not a textbook for leisure or causal reading. The main purpose of this book is to give help for residents in preparing for subspecialty board examination in medical oncology. The other, not less important goal of the book is to be a guide for all the physicians treating tumorous patients.

The publication introducing the author's comprehensive experience perfectly corresponds to the purpose. All the field of oncology is presented and various types of malignomas are discussed in detail, but according to their importance. After the favourable reception of the first edition, the recent second edition, published in 1986, is completely updated and expanded to two volumes reflecting the enormous growth of clinical oncology. The work represents an unparalleled accomplishment in gathering together the hard-core advances from the vast amount of recent literature on malignant diseases.

Since the book is intended for physicians, the author does not deal with the determination of individual forms of diseases. These definitions can be obtained from other textbooks. Instead, the up-to-date information and data on pathology, etiology,

immunology of malignomas are discussed, the recent developments in their diagnosis and treatment are presented. On the basis of several thousand publications, a great amount of information is collected and presented in a concise form.

The book is divided into six main parts which are composed of several chapters. The individual diseases are discussed concerning their etiology, epidemiology, histopathology, diagnosis and staging, treatment and occasionally the supportive care.

In the first part the author deals with haematologic oncology; he introduces the changed concepts of the classification and therapy of lymphomas. The new achievements of the leukaemia and AIDS research are also presented, including information on the role of oncogens in human tumors.

The second part deals with tumors originated from mesenchima, particularly with the soft tissue- and bone tumors. In the third part the neuroectodermal and neurogenic tumors are discussed. Classification of the pigment-cell neoplasms and APUD-omas are also included in this chapter, indicating the author's modern concepts.

The carcinomas are described in part four. Obviously, the largest scope is devoted to this most frequent and most significant group of malignomas. Their incidence and their etiology are consistently discussed in connection with the epithelial tumors of different organs. Detailed information is given on the most important histological features, clinical signs, staging, about efficient diagnostic and therapeutic procedures.

The fifth part is a summary on the principles and practice of modern cancer therapy. Particular emphasis is laid on the presentation of the internationally accepted

and successfully used new protocols in the treatment of malignant melanoma, breast-, lung cancers, gastrointestinal tumors and gynecological malignancies. Side effects, infectious complications and their treatment facilities are also discussed in detail.

Chapter six is an especially useful part of the book. According to the purpose of publication it is devoted to residents preparing to take board examination. This part contains self-assessing tests. A key to the questions is included, even giving references in some instances.

The book is well structured and balanced. Its style is concise, for the sake of brevity — according to the author's words — it is almost telegraphic. In the presentation,

attached to the description of each individual disease, clear-cut tables can be found summarizing the most important characteristics. This presentation assures a good orientation and the book can easily be used in the every-day practice.

This publication containing an enormous amount of information proves the author's great experience in oncology. The book is an excellent manual in training for examinations, but it is also a suitable work for practitioners, giving a versatile review about various types of malignancies. It can also be a useful reference-book for theoretical experts.

K. Lapis and K. Simon

Announcement

GUIDELINES FOR MANUFACTURERS MANUALS FOR USE IN DEVELOPING COUNTRIES

The International Committee for Standardization in Haematology has recommended that the following information should be provided by the manufacturers of instruments intended for customers in areas where service facilities and reagent supplies may be limited.

1. Ideally the manual should be provided in the language of the country and the translation checked by a native speaker; otherwise it should be available in an international language which is most commonly used in that country. It should be written at a level which is understandable to a technical worker who has had the equivalent of five years of high school education.

2. The manual should have 8 sections:

- (a) Description of instrument and its component parts
- (b) Unpacking and setting up for use
- (c) Method of operation including daily maintenance by junior staff
- (d) Method of calibration and quality control
- (e) Service maintenance by senior staff
- (f) Trouble shooting
- (g) Description of ancillary materials (reagents etc.) used in the analytic procedure
- (h) Performance characteristics, and limitations of the instrument.

3. The description of the instrument and its component parts should be amply and clearly illustrated so that each part can be readily identified. The principles of the function of the instrument should be described.

4. In unpacking, if there is more than one package, each package should be numbered, and the instruction should state how to loosen/unwrap the package. Setting-up instruction should include clear description of essential ambient location, power requirement – e.g. what type of plug and how to connect it. It should be remembered that most medical equipment failures in developing countries are due to simple faults such as a blown fuse, a defective electrical connection, or simply the inability of the operator to install the equipment properly.

5. Method of operation should be set out in numbered order. It is desirable to have, in addition, a summary of these numbered instructions on a separated plastic covered card and/or a wall chart.

6. There should be similar clear instructions in numbered order for

(a) Cleaning at end of each day

(b) Instrument shut-down at end of each day

(c) Any special precautions if instrument is not going to be used for a period of time

(d) Avoiding contamination, and dealing with a spill of specimen and/or reagent.

7. There should be clear instructions for the regular maintenance procedures, listed in the correct order of procedure and indicating the time intervals between the maintenance schedules. The degrees of skill required for the different levels of maintenance should be indicated.

8. Adequate descriptions should be given for identifying likely cause of problems with well illustrated instructions on their correction in step by step detail. A circuit diagram should be provided. It might be desirable to advise how a temporary repair can be performed until the next opportunity for a visit by the manufacturer's Service Engineer.

9. The composition of all reagents used in the system should be given with full details of their preparation. The possible use of locally available alternatives to the manufacturer's disposable items should also be indicated. When a non-disposable container is used in place of the disposable one, or a disposable one is to be reused, instructions should be given on how to clean and prepare it for use.

Printed in Hungary

Akadémiai Kiadó és Nyomda, Budapest

INSTRUCTIONS TO CONTRIBUTORS

HAEMATOLOGIA is designed for the publication of original papers, preliminary reports, and reviews which contribute to the advancement in all fields related to haematology and blood transfusion. Manuscripts should be submitted in correct English and are accepted on the condition that they have not been published or accepted for publication elsewhere. Case reports, technical notes, and Letters to the Editor are also welcomed and will be published if appropriate.

Manuscripts should be sent to the Editor-in-Chief:

Prof. Susan R. Hollán
National Institute of Haematology and Blood Transfusion
Daróczi út 24
H-1113 Budapest, Hungary

Three copies of the manuscript should be submitted. They should be typed double-spaced on one side of a good quality paper with proper margins. The first page of the manuscript should contain the following information: (1) title of the paper; (2) authors' names; (3) name of institution in which the work has been carried out; (4) name and full postal address of the author to whom communications regarding the manuscript should be directed; (5) a short title not to exceed 40 characters (including space) to be used as a running head. The second page should contain an **abstract** of 50–100 words, summarizing the reasons for the study, the methods used, the results, and the major conclusions. This page should also contain 4–8 **keywords** placed in alphabetical order. Original papers should not exceed 15 printed pages including tables, figures, and references. Case reports should not be more than four, technical notes and Letters to the Editor not more than two printed pages in length. In the manuscripts the approximate location of tables and figures should be indicated in the margin. The manuscript of original papers should be divided into summary, introduction, materials and methods, results, discussion, acknowledgements and references. Review articles should also be appropriately divided. SI units should be used in the manuscript, except that, for the time being, litre (l) may be used as a unit of volume.

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✓ 315 871

VI. 21
1988

VOLUME 21 • NUMBER 2 • 1988

HU ISSN 0017-6559

HAEMATOLOGIA

International Quarterly of Haematology

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HAEMATOLOGIA

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Daróczy út 24.
H-1113 Budapest
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Copy Editor:
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DM 312,— incl. surface mail
DM 330,— incl. air mail

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VOLUME 21 · NUMBER 2

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

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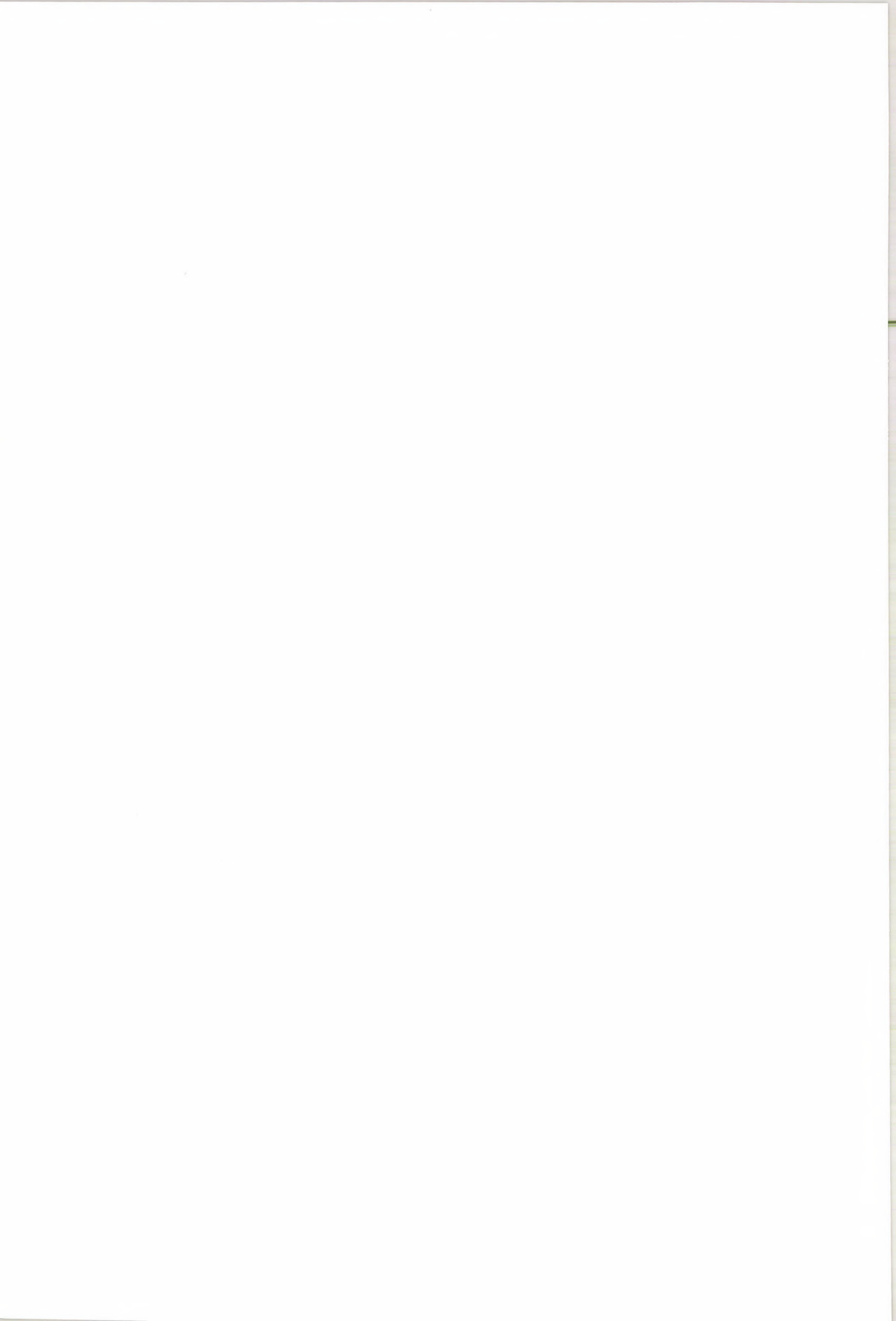
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Serodiagnostic Profiles of HIV and HIV Pathogenesis In Vivo

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(Received 16 April 1987; accepted 23 April, 1987)

Different stages of HIV infection are marked by expression of HIV genes, production of HIV antibodies, formation of antigen/antibody complexes and clearance of such complexes. Transient HIV antigenemia appearing generally 6–8 weeks prior to HIV antibody (HIV-Ab) seroconversion and lasting 3–4 months is generally seen in acute infection. If IgG antibodies to both envelope and core protein persist in the absence of HIV-Ag the short-term prognosis is relatively good. However, HIV-Ag seroconversion may appear at any time after HIV-Ab seroconversion. Progression to AIDS is strongly associated with declining or absent levels of IgG antibodies to p24. Titers of antibodies to HIV p24 below 64 are strongly associated with the presence of HIV antigen and a poor clinical outcome.

HIV antigen may be less efficiently detected with the present assays in sera from regions where the prototype strains of HIV (HTLV-III and LAV) are less prevalent, like Central Africa. Levels of HIV-Ag in serum, and possibly in CSF, can be decreased by nucleoside analogues, like AZT. This indicates HIV-Ag and possibly antibody to HIV core protein p24 as suitable markers for selecting individuals for antiviral therapy as well as monitoring the efficacy of such therapy.

Keywords: HIV, serodiagnosis, HIV core antibody, HIV core antigen, prognostic markers

Introduction

Human immunodeficiency virus (HIV) is the cause of AIDS as well as severe neurological diseases [1–4]. HIV infection may also result in progressive encephalopathy (PE) even without severe immunodeficiency in both children and adults [5, 6]. Brain tissue of HIV infected individuals has transmitted HIV to chimpanzees [7], and HIV antigens and particles have been demonstrated in brain [8] as well as other tissues of AIDS patients. In vitro uninfected CD4+ cells fuse with cells expressing HIV glycoproteins on their membrane forming multinucleated giant cells [9, 10].

Synthesis of HIV envelope and core proteins is activated in vitro by the trans-activator protein tat3 [11]. Tat3 activity is crucial for replication of HIV. In vivo clinical and immunobiological abnormalities develop in a large proportion of long-term HIV antibody seropositive persons [12].

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Recently we have been able to show that serodiagnostic profiles in serum as well as cerebrospinal fluid are strongly associated not only with the present clinical status of the patient but may also be helpful in predicting the clinical outcome of asymptomatic HIV infected individuals [15]. These advances in diagnostics will help us to select individuals [13–19] for antiviral therapy as well as to monitor the efficacy of such therapy. In this paper we will review the several markers that have appeared useful in studying the pathogenesis of HIV *in vivo*.

Materials and Methods

Subjects

Sera originated from several sources. The first group was comprised of homosexual men from Amsterdam. These men were participants in a prospective study on the prevalence and incidence of HIV infection or patients treated at the internal medicine department of the Academic Medical Center of the University of Amsterdam. The second group consisted of haemophiliacs from the Netherlands seroconverting for HIV antibody and followed for at least three years. The third group comprised of HIV-Ab seropositive intravenous drugusers participating in a prospective study in Amsterdam.

Immunoblot analysis of HIV antibodies

Sucrose gradient purified, heat inactivated LAV/HTLV-III was fractionated by electrophoresis using a 12% polyacrylamide slab gel in the presence of sodium dodecylsulphate. The protein bands on the gel were transferred to a nitrocellulose sheet (0.45 μm Schleicher and Schuell, Dassel, W. Germany). Following transfer, the sheet was rinsed with PBS-Tween 0.05% and incubated for two hours in PBS (pH 7.4.) at room temperature. Next the sheet was rinsed with PBS-Tween, dried and stored at 4 °C until use. When used, the sheet was cut into strips and each strip was pre-incubated for one hour at 37 °C in 2.5 ml PBS-Tween, containing 4% normal goat serum. Subsequently 25 μl of heat inactivated test serum was added to each strip separately and incubation was continued for one hour at 37 °C. Following washing with PBS-Tween, the strips were incubated at room temperature for one hour with biotinylated goat anti-human IgG (Vector, Burlingame, California, USA), or with mouse monoclonal antibodies (Bio-Yeda, Israel) if human IgG subclasses were determined. After another washing the strips were incubated for one hour at room temperature with a preformed complex of avidin and biotinylated horse-radish peroxidase (Amersham, Buckinghamshire, UK) if goat antihuman IgG was used. When mouse monoclonals were used an additional step with biotinylated goat-anti mouse IgG was performed prior to incubation with the avidin-HRP complex. Following washing, colour was developed using 4-chloro-1-naphthol as substrate.

Proteins p18, p24 and p55 were considered to be encoded by the gag-gene and proteins gp41, and gp120 by the env-gene.

Proteins p65/53 and p33 were considered to be encoded by pol-gene.

HIV antigen detection

Solid phase enzyme immunoassay (Abbott Laboratories, North Chicago, Ill., USA) was used for assay of serum samples for HIV antigen. Two-hundred microliters of sample were incubated overnight at room temperature with a bead coated with human antibody to HTLV-III. Beads were washed with distilled water; rabbit IgG antibody to HTLV-III added and incubated for 4 hours at 40 °C. Beads were washed as before, then incubated for 2 hours at 40 °C with horseradish peroxidase-conjugated goat antibody to rabbit IgG. After a final wash, the beads were transferred to tubes and 0-phenylenediamine was added. After 30 min at room temperature in the dark, 1 ml of 1 N H₂SO₄ was added to each tube. The A₄₉₀ was read with a Quantum Dual Wavelength spectrophotometer (Abbott, North Chicago). A sample was considered positive if its OD was ≥ 0.050 plus the mean of five replicates of normal human plasma. The assay is most sensitive for the core antigen of HIV and detected approximately 20–30 pg/ml in these assays.

Antibodies to HIV core and envelope proteins

Antibodies were measured by using commercially available, competitive enzyme immunoassays (Abbott Laboratories), referred to as CIA-RA. Briefly, in the first system, beads coated with recombinant HTLV-III core antigen (containing the entire p24 gag-gene product, as well as portions of the p15 and p18 gag-gene products) were incubated with 50 µl of the serum samples and with 200 µl of horseradish peroxidase-conjugated human antibody to HTLV-III for 16–22 hours at room temperature. Beads were washed with distilled water, and colour was developed as described above for the antigen test. Because this is a competitive enzyme immunoassay, intensity of the colour formed was inversely related to the amount of antibody to the HIV core antigen in the sample. The cutoff point was determined as the sum of the negative control mean (n = 3) and the positive control mean (n = 2) divided by two. The same procedure applied to the antibody to envelope antigen assay; however, beads were coated with recombinant HTLV-III envelope antigen (containing the entire p41 env-gene product, as well as 45 amino acids from the gp120 env-gene product). Labeled antibodies in the core and envelope CIA-RA were prepared from patients' sera with high titers to core or envelope, respectively, on Western blot. Core antibodies were titrated using a standard curve.

Detection of HIV specific immune complexes

Two sets of 400 μ l of each serum sample were incubated overnight at 4 °C with an equal volume of 0.2 M EDTA (pH 7.5) and 200 μ l polyethylene glycol (PEG) 12%.

After centrifugation at 8400g for 15 minutes, the supernatants were tested for HIV-Ag and antibodies to recombinant core and envelope antigens. Subsequently the pellet of one tube was dissolved in PBS, the pellet of the other in 6 M guanidine to disrupt the immune complexes. Samples with buffer only and with purified HIV lysate dissolved in PBS or 6 M guanidine were used as controls. The samples were used to coat beads for 6 hours at 40 °C. The coating solution was then washed away and beads were incubated with horseradish peroxidase-labeled human anti-HTLV-III for 2 hours at 40 °C. Beads were again washed and colour was developed by using OPD as substrate similarly to the HIV-Ag assay.

Results*HIV antigen in serum in acute HIV infection*

Transient HIV antigenemia has been observed around HIV-Ab seroconversion in 19% of our series of acutely infected homosexual males and in 13% of HIV antibody seroconverted haemophiliacs (Table 1). HIV-Ag was observed in samples taken three months before the first HIV-Ab positive sample.

In all of these cases antibodies to both the HIV core and HIV envelope proteins appeared.

The short-term prognosis of this group was indistinguishable from the majority of HIV antibody seroconverters who never showed HIV-Ag in any serum sample and remained asymptomatic during the first two years of follow-up. 14% of the homosexual men seroconverting for HIV-Ab became HIV antigenemic approximately at or may be even before HIV antibody seroconversions, and

Table 1
Occurrence and persistence of HIV antigen in acute HIV infections

	HIV antigen status	Number (%)
1. <i>Homosexual men</i>	Negative	36/58 (62%)
	Transient	11/58 (19%)
	Persistent	8/58 (14%)
2. <i>Haemophiliacs</i>	Negative	14/16 (87%)
	Transient	2/16 (13%)
	Persistent	0/16 (—)

remained so during the follow-up period. This group had a poor short-term clinical outcome; 50% of these men developed ARC or AIDS within the first year of follow-up. Antibodies to HIV env proteins became readily detectable in contrast to antibodies to HIV core proteins which had a low titer or were absent.

Figure 1 summarizes the serodiagnostic profiles observed during acute HIV infection. Profile I, showing transient HIV-Ag and IgM antibodies to HIV core and persistence of IgG antibodies to HIV-core and envelope proteins with occasionally circulating HIV specific immune complexes, is the most frequent and has the best prognosis for the time being. Profile II, showing persistence of HIV-Ag in both free as well as complexed configuration and the occurrence and persistence of IgG antibodies to envelope proteins, is far less frequent, and is associated with a grave clinical and immunological outcome.

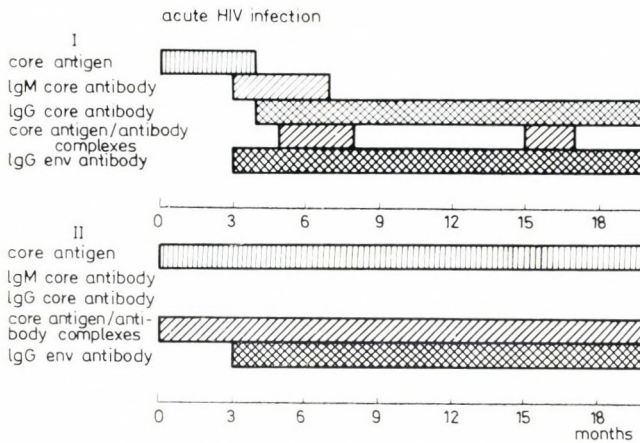


Fig. 1. Serodiagnostic profiles of acute HIV infection

“Flu-like” syndrome may occur in association with both profiles in the early antigenemic phase, prior to HIV-Ab seroconversion. HIV may pass the blood-brain barrier shortly after entrance into the body resulting in transient expression of HIV in the cerebrospinal fluid followed by intra-blood-brain-barrier synthesis of HIV antibody. Symptoms of acute meningitis or meningoencephalitis may accompany this phase occurring at, or shortly after HIV-Ab seroconversion. Generally these symptoms disappear within the first month after HIV-Ab seroconversion.

Preferential decline of antibodies to p24 associated with AIDS

Immunoblot analysis of sequential sera of individuals progressing to AIDS showed a decline of IgG recognition of core proteins, especially p24, while IgG recognition of the envelope proteins gp41 and gp120, as well as of the pol-gene

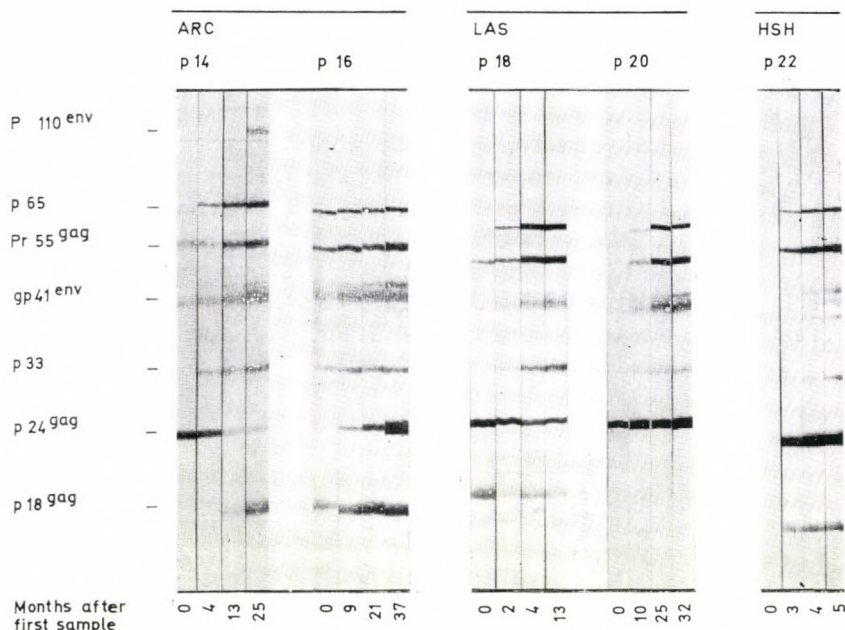


Fig. 2. Immunoblot analysis of sequential sera of patients with ARC, one with LAS and a healthy homosexual man (HSH). Patient 14 progressed to AIDS

products p65/53 and p33 remained constant (Fig. 2). This loss of IgG recognition of p24 was not seen in patients not progressing beyond ARC.

Relationship of circulating HIV antigen, decline of antibodies to HIV core proteins and disease

HIV-Ag was seldom found in free configuration (8%) and somewhat more frequently in complexed form (29%) in sera with detectable levels of antibodies to p24 (Table 2). HIV-Ag was frequently found in free configuration (70%) and less

Table 2

Relationship between HIV antigen and HIV core antibody and the formation of HIV antigen-antibody complexes in serum

Serum antibody status	Number of samples	HIV antigen (%)		
		Serum/supernatant	PEG precipitate	Serum and PEG
Ab to HIV core present	38	1 (3%)	11 (29%)	2 (5%)
Ab to HIV core absent	50	10 (20%)	8 (16%)	25 (50%)

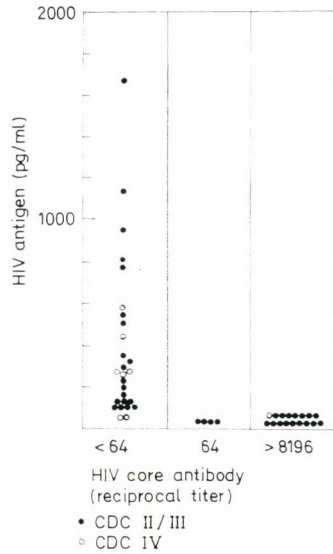


Fig. 3. Relationships between HIV antigen levels, titers of antibodies to HIV core and clinical status

frequently (16%) in complexed form in sera without detectable levels of antibodies to p24.

HIV antigen was not detected in sera with reciprocal anti-p24 titers over 64 (Fig. 3).

Severe clinical disease was strongly associated with low titers of anti-p24 and high levels of HIV-Ag.

Table 3

HIV antigen in serum of HIV antibody positives according to age, geographic origin and stage of infection

Clinical category	Geographic origin	Percentage positive
1. AIDS (CDC IV C&D)		
homosexual men	Europe	86% (30/35)
slim disease	Central Africa	10% (1/10)
2. Constitutional disease (CDC IV A)		
homosexual men	Europe	32% (7/22)
3. Other (CDC II & III)		
homosexual men	Europe	17% (24/145)
haemophiliacs	Europe	0% (0/16)
IV drugusers	Europe	3% (2/60)

HIV antigen in serum according to age, geographic origin and stage of infection

Among adults from Europe or the USA, HIV antigenemia was observed in 86–91% of the cases (Table 3). In contrast HIV antigenemia was seldom present in sera of Central Africans with slim disease.

HIV antigenemia was less frequent among adults with constitutional disease or without symptoms.

Figure 4 illustrates the different serodiagnostic profiles observed in our series and patients. Profile I and III with persistence of IgG antibodies to core and envelope proteins with or without core Ag/Ab complexes were the most frequent. These profiles are strongly associated with subclinical HIV infection. Profile IV represents the transitional state to persistent HIV antigenemia (Profile V). Generally antibodies to p24 disappear. Transition from profiles I–III to IV/V may occur at any time and places the person subsequently at increased risk for AIDS. Profiles IV and V are strongly associated with the progression to AIDS. Profile II (with persistence of antibodies to env proteins, but absent antibody to core) has been noted in several AIDS and ARC patients.

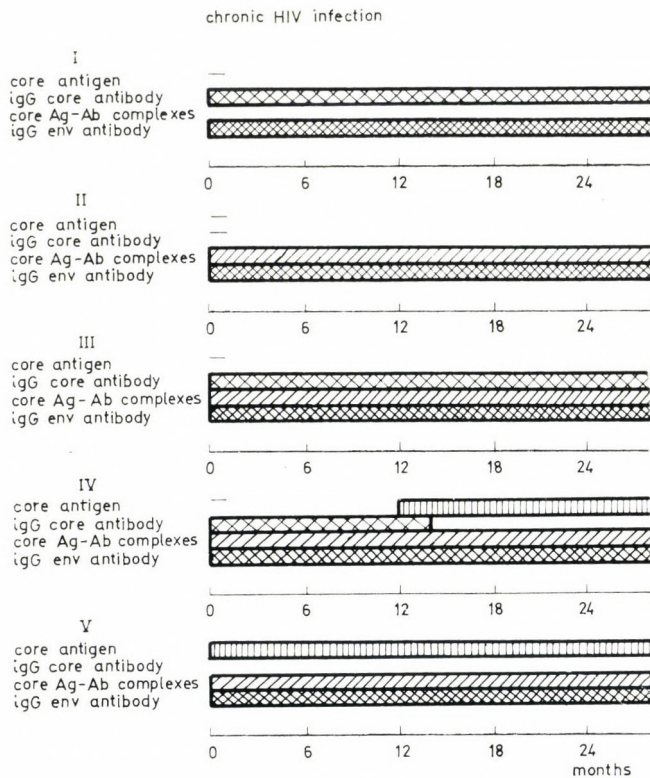


Fig. 4. Serodiagnostic profiles of chronic HIV infection

Discussion

HIV antigen in serum appears to be detectable in pictogram amounts generally six to eight weeks prior to HIV-Ab seroconversion without a window phase with both markers absent [16, 20]. Because of the sensitivity of the current HIV-Ag assays as well as sampling errors approximately 80% of sera taken 3–4 months prior to HIV-Ab seroconversion escape HIV-Ag detection. IgM antibody assays do not appear to solve this problem.

To ascribe a 'flu-like' syndrome firmly to an acute HIV infection paired sera should be tested. In the absence of positive serology for a CMV, EBV, HBV, HAV or Toxoplasma infection the following serological pattern may be considered conclusive:

- 1) HIV-Ag in the absence of anti env/core Ab in the acute phase serum.
- 2) HIV-Ag +/- in the presence of anti env/core Ab in the reconvalescence serum taken 3 months later.

The same philosophy is pertinent to needle stick injuries: HIV-Ab seroconversion is obligatory to avoid problems with the interpretation of the results.

When HIV-Ag persists directly following HIV-Ab seroconversion the clinical prognosis appears to be worse than when HIV-Ag disappears. For screening purposes sensitive assays, including immunodominant epitopes of the viral envelope glycoproteins are mandatory. Antigen testing in low risk groups has still to be evaluated.

For prognosis the most important markers to date are HIV antigen and HIV core antibodies. Two main aspects should be stressed. Both HIV-Ag and HIV core Ab should be quantitated and the consistency of the serodiagnostic profile should be confirmed by testing the individual at least twice in 6 months.

Progression to AIDS is strongly associated with declining or absent levels of antibodies to the HIV core protein p24, as has been subsequently confirmed by Weber et al. [22]. Recently we have been able to show that these antibodies are trapped in immune complexes of core antibody and antigen [23].

The following tentative conclusions can be drawn: Declining or low titers (< 64) of antibodies to p24 in the presence or absence of free circulating HIV antigen bears a grave clinical prognosis when persisting over 6 months.

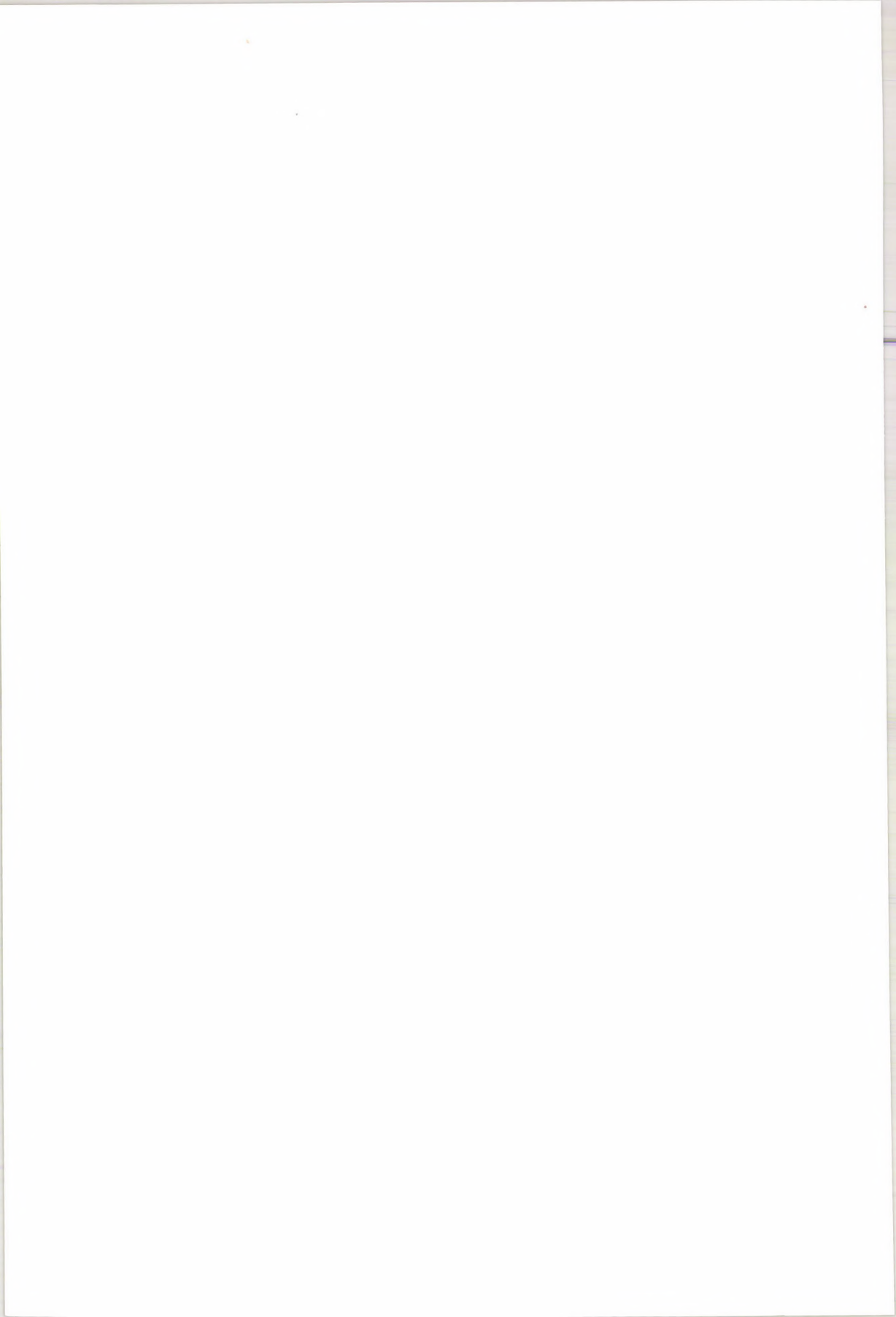
HIV antigen levels decline concomitant with clinical improvement when adequate doses of AZT are administered [24]. No data on changes in titers of antibodies to core proteins are available yet.

This indicates HIV-Ag and possible HIV core Ab as suitable markers for antiviral therapy as well as for monitoring such therapy.

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Prevalence of HIV-Antibodies in Patients with Haemophilia in Hungary

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(Received 7 May, 1987; accepted 15 May, 1987)

Incidence of HIV antibodies have been studied in 617 patients with congenital bleeding disorders. Screening was performed with the Organon ELISA test, repeatedly positive samples were tested with four different confirmatory assays. HIV antibodies were found in 3/356 patients with haemophilia A, 21/114 patients with haemophilia B, 3/123 patients with von Willebrand disease and in 1/24 patients with other types of congenital coagulation disorders. All the 28 seropositive patients were treated with imported coagulation factor, mostly with factor IX concentrates. No patient treated exclusively with nationally produced factor preparations between 1981 and 1985 was found to be anti-HIV positive.

Keywords: HIV, haemophilia, factor IX

Introduction

Haemophiliacs have to be treated with coagulation factor concentrates originating from hundreds to thousands of plasma donors. As a consequence, determination of the prevalence of anti-HIV antibodies in haemophiliacs treated with locally produced preparations can be considered as a good estimate for the prevalence of HIV-infected persons among blood donors and in the total population of a given country [6].

In the present study, anti-HIV antibodies have been measured in 617 patients with congenital bleeding disorders. This includes vast majority of congenital bleeders in our National Registry. Correlation of the prevalence of seropositivity to the type and intensity of the treatment was calculated. Confirmed anti-HIV positivity was found in 28 patients with haemophilia. All the 28 seropositive patients were treated with imported commercial coagulation factor, mostly with factor IX concentrates. No patient treated exclusively with nationally produced factor preparations was found to be anti-HIV positive.

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Materials and Methods

Patients

Blood samples from 356 patients with haemophilia A, 115 patients with haemophilia B, 123 patients with von Willebrand disease, as well as from 23 patients with other congenital coagulation disorders were collected in 25 Haemophilia Care Centers. Sera were separated, heat-treated at 56 °C for 30 min, and stored at -20 °C for a maximum of 4 weeks. After that sera identified with code numbers were transported to the National Institute of Haematology and Blood Transfusion, Budapest for anti-HIV evaluation. After completion of the study, the centers were asked to give the following data: diagnosis, sex, age, use of imported factor concentrate in the last 4 years, type and amount of preparation used as well type and amount of the nationally produced coagulation factor preparations used in 1984. Spouses or regular sexual partners of the anti-HIV positive haemophiliacs were also tested for the presence of HIV antibodies.

Measurement of the anti-HIV antibodies by ELISA technique

Vironostika kit of the ORGANON TEKNIKA (Turnhout, Belgium) was used for the anti-HIV screening. Measurement was performed strictly according to the recommendations of the manufacturer.

Confirmatory investigations

Each repeatedly positive serum sample was tested with four types of confirmatory assays: 1. For revealing false-positivity due to anti-lymphocyte antibodies with a control ELISA plate coated with shed material from uninfected H9 cells (SORIN BIOMEDICA, Saluggia, Italy), 2. with a type 2 (competitive) enzyme immunoassay kit of Wellcome Diagnostics (Temple Hill, England) known to have an outstanding specificity [11], 3. with indirect immunofluorescence (IFA) test using the HIV producer cell line CEM/LAV as antigen (kindly provided by Prof. Chermann, Paris) and as a control, uninfected CEM cells [9], 4. with the Western blot kit of the Du Pont de Nemours firm.

Immunological studies

T cell subsets were determined in 9 confirmed anti-HIV positive children with haemophilia B, in a patient with factor VII deficiency and as control in 8 seronegative children with haemophilia A. T cell subsets were measured in mononuclear cell suspensions isolated from the peripheral blood of patients using indirect immunofluorescence microscopy and monoclonal antibodies from the OKT series (ORTHO, Raritan, USA).

Results

Incidence of anti-HIV positivity among patients with congenital coagulation disorders

Anti-HIV measurements were performed in the sera of 617 patients with haemophilia A, B, von Willebrand disease or other type of congenital coagulation disorders (Table 1). Presence of anti-HIV antibodies were confirmed by all the 4 methods applied in all of the 28 seropositive patients. Repeatedly positive ELISA result was obtained in the sera of an other patient which, however, could not be confirmed. Thus, the confirmation rate (number of confirmed positive samples/repeatedly ELISA positive samples) was 28/29. Most patients (21/28) found to be anti-HIV positive had haemophilia B. Almost one-fifth of the patients with haemophilia B became seropositive, whereas only a minor percentage of the patients with other type of congenital coagulation abnormality tested had HIV antibodies.

Table 1

Incidence of anti-HIV positivity in patients with haemophilia in Hungary

Type of haemophilia	Number of patients tested	Patients with confirmed anti-HIV positivity	
		number	%
Haemophilia A	356	3	0.8
Haemophilia B	114	21	18.3
von Willebrand disease	123	3	2.4
Other	24	1	4.3
Total	617	28	4.5

Only 2 of the 28 HIV antibody positives are females, one of them (who in the meantime, died of gastric cancer) had von Willebrand disease, the other patient, a 15 year old girl has factor VII deficiency. One half of the positives are children and 20/28 are under 20 years of age.

Results of anti-HIV measurements in the regular sexual partners of the seropositive heterosexual haemophiliacs

8 spouses and regular sexual partners of the seropositive adult patients with haemophilia were tested. Two of them were found to have HIV-antibodies.

Study of the association between the treatment with imported coagulation factor concentrate and occurrence of HIV seropositivity

After completion of the study, the haemophilia centers had been asked to give information on the use of imported coagulation factor concentrates in the

Table 2

Incidence of anti-HIV positivity in haemophiliacs treated or not treated with imported coagulation factor concentrate

Type of haemophilia	Patients					
	treated			non-treated		
	with imported factor concentrate in 1981–1985					
	Number of patients tested	Anti-HIV +ve patients		Number of patients tested	Anti-HIV +ve patients	
number		%	number		%	
Haemophilia A	30	4	13.3	326	0	0.0
Haemophilia B	45	20	44.4	69	0	0.0
von Willebrand disease	9	3	33.3	114	0	0.0
Other	1	1	—	23	0	0.0
Total	85	28	32.9	533	0	0.0

patients tested between 1981 and 1985. Data are shown in Table 2. Less than 10 per cent of the patients with haemophilia A were treated with imported concentrates, and HIV antibodies developed only in a small percentage (13%) of these patients. By contrast, two-fifths of the patients with haemophilia B tested were given imported factor concentrates and 20/45 (44%) of them became seropositive. Only a few patients with von Willebrand disease or other congenital coagulation disorders were treated with imported factor preparations. 4/12 of these patients, however, were found to be seropositive. Altogether, 28/85, that is 33 per cent of the imported concentrate-treated patients became anti-HIV positive.

None of the patients treated exclusively with coagulation factor concentrates produced in Hungary were found to have HIV-antibodies, although these patients were exposed, on the average to blood of 62 donors in 1984 and some of them received preparations made from several hundreds of Hungarian blood donors.

Association between the amount of imported factor IX concentrate used and the development of HIV-antibodies

We have got exact data on the use of imported factor IX concentrate in 1981–1985 in the case of 46 patients with haemophilia B (45 patients) and factor VII deficiency (1 patient). 19 of these patients became anti-HIV positive (Group 1) while 27 patients remained seronegative (Group 2). There was a significant difference in the amount of factor IX concentrate administered to the two groups. The patients belonging to Group 1 received more than 6 times as much concentrate as the patients of Group 2 (Table 3).

Table 3

Amounts of imported factor IX concentrate which had been administered in 1981–1985 to haemophiliacs found to be anti-HIV positive and negative in 1986

Patients	Number of patients tested	Amount of imported Factor IX concentrate received/patient	
		mean	± S.D.
Anti-HIV positives*	19	29 130	25 360
Anti-HIV negatives**	27	4 550	6 230

* 18 haemophilia B, 1 factor VII deficiency

** 27 haemophilia B

T cell subsets in HIV seropositive and seronegative children with haemophilia

The absolute number of lymphocytes, as well as the percentage and absolute number of T3+, T4+, and T8+ lymphocytes were determined in the blood of 10 children with haemophilia (9 haemophilia B, 1 factor VII deficiency) found to be HIV seropositive, and as control, in 8 HIV-seronegative children with haemophilia A of the same age as the seropositives (Table 4). In October, 1986 significant difference was found only in the absolute number of T4+ cells. In the group of seropositives this value was about one-half of that of the seronegative children. 3 seropositive patients, but none of the seronegative children, had an absolute T4+ cell number below 400 μ l.

Table 4

T-cell subsets in anti-HIV positive and negative children with haemophilia (October, 1986)

Group	Number of patients tested	Lymphocytes μ l	OKT3 + lymphocytes		OKT4 + lymphocytes		OKT8 + lymphocytes		OKT4+ OKT8+
			%	μ l	%	μ l	%	μ l	
Anti-HIV positives	10	2318 ± 804	54.9 ± 9.7	1280 ± 486	24.7 ± 9.4	576 ± 315	31.9 ± 10.0	738 ± 324	0.85 ± 0.40
Anti-HIV negatives	8	3146 ± 1042	50.9 ± 9.6	1680 ± 575	30.3 ± 10.4	1033 ± 548	27.1 ± 7.8	942 ± 519	1.12 ± 0.26
p	—	0.05 < p < 0.1	n.s.	n.s.	n.s.	< 0.05	n.s.	n.s.	n.s.

n.s. = not significant

Discussion

Near to or more than 50 per cent of the haemophiliacs are anti-HIV positives in West-European countries; and 80–95% in USA (in Australia 56%; in France 48%; in Spain 67,7%) [7]. Very recently similar data were reported from the United Kingdom: 927 haemophiliacs out of the 1264 tested had antibodies to HIV [1].

Since most patients with haemophilia in Hungary are treated with nationally produced coagulation concentrate, it seemed very important to study the prevalence of anti-HIV positivity in Hungarian haemophiliacs. Determinations made in the majority of the registered haemophiliacs indicate that less than 5 per cent of these patients have HIV antibodies. Moreover, none of the 533 haemophiliacs tested who were treated exclusively with nationally produced coagulation factor concentrate or were not treated at all became HIV seropositive, although many patients were exposed to the blood of several hundreds of donors. This finding demonstrates that the vast majority of Hungarian blood donations could be considered safe between 1981 and 1985. This assumption was supported by the initial results of the donor screening. Regular screening of blood donors began in Hungary in February, 1986 and since 1 July a compulsory anti-HIV antibody assay is made from the blood of each donor at each donation. Till 31st December 1986 389000 units were tested. Confirmed positivity was found in 5 donors, all the five were males and admitted retrospectively to be homosexual. Infection of the first full-blown AIDS patient in Hungary (registered in late December, 1986) originated from a blood transfusion during cardiac surgery (coronary bypass) in January, 1985. The patient belonged to no known risk group. Detailed investigation of blood donors revealed one HIV-antibody positive individual, one of the later detected five seropositive homosexual donors.

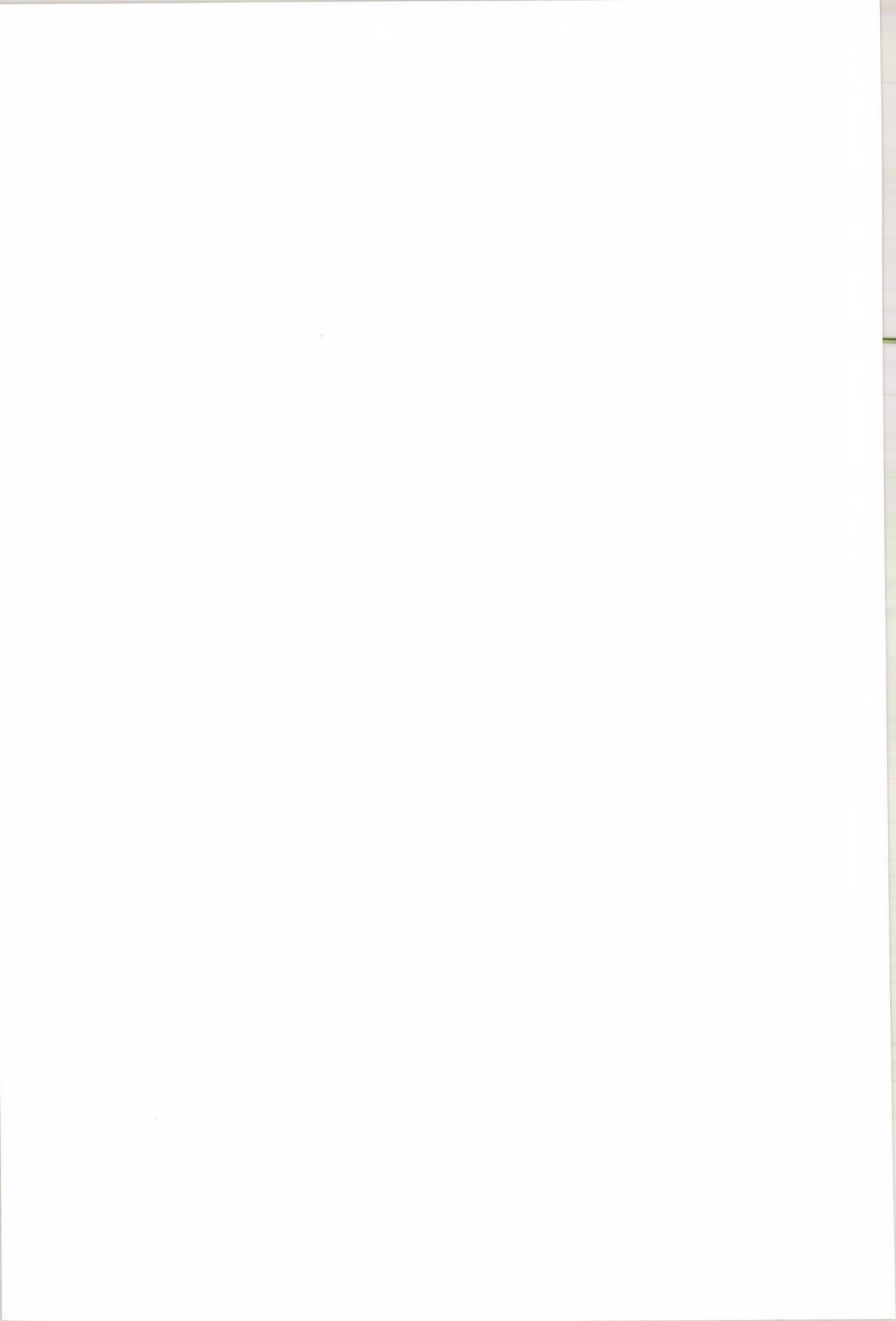
In the USA [5] and UK [1] the rate of seropositivity was considerably higher in patients with haemophilia A than in those with haemophilia B. In contrast to this 20 of the 28 HIV seropositive haemophiliacs in Hungary have haemophilia B. The explanation of this apparently paradoxical finding is simple: while the vast majority of patients with haemophilia A in Hungary have been treated with nationally produced concentrates, due to a temporary shortage of the national PCC (prothrombin complex concentrate) product, a number of patients with haemophilia B especially those treated in a Pediatric Haemophilia Centre received imported commercial factor IX concentrate. Our findings, in agreement with the results of Baselga et al. [3], demonstrate that risk of transmitting HIV infection with factor IX concentrate is similar to that of factor VIII concentrate. When it became clear that these concentrates caused HIV infection, their import was immediately suspended. Seropositive patients were notified and educated on safe-sex practices. Unfortunately, in 2 cases this education was too late: the regular female sexual partners of these patients also became anti-HIV positive. There was a strong positive correlation between the amount of the concentrate used and the development of HIV antibodies: those patients who became

seropositive had received more than 6 times more concentrates than those who remained seronegative. This finding is in line with the results reported by several groups concerning factor VIII [2, 8] and factor IX [3] administration.

We have started a longitudinal immunological and clinical follow-up study in the patients with haemophilia found to be HIV seropositive. Until now none of these patients have developed full-blown AIDS, 2 children who had lymphadenopathy and minor opportunistic infection can be classified in Group III or IVa of HIV infection according to the newest CDC classification system [4]. At the latest immunological evaluation performed in October, 1986, seropositive children had significantly less absolute T4+ cell number than the seronegative haemophiliacs of the same age and 3/10 seropositives had less than 400 T4+ cells/ μ l which is considered to be a critical value [10]. In the future we shall continue the longitudinal study of the HIV antibody positive haemophiliacs using several immunological tests in parallel.

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Equations for the Calculation of Factor VIII Potencies from the Results of Parallel-Line Clotting-Time Assays*

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(Received 30 March, 1987; accepted 29 May, 1987)

An equation was derived: $\ln C_{01} = \ln D_1 - (a_2/a_1)\ln D_2 + (a_2/a_1)\ln C_{02} + 1/a_1(b_1 - b_2)$ which related the potency of a sample (C_{01}) to the potency of a standard (C_{02}) via the dilution factors (D_1 & D_2) that produced common clotting times for the sample and standard. This equation could not be used in practice since C_{01} and b_1 were both unknown. Derivation of a usable formula for calculating sample potencies was found to require the assumption that $b_1 = b_2$. It was found that b is not always a constant in Factor VIII assays and may vary with the nature of the clotting assay mixtures.

Keywords: assay variation, coagulation factor potencies, Factor VIII assays, parallel-line bioassays

Introduction

Early descriptions of clotting factor assays sometimes contained statements saying that linear graphs of clotting-time versus dilution could not be obtained even with logarithmic transformations [1, 2]. However, later improvements led to the use of logarithmic standard curves with the thromboplastin generation test [3, 4] and the activated partial thromboplastin test (APTT) [5], although the original description of the APTT referred to the use of a semilogarithmic graph [6].

Despite these improvements, problems related to variations of slopes [7] and other parameters have continued to plague laboratories that assay clotting factors [8, 9]. Thus it became clear that additional variables may require definition and control before these clotting tests may be considered to be as accurate and precise as possible.

The study to be reported here arose from an attempt to define the mathematical factors that were required for a valid coagulation factor assay when the parallel-line method and logarithmic transformations were employed. Rather than use an approach based on enzyme kinetics, the study was designed to consider the assay from the point of view of the analyst who is presented with clotting-times and dilutions and must derive a potency by comparison with a similar set of data from a standard preparation.

* An abstract describing the results of this study was presented before the 69th Annual Meeting of the Federation of American Societies for Experimental Biology, Anaheim, CA, USA (Fed. Proc. 44: 1845 (1985)).

The results of the current study reinforced some of the assumptions and "rules of thumb" that are employed by those who routinely conduct clotting factor assays. However, it also revealed the requirement for an assumption that has not been previously considered. The results have shown that this is not always a valid assumption, and this condition may invalidate the use of dilution ratios for calculating clotting factor potencies from parallel line assays.

Materials and Methods

Definitions

- a – Slope of a line generated by plotting a transformed clotting-time on the vertical axis versus a transformed dilution factor on the horizontal axis.
- b – Constant introduced into the equation for a straight line. Initially it served a purpose similar to that of an integration constant and was not necessarily equal to the intercept.
- C – Concentration of a clotting factor in units per ml.
- C_0 – Original concentration before starting a dilution series.
- D – Dilution factor. Series of 1 : 4, 1 : 8, 1 : 16, 1 : 32 and 1 : 5, 1 : 10, 6 : 20, 1 : 40 were used after predilution of samples to approximately 1 unit/ml.
- I – The intercept along the vertical axis when $D = 1$ (1 : 1 dilution).
- r – The correlation coefficient obtained from the least squares fit to a straight line. When the data points fall along a perfectly straight line, r equals 1.0.
- T – A clotting-time corresponding to a particular value of D.

Subscripts: Terms related to samples have 1 as a subscript, while a subscript of 2 refers to a standard.

Calculations

Calculations were done with a Texas Instruments TI-59 calculator using built-in linear regression functions [10] for calculating least squares fits to a straight line. The calculator was programmed to accept duplicate clotting-times and single dilution factors. Average clotting-times were calculated, and they and their corresponding dilution factors were transformed as appropriate. The paired data were then entered into the calculator's linear regression subroutine. The parameters for the least squares fit to a straight line were calculated and stored for later retrieval.

Clotting assays

The data for this study were obtained from two laboratories who used the Hyland Clotek and General Diagnostics Coag-A-Mate X2 as clot timers. These laboratories were engaged in the potency testing of factor VIII concentrates via APTT assays. The data were taken from routine test results on a variety of samples in various potency ranges. One of the laboratories used the General Diagnostics Automated APTT reagent, containing micronized silica, and 25 mM CaCl₂; while the second laboratory used a Hyland Diagnostics reagent, containing kaolin, and 30 mM CaCl₂. Samples were prediluted to approximately 1.0 unit/ml. Dilutions were then made and incubated for 3 or 5 min with the activator and phospholipid reagent in factor VIII deficient plasma before CaCl₂ was added to initiate clotting.

Results*Derivation of an equation for calculating concentrations*

In deriving the equation, the following general assumptions were employed.

1. The clotting-time is inversely proportional to the concentration of the coagulation factor, i.e. $T \propto 1/C$.
2. The sample and standard may be considered to be simple dilutions of each other. The result being that dilution series and their associated clotting-times from the sample and standard will generate a pair of parallel lines separated by a distance proportional only to the difference in concentration between the sample and the standard.
3. If two reaction mixtures each contain the same concentration of the coagulation factor, they will produce the same clotting-time, i. e. if $C_1 = C_2$, then $T_1 = T_2$.
4. Within the range of the dilutions used, a straight line may be produced by plotting $\ln T$ on the vertical axis and $\ln D$ on the horizontal axis.

Each of these assumptions may become invalid under certain conditions, but the execution of a valid coagulation factor assay requires at least the first three to be valid. Assumption number 4 will be assumed to be valid for the purposes of the following derivations. It will be seen later that the only critical part of assumption 4 is the use of a logarithm of D .

Assumptions 1 and 4 could be combined since:

$$C = C_0/D$$

and substitution into assumption 1 gave:

$$T \propto D/C_0$$

Assumption 4 then gave:

$$\ln T \propto \ln(D/C_0)$$

or

$$\ln T = a \ln(D/C_0) + b$$

The equation could now be expanded for generating the dilution curve.

A.
$$\ln T = a \ln D - a \ln C_0 + b$$

The intercept (I) was evaluated by setting $D = 1$ (i.e. the sample had not yet entered the dilution series). At this point $\ln D = 0$ and I is related to a, b and C_0 .

Thus:
$$I = b - a \ln C_0 = \ln T(\text{at } D = 1)$$

or

B.
$$b = I + a \ln C_0$$

as antilogs:

C.
$$e^b = e^I C_0^a z T C_0^a \text{ (at } D = 1)$$

If b were a true constant such that $b_1 = b_2$ for a pair of parallel lines in a clotting assay, then from equation B:

D.
$$\ln C_{01} = (a_2/a_1) \ln C_{02} + \frac{I_2 - I_1}{a_1}$$

If the lines were truly parallel so that $a_1 = a_2$ and if the concentrations were given in percentages so that $C_{02} = 100\%$, then equation D would be identical with an equation derived by Margolis et al. [11], if $D = 1$ were taken as the common dilution factor.

Returning to equation A and choosing a common clotting-time for lines from a sample and standard so that $\ln T_1 = \ln T_2$, it follows that:

$$a_1 \ln D_1 - a_1 \ln C_{01} + b_1 = a_2 \ln D_2 - a_2 \ln C_{02} + b_2$$

or

E.
$$\ln C_{01} = \ln D_1 - (a_2/a_1) \ln D_2 + (a_2/a_1) \ln C_{02} + 1/a_1 (b_1 - b_2)$$

Where D_1 and D_2 were the dilutions giving $T_1 = T_2$. Equation E could not be evaluated as b_1 needed to be known before C_{01} could be calculated.

If it were assumed that $b_1 = b_2$, then:

$$\ln C_{01} = \ln D_1 - (a_2/a_1) \ln D_2 + (a_2/a_1) \ln C_{02}$$

or in antilogarithmic form:

F.
$$C_{01} = D_1/D_2 (a_2/a_1) C_{02} (a_2/a_1)$$

Imposing the parallel line assumption so that $a_1 = a_2$, gave:

$$G. \quad C_{01} = C_{02}(D_1/D_2)$$

which was the usual dilution ratio equation for calculating the potency of an unknown from that of a standard.

Evaluation of b

The assumption that b was a constant was crucial for the derivation of a formula for calculating concentrations of factor VIII relative to that of a standard. Therefore the numerical value of b was measured under various conditions to see if b could be established as a constant in factor VIII assays.

An in-house factor VIII control preparation was diluted to produce values of C_0 in the range about 1.0 unit/ml. Dilution curves were then produced for each value of C_0 . The values of a and I were calculated for each line and, along with the corresponding value of C_0 , were entered into equation C. The calculated value of e^b was then plotted against the corresponding value of C_0 . Figure 1 showed that the value of b differed between the two laboratories and varied with the value of C_0 ; even though each laboratory did all of its testing during a single day, using the same reagents and substrate plasma for all assays.

Discussion

Equations

The reader should not conclude that equations D and F will allow one to ignore a lack of parallelism. Equations D and F will permit the calculation of a potency from a pair of intersecting lines, but the validity of the number will be questionable as the lack of parallelism will indicate that the sample and standard are not simple dilutions of each other and that the clotting-times are responding to different controlling factors. Margolis et al. [11] noted that the slope of a dilution curve should be characteristic of the factor being assayed. Therefore if the slopes are not equal the possibility that the clotting-times are being controlled by different limiting factors becomes real.

Equation E was derived without assuming that $a_1 = a_2$ or $b_1 = b_2$. Unfortunately the calculation of C_{01} via equation E required prior knowledge of b_1 . The derivation of equations D and F required the assumption that $b_1 = b_2$, and equation G, an often used formula, required the further assumption that $a_1 = a_2$.

Note that when equation E was derived, the clotting-time ceased to be a factor as the condition that $T_1 = T_2$ eliminated T from the resulting equation. The result was the same whether T or a logarithm of T were used.

The constant, b

Other equations exist for calculating the results of clotting factor assays. Elődi et al. [12] derived a semilogarithmic equation that was employed for studying sources of assay errors. Margolis et al. [11] derived a logarithmic equation that was carried through to a formula for calculating clotting factor concentrations by comparing clotting-times at a common dilution factor. Neither of these groups appear to have included the constant, b .

Hemker et al. [13] approached this derivation from basic enzyme kinetics and generated equations that did not require logarithmic transformations. With these equations a straight line could be obtained when the clotting-time was plotted as the ordinate with the dilution factor as the abscissa. When the dilution factor was set to zero in their equation, the intercept on the ordinate was found to be related to the reciprocal of the enzyme concentration times a constant. As their derivation and the present derivation were based upon different initial assumptions, it is difficult to say if their constant and b are the same. However there are similarities between the terms and these constants may be related.

The derivations provided by Hemker et al. [13] were interesting and informative, as they were based on enzyme kinetic assumptions that were useful for describing the relationship between clotting-enzyme concentrations and clotting-times. However, the present work was based upon the assumptions for parallel line bioassays and the relationship between the present study and theirs was not pursued.

If equation A is taken and b omitted, I , the intercept where $D = 1$, would be negative at values of C_0 greater than 1.0 unit/ml. As $\ln T$ at $D = 1$ equals I and is known to be positive at C_0 values that are greater than 1.0 unit/ml, the existence of b as an additional term in the intercept is inferred.

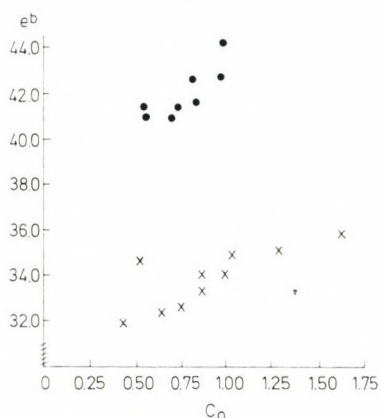


Fig.1. Variation of b as a function of factor VIII concentration (C_0). Equation C (see text) was used to calculate values for b from data obtained from Laboratory A (●) and Laboratory B (X)

From Fig. 1, it appears that b has a non-linear relationship to C_0 , and there are regions where b is fairly constant. These variations could cause problems when a sample is prediluted to several levels. In fact it is possible that the variation of b may contribute to the phenomenon known as the predilution effect. Aronson and Marder [14] described this anomaly, in which the back-calculated potency of a preparation varies with the predilution made before entry into the assay dilution series. Inspection of equation E and Fig. 1 will lead to the conclusion that this situation could arise in a series of assays where b varied with C_0 . From Fig. 1 it appears that the difference between b_1 and b_2 may be minimized by having C_{01} and C_{02} very close to each other. This may be the reason for the laboratory technician's rule that the best assay results are obtained when the sample and standard are very close to each other in potency.

Figure 1 showed that the value of b is dependent upon the composition of the clotting systems (difference between laboratories) and the initial concentration of factor VIII (variation of b with C_0). The failure to eliminate inter-laboratory variations with a monographed assay for factor VIII [9] may have been the result of variations of b among and within the laboratories participating in the study.

If b is not constant or equal to zero, equation B shows that the intercept of an assay line will be dependent upon b as well as C_0 . Thus the distance separating a pair of parallel lines will be dependent upon another factor besides the difference between the concentrations of the sample and standard. The resulting violations of assumptions 2 and 3 would invalidate the use of a parallel line assay system.

It was concluded that the use of semilogarithmic or logarithmic systems led to an equation that could not be evaluated. Derivation of useful equations required the use of assumptions that were demonstrably incorrect under certain conditions. Consequently, the performance of valid coagulation assays was found to require the demonstration that the dilution curves for the samples and the standard were similar enough to minimize variations of the intercept term, b .

Acknowledgements

C. Graf, Y.-C. Hong, G. Jacoby, and R. Vasquez provided the raw data used for the calculation of e^b .

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Lectin-Induced Adhesion of Human Platelets to Glass. Comparison with the Lectin-Induced Aggregation

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(Received 6 April, 1987; accepted 8 June, 1987)

Effects of Phytohaemagglutinin (PHA), Concanavalin A (Con A) and Lens culinaris (Lens) lectins on the adhesion of human platelets to glass surface were studied. All the three lectins tested enhanced platelet adhesion to glass.

Concentration-effect curves showed that maximum effect was reached at about 25–30 $\mu\text{g}/\text{ml}$. In the presence of autologous plasma both spontaneous and lectin-induced adhesion to glass were diminished. The inhibitory effect of KCN, NaN_3 , cytochalasin B and partly of colchicin suggests that lectin-induced platelet adhesion to glass is a process depending on cell metabolism and the state of the cytoskeleton. Only PHA was observed to be a potent inducer of platelet aggregation similar to physiological aggregants. Con A had no effect on platelet aggregation but caused shape change which was blocked by metabolic inhibitors. Neither shape change nor aggregation were induced by Lens up to a concentration of 400 $\mu\text{g}/\text{ml}$. A comparison between the mechanisms of lectin-induced substrate adhesion and lectin-induced aggregation of platelets is described.

Keywords: platelets, lectins, substrate adhesion, glass surface, aggregation

Introduction

Studies on platelet adhesive properties are of interest because of the central role they play in the haemostatic processes and their pathological deviations. When the blood vessel wall is injured, platelets become activated and typical functional alterations [1] can be observed: they begin to adhere to the exposed sub-endothelial connective tissue, which is rapidly followed by aggregation and release of some biologically active substances. In vitro platelets adhere to most surfaces and usually adhesion leads to ADP release. When the investigation is performed in a dynamic system, the formation of platelet aggregates can be observed [2]. These early steps of platelet activation have been a subject of intensive investigation during the past few years.

Plant lectins are a very convenient tool for studying the mechanisms of platelet activation. The effect of their binding to human platelets has been investigated earlier [3, 4, 5, 6, 7]. It has been found that lectins with specificities directed towards mannose and glucose, such as Concanavalin A (Con A), Lens culinaris (Lens) etc., do not induce platelet aggregation, whereas Con A itself can induce a release reaction [3] and some macromolecular events characteristic for platelet activation

[4, 5, 6]. On the other hand, lectins with galactose directed specificity, such as *Ricinus communis*, as well as wheat germ agglutinin (N-acetylglucosamine determinant) and *Phaseolus vulgaris* agglutinin or PHA (N-acetylgalactosamine determinant) were found to be strong inducers of both aggregation and release reaction [3, 7]. There is no clear evidence, however, about the effect of lectins on platelet substrate adhesion, for example to glass or other artificial surfaces.

The aim of the present paper is to investigate the effect of some lectins: Phytohaemagglutinin (PHA), Con A and Lens on human platelet adhesion to glass and to compare it with the lectin-induced aggregation.

Materials and Methods

Materials: The experiments were performed with human venous blood from 7 healthy volunteers who stated that they had had no medication for at least 10 days prior to bleeding. 3.8% sodium citrate in 9 : 1 ratio was used as an anticoagulant. Con A, Lens, colchicin and cytochalasin B were purchased from Sigma; PHA^P was purchased from Difco Lab.; KCN, NaN₃ and formaldehyde were obtained from Merck. All other reagents were commercial preparations of analytical grade.

Isolation of platelets: Citrated blood was centrifuged at 120g for 15 min at room temperature. Platelet-rich plasma (PRP) was transferred to polypropylene tubes and diluted with autologous plasma to 10 cell/ml. Washed platelets were prepared from PRP by centrifugation at 750g for 20 min in the presence of 3 mM EDTA. The cell pellet was gently resuspended in HEPES-buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na₂HPO₄, 1 mM MgCl₂, 5 mM glucose and 10 mM HEPES, pH 7.3), and washed twice more with the same buffer. The final pellet was resuspended to a concentration of 1×10^8 cells/ml in HEPES-buffer without EDTA.

⁵¹Cr labeling of platelets: Platelet suspension in HEPES-buffer was incubated for 30 min with Na₂(⁵¹Cr)O₄ at a concentration sufficient to give approximately 5×10^4 c.p.m. per 0.2 ml platelet suspension. Cells were washed 3 times with HEPES/EDTA-buffer and resuspended to 1×10^8 cells/ml in buffer without EDTA.

In some experiments fixed platelets were prepared as follows: washed cells were resuspended in 2% formaldehyde in buffer saturated with 5% autologous plasma and incubated at 4 °C for 20 min. After two further washings in HEPES-buffer the cells were adjusted to 1×10^8 cells/ml.

Measurement of platelet adhesion: Flat bottomed glass scintillation vials were used for the adhesion test. They were processed as follows: the vials were washed with non-ionic detergent (Triton X-100), rinsed with tap and distilled water followed by soaking in 10% H₂O₂ for 6 hours. After further thorough washing with bidistilled water, the vials were dried at 120 °C for 1 hour. Processed glass vials were filled with 0.2×10^8 ⁵¹Cr-labeled platelets and resuspended in a final volume of 2 ml HEPES-buffer. The vials were incubated for 60 min at 37 °C and counted in a γ -counter (LKB) The solution was then decanted, the vials

rinsed 5 times with buffer and counted again. The percentage of adhesion was calculated according to the formula:

$$\% \text{ ADHESION} = \frac{\text{C.P.M. before washing} - \text{C.P.M. background}}{\text{C.P.M. after washing} - \text{C.P.M. background}} \times 100$$

The experiments were considered correct if no aggregation was found in the decanted solution by microscopic investigation. In some experiments the platelets were preincubated for 1 hour at 37 °C with 2 mM KCN, 10 mM NaN₃, 1 mM colchicin or 20 µg/ml cytochalasin B.

Measurement of platelet aggregation: The aggregation was measured by recording the light transmission through a stirred platelet suspension, according to the method of Born [2]. The increase of optical transmission at 400 nm was registered with an aggregometer, adjusted to produce full-scale deflection for platelet count difference of 0.8×10^8 cells/ml.

The platelet shape change was measured under the same optical conditions and defined as a decrease of optical density (caused by changes in the form of platelets from disc to sphere) within 3 minutes after addition of an aggregant.

Data presentation: Each point is the mean \pm S.E.M. of 4 different platelet preparations. Each measurement was carried out in triplicate. Statistical significance between comparable points was determined using Student's *t* test.

Results

Effect of lectins on platelet adhesion to glass

Preliminary experiments showed that the lectins used did not induce detectable release of ⁵¹Cr at the concentration tested for at least four hours (data not shown).

PHA was found to increase sharply the adhesion of washed platelets to glass surface (Fig. 1). The maximum effect was reached at a lectin concentration of 30 µg/ml: $27.8 \pm 2\%$. This is about 4 times higher than spontaneous adhesion to glass: 6.9 ± 0.7 ($p < 0.05$). At higher concentrations of lectin, a decrease of the PHA-induced adhesion was observed ($19.5 \pm 1.5\%$ at 100 µg/ml PHA). At the same time microscopic investigation revealed single aggregates.

Con A and Lens also amplified the adhesion of platelets to glass to an approximately equal extent (Fig. 1). Maximum adhesion was reached at a concentration of about 25–30 µg/ml which was $19.8 \pm 1.8\%$ for Con A and $18.4 \pm 2.1\%$ for Lens ($p < 0.05$), which is much lower than the PHA-induced one. The concentration-dependent curves for Con A and Lens were flatter.

In a preliminary experiment we found that unwashed platelets (platelet-rich plasma) showed lower adhesion to glass than washed cells. The influence of added autologous plasma on spontaneous and lectin-induced platelet adhesion

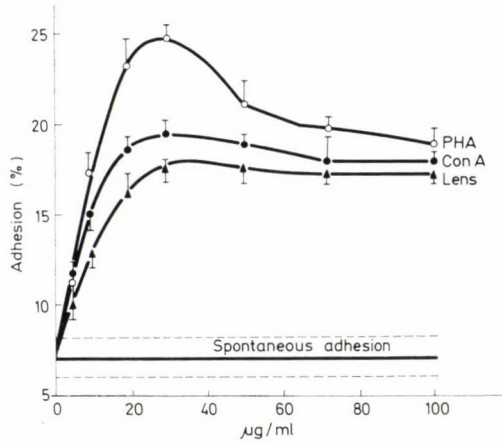


Fig. 1. Effect of PHA, Con A and Lens on human platelet adhesion to glass. Concentration-dependent curves. 0.2×10^8 ^{51}Cr -labeled platelets were incubated in a final volume of 1 ml for 60 min at 37°C in scintillation glass vials. The percentage of adhesion was calculated as described in Materials and Methods. Each point is mean \pm S. E. M. of four different experiments. The measurements were done in triplicate

to glass was tested (Table 1). The results showed that increased plasma concentration diminished spontaneous, as well as Con A and Lens-induced adhesion of platelets to glass surface. The results with PHA (not presented) were considered inaccurate because of the formed aggregates.

Effect of lectins on platelet aggregation

Of the three lectins tested, only PHA induced both change of shape and aggregation (Fig. 2). The degree of aggregation depended on PHA concentration.

Table 1

Spontaneous and lectin-induced adhesion of platelets to glass in the presence of autologous plasma

Autologous plasma	0%	1%	5%	10%	20%
Spontaneous	7.8	7.4	—	4.8	4.1
+ Lens 20 µg/ml	15.3	11.1	9.9	8.7	8.1
+ Con A 20 µg/ml	15.1	9.8	9.3	7.8	7.4

The percentage of platelet adhesion to glass was measured. Cells at a concentration of 0.2×10^8 cells/ml were incubated at 37°C for 60 min in glass vials with HEPES-buffer, pH 7.3, to which autologous plasma was added in the indicated percentages. The final assay volume was 1 ml. Each value is a mean calculated from three experiments.

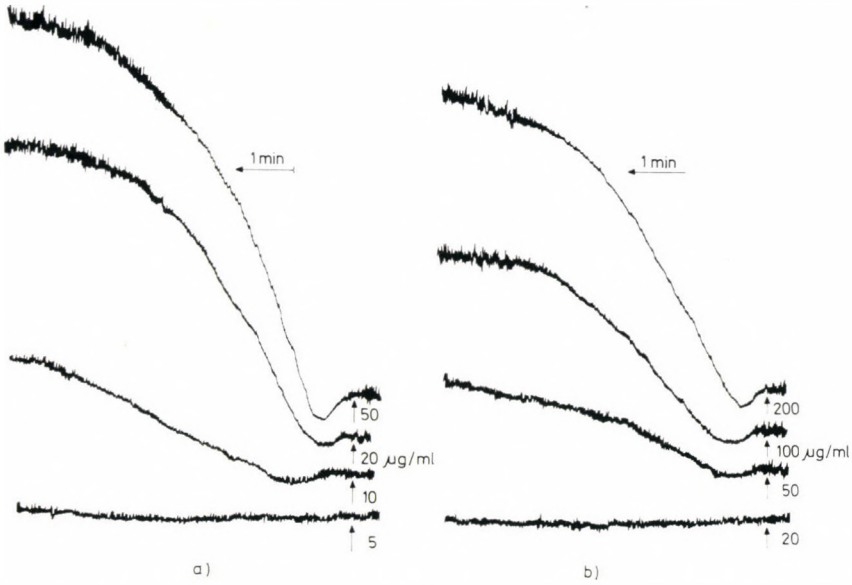


Fig. 2. PHA-induced aggregation of washed platelets (a) and platelets suspended in autologous plasma (b). Platelets were suspended in HEPES-buffer pH 7.3 or in autologous plasma (PRP) at a concentration of 10^8 cells/ml. Aggregation was assayed at 37°C in the presence of different PHA concentrations

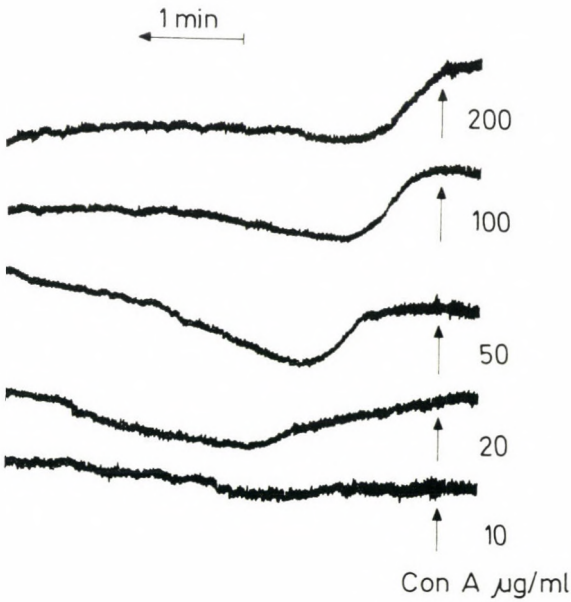


Fig. 3. Con A-induced shape change of washed platelets. The experimental conditions were as described in Fig. 2

Washed platelets showed maximal aggregation at 50 $\mu\text{g/ml}$ (Fig. 2-a), while in the presence of plasma (PRP) a similar effect was reached at 200 $\mu\text{g/ml}$ (Fig. 2-b).

As shown in Fig. 3, Con A produced a well expressed change of platelet shape, but no aggregation. This phenomenon was also concentration-dependent and was "optimal" at about 50 $\mu\text{g/ml}$. At higher concentrations the effect was irreversible.

Lens culinaris did not produce any effect on either platelet aggregation or shape change up to 400 $\mu\text{g/ml}$ (data not shown).

Influence of different "inhibitors" on lectin-induced adhesion and aggregation

The effects of cytochalasin B, colchicin, KCN and NaN_3 on Con-A-induced platelet adhesion were tested. Each compound except colchicin showed statistically significant inhibitory effects (Fig. 4). Similar results were obtained with Lens-induced adhesion (data not shown).

The effect of indomethacin (10 μM), KCN, NaN_3 and chemical fixation of cells with formaldehyde on PHA-induced aggregation is shown on Fig. 5-a. Indomethacin inhibited the aggregation by about 30%, while KCN, NaN_3 and formaldehyde fixation — by about 50%. Fig. 5-b shows the temperature dependence of PHA-induced aggregation of platelets. Neither aggregation nor shape change was observed at temperatures below 10 $^\circ\text{C}$.

Con-A-induced platelet shape change was totally blocked by KCN, NaN_3 and low temperature (Fig. 6).

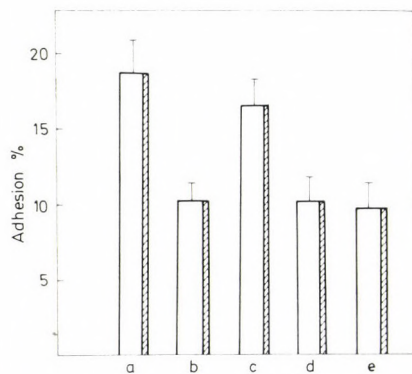


Fig. 4. Effects of some inhibitors on Con A-induced platelet adhesion to glass. Platelets were suspended in HEPES-buffer pH 7.3 at a concentration of 1×10^8 cells/ml. To each vial 0.2. ml cell suspension, 50 $\mu\text{g/ml}$ Con A and one of the following inhibitors were added: cytochalasin B (b), colchicin (c), KCN (d) and NaN_3 (e). The control is signed (a). Each value is mean \pm S. E. M. of four experiments

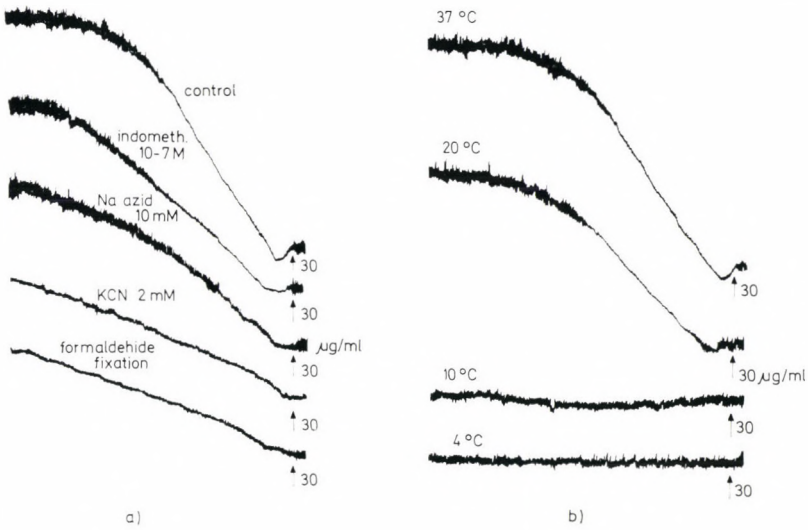


Fig. 5. Effects of inhibitors (a) and temperature (b) on PHA-induced aggregation of platelets. The washed platelets were preincubated with the inhibitors indicated and after two more washings the cells were resuspended in HEPES-buffer pH 7.3. Other experimental conditions were as described in Fig. 2

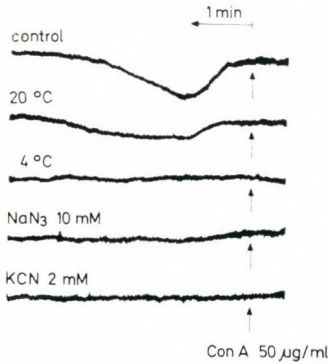


Fig. 6. Effects of temperature and some metabolic inhibitors on Con A-induced shape change of washed platelets. The washed platelets were preincubated with the inhibitors indicated and after two more washings the cells were resuspended in HEPES-buffer pH 7.3. Other experimental conditions were as described in Fig. 2

Discussion

Our results show that the lectins PHA (N-acetylgalactosamine determinant), Con A and Lens culinaris (glucose-mannose determinants) have the ability to amplify adhesion of platelets to glass. PHA had the strongest effect with a maximum at about 30 $\mu\text{g}/\text{ml}$, where the adhesion was nearly 4 fold higher than the spontaneous one (Fig. 1). The decrease of adhesion at a concentration of 100 $\mu\text{g}/\text{ml}$ (or higher) was due to a parallel PHA-induced aggregation which was confirmed by microscopic investigations. It is known [2] that rotation is necessary for aggregate formation, but evidently single aggregates may also be formed under static conditions. The disadvantage of our adhesion system is that it does not differentiate between platelet adhesion and eventual lectin-induced aggregation. That is why experiments on the effect of some inhibitors on lectin-induced adhesion to glass were performed with Con A and Lens only. As shown in Fig. 1, these lectins which did not induce aggregation showed typical saturation-concentration curves.

As far as the mechanism of lectin-induced adhesion to glass is concerned, similarly to lectin-induced agglutination mechanism [8], it could be assumed that lectins as polyvalent ligands bind simultaneously to platelet membrane and glass surface, e.g. the adhesion is carried out by a "bridging action" of the lectin molecule. Such a mechanism, however, is not in agreement with our observations on the effects of different inhibitors. Depression of the lectin-induced adhesion to glass by KCN and NaN_3 (Fig. 4) shows that the level of intracellular ATP is important. Furthermore the inhibitory effect of cytochalasin B (and partly of colchicin) suggests an involvement of the cytoskeletal system in the adhesive process. The low effect of colchicin might be due to the short-term preincubation of platelets with this inhibitor (1 hour). Longer periods (the recommended 4 hours) [9] were not suitable for our non-sterile experimental conditions.

Recently it has been shown that polymerisation of platelet cytoskeletal elements and association of specific platelet surface proteins with the cytoskeleton might be key elements in the process of platelet activation [5, 6]. As the latter is dependent on the metabolic state of the cells and the function of the cytoskeleton, we suggest their participation in the mechanism of lectin-induced adhesion of platelets to glass.

Previous investigations by other authors indicated that Con A can induce platelet activation and membrane glycoprotein phosphorylation, association of membrane proteins with the cytoskeleton [5, 6], rearrangement and capping of the platelet membrane receptors [10] and release reaction [3]. All these data suggest that the effect of lectin-induced adhesion to glass — observed in our laboratory — may be of physiological importance and is dependent on the initial functional state of the platelets.

The presence of autologous plasma significantly decreased both spontaneous and lectin-induced platelet adhesion to glass (Table 1), which may be due to the screening action of plasma proteins. Our preliminary results have shown

that some plasma glycoproteins have similar effect, but further investigations are required for solving this problem.

Our studies show that of the three lectins tested only PHA was a potent inducer of platelet aggregation. This is in agreement with the data of Majerus and Brodie [7]. In the presence of autologous plasma the PHA-induced aggregation was significantly reduced (Fig. 2-b). A ten-fold higher PHA concentration was necessary for inducing the typical platelet aggregation effect in the presence of PRP. We assume a similar screening action of plasma proteins, as that involved in the adhesion process.

The metabolic inhibitors KCN and NaN_3 reduce the aggregation, but not completely (by about 30–35%), while temperatures below 10 °C totally block the process. This suggests that lectin-induced aggregation is also dependent on cell metabolism, but probably a “physical-chemical” cell-lectin interaction takes place during the aggregation. Our findings related to chemically fixed cells are in agreement with this theory (Fig. 2-a).

Indomethacin causes an inhibitory effect of about 30–35%, which suggests an involvement of arachidonic acid metabolic products in the mechanism of lectin-induced aggregation (similar to physiological aggregation).

Our finding that Con A and Lens do not cause platelet aggregation actually confirmed the results of Greenberg and Jamieson [3]. However, Con A induces a well expressed platelet shape change, which is also dependent on the metabolic state of the cells, as shown by its inhibition by KCN and NaN_3 or low temperature (Fig. 6). This effect was described in detail in our previous work [11].

The data reported herein show that the tested lectins have different effects on platelets. While PHA is a potent inducer of aggregation (similarly to the physiological one) Con A causes only platelet shape change, and Lens has no effect on either shape change or aggregation. However, all three of them induce platelet adhesion to glass. This indicates that lectin-induced adhesion is a less specific process than lectin-induced aggregation, and apparently there are differences in the underlying mechanisms. On the other hand, both processes are dependent on cell metabolism, the cytoskeleton and probably the initial state of membranes, which suggests that this might be a possible approach to the functional assessment of platelets in vitro.

Acknowledgement

The authors express their thanks to Mrs. Ivanka Solaková for her valuable technical assistance.

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Subcellular Distribution of Hexokinase in Leukemic and Stimulated Lymphoid Cells of Mice

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(Received 9 April, 1987; accepted 10 June, 1987)

The oxygen consumption, subcellular distribution of hexokinase were studied in normal and leukemic spleen cells of AKR mice. High mitochondrial-bound hexokinase activity and O_2 -consumption were detected in fetal state, in leukemic or BPV-induced lymphoproliferative conditions and after spermine incubation of normal spleen cells. After ConA-treatment, however, all of the hexokinase was found in cytoplasmic fraction and O_2 -uptake was strongly decreased. Studies on respiration of mitochondria isolated from normal and leukemic cells supported the role of mitochondrial-bound hexokinase in elevated O_2 -consumption of leukemic cells.

Keywords: mitochondrial-bound hexokinase, oxygen consumption, leukemic and stimulated lymphoid cells, age dependency

Introduction

Previously, we found increased total hexokinase activity and altered subcellular distribution in leukemic cells. Large amount of hexokinase activity was bound to the mitochondria and elevated O_2 -consumption was observed in leukemic cells. We assumed a possible relationship between mitochondrial-bound hexokinase and elevated leukemic cell respiration [1].

Our aim was to reveal the correlation between mitochondrial hexokinase binding and O_2 -uptake in normal and leukemic cells.

Materials and Methods

18-day-old fetal, newborn and 6–8-week old adult AKR female mice were used. In adult AKR animals the AKR leukemia (AKRL) was maintained by serial passages from mouse to mouse with a leukemic spleen suspension containing 10^6 viable cells [1, 2]. In addition, the following procedures were carried out:

- one group of the animals was treated with *Bordetella pertussis* vaccine (BPV, 9×10^9 germ/animal), and the spleen cells were prepared on the 3rd day after the treatment

- another group of the animals was treated with *Concanavalin A* (ConA, 2,4 mg/animal). The spleen cells were prepared on the 6th day after i.p. inoculation
- 10^7 viable spleen cells of normal AKR mice were incubated in the presence of 1 mM *spermine* for 30 min, at 37 °C.

The subcellular distribution of hexokinase was determined as previously described [1], according to Hommes and Everts [4], and Salotra and Singh [10]. Mitochondria were prepared by the method of Viyayakumar and Weidemann [14]. The respiratory control ratio of all preparations was better than 4 in the presence of 10 mM succinate plus rotenone. O_2 -consumption of intact cells and mitochondria was determined at 37 °C using a Clark-type electrode [1, 3]. The standard incubation medium for mitochondria contained: 110 mM sucrose, 60 mM KCl, 10 mM KH_2PO_4 , 5 mM $MgCl_2$ and 0.5 mM EDTA [7]. The pH was adjusted to 7.4 with HCl. Further additions are given on the appropriate table.

Results

In our first set of experiments the subcellular distribution of hexokinase activity was determined in normal and leukemic spleen cells. The data of Table 1 indicate that most of the hexokinase activity was found in the mitochondrial (8.500 g pellet) and the cytoplasmic (18.000 g supernatant) fractions. Normal and leukemic cells showed significant differences in the hexokinase activity of these fractions. Increased hexokinase activity of the whole homogenate of leukemic cells and altered subcellular distribution were found. In leukemia, a subcellular trans-

Table 1

Distribution of hexokinase activity between the subfractions of normal and leukemic AKR spleen cell homogenate (mU/mg protein \pm SE)

	Normal cells	recovery ^c	Leukemic cells	recovery ^c
850 g pellet	0.9 \pm 0.05	6.6	1.0 \pm 0.1	2.8
8500 g pellet ^a	2.1 \pm 0.1	15.5	18.3 \pm 1.2**	51.9
18 000 g pellet	0.5 \pm 0.01	3.7	1.2 \pm 0.1	3.4
18 000 g supernatant	9.1 \pm 1.1	67.4	12.4 \pm 1.1	35.2
Total activity ^b	13.5 \pm 1.3	93.2	35.2 \pm 1.6**	93.3

^a treated with 0.5% Triton X 100

^b total activity was measured in whole homogenate, treated with 0.5% Triton X 100

^c the values are given as percentage of recovery in each fraction compared to homogenate

** $p < 0.001$ compared to the normal value

Table 2

Hexokinase activity and oxygen consumption in spleen cells of AKR mice in different ages and leukemic conditions

	Hexokinase activity ^a			O ₂ consumption ^b
	8500 g pellet ^c	18 000 g supernatant	total	
18 day old foetus	14.5 ± 1.0**	2.0 ± 0.6**	20.2 ± 1.5*	20 ± 1.5*
Newborn	10.5 ± 1.2**	3.4 ± 0.4**	14.8 ± 1.1	16 ± 1*
6-8 week old adult	2.1 ± 0.1	9.1 ± 1.1	13.5 ± 1.3	13 ± 1
Adult, leukemic	18.3 ± 1.2**	12.4 ± 1.1	35.2 ± 1.6**	18 ± 1*

^a mU/mg protein ± SE (n = 6-6)^b nmoles O₂/min/10⁷ cells ± SE (n = 6-6)^c treated with 0.5% Triton X 100

* p < 0.01 compared to the

** p < 0.001 normal adults

location of hexokinase activity from the cytoplasmic (18.000 g supernatant) to the mitochondrial (8.500 g pellet) fractions was observed.

The influence of age (compared to the leukemic conditions) on subcellular distribution of hexokinase activity and its relation to the O₂-consumption is shown in Table 2. It was observed that the age of the animals plays an important role in total hexokinase activity and its subcellular distribution. The highest total and mitochondrial-bound hexokinase activity was found in fetuses. The O₂-uptake of spleen cells changed similarly to that of the mitochondrial hexokinase activity.

Table 3

Effect of different treatment on subcellular hexokinase distribution and oxygen consumption in spleen cells of AKR mice

	Hexokinase activity ^a			O ₂ consumption ^b
	8500 g pellet	18 000 g supernatant	total	
Control, adult, untreated	2.1 ± 0.1	9.1 ± 1.1	13.5 ± 1.3	13 ± 1
Adult, leukemic ^c	18.3 ± 1.2**	12.4 ± 1.1	35.2 ± 1.6**	18 ± 1*
BPV treated ^c	28.5 ± 1.7**	0.0 ± 1.0	40.2 ± 2.1**	19 ± 1.5*
Spermine inc. ^c	8.9 ± 1.0**	2.6 ± 0.2**	12.6 ± 1.4	21 ± 1.3**
ConA treated ^c	—	33.6 ± 1.8**	34.4 ± 2.0**	4 ± 0.8**

^a mU/mg protein ± SE (n = 6-6)^b nmoles O₂/min/10⁷ cells ± SE (n = 6-6)^c for more details see: "Materials and Methods"

* p < 0.01 compared to the adult

** p < 0.001 controls

We investigated the effect of different factors on subcellular distribution of hexokinase and O_2 -consumption. Table 3 indicates that in leukemic as well as in BPV-induced lymphoproliferative conditions the hexokinase activity is shifted from the cytoplasm to the mitochondria, in spite of an elevated total hexokinase activity.

Incubation of normal spleen cells in the presence of spermine can shift the hexokinase equilibrium towards the mitochondria without any changes in total activity. In all cases the elevated mitochondrial bound hexokinase activity was accompanied by a higher O_2 uptake. In ConA induced "normal" lymphoproliferative conditions we observed an increased total hexokinase activity without extended mitochondrial binding and a strongly decreased O_2 -consumption.

Table 4

Effect of hexokinase on oxygen consumption of mitochondria isolated from normal and leukemic cells^a

	no addition	+ 50 mU hexokinase
Normal	7.43 ± 0.68	13.4 ± 0.95**
Leukaemic	8.35 ± 0.73	15.8 ± 0.93**

^a nmoles O_2 /min/mg mitochondrial protein ± SE (n = 6-6)

Incubation of mitochondria was carried out (1-1 mgs) in standard medium (see: Materials and Methods) with 1 mM ATP and 10 mM glucose. The respiratory substrate was 2 mM succinate plus 1 μ M rotenone.

** p < 0.001 compared to the value measured in absence of hexokinase

These data indicated a possible stimulatory effect of mitochondrial-bound hexokinase on cell respiration. To support this hypothesis, we investigated the O_2 -consumption of mitochondria isolated from normal and leukemic cells. Table 4 shows that there is no difference in O_2 -consumption of normal and leukemic mitochondria. At the same time the O_2 -uptake of both normal and leukemic mitochondria were stimulated to the same extent by adding hexokinase (glucose phosphorylation system).

Discussion

Hexokinase has been considered as a typical example of "ambiquitous" enzyme the intracellular distribution of which may vary rapidly and reversibly between soluble and membrane-bound forms reflecting the change in concentrations of regulating metabolites [6, 8]. High aerobic glycolysis in rapidly growing tumor cells has been reported to be correlated with the increased mitochondrial-bound hexokinase activity [8]. On the other hand, because of direct utilization of

mitochondrially formed ATP the mitochondrial-bound hexokinase can regulate and stimulate the mitochondrial respiration [5, 12, 13].

Our data suggest:

- the mitochondrial-bound hexokinase activity is high in fetal state, in leukemic or BPV-induced lymphoproliferative conditions (this latter resembles leukemic conditions in some aspects [11]), but not in adult state or “normal” proliferative conditions (ConA treatment). High mitochondrial hexokinase activity was observed after spermine incubation of normal spleen cells. Polyamines are known to support the translocation of hexokinase from cytoplasm to the mitochondria [6].
- the changes in subcellular distribution of hexokinase are reversible and rapid (see spermine incubation), although we have no data concerning ConA treatment.
- the observed high mitochondrial-bound hexokinase activity is always accompanied with higher cell respiration.
- in spite of the elevated energy requirement only an elevated cytoplasmic and no mitochondrial-bound HK activity was observed after ConA treatment. As increased cellular activities following lectin stimulation are dependent on glycolysis rather than on respiration [9], our observations support this phenomenon.
- studies on O₂-consumption of both normal and leukemic mitochondria suggested that the increased O₂-uptake of leukemic cells was due rather to the altered subcellular distribution of hexokinase (i.e.: increased mitochondrial binding) than to the different respiratory rates of normal and leukemic mitochondria. Although, both normal and leukemic mitochondria may bind hexokinase, the increased mitochondrial hexokinase activity and the subsequent high O₂-consumption are characteristic for leukemic cells.

The observed findings may reflect the different energy requirements of normal and leukemic states and/or the altered concentrations of metabolites affecting hexokinase equilibrium.

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Granulocytic Progenitor Cells in the Adherent Layer of Human Long-term Bone Marrow Cultures

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(Received 29 October, 1986; accepted 6 March, 1987)

The importance of the adherent layer in long-term mouse bone marrow cultures as a reservoir of the most primitive stem cells is known. The role of the adherent cell layer in long-term human cultures (LTC) is examined from this point of view. Confluent adherent layers developed after about 3 weeks of culture. At that time and weekly thereafter the cellularity and granulocytic-macrophage progenitor (CFU-GM) content of the adherent fraction were determined after trypsinisation. We have documented that CFU-GM were present in the adherent layer of human cultures for at least 8 weeks. These findings emphasize the importance of assessing the progenitor cell content of the adherent layer in long-term human cultures.

Keywords: adherent layer, long-term human cultures, progenitor cells

Introduction

The development of long-term bone marrow cultures is an important advance in our ability to study stem cell proliferation and differentiation, the role of interactions between stromal cells and haemopoietic cells [1]. An adherent layer develops after the inoculation of bone marrow cells which was found to be composed of phagocytic mononuclear cells, lipid-containing, spindle-shaped cells. This layer is essential for the survival of progenitor cells in most species [2], but the role of the single cell types is uncertain yet. In mouse cultures, when all of the non-adherent cells are removed, the conditions are suitable for stem cell maintenance and proliferation [3]. It is assumed that the adherent layer serves as the primary stem cell reservoir. In human bone marrow cultures the evaluation of the non-adherent fractions of LTC shows that primitive progenitors are detectable for periods of 1–3 months [4, 5, 6]. It appears that the adherent fraction of human cultures, similarly to mouse cultures, contains important populations of progenitor cells.

In this report we have assessed granulocytic progenitor cell maintenance in human long-term cultures, and have compared the CFU-GM content of non-adherent and adherent fractions.

Materials and Methods

Patients

The bone marrow samples of 9 haematologically normal individuals were investigated. Samples of normal bone marrows were obtained by aspirating cells from a rib removed from patients undergoing cardiothoracic surgery. The bone marrow samples were transferred to heparinized blood collection vials containing Iscove's medium.

Bone marrow cultures

Long-term cultures (LTC) were established as previously described [1]. Briefly, 2×10^7 nucleated cells were seeded into 25 cm² tissue culture flasks containing 10 ml Iscove's medium (Gibco) supplemented with 10% preselected fetal calf serum (FCS), (Flow), 10% horse serum, antibiotics and 5×10^{-7} M hydrocortison in final concentration. Cultures were incubated at 37 °C in 5% CO₂ atmosphere. At weekly intervals half of the medium and the non-adherent cells were removed and replaced with an equal volume of fresh medium.

Confluent adherent layers developed after about 3 weeks of culture. At that time and weekly thereafter, the cellularity and the number of progenitor cells of adherent fraction and non-adherent compartment were determined. The adherent cells were removed by trypsinization [7]: all the growth medium with the non-adherent cells was removed from culture dishes and 5 ml of 0.1% trypsin solution was added to the flasks, and cultures were incubated for 10 min at 37 °C. One milliliter of FCS was then added to stop further trypsin action, and adherent cells were easily detached by pipetting.

CFU-GM assay

Bone marrow cells were plated at 1×10^5 /ml in Iscove's medium containing 15% FCS and 20% supernatant of bladder carcinoma cell line 5637 as the source of colony stimulating factor (CSF) in 0.3% agar. The plates were scored on day 11. Groups of more than 50 cells were counted as colonies [11].

Results

Nonadherent cell compartment

Cellularity. The cell content of the non-adherent layer declined gradually. After the third week the cell number of supernatant decreased to 3.7×10^6 from the initial 2×10^7 /flask. After the sixth week the cell counts declined to 4.5×10^5 . In the cultures non-adherent cells were detectable until the eighth week (Fig. 1).

Composition. The cellular composition of the supernatant changed during the life of the culture as shown in Table 1. The relative numbers of monocytes and

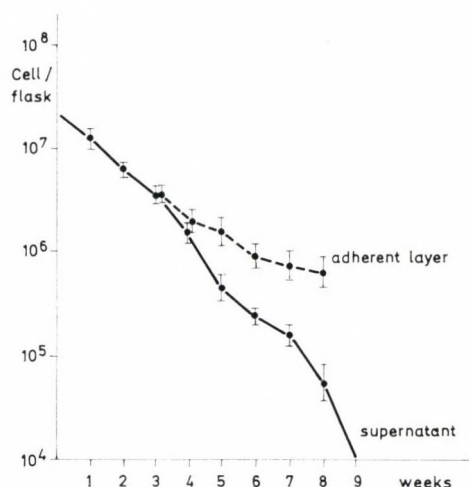


Fig. 1. The cellularity of the nonadherent and adherent layer in long-term cultures

macrophages increased to 30% by the fourth week, and at the same time there was a decline in the proportion of blasts, promyelocytes, myelocytes and metamyelocytes. The relative number of granulocytes remained constant by the fourth week.

Table 1

Composition of non-adherent compartment in long-term cultures

Weeks	% Blast and promyelocytes	% Myelo- and metamyelocytes	% Bands and segments	% Monocytes and macrophages	% Lymphocytes and normoblasts
1.	2 ± 0.81	26.22 ± 3.9	40.77 ± 2.77	9.66 ± 2.94	21.44 ± 2.67
2.	0.77 ± 0.77	15.88 ± 3.17	49.22 ± 3.32	30.44 ± 4.00	4.22 ± 2.14

The proportion of lymphocytes and normoblasts was 21% in the first week, it declined to 10% by the fourth week.

CFU-GM content. The initial CFU-GM number was 1×10^4 /flask, in the first week it was 5.8×10^3 /flask. During the first six weeks of culture there was a steady decline in the numbers of CFU-GM. Granulocyte-macrophage progenitors were detectable until the eighth week (Fig. 2).

The adherent layer

Cellularity. The cell count of adherent fractions in the same experiments remained almost constant between the third and fifth weeks: $(3.7 - 1.5 \times 10^6)$ /flask and thereafter declined gradually. In the eighth week the cell number of the ad-

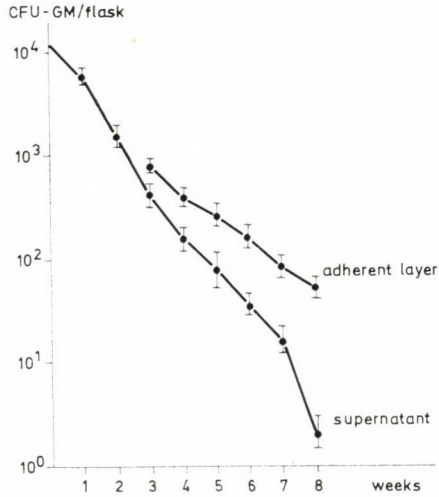


Fig. 2. The CFU-GM content of the long-term cultures

herent layer was 5×10^5 , ten times more than the cell content of the supernatant (Fig. 1).

Composition. By the end of the third week there was a confluent adherent layer with small round cells in association with adherent cells in the areas where the spindle-shaped cells first attached. Hereafter, increasing numbers of lipid containing cells appeared. Wright's-Giemsa staining of adherent cells showed fibroblast-like cells, monocytes and macrophages.

CFU-GM content of cultures declined gradually with time. After 4 weeks of cultures, however, the CFU-GM content of the adherent layer was higher compared to that of the supernatant. The difference in CFU-GM concentrations of the two fractions was highest in the fifth and eighth weeks (10–25 fold).

Discussion

The application of Dexter type murine base marrow cultures for human specimens resulted in CFU-GM maintenance of 6–9 weeks [7, 8, 9].

Improvement of long-term human cultures by combination of fetal calf serum and horse serum and use of intraoperative marrow specimens has been reported [4]. This ensures a prolonged maintenance of granulocytic progenitors. In our experiments we were able to maintain CFU-GM in long-term cultures for at least 8 weeks without recharging. Some data [12] suggest that there are two phases in the life of the cultures. During the initial 3–4 weeks there are several simultaneous changes: the decline in the non-adherent cell count explained by feeding depopulation, attachment of cells to the flask, cell death and maturation.

Determination of the number of progenitor cells present in the adherent fraction necessitated the development of a suitable method for detaching the adherent cells. Trypsination was used based on the observation that satisfactory cell suspension could be obtained by using either trypsin or collagenase [10]. Neither of these agents have significant adverse effects on progenitor cell plating efficiency.

The studies of long-term bone marrow cultures have established two important functions of the cells in the adherent layer. One is the modulation of stem cell proliferation [13] and the other is to provide the initial reservoir of stem cells [3]. Our experiments similarly to others suggest a flow of haemopoietic cells from the adherent to the non-adherent fraction. We have confirmed that CFU-GM in the supernatant arise from the adherent compartment. By the third week there are at least as many CFU-GM in the adherent layer as in the non-adherent fraction. Coulombel [10] reported that the most primitive progenitor cells identified by their high proliferative capacity were found in the adherent layer, and progenitor cells with lower proliferative capacity were found in the non-adherent fraction.

These findings implicate that if only the non-adherent fraction is evaluated the progenitor content of the culture may be underestimated. Changes in the number of total cells and progenitors in the supernatant are influenced by the release of cells from the adherent layer.

The retention of the most primitive progenitor cells in the adherent layer appears to be a feature of long-term human cultures in which haemopoiesis from normal progenitors is maintained. We suggest that assessment of the adherent fraction may be important when analyzing marrow cultures of patients with leukaemia, as primitive normal stem cells, even if present, might not appear in the supernatant.

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Case Reports

Juvenile Chronic Myelogenous Leukaemia and Cleidocranial Dysostosis

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A 6 year-old Caucasian girl was admitted in May 4, 1982 with a 1-month history of haemorrhagic manifestations.

The mother, a maternal aunt and the maternal grandmother had cleidocranial dysostosis.

Physical examination revealed cranial enlargement and prominent frontal bossing, the sagittal suture was depressed and the palate was high and narrow. The right clavicle was hypoplastic and the fingers were long and thin, clinodactyly of the fourth and fifth fingers was also observed. Other significant clinical findings included hepatomegaly of 4 cm, splenomegaly of 6 cm, strabismus and otitis.

Haematological data: Hb 10.9 g/dl, reticulocytes 1.2%, platelet count $5 \times 10^9/l$, WBC count $30 \times 10^9/l$ with 3% blasts, 1% promyelocytes, 58% neutrophils, 17% lymphocytes and 21% monocytes. There were also 2 normoblasts/100 leukocytes. The bone marrow aspirate demonstrated increased cellularity with predominance of granulocytic cells at all stages of maturation with a M : E ratio of 5.6 : 1, megacaryocytes were very reduced in number, lymphocytes 8%. The karyotype was normal. All laboratory values were normal except fetal haemoglobin 65%, Hb A₂ 0% and Hb A 35%, γ globulin 1.82 g/dl and serum muramidase: 22.5 $\mu\text{g/ml}$ (normal values: 8–13). The leukocyte alkaline phosphatase was 54 (normal value 35–50).

Roentgenograms confirmed the clinical finding.

Although the age of onset of the disease was unusual, the clinical and laboratory data indicated juvenile chronic myelogenous leukaemia (JCML), and treatment with 6 mercaptopurine was started on May 19, 1982. Within 1 month there was a decrease of hepatosplenomegaly and of leukocyte count, but the platelet count remained low. On November 29, 1982 the bone marrow was replaced by myeloblasts. The patient failed to respond to chemotherapy with COAP and died 13 months after the diagnosis. Postmortem examination was not performed.

JCML is an uncommon childhood leukaemia characterized by hepatosplenomegaly, leukocytosis, thrombocytopenia, the absence of the Philadelphia chromosome and an increased fetal haemoglobin [1]. Increased levels of γ -globulin have also been observed [2].

JCML has been reported to be associated with congenital malformations,

Von Recklinhausen's neurofibromatosis, hypospadias, cleft palate, craniotabes, dysmorphic facies, cataracts, strabismus, nistagmus [2] and trisomy 8 [3]. Other defects involving skeletal system have been described in leukaemia [4].

Cleidocranial dysostosis is a rare disturbance of bone development of autosomal dominant inheritance [5].

To our knowledge this is the first report of association between cleidocranial dysostosis and JCML.

The occurrence of JCML together with cleidocranial dysostosis may be only coincidental or this congenital disease may be associated with a genetic predisposition to leukaemia like other skeletal malformations.

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Duchenne Muscular Dystrophy and Acute Lymphoblastic Leukaemia

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Duchenne muscular dystrophy is a sex-linked trait which so far has not been reported to be associated with other malignant diseases.

An 8 year old boy was admitted to our Institute due to anaemia. Physical examination revealed lumbar lordosis, weakness of the shoulder and pelvic girdles. The patient had a waddling gait, difficulty in climbing stairs and increased size of calf muscles (pseudo-hypertrophics). Gower's sign was positive.

When the boy was 3 years old, Duchenne muscular dystrophy was diagnosed. An elevated creatine phosphokinase (CPK): 95 μ /l (Normal: 50 μ /l) and muscular biopsy confirmed the clinical diagnosis. Haematological data at the time of admission were: Hb 9.6 g/dl, reticulocytes 1%, platelet count 150×10^9 /l, WBC count 3.7×10^9 /l with 3% myelocytes, 45% neutrophils, 46% lymphocytes and 6% monocytes.

Bone marrow aspiration revealed ALL, L₁ type according to the FAB classification. No immunological markers were detected. The treatment included the use of prednisone and vincristine for induction, prophylactic CNS therapy with methotrexate and dexamethasone, maintenance chemotherapy with 6 mercaptopurine and methotrexate and reinforcement therapy every 3 months of 1 dose vincristine and prednisone for 1 week. Just after the completion of induction therapy, CNS leukaemia appeared, and therefore he received cranio-spinal irradiation. He remained in remission for 48 months and died of pneumonia in another hospital.

Although the etiology of ALL is not known, a pathogenic role of genetic factors is suggested based on the high incidence in identical twins [1].

The only cases when an association between a neoplasm of the haematopoietic system and a neuromuscular disease was found are the 3 subjects with progressive muscular dystrophy with multiple myeloma [2] reported by Kissling et al. No data are available on a possible linkage between the inheritance of genetic factors in ALL and the \times chromosome. To our knowledge this is the first report of an association between Duchenne muscular dystrophy and ALL.

Although this association may be purely coincidental, we cannot exclude the existence of a higher prevalence of ALL in Duchenne muscular dystrophy. In view of this possibility, we thought this case interesting and that it may stimulate further studies to clarify this problem.

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Abstracts

Modulation of erythrocyte vesiculation by amphiphilic drugs. P. Bütikofer, U. Brodbeck and P. Ott (Institut für Biochemie und Molekularbiologie der Universität Bern, Bern, Switzerland). *Biochim. Biophys. Acta* 901, 291 (1987).

Release of acetylcholinesterase-containing vesicles from human erythrocyte membranes induced by dimyristoylphosphatidylcholine (DMPC) was inhibited by exposure of red cells to cationic amphiphilic drugs like tetracaine, chlorpromazine and primaquine which all are known to induce stomatocyte formation. On the other hand, the process was facilitated when red cells were exposed to crenators like the anionic drugs indomethacine and phenylbutazone or when DMPC was added to calcium-loaded red cells. The results suggest that agents which are known to modulate red cell shape do also influence the vesiculation behavior of the cells.

G. Gárdos

Inhibition of calmodulin stimulation of phosphodiesterase and Ca^{2+} , Mg^{2+} -ATPase activities and shape change of erythrocyte ghosts by chloroquine. F. Nagai, K. Ushiyama, I. Kano, A. Nakagawa, T. Nakao and A. Nakajima (Department of Toxicology, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo 160, Japan). *Biochem. Pharmacol.* 36, 3433 (1987).

The effects of chloroquine on calmodulin (CaM)-related enzyme activities and the shape of human erythrocytes have been studied. It was found that the CaM activation of rat brain phosphodiesterase was abolished by the addition of chloroquine. CaM was included in the assay of phosphodiesterase activity at the concentration that gave half-maximal activation. The concentra-

tion of chloroquine that caused 50% inhibition of CaM stimulation of phosphodiesterase was 7×10^{-5} M. The type of inhibition was competitive with respect to CaM. The CaM-stimulated Ca^{2+} , Mg^{2+} -ATPase in erythrocyte membrane was also inhibited by chloroquine, the 50% inhibitory concentration of which was about 2×10^{-4} M. Its mode of action was also competitive with respect to CaM. The shapes of erythrocyte ghosts prepared by hypotonic hemolysis were examined in a solution consisting of 2 mM $MgCl_2$, 154 mM NaCl and 10 mM Tris-HCl (pH 7.4); they were discocytic in the presence of 2 mM ATP and in its absence. They were converted to the invaginated form by the addition of chloroquine in the concentration range of 1×10^{-4} – 5×10^{-4} M. This concentration is similar to that which caused the inhibition of CaM activation of Ca^{2+} , Mg^{2+} -ATPase.

G. Gárdos

Identification and purification of a novel M_r 43,000 tropomyosin-binding protein from human erythrocyte membranes. V. M. Fowler (Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115, USA). *J. Biol. Chem.* 262, 12792 (1987).

A new M_r 43,000 tropomyosin-binding protein (TMBP) has been identified in erythrocyte membranes by binding of ^{125}I -labeled Bolton-Hunter tropomyosin to nitrocellulose blots of membrane proteins separated by sodium dodecyl sulfate-gel electrophoresis. This protein is not actin, because ^{125}I -tropomyosin does not bind to purified actin on blots. Binding of ^{125}I -tropomyosin to this protein is specific because it is inhibited by excess unlabeled tropomyosin but not by

F-actin or muscle troponins. This protein has been purified to 95% homogeneity from a 1 M Tris extract of tropomyosin-depleted erythrocyte membranes by DEAE-cellulose and hydroxylapatite chromatography, followed by gel filtration on Ultrogel AcA 44. The purified protein has a Stokes radius of 3.9 nm and a sedimentation coefficient of 2.8 S, corresponding to a native molecular weight of 43,000. Binding of ^{125}I -tropomyosin to the purified TMBP saturates at one tropomyosin molecule (M_r 60,000) to two M_r 43,000 TMBPs, with an affinity of about $5 \times 10^{-7} \text{ M}$. The TMBP is associated with the membrane skeleton after extraction of membranes with the non-ionic detergent, Triton X-100, and is present with respect to tropomyosin at a ratio of about one for every two tropomyosin molecules. Because there is enough tropomyosin for two tropomyosin molecules to be associated with each of the short actin filaments in the membrane skeleton, the erythrocyte membrane TMBP, together with tropomyosin, could function to restrict the number of spectrin molecules attached to each of the short actin filaments and thus specify the hexagonal symmetry of the spectrin-actin lattice. Alternatively, this TMBP could be homologous to one of the muscle troponins and might function with tropomyosin to regulate erythrocyte actomyosin-ATPase activity and influence erythrocyte shape.

B. Sarkadi

Specific deposition of complement protein C3b on abnormal PNH erythrocytes permits their separation by partitioning. Possible general approach for isolation of specific cell populations. M. K. Pangburn and H. Walter (Department of Biochemistry, University of Texas Health Center, Tyler, TX 75710, USA). *Biochim. Biophys. Acta* 902, 278 (1987).

The deposition of complement proteins on a cell surface has previously been shown to reduce the cell's partition ratio in a two-polymer aqueous phase system. This phenomenon has now been extended to segregate,

by partitioning, subpopulations of erythrocytes from patients with paroxysmal nocturnal hemoglobinuria (PNH). Purified components of the complement system were employed to deposit the protein C3b specifically on abnormal erythrocytes which lacked the membrane-associated complement regulatory protein DAF. As few as 2100 C3b/cell reduced the partition ratio and 24000 C3b/cell resulted in resolution of the C3b-bearing and non-bearing human red cells. It was found that the proportion of cells separated did not equal the proportion of cells lysed by complement in the acidified serum lysis test when blood from three of the five patients was examined. The results indicate that the defect giving rise to DAF⁻ cells may be, but is not necessarily, coexpressed with defects affecting other membrane-associated regulatory factors. A broader application of the method using monoclonal antibodies to direct purified complement components to specific cell populations should permit their isolation in large quantities.

B. Sarkadi

Alterations of electrolytes in serum and erythrocytes after myocardial infarction. H. Ising, T. Günther, F. Bertschat, K. Ibe, V. Stoboy and E. Heldman (Institute for Water, Air, and Soil Hygiene of the Federal Health Office, Berlin-West). *Magnesium* 6, 192 (1987).

Electrolyte concentrations in serum and erythrocytes of 56 myocardial infarction (MI) patients and 50 patients having had an orthopedic operation or an accident were measured up to 2 weeks after the event. Several hours after the MI, considerable percentages of patients had had pathologically low serum concentrations of either K, Ca or Mg. In the erythrocytes of the MI group, K was decreased and Ca increased during the entire period of investigation, whereas, the serum electrolytes K and Ca had normalized by day 15, at which time only half of the patients with pathologically low serum Mg had reached the normal Mg range. By correlation analysis it was shown that

the more the Ca concentration in the serum was decreased after MI, the less was the concentration of brain muscle creatine kinase.

Ilma Szász

Diamide stimulates calcium-sodium exchange in dog red blood cells. J. C. Parker (Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514, USA). *Am. J. Physiol.* 253, C580 (1987).

Calcium influx can be stimulated in dog red blood cells by preexposure to diamide under certain conditions. Diamide-activated calcium influx resembles swelling-induced Ca^{2+} - Na^{+} exchange in several respects. These include saturation of calcium influx at external calcium levels > 0.5 mM, suppression of calcium influx by external sodium, and inhibition by quinidine. The ability of diamide to stimulate this transport pathway depends critically on the ionic composition of the medium in which the cells are bathed at the time of diamide exposure. The effect is greatest if the diamide preincubation is conducted in a hypotonic lithium chloride medium containing at least $1 \mu\text{M}$ calcium. Stimulation of Ca^{2+} - Na^{+} exchange is seen at diamide concentrations (0.10–0.33 mM) that are lower than those reported to cause major spectrin cross-linking, glutathione depletion, Ca^{2+} -ATPase inhibition, or ion channel formation. The results suggest that dog red cells have a large latent capacity for Ca^{2+} - Na^{+} exchange.

G. Gárdos

Action of erythropoietin in vitro on rabbit reticulocyte membrane Ca^{2+} -ATPase activity. W. D. Lawrence, P. J. Davis and S. D. Blas (Hematology and Endocrinology Divisions, Department of Medicine, State University of New York, Buffalo, N. Y. 14215, USA). *J. Clin. Invest.* 80, 586 (1987).

The mechanism of action of erythropoietin is thought to require specific interaction with the target cell surface and involve alteration of cellular calcium metabolism.

Using the rabbit reticulocyte membrane as a model of the immature red cell membrane, the *in vitro* effects of human recombinant erythropoietin on membrane Ca^{2+} -ATPase (calcium pump) activity were investigated. Erythropoietin in a concentration range of 0.025 to 3.0 U/ml progressively decreased membrane Ca^{2+} -ATPase activity by up to 64% ($P < 0.01$). These concentrations have been shown by others to stimulate *in vitro* erythroid growth. The action of erythropoietin on reticulocyte Ca^{2+} -ATPase required an incubation time of 1 h before enzyme assay for maximum effect and was neutralized by antierythropoietin antiserum. Other nonhemopoietic growth factors (epidermal growth factor, insulin) had no effect in this assay. Ca^{2+} -ATPase activity of membranes prepared from rabbit mature red blood cells was not inhibited by erythropoietin. The novel effect of erythropoietin on reticulocyte membrane Ca^{2+} -ATPase activity is a mechanism by which erythropoietin can influence cellular Ca^{2+} metabolism.

Ilma Szász

Conformational differences between the E_1 and E_2 states of the calcium adenosinetriphosphatase of the erythrocyte plasma membrane as revealed by circular dichroism and fluorescence spectroscopy. J. Krebs, M. Vasak, A. Scarpa and E. Carafoli (Laboratory of Biochemistry, Swiss Federal Institute of Technology, Zurich, Switzerland). *Biochemistry* 26, 3921 (1987).

Different conformational states of the purified plasma membrane Ca^{2+} -ATPase from pig erythrocytes have been detected by circular dichroism (CD) and fluorescence spectroscopy. The helical content of the enzyme decreased by about 10% in the transition from the Ca^{2+} high-affinity form ($10 \mu\text{M}$ free Ca^{2+} = E_1 state) to the VO_4^{3-} -inhibited state ($20 \mu\text{M}$ VO_4^{3-} = E_2 state). The changes in the CD spectra did not show full reversibility upon reversing the E_1 - E_2 transition, whereas those in the fluorescence spectra did. A temperature-dependent loss of α -helical content in the presence of Ca^{2+} was also observed. Intrinsic fluorescence measurements revealed an increase in

fluorescence intensity upon addition of Ca^{2+} . The change was fully reversed by ethylene glycol bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid. The increase in fluorescence intensity was partly reversed by adding ATP, an effect which is suggested to correspond to the " Ca^{2+} -occluded" form of the ATPase. The steady-state level of the fluorescence intensity was stable for several minutes in the presence of $100 \mu\text{M}$ ATP. By contrast, the decrease of fluorescence intensity induced by limiting concentrations of ATP ($=1 \mu\text{M}$) was only transient, indicating the decomposition of the phosphorylated intermediate of the ATPase and the reestablishment of the Ca^{2+} high-affinity form of the enzyme.

B. Sarkadi

Inhibitors of inositol trisphosphate-induced Ca^{2+} release from isolated platelet membrane vesicles. S. M. Seiler, A. J. Arnold and H. C. Stanton (Department of Cardiovascular Biology, Bristol-Myers Pharmaceutical Division, Evansville, IN 47721, USA). *Biochem. Pharmacol.* 36, 3331 (1987).

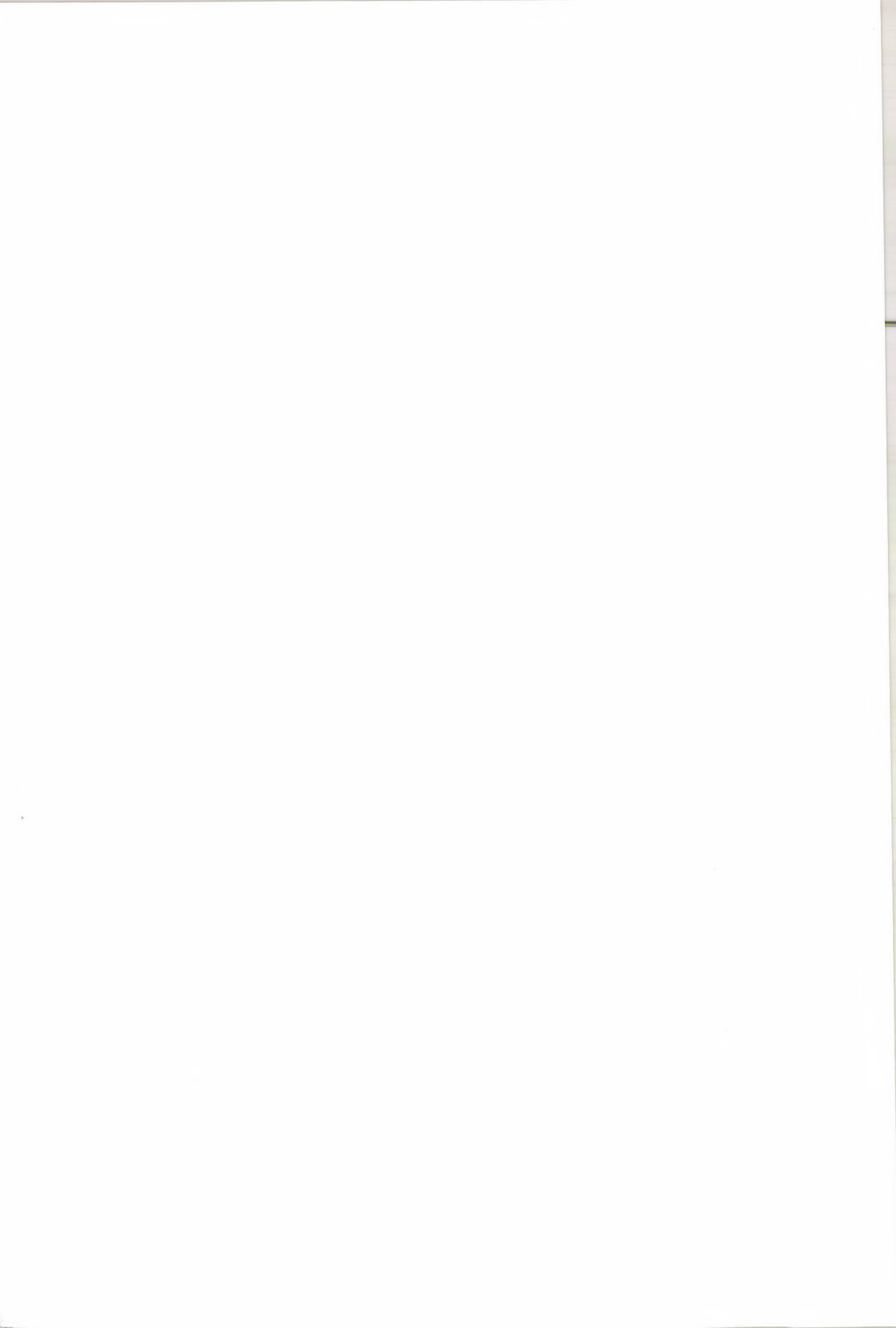
Platelet membrane vesicles accumulated Ca^{2+} in an ATP-dependent fashion, and

25–50% of the accumulated Ca^{2+} was released by the addition of $10 \mu\text{M}$ inositol 1,4,5-trisphosphate (IP_3). The concentration of IP_3 required for half-maximal Ca^{2+} release was approximately $0.5 \mu\text{M}$. The inhibition of IP_3 -induced Ca^{2+} release from these membrane vesicles by various agents was examined. On the plasma membrane Ca^{2+} channel blockers, cinnarizine and flunarizine were found to be potent inhibitors of IP_3 -induced Ca^{2+} release while having no effect on ATP-dependent Ca^{2+} uptake. The IC_{50} value for both cinnarizine and flunarizine as inhibitors of IP_3 -induced Ca^{2+} release was below 10^{-6}M . Nifedipine, verapamil, bepridil, and diltiazem did not significantly inhibit IP_3 -induced Ca^{2+} release at the highest concentration tested ($50 \mu\text{M}$). The "intracellular Ca^{2+} antagonists" ryanodine, TMB-8 (8-N,N-diethylamino)octyl-3,4,5-trimethoxybenzoate), dantrolene, trifluoperazine and chlorpromazine were not inhibitors of IP_3 -induced Ca^{2+} release at $50 \mu\text{M}$. The local anesthetics benzocaine and lidocaine weakly inhibited the IP_3 -induced Ca^{2+} -release with IC_{50} values of approximately 5 and $50 \mu\text{M}$, respectively, whereas other local anesthetics tested were less potent inhibitors. The potent inhibitors described may prove useful as probes of the IP_3 -induced Ca^{2+} release channels.

Ilma Szász

Printed in Hungary

Akadémiái Kiadó és Nyomda Vállalat, Budapest



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VOLUME 21 • NUMBER 3 • 1988

HU ISSN 0017-6559

AEMATOLOGIA

International Quarterly of Haematology

EDITOR-IN-CHIEF:

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VSP
P.O.B. 346
3700 AH ZEIST, The Netherlands

Subscription price for volume 21:

DM 312,— incl. surface mail
DM 330,— incl. air mail

Haematologia is covered in *Current Contents/Clinical Practice*, *ISI/Biomed* and *the SCI*.

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VOLUME 21 · NUMBER 3

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

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Morphometry of Human Blood Leukocyte Ultrastructure: Its Potential Value in Haematology

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(Received 14 May, 1987; accepted 5 August, 1987)

A review of the literature on ultrastructural morphometry of human blood leukocytes has been carried out. It is concluded that: (1) Blood leukocytes are particularly suitable for morphometric study. (2) Morphometric methods have proved valuable in defining differing cytological features of cells in various lymphoid malignancies, and in demonstrating ultrastructural differences (which could not otherwise have been detected) in monocytes and eosinophils corresponding to known functional changes. (3) Appropriate and valid numerical procedures are essential for determining morphometric equations and statistical probabilities; both morphometric measurement and statistical analysis are made easier by the use of computers. (4) Ultrastructural morphometry should ultimately find an important place in clinical haematology.

Keywords: granulocytes, leukocytes, lymphocytes, monocytes, morphometry, stereology, ultrastructure

Introduction

Blood leukocytes are particularly suitable for morphometric study, as many of the normal limitations of these methods are minimized. A sample of blood cells is likely to be representative of the whole population and leukocytes are presented in random orientation; it is relatively easy to identify the cells to be studied and they are usually at a similar stage of maturity. It is therefore not surprising that in recent years there have been increasing numbers of morphometric studies of human leukocyte ultrastructure in health and disease. However, the references are scattered throughout the literature. The extent of study of the different types of leukocyte varies widely and the minimal conditions for numerical validity have rarely been considered. It seemed important to review this work and to consider the use of appropriate numerical procedures, in order to highlight the potential value of the method in providing the haematologist with information not available from subjective assessment.

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Lymphocytes

Considerable attention has been given to the ultrastructural morphometry of normal blood lymphocytes. In earlier studies, quantitative measurements on separated lymphocytes of normal subjects were obtained as a basis of comparison with those of lymphoid cells in malignant states [1] together with the changes they undergo in culture after phytohaemagglutinin (PHA) stimulation [2]. It has subsequently proved profitable to consider different lymphocyte subsets separately. A stereological model cell was described in relation to small T lymphocytes obtained after passing mononuclear blood cells through a nylon-wool column [3] and this enabled changes associated with their activation with PHA to be defined [4]. Differences between T and non-T lymphocytes have also been quantitated using cells separated by E-rosetting techniques. In a series of 6 healthy volunteers, the average T cell had a smaller cell volume and cell surface area, a lower nuclear euchromatin-heterochromatin volume ratio and a smaller volume and surface area of rough endoplasmic reticulum (RER) [5]. Statistically significant differences were reported between T and B cells (identified by Fab-peroxidase labelling) [6] but cells from only a single donor were studied. Morphometric studies of T cell subsets using monoclonal antibodies and fluorescent activated cell sorting [7] have shown that the $T8^+$ suppressor/cytotoxic lymphocytes have larger cell volumes and surface areas, and a smaller nuclear:cytoplasmic (N:C) ratio than the $T4^+$ helper/inducer cells. They also have a larger Golgi zone, a more extensive RER and mitochondrial component and a greater amount of heterogeneous granules. These differences may be the morphological counterpart of the differing functions of the two subsets.

In studies of patients with chronic lymphocytic leukaemia (CLL) [1, 8–11], the size of the circulating lymphocytes has been of particular interest. In these studies the mean volume of CLL cells was within the normal range [11] although a bimodal distribution into small and large cell types with mean sizes outside this range has been suggested [1] and mitogen-transformed CLL cells have been reported to be smaller than expected [12]. B cell types of CLL, as anticipated from the studies of normal cells, appear to have a more irregular plasma membrane (shown by relating the cell surface area to that of a sphere of equivalent volume) and a larger volume of RER than their T cell counterparts [11]. In hairy cell leukaemia, the cells have been shown to have volumes significantly greater than normal [1] and have the largest surface areas, the most irregular plasma membranes and the lowest N:C ratios amongst the chronic leukaemias and malignant lymphomas [11].

In polymorphocytic leukaemia, the cells may be distinguished from CLL cells by their larger volume, lower N:C ratio and proportionately less heterochromatin [11]. They have also been reported to have a lower nuclear contour index (perimeter/ $\sqrt{\text{area}}$) than normal lymphocytes, with an increased number of mitochondrial profiles per section [13].

In B cell non-Hodgkin's lymphomas (NHL), the circulating centrocytes

may be distinguished from the cells of other B type malignancies by the high N:C ratio, the high degree of nuclear irregularity and the lower amount of RER [11]. In Sézary syndrome, morphometric assessment of nuclear shape expressed as the nuclear contour index may be helpful in establishing the diagnosis [14].

Ultrastructural morphometry has also provided quantitative data on the circulating blasts of patients with acute lymphoblastic leukaemia (ALL) [15–19], but here morphometry appears to be of less value as an aid to diagnosis and classification than in the more chronic states. When immunologically-defined subsets of lymphocytes have been compared, no differences were found in respect of the volumes of the cell, nucleus, nucleolus or hetero- and eu-chromatin or in the surface areas of the cell and nucleus, although there was a greater volume of RER in the B-derived cells and a greater volume of dense granules in the T-cell types [19].

It may be noted here that morphometric analysis has also been applied to the study of lymphoid cells in other sites, and some of the ideas would be valid for application to blood cells. For example, assessments of nuclear contour index or nuclear "form factor" ($4\pi \times \text{area}/\text{perimeter}^2$) have been found useful in the diagnosis of T cell lymphomas involving lymph nodes [20] or skin [21, 22], especially when combined with immunohistochemistry [23]. Techniques for stereological study of RER were developed and applied to marrow myeloma cells in different immunological types of myelomatosis [24] while ultrastructural analysis of the RER of jejunal plasma cells showed increased RER length in coeliac patients [25]. Light microscopy preparations have sometimes proved adequate for morphometric studies of lymphoid cells in the tissues. This has been particularly the case where changes in nuclear size and contour are helpful in diagnosis or classification. For example in sections of lymph node biopsies in non-Hodgkin's lymphomas, good correlation has been reported between morphometric findings and histopathological categories [26, 27], while in CLL, morphometric analysis of nuclear area distribution curves by skewness coefficient was found to be useful in identifying patients with a poorer prognosis [28]. However, using imprint preparations of lymph nodes and other tissues, morphometry proved less valuable in classifying the cell type in individual cases of non-Hodgkin's lymphoma, although significant differences were present between the centroblastic and B immunoblastic groups [29]. Smears of bone marrow cells have also been studied by these techniques in an effort to develop a computer-assisted method for classification of cells in ALL of childhood [30]. Such techniques seem both rapid and standardized and might give a meaningful prediction of survival.

Monocytes

While there is considerable evidence of dysfunction of mononuclear phagocytes in disease states [31], there has been no work until recently on the morphometry of blood monocytes, although some basic morphometric data has been reported for leukaemic monoblasts [16, 17, 32]. In morphometric studies of blood

monocytes carried out by us on 20 healthy subjects, 23 patients with Hodgkin's disease and 12 with non-Hodgkin's lymphoma [33, 34], 24 different measurements were made. The results were examined by statistical procedures which included multivariate analysis and showed that the mitochondrial contribution to the cellular ultrastructure was significantly less in Hodgkin's disease than in the other two groups, where a smaller total volume and surface area were found. This change appeared to be due to a reduction in the number of mitochondria (rather than their size). This finding, suggesting a reduced energy potential [35], would be consistent with previous studies which have shown decreased monocyte function in Hodgkin's disease [36–38]. In the above morphometric studies [33, 34], there were also significant changes in non-Hodgkin's lymphoma, which implied the presence of larger, more leptochromatic nuclei. This would be consistent with the nuclear changes present in skin window inflammatory macrophages in this condition [39]. The findings recall the diversity of functional changes which have been reported in monocytes in malignant disease presumably reflecting an interaction of factors of host and tumour origin [31]. The use of morphometric techniques should have an important place in detecting the presence of structural changes in monocytes in disease states.

Morphometric studies of macrophage development may also have important applications. Quantitative methods were recently used to measure changes taking place in normal human blood monocytes during suspension culture [40], and similar methods are currently being employed to see whether the pattern of differentiation is disturbed in malignant lymphoma. In a semiquantitative investigation of cultured blood monocytes in childhood ALL [41], an arbitrarily defined cellular differentiation index suggested that there was impairment of morphological differentiation.

Granulocytes

A particular advantage of blood granulocytes is that they can be readily identified by their specific granules, thus permitting the use of sections where the nucleus is not present, and avoiding the need for correction for nuclear-biased sampling. Some basic morphometric data for neutrophils and eosinophils in healthy subjects have been reported [42–44]. In a recent morphometric study of circulating eosinophils in 18 healthy individuals, 24 different ultrastructural parameters were examined. No significant differences were found between the male and female subjects [45]. In unpublished observations, the same methods have been used to study the blood eosinophils of a patient with idiopathic hyper-eosinophilic syndrome and, applying appropriate multiple comparison procedures, some significant differences were found which could not have been determined on subjective analysis of the micrographs alone. The most important findings are summarized in Table 1. The cells in hypereosinophilic syndrome were significantly larger and had a bigger surface area, the increase in surface area being much greater than would be expected from the increased cell volume. Increased numbers

Table 1

Morphometric characteristics (mean values) of blood eosinophils

	Normal [45]	Hyper- eosinophilic Syndrome
<i>Whole cell</i>		
Volume (f1)	255	370*
Surface area (μm^2)	228	330*
Excess surface membrane (μm^2)	34	81*
<i>Nucleus</i>		
Volume (f1)	51	62
Surface area (μm^2)	116	120
<i>Mitochondria</i>		
Volume (f1)	4.4	6.9*
Surface area (μm^2)	57	84*
Profiles/section	5.2	6.3*
Individual profile area (μm^2)	0.11	0.13
<i>Granules</i>		
Volume (f1)	43	30*
Surface area (μm^2)	408	331*
Profiles/section	27	22*
Individual profile area (μm^2)	0.20	0.16*

* Significant difference from normal.

of mitochondrial profiles were present whereas there were fewer granule profiles and these were of smaller size. The changes suggested premature activation of circulating eosinophils in hypereosinophilic syndrome. The larger cell size and the excess surface membrane (evidence of enhanced surface membrane activity) would be consistent with this concept, while an increase in mitochondrial numbers is known to be a concomitant of enhanced metabolic activity [35]. These findings may be related to the presence of abnormal proportions of light density eosinophils with enhanced function which has previously been reported in hypereosinophilic syndrome [46, 47]. The presence of fewer granule profiles (Table 1) would be consistent with inappropriate degranulation. A morphometric analysis of human granulocyte degranulation in vitro [48] has shown that there is a selective loss of larger granules and this may account for the smaller size of the profiles here.

A few quantitative measurements have been made of granulocyte precursor cells (myeloblasts and promyelocytes) in acute leukaemias. There were significantly more granules and long endoplasmic reticulum in leukaemic myeloblasts when compared with normal myeloblasts [17] but the mitochondria were approximately equal in number and profile area [49]. Differences were also shown in the pattern

of ultrastructural features in different "paraleukoblasts" in four forms of acute leukaemia [16]. In a recent study of six patients with acute myeloid leukaemia [50], planimetry was used to demonstrate differences in myeloperoxidase-deficient polymorphs in blood smears.

It is surprising that so few studies of granulocyte morphometry have been made in clinical states. Changes in cell size, nuclear segmentation and granule morphology are known to occur in both inherited and acquired conditions e.g. in Pelger – Huët anomaly, vitamin B12 deficiency, chronic granulocytic leukaemia and toxic states, and it seems likely that morphometry would provide valuable additional information in these conditions.

Numerical procedures

For quantitative studies, appropriate and valid procedures are required both to determine morphometric equations and to assess statistical probabilities. It is apparent from the literature reviewed above, that these requirements have not always been met. Textbooks of morphometry tend to concentrate on the morphometric equations and some discussion may therefore be useful in relation to the valid analysis of results and probabilities.

The validity of the analysis rests upon the use of unbiased estimators, and procedures theoretically derived for this purpose only yield unbiased results if all the statistical test assumptions have been fulfilled. Prior to statistical analysis, results expressed as percentages (e.g. mitochondrial volume fractions) may need to be transformed to ensure independence of variance to the mean; the normality of distribution and homogeneity of variances should also be tested where appropriate; and ratios where numerator and denominator both vary as normal distributions should be excluded (as these generate Cauchy distributions which do not have finite means and variances, thus invalidating common sampling assumptions).

Statistical tests such as the Student-*t* test are only valid provided certain *a priori* conditions are met (as mentioned above) and single comparisons are to be made. Where more than one comparison is to be carried out, multiple comparison procedures are essential to avoid false conclusions regarding significant changes. Such procedures include the Dunn – Šidak procedure [51] and Multivariate Analysis [52 – 54]. The Dunn – Šidak procedure makes use of the fact that the overall significance level (*S*) for comparing a number of parameters (*K*), at the individual significance level of α , is given by the Bonferroni relation:

$$S = 1 - (1 - \alpha)^K$$

The individual significance levels (α) are determined from this relation, after setting the desired overall significance level (*S*). For example in a recent study of eosinophil granulocytes [45] where 13 independent parameters were being compared in 9 male and 9 female subjects, individual significance levels (α) had to

be set at 0.002 to give an overall significance level (S) of <0.05 . Similarly when adequate sample size is being calculated for a morphometric study where multiple comparisons are to be made, the Bonferroni relation should be used to determine the individual significance levels in the calculations.

Both Univariate and Multivariate Analysis of Variance (ANOVA and MANOVA) are applicable when comparing several parameters in the presence of a number of other variables. Each has a different role. For example, ANOVA was suitable for a morphometric study of human macrophage development *in vitro* where 9 cell measurements were individually studied at 4 time intervals [40] while MANOVA was required for a morphometric study of blood monocytes where properties of the cell, cytoplasm, nucleus, intranuclear chromatin and mitochondria were simultaneously analysed for three data sets, i.e. normal, Hodgkin's disease and non-Hodgkin's lymphoma, allowing full comparisons to be made between the sets (normal v Hodgkin's disease; normal v non-Hodgkin's lymphoma; and Hodgkin's v non-Hodgkin's lymphoma) [34].

The increasing availability of modern computers upon which sophisticated statistical packages can be implemented, has made the application of valid numerical procedures much easier. However, it must be emphasized that all the test assumptions pertinent to the particular package must be met if valid conclusions are to be drawn.

Summary and Conclusions

Morphometry of peripheral blood leucocytes has led to clearer definition of the characteristics of different cell types and hence a greater understanding of the ultrastructural changes associated with disease states. In particular, the distinctive features of different types of circulating lymphocyte and the T cell subsets have been quantitated, and it has been possible to define the differing cytological features of cells in chronic lymphocytic, hairy cell and prolymphocytic leukaemias, and in Sézary syndrome and non-Hodgkin's lymphoma. With regard to blood monocytes, subtle ultrastructural changes have been demonstrated which correspond to known functional defects in malignant lymphoma, and the study of defective monocyte differentiation *in vitro* appears promising. Surprisingly few morphometric studies of granulocytes have been made, but cellular changes have been defined in the circulating eosinophils of hypereosinophilic syndrome and in the blast cells of acute myeloid leukaemia.

The importance of using valid statistical procedures for the analysis of results has been emphasised. Population variances and normality of distribution must be taken into account and multiple comparison procedures are often essential.

Blood leukocytes are particularly well-suited to morphometric study and the increasing use of computers makes both morphometric measurement and the analysis of results much easier. While the application of quantitative methods is still mostly at the research stage in characterising leukocyte changes in disease, they will ultimately find a place in everyday haematological practice.

Acknowledgements

We thank the Trent Regional Health Authority and Weston Park Hospital for provision of facilities: the Yorkshire Cancer Research Campaign for financial support: Mrs P. Burke for secretarial assistance.

Appendix

Standard evaluation procedures and statistical methods used in the authors' work

All studies are made on cells in random orientation, samples being collected and processed for electron microscopy by identical methods. Cells are selected for examination in an independent uniform random manner, the magnification of each micrograph being obtained by reference to a test graticule. For morphometric analysis, micrographs are covered by a transparent test grid bearing a rectangular lattice of known spacing, and point and intersect counting made on the features to be studied. Areal fractions (numerical equivalent to volume fractions) and surface-to-volume ratios are calculated from conventional formulae, compensating for non-equatorial sectioning and (where appropriate) for nuclear-biased sampling [33]. Adequacy of sample size is verified by ensuring that relative standard errors do not exceed 0.05 [45]. (In particular circumstances, pattern analysis is also used to estimate the distribution and relative amounts of nuclear heterochromatin and euchromatin [33, 34]). The raw data are tested for normality and equality of variances and when these are not shown, the data are logarithmically transformed for the subsequent analysis [40]. Statistical analysis is only carried out on the measurements which are considered to be independent of one another [40]. The method used depends on the design of the particular investigation. We are currently using a SPSS-X statistical package run on a main-frame computer to carry out both multivariate and univariate analysis of variance in an investigation of macrophage development in malignant lymphoma where data for 9 parameters are being analysed at 5 culture intervals in 3 subject groups and the effects of age, sex, cellular immune status, histological type and disease stage taken into account. Other examples of less complexity are given in the section on 'numerical procedures' above.

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Differentiation Induction Therapy of Acute Myelogenous Leukaemias

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(Received 8 July, 1987; accepted 27 August, 1987)

This article reviews the progress achieved in the field of inducing differentiation in human myeloid leukaemia *in vitro* and the need for applying this model in leukaemia therapy *in vivo*. The pre-clinical evaluation of differentiating agents in human myeloid leukaemia is analysed. The clinical experience with differentiation induction therapy in acute myeloid leukaemia is reviewed. This review examines the prospective application of this new form of therapy in patients with acute myeloid leukaemia.

Keywords: chemotherapy, differentiation, monoblastic leukaemia, myeloblastic leukaemia

Introduction

The prevailing dogma of the 1950s and 1960s was "once a cancer cell, always a cancer cell" [1]. Studies of differentiation of cancer cells were not particularly encouraged at that time [2]. In the mid 1960s, an intensive study began of peptide growth factors (colony stimulating) and small effectors that could induce differentiation of myeloid and erythroid leukaemia cells [3]. This approach has had a major impact in leukaemia research [4]. The important discovery in 1971 that dimethylsulfoxide (DMSO) induced erythroid differentiation in the Friend murine erythroleukaemia cells (FMEL) led to a decade of intense research on the small effector molecules [5–9]. This research has been directed towards identifying new inducers and helping to understand the biological and biochemical mechanisms involved in the induction of differentiation in leukaemic cells. Since then, the list of inducing agents has been greatly extended (Table 1). Human acute myeloid leukaemia (AML) arises from a neoplastic transformation at the pluripotent stem cell level, leading to a block in cell maturation at the recognizable stage of myeloblast or promyelocyte [10, 11]. The leukaemic blasts are unable to mature to functional end cells, they remain in the proliferative pool and rapidly accumulate [12]. It seems likely that leukaemic cells fail to differentiate because of an alteration of specific genes or their products that are obligatory for differentiation. Another possibility may be a disturbed cellular mechanism that is normally required for a response to exogenous differentiating factors. Extensive

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Table 1
Inducers of human myeloid leukaemic cells differentiation in culture

Differentiation inducer	Concentration	Target cells	Mature cells	References
<i>I-Polar-Planer Compounds</i>				
Dimethylsulphoxide (DMSO)	1.25 %	HL-60 & fresh AML	Granulocytes	21-25
Dimethylformamide (DMF)	60-100 mM	HL-60	Granulocytes	22, 25, 26
N-methylacetamide	20 mM	HL-60	Granulocytes	22, 25, 26
N-methylformamide	150-180 mM	HL-60	Granulocytes	22, 25, 26
1-methyl-2-piperidone	4 mM	HL-60	Granulocytes	22, 25, 26
Piperidone	37.5 mM	HL-60	Granulocytes	22, 25, 26
Butyric acid	0.06 mM	HL-60 & fresh AML	Granulocytes	21, 23, 26, 27
Hexamethylene bisacetamide (HMBA)	2-5 mM	HL-60	Granulocytes	21, 22, 24, 26
Diethylacetamide	10 mM	HL-60	Granulocytes	26
N-ethylformamide	100 mM	HL-60	Granulocytes	26
<i>II-Vitamins Analogues:</i>				
Retinoic acid	1 μ M	HL-60 & fresh AML	Granulocytes	23, 24, 28, 29, 30, 31
1,25-dihydroxyvitamin D ₃	1 μ M	HL-60 & fresh AML	Macrophages	32-36
<i>III-Cytotoxic Drugs:</i>				
<i>Nucleosides</i>				
Cytosine arabinoside (Ara-C)	2 μ M	HL-60 & fresh AML	Monocytes	22, 24, 37, 38, 39, 40, 41
5-azacytidine	1 μ M	HL-60 & fresh AML	Both	38, 42, 43
2-deoxy-5-azacytidine	1 μ M	HL-60 & fresh AML	Both	38, 42, 43
2-B-D-ribofuranosyl-selenazole-4-carboxamide	2 μ M	HL-60	Both	39, 44
<i>Anthracyclines</i>				
Daunomycin I	25 ng/ml	HL-60	Granulocytes	21, 32, 39, 45, 46
Mitomycin C	20-40 nM	HL-60	Granulocytes	31, 45, 46
Aclacinomycin A	8 M	HL-60	Granulocytes	24, 46
Actinomycin D	5 ng/ml	HL-60 & fresh AML	Granulocytes	24, 39, 46
Methotrexate	10 nM	HL-60	Granulocytes	31, 38
6-thioguanine	1.5 μ M	HL-60	Granulocytes	21

Table I (continued)

Differentiation inducer	Concentration	Target cells	Mature cells	References
Homo-harringtonine	0.01 $\mu\text{g/ml}$	HL-60 & fresh AML	Monocytes	47
Hydroxyurea	8 $\mu\text{g/ml}$	HL-60	Granulocytes	21, 22
Vincristine	5 ng/ml	HL-60	Granulocytes	21, 22
Bromodeoxy-uridine	3 $\mu\text{g/ml}$	HL-60	Granulocytes	45, 48
<i>V-Phorbol Esters &</i>				
<i>Teleocidin:</i>				
Phorbol myristate acetate (PMA)	16 nM	HL-60 & fresh AML	Macrophages	21, 23, 24, 30, 49, 50, 51, 52, 53
Teleocidin	50 nM	HL-60	Macrophages	51, 53
<i>V-Lymphokines & Cytokines:</i>				
Alpha-A-interferon	5,000 U/ml	HL-60	Monocytes	54, 55
Alpha-D-interferon	1,000 U/ml	HL-60	Monocytes	54, 55
Gamma interferon	1,000 U/ml	HL-60	Macrophages	36, 54, 55, 56, 57
<i>VI-Others</i>				
Alkyllysophospholipids	1 $\mu\text{g/ml}$	HL-60	Both	23, 58
Glycosphingolipid (GM3)	50 nmol/ml	HL-60	Monocytes	59
Tunicamycin	0.5 $\mu\text{g/ml}$	HL-60	Granulocytes	60
Calmodulin antagonists	1–5 mM	HL-60	Granulocytes	61
Azobenzene carboxylic acids	5 μM	HL-60	Granulocytes	62
Stilbenecarboxylic acids	5 μM	HL-60	Granulocytes	2
Phenylcarbamoylbenzoic acid	NR*	HL-60	Granulocytes	63
Polyeenamides	NR	HL-60	Granulocytes	63
Arphamenine A	100 $\mu\text{g/ml}$	HL-60	Granulocytes	64
Forphenicine	10 $\mu\text{g/ml}$	HL-60	Granulocytes	64

* NR=not reported

reports of inducing differentiation in leukaemic cells clearly demonstrate that the maturation block is reversible [13]. The arrest of cell proliferation by induction of differentiation to mature cells bypasses the genetic changes which cause malignancy, thus, by inducing differentiation, the multiplication of cells is stopped. This phenomenon has changed our ideas on the treatment of acute leukaemias [14–17]. An alternative approach to the conventional cytotoxic therapy would be to use differentiating agents capable of inducing a terminal differentiation of leukaemic cells to mature forms with no further proliferative potential [18]. It is anticipated that this differentiation induction therapy could be applied using drug

doses lower than those required for successful cytotoxic therapy. Therefore, the induction of differentiation would be of therapeutic value both in reducing the body burden of leukaemic cells and in minimizing the major toxicities caused by conventional cytotoxic therapy [19].

The management of acute myeloid leukaemia (AML), in spite of the appreciable progress since the early 1960s, has failed to achieve results similar to those in childhood acute lymphoblastic leukaemia. Although the median survival time for those who achieve a remission is now about 2 years, the majority of patients with AML still die within 18 months of diagnosis [20]. This supports the application of a new differentiation induction therapy in AML, which at the very least may represent a way to achieve the same limited results of our current cytotoxic therapies with much less distress to the patient.

Pre-Clinical Evaluation of Myeloid Leukaemia Differentiation Inducers

Two leukaemic cell lines: the Friend murine erythroleukaemia virus-induced cell line (FMEL) and the human acute promyelocytic leukaemia cell line (HL-60) were extensively studied. When exposed to many differentiating agents *in vitro*, both (FMEL) and (HL-60) cell lines lose their proliferative capacity and develop the morphological, biochemical and functional characteristics of their mature counterparts. Both cell lines are at a more mature developmental stage than the typical acute leukaemia cells and may, in fact, be primed to differentiation. Therefore caution is necessary when extrapolating results from these models for possible future clinical trials with differentiating agents. Also, these cell lines represent a single population of leukaemic cells, while fresh myeloid leukaemic cells from patients are a heterogeneous group of malignant cells [65]. Thus, a pre-clinical screening of the response of fresh human leukaemic cells in primary culture from patients with AML towards various differentiating agents might provide a basis for an *in vivo* clinical application. Only few studies with fresh human myeloid leukaemic cells from patients in primary culture have been reported.

In the proposed clinical trials with differentiating agents, a terminal differentiation of the leukaemic cells should be achieved, i.e. almost all leukaemic cells mature with no residual blasts. In order to overcome the maturation arrest, treatment of the leukaemic cells with a selective combination of differentiating agents is one of the possibilities. Recently some reports suggested the usefulness of this method [66, 67, 68]. A pre-clinical screening of the response of fresh human leukaemic cells from patients with AML in primary culture using several combinations of chemotherapeutic agents would be of great interest in supporting the use of maturation induction as an alternative to current anti-leukaemic therapy.

Clinical Trials: Experience and Prospects

Many clinical studies applying the differentiation induction therapy in patients with acute myeloid leukaemia have been published either as anecdotal case reports, or as an abstract and/or small clinical trial. These patients were in relapse, either refractory to the conventional cytotoxic treatment or very old (age > 70), or cytotoxic therapy was contraindicated (e.g. hypoplastic bone marrow) [69–98].

Cytosine arabinoside (Ara-C), the drug of choice in the treatment of AML has been used in a subcytotoxic low doses. 60% of patients achieved a complete remission. The attractiveness of low doses of Ara-C in terms of minimal toxic side effects and potential use as an outpatient form of treatment guarantees continued drug trials. Two patients with acute promyelocytic leukaemia (APL) were treated with 13-cis retinoic acid: one achieved complete remission and one showed partial remission. Daily and intermittent administration of acalainomycin-A in low noncytotoxic doses achieved a complete remission in a patient with AML. On the other hand, sodium butyrate failed to achieve any remission in 8 patients and showed only a partial remission in a child with refractory AML. This disappointing result of butyrate therapy is due to the fact that the concentrations known to be effective *in vitro* (600 μ M) cannot be given *in vivo* because of their toxicity. The same problem arises with other polar-planar compounds such as dimethylsulphoxide (DMSO) and dimethylformamide (DMF). It is obvious that these compounds are in the early stage of development as drugs and caution is needed when using them in clinical trials.

In two studies, 6 patients were treated with a differentiating agent in combination with chemotherapeutic agents, biological response modifiers and/or other inducers. Alpha-interferon in combination with cimetidine resulted in a complete remission in an elderly female patient with AML. Five patients were treated with a combination of 13-cis retinoic acid and 1,25 dihydroxy-vitamin D₃ in addition to alpha-interferon and/or low dose cytosine arabinoside (Ara-C). 60% of patients achieved a complete remission. The evaluation of this form of combination therapy should be performed in larger randomized clinical trials.

Results from these limited clinical studies showed a complete remission in 60% of patients. The patients with AML who seem most likely to benefit from this differentiation induction therapy are:

- a) those aged 60 or more
- b) those in a relapse and refractory to conventional cytotoxic therapy
- c) those in whom cytotoxic therapy is contraindicated, e.g. hypoplastic.

Further larger, controlled clinical trials based on differentiation induction therapy for the above listed AML patients are necessary for introducing this type of therapy.

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Role of Actin Polymerization in Monocyte Phagocytosis of Yeast Cells Effect of Cytochalasin B

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(Received 1 April, 1987; accepted 4 August, 1987)

The role of the cortical actomyosin-like contractile system in monocyte phagocytosis was studied by means of inhibition with cytochalasin B. Monocyte phagocytosis of yeast cells was assayed with a fluorescence extinction technique of surface-located phagocytosis which distinguishes between adherence and ingestion phases of total phagocytosis. Cytochalasin B, 10 $\mu\text{g}/\text{ml}$, inhibited monocyte phagocytosis by 20–30%. The cytochalasin inhibition was significant and restricted to the ingestion phase. The finding supported the hypothesis that the contractile structures of the cell have a contributory role in IgG-mediated monocyte phagocytosis on a surface. It is suggested that the cytochalasin effect reflected the role of chemotaxis in monocyte phagocytosis assayed by the present technique.

Keywords: monocyte, phagocytosis, motility, cytochalasin B

Leucocyte phagocytosis as a part of the host defence against infections is a complex process comprising leucocyte adherence to the vascular endothelium, locomotion through tissues, attachment (adherence) to invading microorganisms, followed by engulfment and lysis of the microorganisms. It should be emphasized that a supporting surface appears to be essential for the mentioned activities of the leucocyte [1, 2].

Most tests of leucocyte phagocytosis elucidate only one or a few of the partial processes occurring *in vivo*. A comparatively complete and simple method was introduced by Hed [3] for the study of neutrophil phagocytosis. He confined phagocytosis to a glass surface with scattered neutrophils and an excess of yeast cells. The initial gripping of yeast cells (adherence) was discriminated from completed engulfment of yeast cells by a fluorescence quenching technique. This technique measures a combination of neutrophil chemotaxis, adherence and engulfment of target cells. Unfortunately, only the adherence step and the engulfment step can be assessed directly.

We have adopted the Hed technique for the study of monocyte phagocytosis [4]. To our knowledge, the role of chemotaxis in monocyte phagocytosis measured by this technique has hitherto not been analysed quantitatively. The aim of the

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present study was to quantitate this role by inhibition of the contractile structures of the monocytes with cytochalasin B (CB). The experiment was thus designed with one CB concentration subinhibitory for leucocyte locomotion, 1 $\mu\text{g/ml}$, and one CB concentration completely inhibitory, 10 $\mu\text{g/ml}$ [5, 6, 7].

Material and Methods

Preparation of yeast cell suspension

Baker's yeast (*Saccharomyces cerevisiae*) was suspended in saline, washed twice, boiled 30 min, washed twice, and labelled with FITC in a carbonate buffer, pH 10.2, 10^8 cells per ml of FITC buffer with a FITC concentration of 0.1 mg/ml for 60 min at $+37^\circ\text{C}$. The cells were washed three times with saline, re-diluted to 10^8 cells per ml, and frozen at -17°C in 0.5 ml aliquots. Before the experiment, the FITC-labelled yeast cells were opsonized with heat-inactivated human serum which had been stored at -70°C for less than two weeks. Serum, 0.5 ml, was mixed with 0.5 ml of the FITC-labelled yeast cells, and the mixture was incubated at $+37^\circ\text{C}$ for 30 min. The cell suspension was then washed twice and resuspended in 2 ml of saline.

Complete medium

The complete medium consisted of human serum, 165 ml, L-glutamine 20 mM, 7 ml, Ham's F 10 medium 500 ml (Flow Laboratories, Irvine, Ayrshire, Scotland), amphotericin B 1.7 $\mu\text{g/ml}$, streptomycin 4 $\mu\text{g/ml}$, and benzylpenicillin 2.4 $\mu\text{g/ml}$.

General experimental conditions

The monocyte migration (chemotaxis) over the glass surface toward the yeast cell could not be measured separately in the assay. Yeast cell adherence to the monocyte was defined as visible membrane contact between a brown yeast cell and a monocyte. Yeast cell engulfment by a monocyte was defined as the presence of a fluorescent yeast cell within a monocyte. Adherence and engulfment together made up the total phagocytosis. The distance between the yeast cells on the slide was estimated to be 20–50 μm , but the yeast cells often lay in clusters. The distance between monocytes was estimated to be 100–500 μm . The number of ingested yeast cells per monocyte ranged from 0–12. An excess of non-phagocytosed yeast cells remained in the preparation after stopping phagocytosis.

Blood sampling

Heparinized blood, 16 IU/ml, was obtained from healthy blood donors from the Blood Transfusion Centre of the hospital.

Isolation of mononuclear leucocytes

Mononuclear leucocytes (L-MNs) were isolated from peripheral blood by the one-step metrizoate-Ficoll procedure described by Böyum 1968 [8], using Lymphoprep® (Nyegaard, Oslo, Norway). The L-MNs were then washed twice in "complete medium" and resuspended to a concentration of 5×10^6 cells per ml.

Incubation for monocyte adherence to the glass surface

L-MNs, 1×10^6 cells in 200 μ l complete medium, were then seeded on sterile slides in Petri dishes and incubated in a humidified chamber with 5% CO₂ at 37 °C for 30 min to allow the monocytes to adhere to the glass surface. For the removal of lymphocytes from the preparation, the slides were then rinsed with pre-warmed complete medium at 37 °C.

Table 1

The effect of cytochalasin B on adherence, engulfment, and total phagocytosis of yeast cells by monocytes. D: cell donor. S: sex. C1: control cells without DMSO. C2: control cells with DMSO 0.25% v/v. T1: test cells with cytochalasin B1 μ g/ml. T2: test cells with cytochalasin B10 μ g/ml. \bar{x} : arithmetic mean. Q₁ - Q₃: interquartile range. Figures denote number of yeast cells associated with 50 monocytes

D	S	Age	Adherence			
			C1	C2	T1	T2
1	f	39	15	12	12	27
2	f	43	27	15	27	14
3	f	26	14	32	17	13
4	f	43	11	33	11	12
5	m	33	18	7	13	15
6	m	33	16	14	7	8
7	m	25	15	12	24	28
8	m	54	18	15	12	8
9	m	60	15	17	6	21
10	m	25	19	21	9	17
11	m	64	29	6	11	15
12	m	59	44	26	36	36
13	f	40	8	12	20	15
14	m	39	9	24	17	17
15	m	32	12	36	29	24
16	m	50	31	12	9	17
17	m	52	26	7	11	2
18	f	21	42	19	40	25
\bar{x}		41	21	18	17	17
Median		40	17	15	13	16
Q ₁ - Q ₃		31 - 53	14 - 28	12 - 25	11 - 25	13 - 25
Range		21 - 64	8 - 44	6 - 36	6 - 40	2 - 36
Significance			p = 0.478			

Table 1 (continued)

D	S	Age	Engulfment			
			C1	C2	T1	T2
1	f	39	125	141	95	87
2	f	43	63	86	118	83
3	f	26	91	113	46	99
4	f	43	85	88	85	79
5	m	33	84	82	63	59
6	m	33	85	79	64	91
7	m	25	115	112	95	43
8	m	54	67	96	95	66
9	m	60	90	72	100	60
10	m	25	121	109	99	39
11	m	64	100	106	131	122
12	m	59	67	57	99	49
13	f	40	117	127	94	68
14	m	39	144	141	103	111
15	m	32	89	60	94	55
16	m	50	97	115	115	59
17	m	52	43	43	65	36
18	f	21	70	123	126	79
\bar{x}		41	92	97	94	71
Median		40	90	101	95	67
Q ₁ - Q ₃		31-53	69-116	77-117	80-106	54-88
Range		21-64	43-144	43-141	46-131	36-122
Significance			p = 0.003			
			p = 0.840			

Cytochalasin B and DMSO

Cytochalasin B (Sigma, St. Louis, Mo., USA) was dissolved in dimethylsulphoxide (DMSO, Sigma), 4 mg/ml and stored at -20°C . Prior to the experiment, cytochalasin B was diluted in phosphate buffer, pH 7.4, and added to the test solutions to final concentrations of 1 and 10 $\mu\text{g}/\text{ml}$ (2 and 20×10^{-6} M). The corresponding DMSO concentrations were 0.025 and 0.25% v/v. Thus, a control of the DMSO effect was introduced (C2, DMSO 0.25%, Table 1).

Pre-incubation with cytochalasin B

Control slides were again incubated in complete medium for 30 min at $+37^{\circ}\text{C}$ in 5% CO_2 in humidified chambers. Test slides were incubated likewise with cytochalasin B, 1 or 10 $\mu\text{g}/\text{ml}$.

Table 1 (continued)

D	S	Age	Phagocytosis			
			C1	C2	T1	T2
1	f	39	140	153	107	114
2	f	43	90	101	145	97
3	f	26	105	145	63	112
4	f	43	96	121	96	91
5	m	33	102	89	76	74
6	m	33	101	93	71	99
7	m	25	130	124	119	71
8	m	54	85	121	107	74
9	m	60	105	89	106	81
10	m	25	140	130	108	56
11	m	64	129	112	142	137
12	m	59	111	83	135	85
13	f	40	125	139	114	83
14	m	39	153	165	120	128
15	m	32	101	96	123	79
16	m	50	128	127	124	76
17	m	52	69	50	76	38
18	f	21	112	142	166	104
\bar{x}		41	112	116	111	89
Median		40	108	121	111	84
Q ₁ - Q ₃		31 - 53	100 - 129	92 - 140	91 - 127	74 - 106
Range		21 - 64	69 - 153	50 - 165	63 - 166	38 - 137
Significance			p = 0.004			
			p = 0.765			

Phagocytosis experiments

Yeast cells labelled with fluorescein isothiocyanate, FITC (Merck, Darmstadt, FRG), and opsonized with human serum were added to control slides and test slides, 2.5×10^7 yeast cells in 200 μ l saline. The yeast cell suspension added to the test slides contained cytochalasin B, 1 or 10 μ g/ml. Incubation was performed for 30 min at +37 °C in 5% CO₂. The phagocytosis was stopped by dipping the slides in ice-cold saline. The fluorescence of non-ingested yeast cells was then quenched by dripping crystal violet in saline (Merck, Darmstadt, FRG), 0.5 mg/ml, onto the slides. The staining time was 10 s. The stain was decanted and the preparation was sealed with a coverslip. Monocyte-adherent yeast cells (brown) and ingested yeast cells (fluorescent) were then counted in 50 consecutive monocytes at 320 \times magnification with a Zeiss incident-light microscope. Control and test samples from a cell donor (N = 18) were run simultaneously (Table 1, Fig. 1).

Glassware

The glassware was thoroughly washed, rinsed in demineralized water and 70% ethanol, and autoclaved at +120 °C for 20 min.

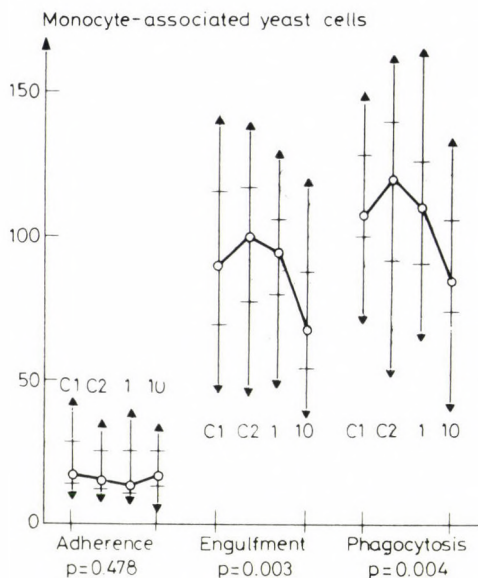


Fig. 1. Cytochalasin B influence on yeast cell adherence to monocytes, monocyte engulfment of yeast cells, and total phagocytosis (the sum of adherence and engulfment). Data from 18 cell donors (Table 1). Median (○), interquartiles (—) and extreme values (arrows). C1: control without DMSO. C2: control with DMSO 0.25%. T1: cytochalasin B 1 $\mu\text{g}/\text{ml}$, T2: cytochalasin B 10 $\mu\text{g}/\text{ml}$

Statistics

The Friedman two-way analysis of variance was calculated according to Siegel 1956 [9]. The basic figures were provided by the numbers of yeast cells associated with 50 monocytes.

Results

The basic data are shown in Table 1 with a graphical representation of central tendencies and dispersion of individual values in Fig. 1.

Cytochalasin B, which has low water solubility, had to be dissolved in DMSO, maximum final concentration 0.25% v/v. This DMSO concentration did not affect monocyte phagocytosis significantly (Fig. 1, Table 1).

The data of Table 1 suggest that cytochalasin B, 10 $\mu\text{g}/\text{ml}$, inhibited monocyte phagocytosis of yeast cells significantly to 20–30%. The cytochalasin B inhibition of monocyte phagocytosis was confined to the engulfment process. Adherence was unaffected. Cytochalasin B, 1 $\mu\text{g}/\text{ml}$, had no effect on the different steps of total monocyte phagocytosis.

Discussion

The main finding of the present study was that cytochalasin B, 10 $\mu\text{g/ml}$, inhibited the ingestion phase of monocyte phagocytosis by 20–30% (Table 1, Fig. 1). It is reasonable to assume that this inhibition reflects the relative importance of cell locomotion in monocyte phagocytosis measured by the present assay, since leucocyte locomotion was reported to be arrested by such CB concentrations [5, 6, 7].

The present findings are in agreement with previous results on neutrophil phagocytosis using the same technique [10] and on monocyte phagocytosis using other techniques [6, 11]. However, methods are reported in which cytochalasin B appeared not to inhibit macrophage phagocytosis [12].

Cytochalasin B is used extensively as a probe for actin polymerization due to its ability to cap the fast-growing barbed end of the fibrous actin molecule [13]. The various *cytochalasins* (from Greek *chhalasis*, relaxation) were initially thought to interfere with cell locomotion and the cytoplasmic cleavage of cell division by paralysis of contractile processes [14]. Further investigations showed, however, that the presence of cytochalasin B in the cell medium induced a cell contraction [5, 6, 7, 15], which was reversible only after CB removal.

In addition to interference with the "relaxation" phase of contractile processes by actin capping, cytochalasin B is reported to be a potent inhibitor of hexose influx into the cells [5, 6]. Control experiments suggested, however, that the CB inhibition of leucocyte phagocytosis could not be explained by CB inhibition of hexose uptake [5, 6].

The above-mentioned observations are compatible with the view that the variable CB effects on leucocyte phagocytosis, reports range from 0–99% [6, 12], reflect the relative importance of leucocyte locomotion in different experimental settings.

Like cytochalasin B, the main microtubule antagonists suppress monocyte phagocytosis by 20–30% when measured by the present technique [4, 16, 17]. The observed effect of cytochalasin B and microtubule antagonists is consistent with the hypothesis that actin kinetics and microtubule kinetics play a contributory role in the contractile events of IgG-mediated phagocytosis (cf. 10, 18).

Acknowledgements

We thank Ms. Lena Backlund for skillful technical assistance. The study was supported by the Swedish Cancer Society No. 2234-B87-02X, Lion's project No. 386/85 and the National Rheumatism Society.

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Changes of Contact Phase Proteins During Haemodialysis

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(Received 24 April, 1987; accepted 22 June, 1987)

Functional and immunological activities of factors XII and XI, Prekallikrein, high molecular weight kininogen and C-1-esterase inhibitor were studied in eight patients before, during and after haemodialysis. During and after dialysis sessions a significant decrease in the functional activities of Prekallikrein and high molecular weight kininogen ($p < 0.05$) could be detected, whereas their immunological levels did not change. Furthermore C-1-esterase inhibitor increased after dialysis ($p < 0.05$). These results indicate activation of the kinin system during haemodialysis.

Keywords: contact phase proteins, haemodialysis

Introduction

The contact of blood with certain surfaces is the triggering mechanism of a set of enzymatic reactions involving different systems such as blood coagulation, complement, the fibrinolytic pathway and the kinin system [1]. During haemodialysis, blood is in contact with foreign surfaces, and the data regarding a possible activation of the kallikrein-kinin system in such circumstances are contradictory [2, 3, 4]. Kinins are generated through the enzymatic cleavage of kininogens by kallikrein, and the activity is induced by its precursor, prekallikrein (PK) by the action of activated Hageman factor (FXII) [5].

The present study was undertaken to investigate the potential changes in the functional and immunologic activities of plasma proteins, such as factors XII and XI, PK, high molecular weight kininogen (HMWKg) and C-1-esterase inhibitor (C-1-INH), all of them involved in the plasma contact activation system, in patients submitted to haemodialysis.

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Materials and methods

Patients and haemodialysis sessions

Our study included eight patients (females and males: age range 19–78 years, mean 58) who were attending the renal unit of the University Hospital. According to conventional clinical, radiological and histological criteria, chronic glomerulonephritis was diagnosed in all patients. The coagulation screening test (prothrombin time, activated partial thromboplastin time and thrombin time) were normal before the onset of our study (data not shown). The patients were on regular haemodialysis (12 hours per square meter per week over three sessions per week). Each dialysis session lasted approximately 4 hours. Dialyses were performed using hollow fiber cartridges. The blood flow rate was over 300 ml per minute, and the water flow rate was 500 ml per minute. Intravenous heparin was given at a loading dose of 2500 U followed by 1000 U hourly to maintain an adequate anticoagulation status throughout dialysis. This dose of heparin was sufficient to obtain a recalcification time between 2.5 and 3.5 fold the control value throughout the dialysis session. None of these patients had hypotension or any other complications during dialysis. All patients were aware of the nature and purpose of the study, and all gave informed consent according to the Declaration of Helsinki.

Blood samples

Whole blood samples were taken before the onset of haemodialysis, 2 hours after and immediately after termination of the sessions. The blood samples were mixed with 0.13 M sodium citrate at a 9:1 volume ratio and platelet-poor plasma was obtained as previously reported [6]. The samples were stored at -80°C until use. Pooled normal plasma was similarly prepared from 20 normal donors.

Coagulation assays

Procoagulant activities of factors XI, XII and HMWKg were assayed in a one-stage test using platelet-poor plasma as substrate obtained from patients congenitally deficient in the respective factors (supplied by Behringwerke, Marburg A. G., FRG) as reported previously [6]. HMWKg-deficient plasma was obtained from a patient with a congenital Fitzgerald trait previously reported [6]. The enzymatic activity of PK was measured using the synthetic chromogenic substrate S-2302 (Kabi Vitrum, Stockholm, Sweden) as described [7].

Quantitative immunological determinations of HMWKg, Pk and C-1-INH were determined by electroimmunoassay as previously reported [6]. Antisera were obtained from Nordic Laboratories (Tilburg, The Netherlands) except the C-1-INH antibody which was purchased from Behringwerke (Marburg A. G., FRG).

Statistical analysis

Results are expressed as mean values \pm SEM. The plasma parameters obtained before, during and after dialysis were compared by Student's *t*-test for paired data.

Results

Table 1 shows the mean values (\pm SEM) of the different parameters studied before, during and immediately after the termination of the dialysis session. Haemodialysis did not modify the functional and immunological activity of FXII. However, during and immediately after dialysis a significant decrease in PK and HMWKg activities ($p < 0.05$) was observed. The immunological levels of both proteins were not modified significantly. The functional activity of FXI only decreased during dialysis ($p < 0.05$) and returned to normal values thereafter. At the end of the procedure C-1-INH showed a significant increase compared to pre-dialysis levels.

Discussion

Data regarding the role of haemodialysis in the activation of contact phase proteins are contradictory [2-4]. Wardle and Piercy [2] reported an increase in plasma kallikrein activity together with a decrease in PK and FXII levels in patients after haemodialysis. On the other hand, Deutsch et al. [3] found differences in the plasma levels of some proteins involved in the contact phase in patients with

Table 1

Values (Mean \pm SEM) obtained for the different proteins studied before, during and immediately after dialysis

	HMWKg		PK	
	Coag.	Antigen.	Coag.	Antigen.
Before dialysis	111 \pm 4	102 \pm 3	97 \pm 7	97 \pm 4
During dialysis	64 \pm 13*	113 \pm 6	75 \pm 11*	89 \pm 8
After dialysis	78 \pm 13*	96 \pm 5	71 \pm 6*	89 \pm 7

	FXII		FXI	C-1-INH
	Coag.	Antigen.	Coag.	Antigen.
Before dialysis	84 \pm 6	90 \pm 2	93 \pm 5	92 \pm 6
During dialysis	72 \pm 7	81 \pm 6	60 \pm 9*	128 \pm 7*
After dialysis	96 \pm 10	85 \pm 4	74 \pm 3	139 \pm 9*

* $p < 0.05$ compared to pre-dialysis values

chronic renal impairments who were on haemodialysis and who were not. However, they did not find alterations in the contact phase after dialysis. Recently Vaziri et al. [4] suggested that dialysis does not induce alterations in the proteins of this system. In our opinion the discrepancies in the results of different authors may be due to the fact that different methods were applied, further on that functional or immunological assays were not uniformly used.

In our study, if PK and HMWKg had been evaluated only by immunological techniques, no abnormalities would have been detected. However, according to functional assays there was a marked decrease in both proteins, with a significant increase in C-1-INH after dialysis. It is unlikely that the decrease in the activities but not in the antigen concentrations of these proteins could be accounted for by the presence of heparin during dialysis since this discrepancy could not be observed in the properties of FXII. Moreover, no change was observed in the functional and antigenic activities of other coagulation proteins investigated, such as factor II and IX (data not shown). Like Vaziri et al. [4], we observed that functional and immunological activities of FXII remained unaltered during dialysis. In other situations where an evident activation of the contact phase has been observed, no changes are reported in FXII levels; the finding has been interpreted as a lack of sensitivity of the method used in the study, which was unable to distinguish the various activated and inactivated forms of this protein [8].

In spite of the alterations found during and after haemodialysis, in the contact phase no clinical anomalies were observed in our series. In view of these results, we feel that the study of the contact phase is of great importance in patients undergoing dialysis, who display severe haemodynamic complications — such as hypotension — in which the genesis of kinins by activation of the prekallikrein-kinin system may play an important role in the pathogenesis of this complication.

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Immunological Markers in a Coexisting Chronic Lymphocytic Leukemia and Hodgkin's Disease

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(Received 23 October, 1986; accepted 30 April, 1987)

A 57 year old patient in whom Hodgkin's disease (HD) and Chronic Lymphocytic Leukemia (CLL) was simultaneously diagnosed is described. The infiltration of peripheral blood and bone marrow by mature lymphocytes, with special immunological phenotype (SIg negative, mouse rosette positive and monoclonal antibodies B1+, B4+) consistent with B-CLL and histological findings of lymphnode and axillary mass biopsies were typical of HD. The immunological study of the cell suspension from the axillary mass displayed a phenotype similar to that of the peripheral blood lymphocytes. Whether HD and CLL are two processes of fortuitous association, or a single clinical entity remains to be elucidated. The immunological findings in our patient suggest a common origin for both disorders and that HD could sometimes be the result of a B cell proliferation.

Keywords: Hodgkin's disease, Chronic Lymphocytic Leukemia, immunological markers

Introduction

The finding of Hodgkin's disease and Chronic Lymphocytic Leukemia simultaneously in the same patient has been occasionally described [10, 11]. Hodgkin's disease appears commonly in the late course of CLL, perhaps as a secondary effect of long term chemotherapy. However, the concurrent diagnosis of both diseases is extremely rare [11]. The characterization of tumour cells by immunological markers may be a useful tool in the study of these cases.

Case Report

A 57 year old male (occupation: wood-carver) was admitted because of the progressive growth of a mass in the left axilla over the previous two months. The patient complained of episodes of nocturnal sweating, but of no other systemic symptoms. Physical examination revealed a diffuse infiltration of the soft tissue in the left arm by a mass of 15 × 15 cm. Adhering to the outer part of the axilla, and extending to the lower third of the arm, the mass showed signs of inflammation.

Further on, a palpable left axillary lymphadenopathy (2 cm) and two unmovable tender epitrochlears (2 cm) were detected. No other lymphadenopathies were found. The patient had splenomegaly and hepatomegaly of 8 and 6 cm, respectively. A blood count showed: Hb 14.5 g/dl, platelets $188 \times 10^9/L$, WBC $33.2 \times 10^9/L$ with 11 granulocytes, 2 monocytes and 87 morphologically mature lymphocytes with abundant smudge cells. Other investigations showed hypogammaglobulinemia with IgG of 605 mg/dl, IgA of 55.6 mg/dl and IgM of 45.5 mg/dl. The immunological study of peripheral blood cells using classical markers and McAbs [6, 24] demonstrated an immature Blymphoid phenotype: EAET rosettes 3%, mouse-cell rosettes 44%, surface Igs negative, Cris-1 (CD 5) 13%, Han-PC1 (anti-plasma cell) negative, B1 (CD 20) 65%, B4 (CD 19) 58% and GRB1 (HLA-DR) 87% [27]. The bone marrow biopsy showed a lymphocytic infiltration of a nodulo-interstitial pattern [23], Reed-Sternberg (RS) cells were not observed. The hepatic biopsy revealed lymphoid infiltration by small lymphocytes, no eosinophils or RS cells were observed. A diagnosis of B-CLL in stage II according to Rai's classification [21] was made. The axillary lymphadenopathy was removed. Infiltration by lacunar cells and – to a lesser extent – typical RS cells with large and birefringent bands of sclerotic tissue surrounding lymphoid nodules were found. A diagnosis of Nodular Sclerosis (NS) type HD was established.

A biopsy was also performed on the axillary mass; on examination an infiltration of the soft tissues by mononuclear Hodgkin cells and to a lesser extent by RS cells, with abundant eosinophils and scarce plasma cells was found. Immunological analysis of the cell suspension of the axillary mass revealed predominantly B markers: E rosettes 3%, mouse rosettes 6%, surface Ig negative, Ki-1 (CD 30) 2%, B1 (CD 20) 70%, B4 (CD 19) 60%, and GRB1 (HLA-DR) 55% [27]. A diagnosis of NS-type HD in stage IIe-B (Ann-Arbor) was established [2]. The patient was given a dose of 600 mg/day cyclophosphamide for three days and subsequently six courses of ABVD chemotherapy (Adriamycine, Beomycine, Vincristine and Daunoblastine) was administered with an excellent response.

Discussion

A patient suffering simultaneously from Hodgkin's disease (HD) and Chronic Lymphocytic Leukemia (CLL) is described. The finding of HD in long term survival CLL patients has been occasionally described and a possible role of alkylating agents has been claimed [10, 11]. However, the concurrent diagnosis of both diseases is extremely rare and to our knowledge only two other cases have been reported [11].

Immunological markers, however, have not been studied in any of the previous cases. The differential diagnosis between HD and CLL or Well Differentiated Lymphocytic Lymphoma (WDLL) is sometimes difficult since RS-like cells have been reported in the latter two diseases [16]. A further difficulty arises when

the histological type of HD is of lymphocytic predominance. In our patient the diagnosis of HD is unquestionable because a typical nodular sclerosis (NS) was established. This histological pattern has only been found in one other case of HD appearing in the course of a CLL [10]. The infiltration of peripheral blood and bone marrow by morphologically mature lymphocytes with a special immunological phenotype (positive for B-cell markers B1, B4, and mouse rosettes) is consistent with B-CLL. The absence of SIg may be accounted for by its weak expression observed in some cases of B-CLL (10% B-CLL are SIg negative) [3]. However, as mentioned above, biopsy of both the adenopathy and of the axillary mass was typical for nodular sclerosis seen in Hodgkin's disease; an unexpected finding was that most of the cells obtained from the axillary mass had B-lymphocyte markers.

Our studies and those of others [1, 9, 19], however, showed that lymph nodes affected by HD usually consist of T-lymphocytes. Furthermore, our observation that axillary mass lymphocytes showed the same aberrant phenotype as the peripheral lymphocytes (SIg negative in contrast to the normal SIg+ B lymphocytes [28]) seems to support the hypothesis of a common progenitor B-cell. A second possibility is that B-CLL cells contaminated or infiltrated the Hodgkin's tissues, and we were dealing with a case of composite lymphoma — HD and non-Hodgkin's lymphoma in the same tissue — a rare finding, though occasionally described [15]. However, this hypothesis was not confirmed by the histological findings which showed a single type of tumoral infiltration: NS Hodgkin's disease. The difference in the mouse rosette receptors might be explained in terms of the variability of this marker depending on the tissue studied [4].

These results also involve the origin of RS cells, a controversial topic hitherto unsolved. Studies by Stein et al. [26] indicate that they are derived from activated lymphocytes of T or B origin. Recently Poppema et al. [20] suggested a B origin for RS cells. Although our studies have been performed on suspension cells, it seems that immunohistological analysis may be useful to assess the phenotype of the Reed-Sternberg cells.

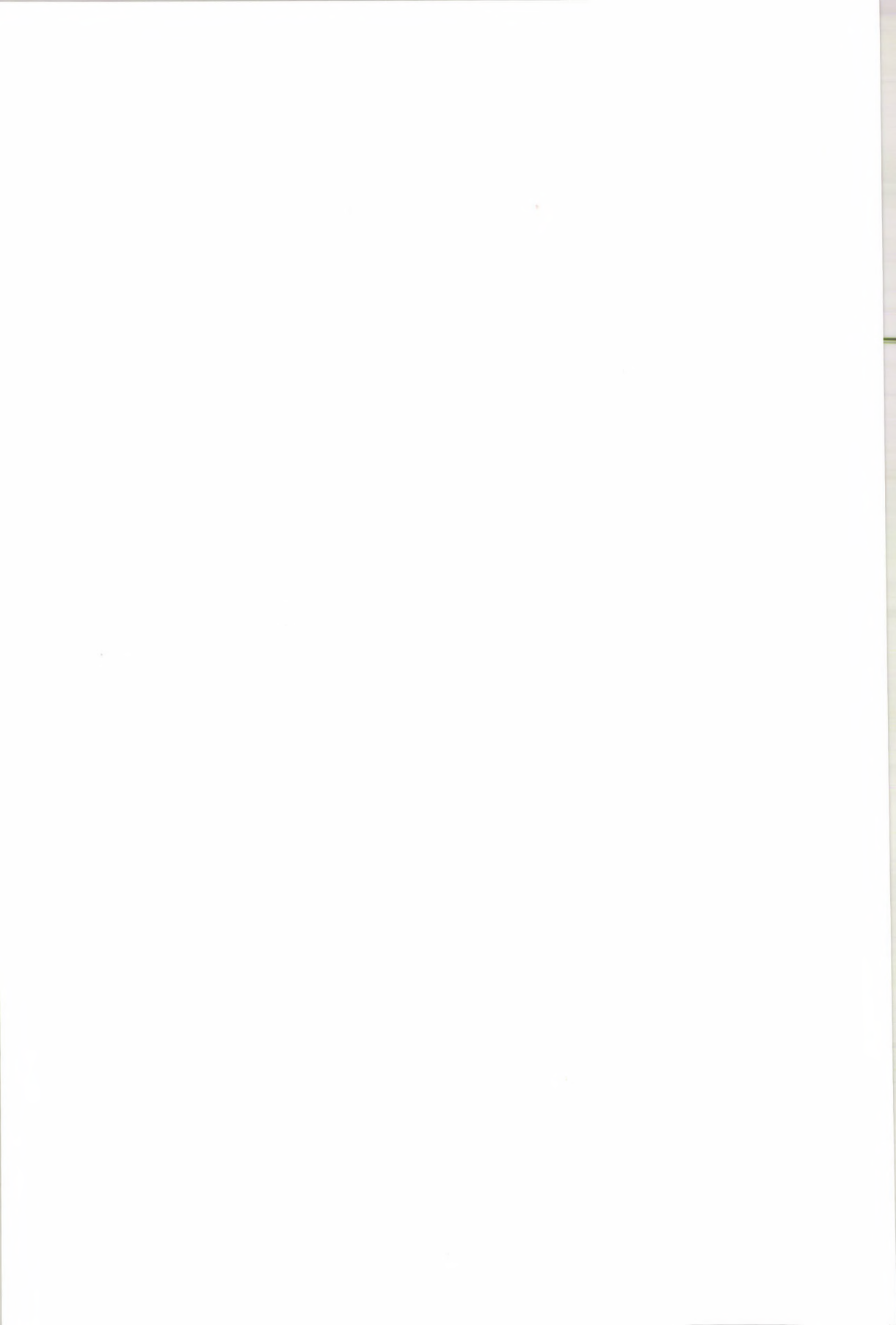
Although the former possibility is attractive, other studies — including chromosomal analysis and gene rearrangement techniques — seem necessary for determining whether we are dealing with two related diseases arising from a common progenitor B cell, or with a fortuitous association of B-CLL with HD.

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The Interaction between Fibrinogen and $^3\text{H-L-arginine}$ Cationic Peptides Derived from Fibrosarcoma in the Presence of Thrombin

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(Received 3 January, 1987; accepted 14 April, 1987)

It has been found that cationic protein breakdown product — $^3\text{H-L-arginine}$ labelled peptide fraction — interacts with fibrinogen in the presence of thrombin. The formation of the fibrin clot under these conditions makes the clot resistant to the fibrinolytic action of plasmin.

Keywords: fibrosarcoma induced by methylcholanthrene, fibrinogen, interaction with $^3\text{H-L-arginine}$ labelled peptide, proteolytic degradation of cationic protein

Introduction

In soluble cytoplasmic proteins of various tumor cells cationic protein mixture with a heterogeneous arginine content has been observed [4, 6, 7]. Proteins containing arginine residues have been observed in tumor ascitic fluid as well [9]. Their amount in ascitic fluid is less than in cytoplasmic fraction.

Cationic proteins labelled with $^{14}\text{C-arginine}$, derived from cytosol of Ehrlich ascites tumor cells are occluded by fibrin clots under the influence of thrombin [8]. In vitro, in the presence of thrombin fibrinogen is bound strongly by cationic proteins rich in $^3\text{H-L-arginine}$ isolated from fibrosarcoma, induced by methylcholanthrene [15]. The effect of this reaction depends on the concentration of cationic proteins built into the fibrin clots and showing antifibrinolytic action [5].

Our work was aimed at studying in vitro the effect of proteolytic degradation products of $^3\text{H-L-arginine}$ labelled cationic protein, isolated from fibrosarcoma, on the thrombin-induced fibrin clot.

Materials and Methods

Bovine fibrinogen (fibrin-stabilizing factor-free) was purified by the method of Kekwick et al. [12]. Thrombin was produced by the Warsaw Serum and Vaccine Plant, Poland, and is expressed in NIH units.

$^3\text{H-L-arginine}$ -rich cationic proteins were isolated from cytosol of fibrosarcoma induced by methylcholanthrene in rats using a method described in detail

elsewhere [6]. The purification of cationic protein mixture was carried out on SP-Sephadex column rechromatography. The chromatographic fractions were eluted with a linear NaCl gradient. The peak with the highest amount of $^3\text{H-L-arginine}$ and the highest specific radioactivity (2150 cpm/mg) was used for proteolytic degradation. This protein had an isoelectric point at basic range $\text{pH}(\text{pI} - 8.6)$ and a molecular weight of about 50 KD.

The enzymatic degradation of cationic protein was performed at pH 7.6. The incubation mixture contained two volumes of protein (2 mg/ml) and one volume of trypsin (0.5 mg/ml) both dissolved in 0.1 M phosphate buffer. The incubation at 37°C was stopped after 60 min by addition of $0.8\ \mu\text{g}$ of trypsin inhibitor IP-315.

Protein degradation products (PDP) were fractionated on CM-cellulose column equilibrated previously with 0.01 M phosphate buffer at pH 7.6. PDP were eluted with a linear NaCl gradient from 0.1 M to 0.6 M. 3 ml fractions were collected at flow rate of 30 ml per hour. Three peaks were obtained: first a peak of low radioactivity (430 cpm/mg) with about 20% of total protein content calculated from total $^3\text{H-arginine}$ radioactivity of cationic protein. The second peak was of medium radioactivity (640 cpm/mg) with 30% of total protein content. The third showed the highest radioactivity (1070 cpm/mg) with a 50% of total protein content. The latter peak migrated to the cathode during electrophoresis at pH 2.7; this indicated a cationic nature. During electrophoresis at pH 2.7 the first and second peaks remained on the start line. In all peaks α -amino nitrogen was measured [3]. $^3\text{H-L-arginine}$ labelled fractions of the single peaks were pooled, and concentrated by lyophilization.

Interaction of all $^3\text{H-L-arginine}$ labelled peptide fractions with fibrinogen was assayed in the presence of thrombin by measuring total radioactivity in the clot on a β -liquid scintillation spectrometer. For this purpose a system of 0.25 ml fibrinogen 0.5%, 0.25 ml peptides (of various concentration) and 0.5 ml thrombin (20 U/ml) was incubated at 37°C for 30 min. The formed clot was separated from the solution by means of a silk filter, and washed three times with 0.9% NaCl and subsequently with distilled water. The clot was solubilized in 1 ml 2N NaOH by heating at 100°C for 5 min. Samples (0.3 ml) neutralised with HCl were added to 3 ml of Bray's solution [1] and ^3H -radioactivity in all samples was measured (Nuclear Chicago).

The effect of peptide fractions on the fibrinolytic activity of plasmin in fibrin clots was evaluated in a system containing: 0.25 ml plasmin (0.1%) + 0.25 ml peptides (of various concentrations). After a 5 min incubation, 0.25 ml fibrinogen (0.5%) and 0.25 ml thrombin (40 U/ml) were added, and from that moment the clot lysis time was measured.

Results

Figure 1 shows that, in the presence of trypsin, the $^3\text{H-L-arginine}$ labelled cationic protein was separated into three peaks on the CM cellulose column. The

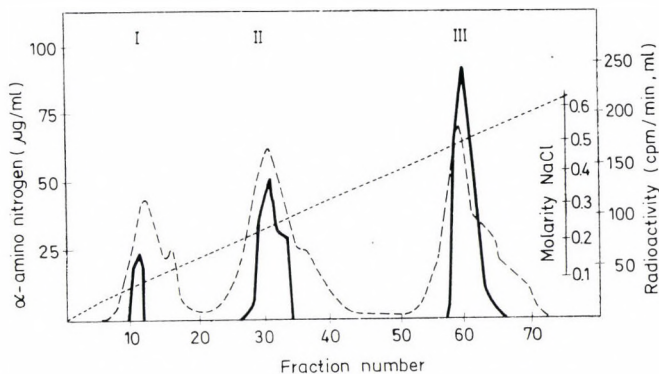


Fig. 1. Fractionation of ^3H -L-arginine labelled protein degradation products on CM-cellulose column (1.4×21 cm). They were applied to a column equilibrated previously with 0,01 M phosphate buffer at pH 7.6. The elution was carried out with a linear NaCl gradient from 0,1 M to 0,6 M. 3 ml fractions were collected at a flow rate of 30 ml per hour

first peak was easily eluted by 0.1 M NaCl and it contained a low amount of α -amino nitrogen (40 $\mu\text{g}/\text{ml}$) with low total radioactivity (50 cpm/ml). The second peak was eluted by increasing ionic strength and contained more radioactivity (144 cpm/ml). The third peak eluted by 0.5 M NaCl contained about 102 μg of α -amino nitrogen and showed the highest total radioactivity (250 cpm/min, ml).

Table 1 illustrates the effect of ^3H -L-arginine labelled peptides on the fibrinogen in the presence of thrombin, at a constant concentration of fibrinogen 1.25 mg/ml. Only fibrin clots formed in the presence of peptide fraction III showed radioactivity depending on the concentration of peptides and their specific radioactivity (Fig. 2). This peptide fraction interacted entirely with fibrinogen even

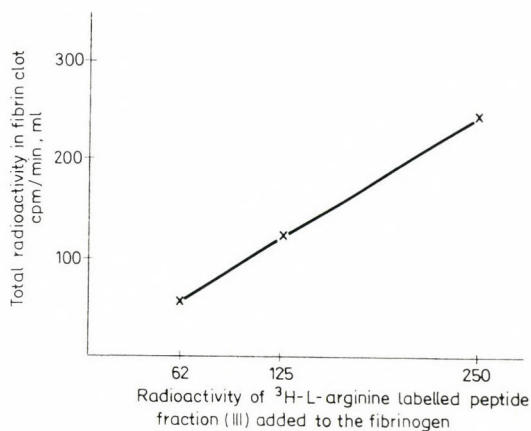


Fig. 2. The effect of ^3H -L-arginine labelled peptide fraction (III) on the radioactivity in the thrombin-induced fibrin clots

Table 1

The interaction between fibrinogen and ³H-L-arginine labelled peptides (obtained by proteolysis of cationic protein derived from fibrosarcoma) in the presence of thrombin

	Peptides		Total radioactivity of peptides in fibrin clot cpm/min, ml	Fibrinolysis time (s)
	Final concentration α -amino nitrogen μ g/ml	Radioactivity cpm/min, ml		
Fraction I	40	50	1	720
	20	25	0	720
Fraction II	75	144	2	720
	35	72	0	720
Fraction III	102	250	246	1130
	51	125	121	890
	25	62	58	790
	0	—	—	720

The results represent the mean values of three experiments for each of the peptide concentrations. The radioactivity of the samples after subtraction of the background.

at a very low concentration, and showed antifibrinolytic action. The final concentration (102 μ g) of this fraction increased the fibrinolysis time from 720 to 1130 s (Table 1).

The remaining two peptide fractions at concentrations of 70 μ g and 40 μ g of α -amino nitrogen content did not interact with fibrinogen in the presence of thrombin and did not show antifibrinolytic action.

Discussion

Our results demonstrate that not all degradation products of cationic protein derived from fibrosarcoma interact with fibrinogen in the presence of thrombin. Only the peptide fraction with the highest radioactivity of ³H-L-arginine and showing pI at basic range of pH (8.9) is built into the structure of the thrombin-induced fibrin clots. They cannot be removed from the clots by repeated washings. It seems that the cationic peptide fraction after interaction with fibrinogen in the presence of thrombin alters the clot structure and makes it resistant to the fibrinolytic action of plasmin.

The remaining two peptides showing neutral character and low molecular weight are not built into the structure of the thrombin-induced fibrin clots, and do not show antifibrinolytic action.

This phenomenon may be of some importance in vivo. In various pathological conditions, particularly in several invasive tumors e.g. fibrosarcoma, infiltration

of tumor cells by host cells such as lymphocytes, granulocytes and macrophages cytolysis takes place [11]. From damaged and disintegrated tumor cells and from aggregating blood platelets many substances (e.g. cationic proteins and peptides) may get released into the extracellular space, into smaller capillaries and into the blood circulation [10]. Their cationic character and low molecular weight facilitate penetration into the blood circulation, and the microenvironment may be locally modified [14].

The interaction of PDP with fibrinogen in the presence of thrombin and the formation and deposition of fibrin or fibrin-like material under clinical conditions is biologically important and essential for the growth of tumor, its invasion and metastasis [13]. The fibrin network provides the matrix into which new capillaries can grow.

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In-Vivo Macrophage-Erythrocyte-Rosettes within Haemolymph Nodes as Evidence of Loss of Self-tolerance

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Many earlier authors have observed autologous erythrocyte-rosette formation within reddish haemolymph nodes but how and why these are formed has not been studied.

In this study we traced the histogenesis of this phenomenon. Since macrophage-erythrocyte-rosettes (MER) mostly occur in the deep sinus we suggest that changes in the red blood cells caused by stagnation, aging or the loss of viability due to deoxygenation may result in development of some unusual sites on these cells. Polar formation of macrophage processes around each rosette erythrocyte in-vivo suggests loss of self-tolerance.

Keywords: macrophage-erythrocyte-rosettes, haemolymph node, autologous erythrocytes, in-vivo rosettes

Introduction

Erythrocyte-rosette formation is normally demonstrated in vitro and involves receptor-specific radial orientation of heterologous erythrocytes around a central leukocyte. The observation of this phenomenon in-vivo within some peculiar reddish nodes [2] was the starting point of our present study.

These nodes are commonly found in the connective tissues around the kidneys, thyroid glands and the spleen. They have been described in sheep, horse, pig, rat and even in humans, showing various degrees of reddish intensity [1, 5, 6, 10, 11]. The term "haemolymph" means the unusual mixture of abundant stagnating erythrocytes with the lymph.

Recently we established that the red cells within haemolymph sinuses reach these sites via the orifice of afferent lymph vessels [3].

The aim of our present work was to determine how and why in-vivo rosettes become formed with autologous erythrocytes.

Materials and Methods

Group I

IO AS and IO DA strains of rats were used. Under ether anaesthesia the vascular system of each animal was perfused with Ringer Lactate solution through a catheter in the descending aorta with outlet in the atrium. The perfusate was replaced with 1.5% buffered glutaraldehyde. This technique allowed rapid fixation of the organs and tissue in-situ. The reddish nodes posterior to the hila of both renal vessels were removed and processed for both light and electron microscopes.

Group II

In this group 5 DA and 5 AS rats were given ether overdosage. The reddish nodes were quickly removed and fixed in Carnoy's solution. They were processed and fixed for light microscopy.

Results

All animals examined had haemolymph (HLN) nodes); those situated posterior to the renal vessels were further studied.

Variation of technique did not cause any difference in the two groups, therefore only the features related to histogenesis of the rosettes were followed.

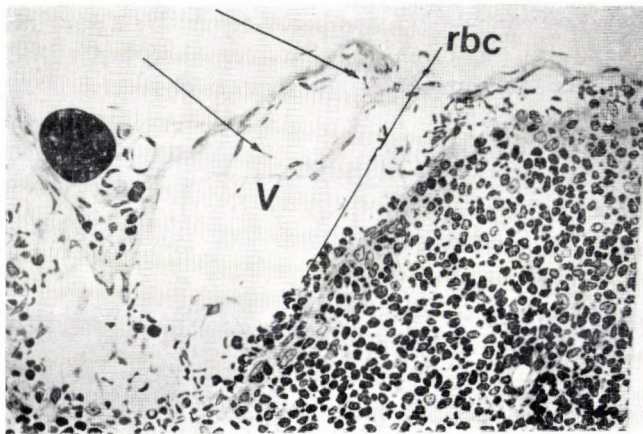


Fig. 1. Section through the peripheral area of the haemolymph nodes. The arrows point to freely suspended erythrocytes (rbc) and V is the sinus. ($\times 60$)

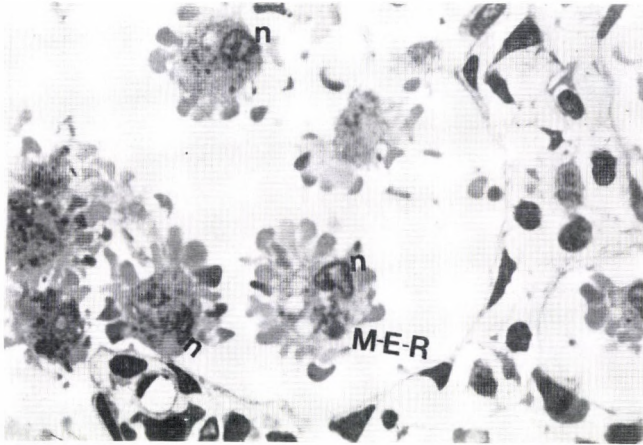


Fig. 2. Macrophage-erythrocyte-rosettes (MER) in the deep sinus. Most of the red cells are spindle-shaped. n = nucleus of the macrophage. ($\times 120$)

In normal circumstances the red blood cells entering the HLN (which is the orifice of the afferent lymphatics) and then spreading in the peripheral sinus are freely suspended. These cells have a characteristic biconcave disc shape. Other cells, such as lymphocytes and macrophages (normally found in the lymph and/or in the blood) are also freely impeneded in this area.

The erythrocytes and leukocytes of the deep sinus were examined and some histological peculiarities were found. Variable numbers of the red blood cells

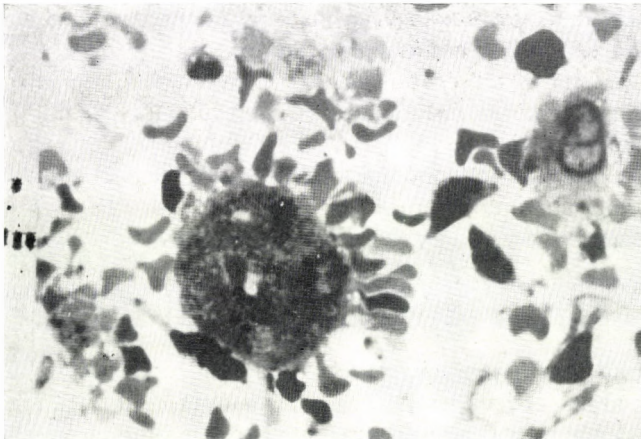


Fig. 3. MER showing haemosiderin granules within the cytoplasm of the central leukocyte. ($\times 360$)

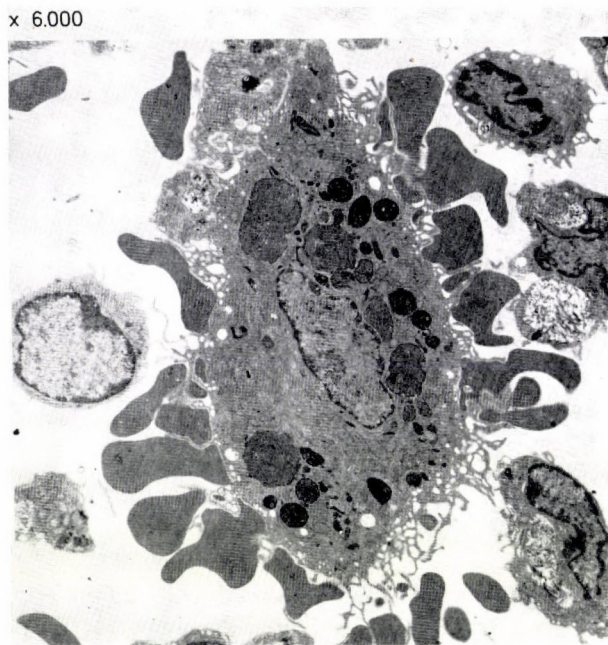


Fig. 4. Electron photograph of MER. The cytoplasmic processes of macrophages form around the pole of each rosetting erythrocyte. ($\times 6000$)

formed a radially arranged pattern along the margins of the leukocytes. Most rosetting erythrocytes lost their biconcave disc shape (seen at the peripheral sinus) and assumed spindle, comma or other shapes. The pole of these erythrocytes was usually connected to the cytoplasmic margin and processes of the leukocytes. These erythrocytes were intensively basophilic.

The cytoplasm of the central leukocyte involved in rosette formation had debris including haemosiderin granules and irregular processes. They belonged to the macrophage series.

Features of active immunological stimulation such as nodules, blast cells or T-immunological stimulation such as nodules, blast cells or T-immunoblasts were rarely seen.

Discussion

When blood and lymph circulation are normal, erythrocytes do not directly bind to the margins of leukocytes. The present work confirmed reports of many earlier authors who found and demonstrated rosette binding within HLN [5, 6, 10]. As an extension of these reports the present study traced the histogenesis of this in-vivo rosette formation.

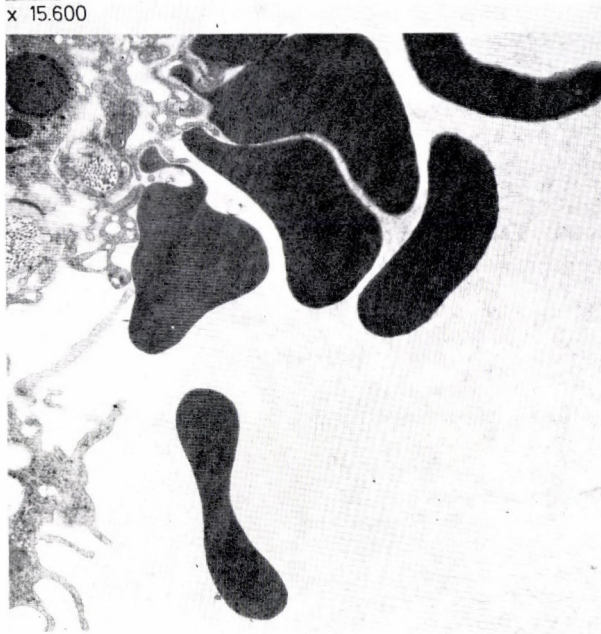


Fig. 5. Electron photomicrograph of MER. Rosetting erythrocytes have lost their typical shape while the isolated red cell at the bottom of the illustration still retained its shape and is probably still viable. ($\times 15,600$)

We observed that the erythrocytes that had recently entered the HLN were freely suspended in the peripheral sinus and kept their typical biconcave disc shape. At the deep sinus they lost these properties and bound to the margins of the macrophages. The question is what factor(s) are responsible for the formation of MER?

A change of technique in the present study did not affect the genesis of MER.

Similarly, reports from many laboratories using different methods did not preclude the demonstration of MER within HLN. Furthermore, the presence of abundant haemosiderin deposits within the cytoplasm of the macrophages are considered physiological *in-vivo* and are not due to experimental intervention.

The next question is what factor(s) are peculiar to the milieu in the deep sinus predisposing to MER formation? It is known that macrophages normally phagocytose effects red blood cells, but the latter cells do not form rosettes before phagocytosis, and it is not a histological feature of the spleen. In our opinion, changes occurring in the red blood cells during their migration are involved in this process

This interpretation is supported by our observation that rosetting erythrocytes appeared oriented edge-on to the processes of the macrophages, similarly to the receptor-specific orientation seen in the *in-vitro* studies. It is known that

spontaneous formation of MER with xenogenic red blood cells is receptor specific and can be depressed by antimacrophage antibodies [4]. Similarly, adherence of red blood cells to autologous T cells has been reported [8]. In vitro, the binding characteristics of erythrocytes to leukocytes change, depending on the storage conditions, age and the treatment of the red blood cells before use [9]. Based on our present in-vivo observations, we conclude that red cell stagnation, aging or the loss of viability due to deoxygenation may result in unusual sites on the surface of sinus erythrocytes. The sinus macrophages might recognize these sites as foreign and stretch out processes to engulf red blood cells (i.e. loss of self-tolerance).

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Abstracts

Action of long-chain fatty acids in vitro on Ca²⁺-stimulatable, Mg²⁺-dependent ATPase activity in human red cell membranes. F. B. Davis, P. J. Davis, S. D. Blas and M. Schoenl (Endocrinology Division, Department of Medicine, State University of New York at Buffalo School of Medicine, Veterans Administration Medical Center, Buffalo N. Y. USA). *Biochem. J.* 248, 511 (1987).

Human red cell membrane Ca²⁺-stimulatable, Mg²⁺-dependent adenosine triphosphatase (Ca²⁺-ATPase) activity and its response to thyroid hormone have been studied following exposure of membranes *in vitro* to specific long-chain fatty acids. Basal enzyme activity (no added thyroid hormone) was significantly decreased by additions of 10⁻⁹–10⁻⁴M-stearic (18 : 0) and oleic (18 : 1 *cis*-9) acids. Methyl oleate and elaidic (18 : 1 *trans*-9), palmitic (16 : 0) and lauric (12 : 0) acids at 10⁻⁶ and 10⁻⁴ M were not inhibitory, nor were arachidonic (20 : 4) and linolenic (18 : 3) acids. Myristic acid (14 : 0) was inhibitory only at 10⁻⁴ M. Thus, chain length of 18 carbon atoms and anionic dulin. To investigate the mechanism by which cetiedil modifies calcium-mediated erythrocyte function, the effect of the drug on the active transport of calcium into inside-out erythrocyte vesicles was examined and its influence on the activities of phosphodiesterase and Ca-ATPase studied. Cetiedil, in the presence of calmodulin, significantly inhibited calcium transport into inside-out vesicles that were prepared with erythrocytes from normal controls and from patients with sickle cell anemia. However, in the absence of calmodulin, no inhibition was

observed. Likewise, cetiedil inhibited calmodulin-stimulated, but not basal, activities of phosphodiesterase and Ca-ATPase. These data, along with previous reports, suggest that cetiedil does not act by lowering the intracellular calcium content. It is, therefore, likely that the beneficial effect of cetiedil is due to its ability to protect the red cell from the deleterious consequences of an elevated concentration of intracellular calcium.

G. Gárdos

Inhibition of erythrocyte calcium transport by cetiedil. S. N. Levine, L. R. Berkowitz, E. P. Orringer (Department of Medicine, Section of Endocrinology, Louisiana State University Medical Center, Shreveport, La, USA.) *Pharmacology* 36, 44 (1988).

The elevated calcium content found in red cells from patients with sickle cell anemia may be of pathophysiologic importance in the hemolysis and vasoocclusion which characterize this disorder. Cetiedil, an anti-sickling agent, has been reported to inhibit the activity of enzymes that are stimulated by the calcium regulatory protein calmodulin. The principal determinants of inhibitory activity. Introduction of a *cis*-9 double bond (oleic acid) did not alter the inhibitory activity of the 18-carbon moiety (stearic acid), but the *trans*-9 elaidic acid did not cause enzyme inhibition. While the predominant effect of fatty acids on erythrocyte Ca²⁺-ATPase *in situ* is inhibition of basal activity, elaidic, linoleic (18 : 2) and palmitoleic (16 : 1) acids at 10⁻⁶ and 10⁻⁴ M

stimulated the enzyme. Methyl elaidate was not stimulatory. These structure-activity relationships differ from those described for fatty acids and purified red cell Ca^{2+} -ATPase reconstituted in liposomes. Thyroid hormone stimulation of Ca^{2+} -ATPase was significantly decreased by stearic and oleic acids (10^{-9} – 10^{-4} M), but also by elaidic, linoleic, palmitoleic and myristic acids. Arachidonic, palmitic and lauric acids were ineffective, as were the methyl esters of oleic and elaidic acids. Thus, inhibition of the iodothyronine effect on Ca^{2+} -ATPase by fatty acids has similar, but not identical, structure-activity relationships to those for basal enzyme activity. To examine mechanisms for these fatty acid effects, the action of oleic and stearic acids on responsiveness of the enzyme to purified calmodulin, the Ca^{2+} -binding activator protein for Ca^{2+} -ATPase was studied. Oleic and stearic acids (10^{-9} – 10^{-4} M) progressively inhibited, but did not abolish, enzyme stimulation by calmodulin (10^{-9} M). Double-reciprocal analysis of the effect of oleic acid on calmodulin stimulation indicated noncompetitive inhibition. Addition of calmodulin to membranes in the presence of equimolar oleic acid restored basal enzyme activity. Oleic acid also reduced ^{125}I -calmodulin binding to membranes, but had no effect on the binding of [^{125}I]T₄ by ghosts. The mechanism of the decrease by long chain fatty acids of Ca^{2+} -ATPase activity *in situ* in human red cell ghosts thus is calmodulin-dependent and involves reduction in membrane binding of calmodulin.

B. Sarkadi

Elevated intracellular Ca^{2+} affects $\text{Li}_i\text{-Na}_o$ countertransport in human red blood cells. G. Agam, P. Hatzav, S. Abekasis, A. Loven and A. Livne (Department of Biology, Ben-Gurion University of the Negev, Beer Sheva, Israel). *Biochim. Biophys. Acta* 904, 207, (1987).

Changes in cytoplasmic Ca^{2+} concentration and in $\text{Li}_i\text{-Na}_o$ countertransport activity have been shown to be associated with essential hypertension. Elevated intracellular free [Ca^{2+}], as well as abnormalities of Ca^{2+} binding and transport have been reported in

cells from different tissues of hypertensive laboratory animals and essential hypertensive patients. Similarly, enhanced rates of $\text{Li}_i\text{-Na}_o$ countertransport and the modified pattern of the temperature dependence of this activity in red blood cells from essential hypertensive patients have been previously demonstrated. The aim of the present study was to investigate possible interaction between changes in intracellular free [Ca^{2+}] and the $\text{Li}_i\text{-Na}_o$ exchange in human red blood cells. The ionophore ionomycin was used to allow Ca^{2+} incorporation into the cells in a dose-dependent manner. The elevation of intracellular [Ca^{2+}], in turn, resulted in enhanced Li^+ efflux from the cells. At 3 μM , ionomycin selectively and significantly enhanced the $\text{Li}_i\text{-Na}_o$ countertransport but not Li^+ leakage from the cells. EGTA totally abolished the effect of ionomycin, indicating that the effect is directly related to Ca^{2+} . As low as 0.4 μM Ca^{2+} caused a statistically significant effect. The maximal effect of Ca^{2+} on the $\text{Li}_i\text{-Na}_o$ countertransport was achieved around the external pH range of 6.8–7.5. In contrast, the leakage of Li^+ was significantly enhanced by Ca^{2+} at a pH of 7.4 and above. Ca^{2+} did not affect the K_m of the $\text{Li}_i\text{-Na}_o$ countertransport for Li^+ . Amiloride, which inhibits Na^+/H^+ exchange, inhibited only 10% of the Ca^{2+} -enhanced countertransport. It is concluded that Ca^{2+} may play a role in the regulation of $\text{Li}_i\text{-Na}_o$ countertransport in erythrocytes.

B. Sarkadi

Inhibition by nucleosides of glucose-transport activity in human erythrocytes. S. M. Jarvis (Biological Laboratory, University of Kent, Canterbury, Kent, UK). *Biochem. J.* 249, 383 (1988).

The interaction of nucleosides with the glucose carrier of human erythrocytes was examined by studying the effect of nucleosides on reversible cytochalasin B-binding activity and glucose transport. Adenosine, inosine and thymidine were more potent inhibitors of cytochalasin B binding of human erythrocyte membranes than was d-glucose [IC_{50} (concentration causing 50% inhibition) values of 10, 24, 28 and 38 mM, respectively].

Moreover, low concentrations of thymidine and adenosine inhibited d-glucose-sensitive cytochalasin B binding in an apparently competitive manner. Thymidine, a nucleoside not metabolized by human erythrocytes, inhibited glucose influx by intact cells with an IC_{50} value of 9 mM when preincubated with the erythrocytes. In contrast, thymidine was an order of magnitude less potent as an inhibitor of glucose influx when added simultaneously with the radioactive glucose. Consistent with this finding was the demonstration that glucose influx by inside-out vesicles prepared from human erythrocytes was more susceptible to thymidine inhibition than glucose influx by right-side-out vesicles. These data, together with previous suggestions that cytochalasin B binds to the glucose carrier at the inner face of the membrane, indicate that nucleosides are capable of inhibiting glucose-transport activity by interacting at the cytoplasmic surface of the glucose transporter. Nucleosides may also exhibit a low-affinity interaction at the extracellular face of the glucose transporter.

G. Gárdos

Volume-sensitive Cl-dependent K transport in human erythrocytes. W. C. O'Neill (Department of Medicine, Emory University School of Medicine, Atlanta, Georgia, USA.). *Am. J. Physiol.* 253, C883 (1987).

Passive K fluxes, measured with ^{86}Rb , were investigated in osmotically swollen human erythrocytes. K influx and efflux increased progressively with increased hypotonicity up to 167 mosmol/kg. No increase in K flux was seen when NO_3 or methyl SO_4 were substituted for Cl. Substitution of choline or N-methylglucamine for external Na reduced the K flux in swollen cells by only 22%, compared with a 60% reduction in euvolumic cells. However, the magnitude of this Na-dependent component was slightly, but significantly, higher in swollen cells. The presence of Na-dependent K influx in swollen cells was confirmed by measurements of Na influx demonstrating a K-dependent Na influx of similar magnitude in isovolumic and swollen cells. The volume-sensitive K flux was inhibited by bumetanide,

but significantly less so than was Cl-dependent flux in isovolumic cells (half-maximal inhibition at 1.0×10^{-4} vs. 5.8×10^{-7} M). Kinetic analysis revealed that Cl-dependent K influx had a lower affinity for external K in swollen cells than in euvolumic cells (K_m was 29.8 vs 6.1 mM). The increased K flux in swollen cells was found to be transient, decreasing substantially and reverting back to a predominantly Na-dependent and more bumetanide-sensitive form after 2 h. The results indicate that swelling of human erythrocytes activates a transient Cl-dependent K flux that differs significantly from that in isovolumic cells in that it is less Na-dependent, less sensitive to bumetanide, and has a lower affinity for K. Na-K cotransport is either unaffected or slightly increased in swollen cells. The altered flux in swollen cells would thermodynamically favor a volume-regulatory KCl efflux.

G. Gárdos

Formation of light irreversibly sickled cells during deoxygenation-oxygenation cycles. K. Horiuchi and T. Asakura (Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA.). *J. Lab. Clin. Med.* 110, 653 (1987).

Changes in density, morphology, and monovalent cation content of sickle (SS) cells caused by repetitive sickling were investigated *in vitro* with a device by which red cells can be exposed to cycles of deoxygenation and oxygenation (d-o cycles). Experiments were done using the discocyte-rich, less dense fractions of SS cells. When such cells were subjected to d-o cycles in the presence of 2 mmol/l Ca^{2+} , most became dense within 4 hours. The density of these cells was slightly lower than usual for irreversibly sickled cells (ISCs), and because they reverted to the discoidal shape after oxygenation, they were not considered ISCs. However, on further d-o incubation, they did assume the morphology and density typical of ISCs. After an additional incubation (20 hours), most cells decreased in density, became very fragile, and hemolyzed easily. Such light ISCs could also be formed from freshly prepared dense cells on

d-o incubation. Once light ISCs were formed, they did not revert to dense cells on further incubation. It should be noted that light ISCs created *in vitro* tended to aggregate much more easily than ordinary (heavy) ISCs did, suggesting that the membrane of these cells may be very adhesive.

G. Gárdos

A spin-label study of membrane proteins and internal microviscosity of erythrocytes in hereditary spherocytosis. G. Bartosz, M. Gaczynska, E. Grzelinska and L. Judkiewicz (Laboratory of Biophysics of Development and Aging, Chair of Biophysics, University of Lodz, Lodz, Poland). *Life Sci.* 41, 2285 (1987).

Electron spin resonance (ESR) spectra of erythrocyte membranes of patients with hereditary spherocytosis (HS) and of healthy controls labelled with a maleimide spin label did not differ significantly both before and after prolonged incubation at 37 °C. It suggests that the different behaviour of spin-labelled HS erythrocyte membranes upon incubation at a higher temperature reported previously is due indeed to structural abnormalities of HS red cell membranes and not to alterations in their proteolytic activity. Measurements of the rotational correlation time of Tempamine spin probe demonstrated a significant elevation of internal microviscosity of erythrocytes in HS, more pronounced in non-splenectomized patients.

Ilma Szász

Ethanol-induced alterations in human erythrocyte shape and surface properties: Modulatory role of prostaglandin E₁. R. W. McLawhion, Y. Marikovsky, N. J. Thomas and R. S. Weinstein (Department of Pathology, Rush Medical College, Chicago, Illinois, USA). *J. Membrane Biol.* 99, 73 (1987).

Exposure of human erythrocytes to ethanol (1 to 20% by vol) in Ca²⁺ and Mg²⁺-free phosphate-buffered saline, pH 7.4, transformed biconcave discs into spiculated echinocytes within 3 min at 25 °C. The effects of ethanol were concentration- and

time-dependent, but reversible by washing in the incubation buffer system within 60 min of initial exposure to ethanol. After prolonged ethanol exposure (180 min), washing of cells resulted in the formation of stomatocytes (cup-forms). Ethanol-induced echinocytosis was also accompanied by a 30% enhancement in the agglutinability of erythrocytes by ligands with high affinity for negative surface charge (poly-L-lysine and wheat germ agglutinin 20 µl/ml) without any alterations in surface charge topography. Concomitant exposure of erythrocytes to prostaglandin E₁ (100 nM) selectively prevented the enhancement of ligand-mediated agglutinability, but did not modify cell shape. These data indicate that certain erythrocyte surface properties may not be directly influenced by cell shape and suggest a unique modulatory action of prostaglandin E₁ on shape-transformed cells.

Ilma Szász

The effect of 2,3-diphosphoglycerate on the tetramer-dimer equilibrium of carbon monoxide hemoglobin in dilute solution. Correlation between sedimentation and kinetic behavior. R. D. Gray and W. L. Dean (Department of Biochemistry, University of Louisville School of Medicine, Louisville, Kentucky, USA.). *J. Biol. Chem.* 262, 15890 (1987).

The effect of 2,3-diphospho-d-glycerate on the sedimentation coefficient of carbon monoxide hemoglobin was correlated with the fraction of rapidly reacting hemoglobin observed subsequent to flash photolysis at 23 °C at pH 7.30 in buffers of 0.1 M ionic strength. Concentrations of the organic phosphate up to about 5 mM resulted in an increase in $s_{20,w}$, consistent with an increase in the fraction of tetrameric hemoglobin. A decrease in rapidly reacting hemoglobin paralleled the increase in the sedimentation coefficient. Between 5 and 20 mM 2,3-diphosphoglycerate, $s_{20,w}$ decreased, suggesting that dissociation to dimers was enhanced. An increase in rapidly reacting hemoglobin was also observed in this concentration range. Similar sedimentation results were obtained with oxyhemoglobin at pH 7.00 and carbon monoxide hemoglobin at pH 7.06. Assuming single binding sites on each species, the dissociation

constants for 2,3-diphosphoglycerate binding to tetrameric and dimeric HbCO. are 0.2–0.3 mM and 2–5 mM at pH 7.30. This biphasic effect of this physiologically important organic phosphate on the state of aggregation of R state hemoglobin has not been previously reported, but it is similar to

that previously noted with inositol hexaphosphate, which enhanced tetramer formation at low concentrations, while at higher concentrations it promoted hemoglobin dissociation to dimers.

Ilma Szász

— MAGYAR
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Book Review

THE ACUTE LEUKEMIAS: *Biologic, Diagnostic, and Therapeutic Determinants*. (Hematology Series/6). Edited by Sanford A. Stass. 1987. 496 pages, bound, illustrated. \$ 115.00 (US and Canada); \$ 138.00 (All other countries). ISBN: 0-8247-7766-2. (Prices subject to change without notice.)

In the past five years, developments in immunology, cytogenetics, biochemistry, and molecular biology have opened up new understandings of acute leukemia never before imagined, refining our ability to understand the biology, characterize, and diagnose this disorder. The identification of nonrandom phenotypic and biologic features, with important clinical and prognostic implications, requires that hematologists, pathologists, and oncologists keep abreast of these advances in order to grasp ongoing and prospective findings.

Authored by nearly 30 leading authorities in the field, *The Acute Leukemias* unifies the current complex and multifaceted

approach to the characterization of acute leukemia and places in perspective the usefulness of the divergent available studies for the evaluation of the leukemic cell.

Topics discussed include specialized morphologic and cytochemical studies... flow cytometry... cytogenetics... immunologic phenotyping... biochemical markers... molecular biology... therapeutic implications of leukemic cell characterization... DNA technology, using oncogene and lineage-associated cells... and nonrandom chromosomal studies.

READERSHIP: Hematologists; hematopathologists; pathologists; oncologists; fellows and residents in pathology and hematology; epidemiologists and biometricians; virologists and molecular biologists; cytogeneticists; medical technologists in hematology and biotechnology, graduate and medical students in hematology, oncology, or pathology; and professional seminars in hematopathology.

Announcement

THEORY OF REFERENCE VALUES

Observed values are meaningful only if assessed against comparable reference individuals and when a comparable analytic method is used. These are known as "reference values". They are more informative than the traditional "normal values" which may be misleading in some circumstances. The principles on which reference values are based, protocols for collecting data and the statistical procedures which should be used for establishing reference values have been described in a series of papers which have been prepared jointly by the International Federation of Clinical Chemistry (IFCC) and the International Committee for Standardization in Haematology (ICSH). These recommendations have been published as follows:

The concept of Reference Values. *Journal of Clinical Chemistry and Clinical Biochemistry*, 1987, 25, 337–342.

Statistical treatment of collected reference values. Determination of reference limits. *Clinical Biochemistry*, 1987, 25, 645–656.

Presentation of observed values related to reference values. *Journal of Clinical Chemistry and Clinical Biochemistry*, 1987, 25, 657–662.

Reprints are available from IFCC Scientific Committee (Dr H E Sollberg), Dept. of Clinical Chemistry, Rijkshospitalet, Oslo, Norway or from ICSH Secretariat (Dr R L Verwilghen), University Hospital, Herestraat 49, B 3000 Leuven, Belgium.

Printed in Hungary

Akadémiai Kiadó és Nyomda Vállalat, Budapest

INSTRUCTIONS TO CONTRIBUTORS

HAEMATOLOGIA is designed for the publication of original papers, preliminary reports, and reviews which contribute to the advancement in all fields related to haematology and blood transfusion. Manuscripts should be submitted in correct English and are accepted on the condition that they have not been published or accepted for publication elsewhere. Case reports, technical notes, and Letters to the Editor are also welcomed and will be published if appropriate.

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Prof. Susan R. Hollán
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References to periodicals should be typed double-spaced and mention: (1) name(s) and initials of all the authors; (2) title of the paper; (3) international abbreviation of the periodical; (4) volume; (5) first page number; (6) year of publication in parentheses. Thus: 4. Liebermann, J. E., Gordon-Smith, E. C.: Red cell pyrimidine 5'-nucleotidase and glutathione in myeloproliferative and lymphoproliferative disorders. *Brit. J. Haemat.* 44, 425 (1980).

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VOLUME 21 • NUMBER 4 • 1988

HU ISSN 0017-6559

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HAEMATOLOGIA

International Quarterly of Haematology

EDITOR-IN-CHIEF:

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HAEMATOLOGIA

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Manuscripts should be sent to the Editor-in-Chief:

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National Institute of Haematology and Blood Transfusion
Daróczi út 24.
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VSP
P.O.B. 346
3700 AH ZEIST, The Netherlands

Subscription price for volume 21:

DM 312,— incl. surface mail
DM 330,— incl. air mail

Haematologia is covered in *Current Contents/Clinical Practice*, *ISI/Biomed* and the *SCI*.

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VOLUME 21 · NUMBER 4

HAEMATOLOGIA

INTERNATIONAL QUARTERLY OF HAEMATOLOGY

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Density Distribution of Red Cells and Prognostic Significance in 50 Patients with Homozygous Sickle-Cell Disease

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(Received 1 June, 1987; accepted 24 July, 1987)

The sickle cell disease is characterized by a heterogeneous clinical and biological expression. In order to evaluate the prognostic significance of the red blood cell density distribution: D50 (median cell density of the distribution), R60 (middle density range in which 60% of the cells can be found), F4 and F5 (proportion of cells with density higher than 1.110 and 1.120 g/ml, respectively) have been determined in 50 patients with homozygous sickle cell disease. The α gene status was determined in 27 patients. All patients have been included in an original score of severity fitted to infancy and childhood. A positive correlation has been found between D50 and the clinical score. This result illustrates the potential clinical importance of this parameter as well as other biological indices such as the haemoglobin F level, the α gene status and the haplotypes of the β -like gene cluster.

Keywords: clinical score, haemoglobin, red cell density, sickle cell disease

Introduction

An important heterogeneity is evident among patients with sickle cell disease in regard to their clinical and biological features and the disease severity is not easily predictable. Biological indices of prognosis might help to determine a high risk group of patients thus making it possible that a close clinical monitoring, genetic counseling and preventive treatment be applied to this group.

Hitherto, it is known that high fetal haemoglobin (Hb F) level [1, 2, 3], α thalassemia [4, 5, 6] and particular haplotypes of the β -like gene cluster [7] modulate the disease severity. The distribution of red cell densities is one of the numerous biological factors which characterizes the sickle cell disease. Compared to normal profile, the distribution of sicklers is differing from one patient to another, but constant in the same patient outside crisis [8].

The aim of this study is to evaluate the eventual influence of red-cell densities on the clinical severity of the disease. To achieve this objective we correlated a clinical severity score with the parameters characterizing the density profile.

* To whom correspondence should be addressed.

Patients and Methods

Patient population

50 patients with sickle cell disease treated at the Enfants Malades Hospital (Paris) were chosen for this study. The patients, 26 males and 24 females, were native of 15 different countries of Africa and the West-Indies and ranged from 15 months to 27 years in age. At the time of the study, all patients were in a clinically steady state and had not been transfused for at least four months.

Haematological data

Haematological data (haemoglobin level Hb-g/dl, mean corpuscular volume MCV-fl) were determined using the Coulter counter S Plus. Reticulocyte counts were performed on thin smears after methylene blue staining; 1000 cells were counted under 100 \times oil immersion and the percentage of reticulocytes calculated. Hb F level was determined by the alkali denaturation method of Pembrey et al. [9]. Homozygosity for HbS was determined by two electrophoretic methods, solubility test and family studies when possible.

Clinical severity score

A clinical score was assessed by a retrospective review of the course of each patient (Table 1). Eight clinical parameters were used: age of diagnosis, age at the time of the study, number of hospitalizations, major organ involvement, sequestration crisis, splenectomy for hypersplenism, infections, acute pulmonary episodes. Points were assigned for each parameter from 0 to 4 depending on the severity conferred upon the considered symptom according to the literature [10, 11, 12, 13] and our experience. Furthermore, the patients were divided into 3 groups of increasing clinical severity; score 0–4: group 1, score 5–9: group 2, score 10–15: group 3.

Density determination

The details of the technique used to determine the density distribution were previously described [14]. Briefly, venous blood samples were collected in EDTA. Phtalate esters were prepared by mixing dimethyl phtalate (sp. gr. 1.090) and N-butyl phtalate (sp. gr. 1.045) to obtain 16 densities ranging from 1.075 to 1.135 according to the method of Danon and Marikovsky [15]. Four parameters were used to characterize the profile according to Noguchi et al. [16]: D50 (median cell density of the distribution), R60 (middle density range in which 60% of the cells

Table 1

Clinical score. Clinical parameters used for the classification of 50 patients with sickle cell disease and the point assigned to each of them

1. AGE OF DIAGNOSIS	4. NUMBER OF HOSPITALIZATIONS
a) <1 year: 3	a) <1/year: 0
b) 1 to 2 years: 2	b) 1/year: 1
c) 2 to 5 years: 1	c) 2 to 3/year: 2
d) >5 years: 0	d) >3/year: 3
e) family investigation: 0	
2. AGE AT THE TIME OF THE STUDY	5. INFECTIONS
a) <5 years: 4	a) osteomyelitis: 3
b) 5 to 10 years: 3	b) septicaemia: 3
c) 10 to 15 years: 2	c) meningitis: 3
d) 15 to 20 years: 1	d) recurrence of a, b, c: 3
e) >20 years: 0	
3. MAJOR ORGAN INVOLVEMENT	6. ACUTE PULMONARY EPISODE
a) neurologic complication: 3	a) mild: 1
b) bone infarction: 3	b) severe: 3
c) priapism: 3	c) recurrence of a: 1, b: 3
d) leg ulcer: 3	
e) gallstone: 2	7. SEQUESTRATION CRISIS: 3
f) others: 2	8. SPLENECTOMY FOR HYPERSPLENISM: 3

can be found), F4 and F5 (portion of cells in which density is higher than 1.110 and 1.120 g/ml, respectively). Eleven normal subjects were studied in order to specify the normal values of red cell densities.

Irreversibly sickled cells (ISC) counting

The ISC was examined by light microscope according to the method of Clark [17], modified as follows: the percentage of ISC among 500 erythrocytes was determined by a single observer after the cells were fixed in 1% glutaraldehyde-0.1 M Sorensen buffer.

Determination of α -globin genotype

The α -globin genotypes were determined in 27 patients using Southern blot hybridization restriction endonuclease mapping of genomic DNA extracted from

peripheral blood leukocytes [18]. The restriction patterns were obtained using Bam HI enzyme, and the fragments separated in agarose gel were hybridized with a genomic α Pst 1 probe after transfer on a membrane.

Statistical method

All statistics were done using a Statworks program on Mackintosh computer.

Results

50 patients with homozygous sickle-cell disease were analyzed. The distribution of clinical and biological variables are summarized in Tables 2 and 3. As expected, the parameters of the density distribution of the patient population differed from controls (Table 4). We examined the relationships 1) between the clinical score and the biological data and 2) between the biological factors themselves.

Table 2

Incidence of the clinical findings in 50 patients with sickle cell disease. This table shows the distribution of the patients according to each parameter

1. AGE OF DIAGNOSIS	4. NUMBER OF HOSPITALIZATIONS
a) <1 year: 14	a) <1/year: 23
b) 1 to 2 years: 8	b) 1/year: 23
c) 2 to 5 years: 18	c) 2 to 3/year: 3
d) >5 years: 7	d) >3/year: 1
e) family investigation: 3	
2. AGE AT THE TIME OF THE STUDY	5. INFECTIONS
a) <5 years: 13	a) osteomyelitis: 7
b) 5 to years: 15	b) septicaemia: 5
c) 10 to 15 years: 12	c) meningitis: 4
d) 15 to 20 years: 8	d) recurrence of a), b) or c): 1
e) >20 years: 2	
3. MAJOR ORGAN INVOLVEMENT	6. ACUTE PULMONARY EPISODE
a) neurologic complication: 3	a) mild: 8
b) bone infarction: 4	b) severe: 13
c) priapism: 1	c) recurrence of a: 3, b: 1
d) leg ulcer: 3	
e) gallstone: 3	7. SEQUESTRATION CRISIS: 2
f) others: 1	8. SPLENECTOMY FOR HYPERSPLENISM: 1

Table 3
Haematological data in 50 patients

	n	m \pm s.d.
Hb (g/dl)	50	7.9 \pm 0.1
MCV (fl)	50	87 \pm 9
Reticulocytes ($\times 10^3$ /cu.mm)	49	402 \pm 164
Hb F (%)	44	9.1 \pm 7
Irreversibly sickled cells (%)	34	6 \pm 1

Number of determinations (n), mean value (m) and standard deviation (s.d.).

Table 4
Red cell density distribution of 50 patients and 11 controls

Parameters	Normal controls (m \pm s.d.)	Patients with sickle- cell disease (m \pm s.d.)	p
D 50	1.102 \pm 0.002	1.101 \pm 0.005	n.s.
R 60	0.007 \pm 0.001	0.016 \pm 0.006	<0.001
F 4	6.5 \pm 6	20 \pm 1.5	<0.001
F 5	0.5 \pm 0.25	7 \pm 6.5	<0.001

Mean (m), standard deviation (s.d.), p = statistical significance, n.s. = non significant

Table 5
D 50, R 60, F 4 and F 5 according to the number of α globin gene in 27 patients

α -genes	D50	R60	F4	F5
$\alpha\alpha/\alpha\alpha$	1.101	0.018	21.9	7.9
n = 14	\pm 0.004	\pm 0.007	\pm 12.2	\pm 7
$-\alpha/\alpha\alpha$	1.101	0.014	16.2	5.6
n = 9	\pm 0.004	\pm 0.004	\pm 10.5	\pm 5.3
$-\alpha/-\alpha$	1.099	0.017	6.5	0.5
n = 2				
$\alpha\alpha\alpha/\alpha\alpha$	1.097	0.012	26.5	1
n = 2				

Number of determinations (n), mean value (m), standard deviation (s.d.).

Positive correlations were found between MCV and the number of α genes ($p < 0.0005$), F5 and D50 ($p < 0.003$) and between F4 and R60 ($p < 0.05$). A negative correlation between D50 and the number of α genes ($p < 0.04$) was noted (Table 5). No relationship between the clinical score and the haemoglobin level, MCV,

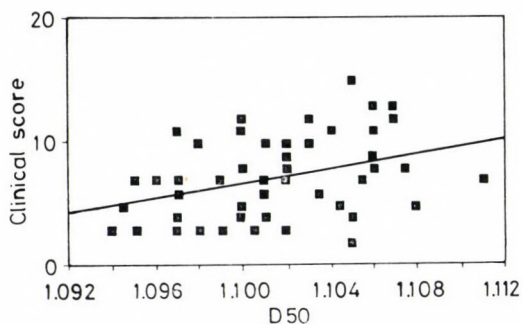


Fig. 1. Distribution of the clinical score and D 50 of the red blood cell density profile

Table 6

Value of D 50 when dividing the 50 patients into 3 groups of increasing clinical severity

	n	m \pm s.d.
Score 0 to 4	15	1.099 \pm 0.003
Score 5 to 9	21	1.102 \pm 0.004
Score 10 to 15	14	1.103 \pm 0.003

Number of patients in each group (n), mean (m), standard deviation (s.d.).

reticulocytes, irreversibly sickled cell levels was found. The haemoglobin F level was not related to the severity index. Likewise, there was no significant difference in the clinical severity among patients with and without α -thalassaemia. We established a positive correlation between D50 and the clinical score ($p < 0.003$) as shown in Fig. 1. The parameters F4, F5 and R60 were not related to the severity index. When dividing patients into 3 groups of disease severity, the value of D50 increased from 1.099 ± 0.003 for the first group, to 1.102 ± 0.004 for the second and to 1.103 ± 0.003 for the third (Table 6).

Discussion

The determination of the red cell density introduced by Danon and Markowsky [15] and applied afterwards to the study of the sickle cell disease is a simple technique. It is, moreover, reproducible when using standard conditions of sample, temperature and centrifugation. Since the preparation of the mixture requires great precision, these conditions must be strictly observed in order to obtain comparable laboratory results.

It is well documented that the sickle red cells' distribution is different in different patients, but is constant in the same patient [8] except in the case of crisis,

an event during which all dense cells disappear [19]. Some biological parameters as Hb F level and α gene status modify the density distribution [14] and are known to influence the prognosis of the patients affected with sickle cell disease [1, 2, 3, 4, 5, 6]. These ascertainments brought us to search for a relationship between the density profile and the clinical severity of the disease.

The establishment of a clinical score is essential in the sickle cell disease for several reasons. Such a score would enable the physician to notice the serious cases among patients and to adapt an adequate therapeutic attitude. A score, also, should offer the possibility of studying the effect of definite biological parameters on the clinical severity in a well defined group of patients. Such scores have been proposed previously [12, 13]. Steinberg's score [12] is better suited to adult. Likewise, it seems to us that certain clinical events which failed in the previous evaluations must be included in a new score. We will insist on the age of the patient at the time of the study. Some acute infection episodes, such as meningitis or septicaemia due to germs other than pneumococcus and as hyperplenism should be included in a clinical score. All these criteria appear to be objective and easily available. However, this score is still an a posteriori approach of the clinical severity. The inevitable limits of this method are as follows: 1) all the lethal forms are excluded from the series of patients; 2) in young patients the follow-up is very short and complications that may occur in the future are not taken into account. For these reasons we give a high score to the age of young patients and to certain complications usual in infancy and childhood.

Concerning the correlations, we first studied the relationship existing between some biological factors. The correlations noted between F4 and R60 on the one hand and between the number of α globin genes and D50 on the other have been described [14]. In this study, the values of D50 in patients with 5 α globin genes are identical to those measured in patients with 2 genes. This result might be explained by the low number of patients studied. The strong positive correlation between MCV and the α gene status ($p < 0.0005$) is as expected because the reduction in α globin chain synthesis induces low MCV. The correlation between D50 and F5 indicates that the patients with higher D50 have the greater level of dense cells, especially cells with density over 1.120 g/ml. It is difficult to determine the effect of haemoglobin F level on the other parameters because of the variation of haemoglobin F which disappears slowly in children with sickle cell disease till 5 or more years of age. Secondly, we studied the relationship between our clinical score and the biological parameters. The most interesting result is the closed positive correlation ($p < 0.003$) observed between the severity index and the median cell density D50. This fact appeared clearly when the patients were divided into 3 groups of severity. Lower values of the D50 were found in patients with mild sickle cell disease. However, this result probably needs to be confirmed in a more extensive population.

The red cell density profile, particularly the median cell density (D50), might be an index of prognosis of sickle cell disease. The displacement of the distribution to higher level and the appearance of dense cells in which the fraction of haemo-

globin S polymers is high, could influence the expression of the sickle cell disease [20, 21]. As for Hb F level, α genes status, haplotypes and other haematological data, the profile of the red cell density might contribute to establish an index of prognosis in sickle cell disease.

Acknowledgements

We are grateful to Valérie Fouquet for excellent assistance in the preparation of this manuscript.

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Lymphocyte Surface Ferritin in Malignant and Inflammatory Diseases

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(Received 3 August, 1987; accepted 10 September, 1987)

We developed a lymphocyte ferritin antibody-binding test (LFABT) to measure lymphocyte surface ferritin (LSF) and used it in cases of malignant and other diseases associated with elevated serum ferritin. LSF was elevated in 33 of 83 patients with a variety of malignant neoplasms in all stages of disease. LSF was also elevated in 2 of 5 patients with infectious mononucleosis, but was normal in all 15 patients with rheumatoid arthritis, bacterial infections and hemochromatosis. LSF and serum ferritin levels do not correlate. These findings suggest the usefulness of LFABT as a diagnostic tool and demonstrate the biologic significance of LSF.

Keywords: ferritin, tumor markers, iron proteins, lymphocyte surface markers

Introduction

Serum ferritin levels are often high in patients with malignant diseases and may be a useful marker of disease activity [1]. The elevated serum ferritin has a very limited diagnostic value because levels are also high in cases of infection, chronic inflammation, liver disease and iron overload. Acidic isoferritins may be disproportionately elevated in the serum of cancer patients, therefore this may be more specific [1, 2].

Peripheral blood leukocytes normally synthesize ferritin. Lymphocyte ferritin synthesis is increased in Hodgkin's disease and leukaemia [3, 4]. Ferritin can be found on the lymphocyte surface, and it is assumed that increased lymphocyte surface ferritin (LSF) may be a sensitive and specific finding in malignancy, particularly in early breast cancer [5, 7]. We have developed a sensitive, reproducible lymphocyte ferritin antibody-binding test (LFABT). This has been used in a large number of cancer patients to confirm or refute previous observations in breast cancer and Hodgkin's disease and to survey other malignant diseases. Prior studies have not proved that elevated LSF is a specific marker of malignancy, so we have studied patients with benign disorders, too (with inflammations, infections and

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Table 1
Elevated LSF with disease

	n	Elevated LSF (P/C > 2)
Hodgkin's disease		
Stage I—II	4	3
Stage III—IV	10	6
In remission	2	0
Breast cancer		
Stage II	5	0
Stage III—IV	17	9
Lung cancers	14	5
Non-Hodgkin's lymphomas	11	5
Chronic lymphocytic leukemia	7	1
Other malignancies	13	4
Benign disorders		
Mononucleosis infections	5	2
Bacterial infections	4	0
Rheumatoid arthritis	9	0
Hemochromatosis	2	0

n = number of patients studied; LSF = lymphocyte surface ferritin; P/C = patient to control ratio

iron overload). To our best knowledge so far the possible correlations between LSF and serum ferritin and the question whether elevated LSF is due to increased serum ferritin and merely reflects it, have not been studied.

Materials and Methods

Patients

103 patients and 45 normal controls were studied. Diagnosis of cancer was confirmed histologically, the disease was in a clinically active stage, and no coincident infection was present. Most patients were in the early stages of treatment; twenty percent were untreated. Patients listed with "other" malignancies (Table 1) mostly had advanced disease and had primary carcinomas of ovary, colon, kidney, testes and thyroid, soft tissue sarcomas and one had acute myeloid leukaemia.

Lymphocyte Ferritin Antibody-Binding Test (LFABT)

Mononuclear leukocytes were isolated from freshly-obtained heparinized venous blood by standard isopycnic flotation [8]. Lymphocyte concentration was adjusted to 1.5×10^6 cells/ml (suspensions contained 1–10% monocytes).

In 12×100 mm glass tubes, 0.33 ml of cell suspension were mixed with 0.2 ml of a high affinity ^{125}I -labeled rabbit anti-human spleen ferritin antibody (Ramco Laboratories, Houston, Texas). Triplicate samples were incubated 15 minutes at 4°C , then 45 minutes at 37°C with 5% CO_2 . Cells were washed five times in phosphate buffered saline, then resuspended in 1 ml buffered saline. The radioactivity of the cell suspension was determined (Packard Autogamma 500 C).

The quantity of anti-ferritin antibody binding was determined by subtracting the radioactive counts of a blank tube (to which antibody but no cells had been added) from the counts of the test sample. In each experiment, at least one normal control was studied simultaneously, and a patient to control ratio (P/C) was calculated. The results are expressed in this manner to correct for differing specific activities among antibody lots and to correct for decay of radioactivity over time in a given lot of antibody. Serum ferritin assays were done on the same blood specimens used in the LSF assay, employing immunoradiometric Fer-iron kit (Ramco, Houston, Texas).

Results

The LFABT was highly reproducible: multiple determinations on the same blood specimen showed a standard deviation from the mean of only 0.085%. When eight different normal controls were studied simultaneously, the standard deviation from their mean was 48% of the mean and no normal was more than twice the mean. We defined a P/C ratio of 2.0 or more to be elevated (statistically only 2% of normals are expected to fall above this level).

Table 1 summarized our findings in the patients with malignancies and benign disorders. 33 of 83 cancer patients (40%) had elevated LSF. 2 of 5 patients with infectious mononucleosis had elevated LSF, but no elevations were detected in the 15 patients with bacterial infections, rheumatoid arthritis and hemochromatosis.

Figure 1 plots LSF versus serum ferritin concentrations in the patients with cancer. There is no correlation ($r = 0.14$, $p > 0.1$). Similarly, there was no correlation of LSF and serum ferritin in patients with benign disorders ($r = 0.02$, $p > 0.1$).

Discussion

Applying our sensitive, reproducible LFABT we found that LSF is frequently elevated in cancer patients. LSF is elevated in a variety of different neoplasms in all stages of disease. To date this study involves the largest number of cancer patients and the largest number of patients with benign diseases. 2 of 5 patients with infectious mononucleosis had elevated LSF, but there were no elevations in patients with bacterial infection, rheumatoid arthritis or hemochromatosis.

Prior studies have not involved correlations between LSF and serum ferritin. Thus, the possibility has not been excluded that elevated LSF in cancer might reflect, at least partly, nonspecific adherence of increased serum ferritin to mono-

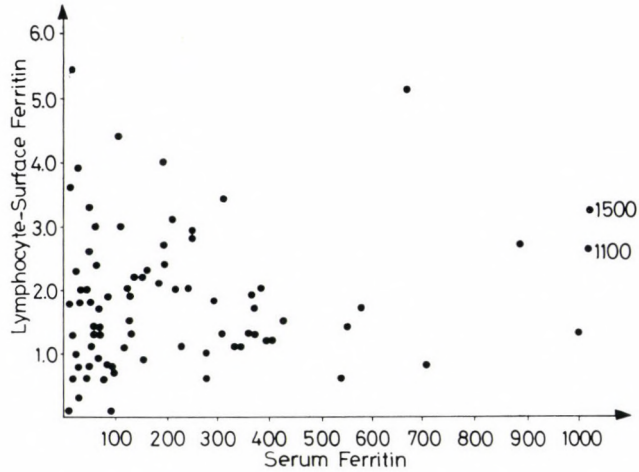


Fig. 1. Absence of correlation between LSF and serum ferritin in cancer patients ($r = 0.14$, $p > 0.1$)

nuclear leukocyte surfaces. Based on our present work, LSF and serum ferritin do not correlate but are independent biologic variables. It should be remembered that our anti-ferritin antibody is directed against a spleen-derived basic isoform. In cancer patients, an acidic isoform (heart-like, HeLA-like) may be disproportionately elevated in serum [2], so future study of this isoform on lymphocyte surfaces may be of interest.

Moroz and Giler have suggested that determination of LSF is clinically useful for diagnosis of early breast cancer [5, 6, 7]. Our findings indicated that LSF was not consistently elevated in early breast cancer and was often elevated in advanced breast cancer. A number of factors could contribute to the differences. An important factor might be the patients' treatment status (our patients were postoperative and theirs preoperative). In the future, the issues could be clarified by long-term serial LSF determinations in patients before and after surgery and monitoring of therapy and course of the disease. Another factor that may contribute to differences between the studies is the different assay techniques for LSF and the different manners of expressing results. Previous studies have determined a percentage of ferritin-bearing lymphocytes by fluorescent or cytotoxic assays [5, 6, 7, 9], or have used a more quantitative but cumbersome LSF assay involving surface protein iodination, cell lysis, electrophoresis and immunoprecipitation [10]. Our simple LFABT determines mean surface ferritin, averaging all peripheral blood mononuclear leukocytes. Conceivably, mean LSF and percent LSF bearing lymphocytes may be disparate with some disorders.

The question of the clinical utility of the test, particularly its sensitivity, arises in relation to our observation of elevated LSF in malignancy. Sensitivity could be increased by lowering our upper normal threshold, but that would

diminish specificity. In this case the results would be similar to Papenhausen's, i.e. a good majority of cancer patients would show elevated LSF, but so would some patients with benign lesions and some normal controls [9]. The diagnostic utility is further reduced by our finding that elevated LSF is not specific for any particular type or stage of malignant disease. While LSF is not a function of serum ferritin and is not elevated in most patients with benign inflammatory disease, there were elevations in cases of infectious mononucleosis.

Studies have suggested that LSF 1) correlates with quantitative and qualitative T-cell deficiencies in Hodgkin's disease [5, 10], 2) can be modulated by the immunopotentiating drug levamisole [10], 3) is carried by a specific T-cell suppressor subpopulation in breast cancer [11], and 4) directs abnormal lymphocyte migration patterns in lymphomas [12]. We suggest that it may be more fruitful to reveal the pathophysiologic causes and significance of LSF than attempting to use it as a diagnostic test for malignancy.

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Familial Chronic B-cell Malignancy Hairy Cell Leukaemia in Mother and Daughter

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(Received 16 June, 1987; accepted 25 August, 1987)

Two familial cases of hairy cell leukaemia are reported: a daughter, 44-years-old, with a very unusual ultrastructural pattern found in hairy cells, the "tubuloreticular inclusions", and her mother, 71-years-old, who was affected six years later. Routine laboratory investigations, cytochemical and cytogenetic studies including HLA typing, as well as *in vitro* proliferative response of peripheral blood mononuclear cells (PBMC) to polyclonal mitogens and to exogenous interleukin 2, were performed. The immunological characterization by assessing the cell surface phenotypic markers with monoclonal antibodies and transmission electron microscopy (TEM) investigations were also carried out. In case 2 all tests were performed both on PBMC and on the bone marrow cells. To the best of our knowledge this is one of the first reports of such familial association. The possibility that genetic factors might play a role in the etiology of leukaemia in man is discussed: in our two cases, however, cytogenetic studies did not support this, while HLA typing revealed a non-significant association of HCL with DQw3 allele. Alternatively, an environmental factor has been considered, and a viral infection—perhaps by a retrovirus of the HTLV family has been suggested as tubuloreticular inclusions have been found in both hairy cell leukaemia, as reported, by us, and AIDS-LAS. However, a long time elapsed between the manifestation of HCL in the daughter and in the mother, and as the two patients had not been living together at that time, the possibility of a viral transmission seems minimal. The results of TEM and of immunological investigations are presented and discussed. Both, but particularly the latter, support the B cell nature of the hairy cell.

Keywords: cell immunophenotyping, chronic B cell malignancy, familial incidence, hairy cell leukaemia, HLA typing, tubuloreticular inclusions, ultrastructural findings

Introduction

Hairy cell leukaemia (HCL) is a lymphoproliferative disorder, which accounts for 2% of all leukaemias. It is characterized by splenomegaly, pancytopenia and by the presence of characteristic mononuclear cells with irregular

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cytoplasmic border and often hairy-like cytoplasmic projections in the bone marrow, spleen and peripheral blood [1–3]. Due to neutropenia, monocytopenia and impaired T cell function, infections are very common and are often the cause of death [4, 5]. In spite of controversy on the origin of hairy cells (HCs) [6–8], the majority of investigators now agree that HCs are neoplastic B lymphocytes, closely related to B-chronic lymphocytic leukemia (CLL) cells [9–13]. Evidence indicates that B-CLL, hairy cell leukaemia, prolymphocytic leukaemia and Waldenstrom's disease are linked by several biological and clinical aspects [14]. They appear as a distinct subgroup among the chronic B cell malignancies, clearly differing from follicular center tumours, and it seems reasonable to suggest that the target of the transforming event might be the same B cell population [15].

As for the familial incidence of HCL, very few cases have been previously reported: the most interesting report [16] described a family of 11 siblings in which three brothers with the HLA A1, B7 haplotype developed HCL within a seven-year period. Another report [17] examined a father and a son with HCL. HLA typing revealed both patients to be A1, 3 and B8, 14. A third report [18] described a family in which members of three generations have been affected by haematologic malignancies: the father with chronic myelogenous leukaemia, the proband with HCL and his brother x, with CLL, and a nephew with cutaneous and CNS lymphoma: this was the first reported case of HCL occurring with other haematologic malignancies within a family. To the best of our knowledge, in the complete case list of over 400 patients reported by the Italian Cooperative Group of Hairy Cell Leukaemia (ICGHCL), in which our group also takes part, no familial case was reported.

Two cases of HCL are described here: the first affecting a daughter with a very unusual ultrastructural pattern found in HCs, previously reported by us [19], and the second affecting her mother who was affected about six years later.

Case Reports

Case 1, L. A., a 44-year-old woman, was hospitalized in November, 1980 due to splenomegaly (7 cm under costal margin) and complaints of fatigue since two months. Diagnostic laparotomy and splenectomy were performed. An enlarged spleen was found and several lymph nodes around the hilum of spleen. The cytologic and histologic pattern of spleen showed a high number of typical HCs. Before splenectomy, leukopenia (WBC count $2.6 \times 10^9/l$), thrombocytopenia (platelet count $45 \times 10^9/l$) and a mild anaemia (Hb 11.4 g/dl, RBC count $3.6 \times 10^{12}/l$) were found. Soon after splenectomy the patient was referred to us. The haematological status at this time was: a mild anaemia (Hb 11.5 g/dl) and leukopenia (WBC count $3.5 \times 10^9/l$) which persisted for the subsequent years, whereas the thrombocytopenia disappeared. The differential leukocyte count showed 50% of lymphoid cells, 10% of which were HCs, and 2% monocytes. In the bone marrow aspirate 16% of the nucleated cells were HCs; the marrow histology displayed

wide areas of adipose tissue and haemorrhagic infiltrates. Cytochemical findings: over 30% of peripheral mononuclear cells showed tartrate-resistant acid phosphatase (TRAP) activity. In the following years the patient suffered from "flu-like" mild intercurrent infectious episodes. No therapy was applied until November, 1985 when treatment with human lymphoblastoid alpha-2 Interferon (IFN) according to Treatment Protocol of the Italian Cooperative Group for HCL was started, due to the mild persistent leukopenia and moderate marrow infiltrate of HCs (approximately 10% of nucleated cells).

The WBC count raised to over $5 \times 10^9/l$ with neutrophils over $2 \times 10^9/l$, the HCs lowered to 2% in peripheral blood and to less than 10% in the bone marrow. Having achieved a minor response, the patient was assigned to maintenance therapy until one year of treatment was completed. At the end of IFN treatment, the patient was well and active (Grade 0 Performance Status, WHO) and the response was assessed as a minor, but significant, response (MR) (Hb 13 g/dl, WBC count $6.5 \times 10^9/l$, platelets $400 \times 10^9/l$, neutrophils $3 \times 10^9/l$, HCs in peripheral blood 5%).

The bone marrow aspirate yielded marrow blood without particles of marrow with HCs < 5%. The percutaneous trephine biopsy of the bone marrow showed a discrete marrow fibrosis with a significant hyperplasia of argyrophilic reticular fibrilles stained by silver-impregnation method. HTLV-III antibody in the serum was not detected. A cytogenetic study including both routine and banding methods was performed: the karyotype was numerically and structurally normal. The HLA typing revealed the patient to be an A2, 31; Cw3, -; B51, 60; DR4, 8; DRw52, 53; DQw3. A sister of the patient, healthy, revealed the identical HLA pattern.

At the last follow-up (February, 1987) the haematological and clinical findings were unaltered.

Case 2, P. G., a 71-year-old woman, mother of L. A. (Case 1), came to our attention in June, 1986, as a result of a chance finding of moderate leukocytosis (WBC count $16-18 \times 10^9/l$) with relative (about 50%) and absolute lymphocytosis (over $9 \times 10^9/l$); this haematological abnormality appeared 2 months afore. The Hb, RBC and platelet counts were in the normal range. All other laboratory findings were normal, in particular the serum immunoglobulins, as well as the skin test response to recall antigens. She was in good clinical condition and physical examination was normal: a mild splenomegaly was found, confirmed by ultrasonic ecotomography and only small lymph nodes were palpable in the left latero-cervical region. The cytochemical staining for TRAP activity showed a moderate amount of positive cells: approximately 20% with score + or ++, 1% with score 5+ in the peripheral blood smear and several cells were 1+ - 3+ in the marrow aspirate smear. It is to be noted that TRAP (isoenzyme 5) is often present in HCs, but the activity may be weak or absent in some cases and is often partially and sometimes completely inhibited by tartrate [20]. The cytochemical staining for PAS, both on peripheral blood and on bone marrow lymphocytes, showed a weak positivity with both diffuse and granular reactions in HCs. The bone marrow

aspirate showed a marked, diffuse increase of lymphoid cells (approximately 50% of nucleated cells), most of which were HCs; the erythroid, myeloid and megakaryocytic series were normal. The percutaneous trephine biopsy of bone marrow showed a discrete marrow fibrosis with reduction of normal haemopoietic marrow and a diffuse increase of reticular fibrilles stained by silver-impregnation method. No HTLV-III antibody was detected in the serum. The karyotype was numerically and structurally normal. The HLA typing revealed the patient to be A2, 24; Cw2, -; B14, 51; DR1, 4; DRw53; DQw1,3.

At a nine month follow-up (February, 1987) the patient was well and active (Grade 0, WHO). The haematological picture was similar to that of the one observed at the time of diagnosis, but the peripheral blood lymphocyte differential count decreased to approximately 35% of all leukocytes, with 20% of HCs. The mild splenomegaly persisted unchanged. As there was no indication to start any treatment, we decided to continue the patient's follow-up.

Methods

Laboratory investigations

The following in vitro investigations were performed:

- in vitro proliferative response of peripheral blood mononuclear cells (PBMC) to polyclonal mitogens PHA, Con A, PWM and to exogenous interleukin 2 (IL 2) either by PHA-activated or non-PHA-activated PBMC with the microtitre plate technique, according to our previously described method [21, 22];
- immunological characterization of PBMC and, in Case 2, also of bone marrow mononuclear cells (BMMC) by assessing the cell surface phenotypic markers with a very large panel of monoclonal antibodies (MoAb) according to our previous work [23]. An optical Flow Cytometry apparatus (Ortho Spectrum III) was utilized.

Transmission electron microscopy (TEM) investigations

These were performed on PBMC and in case 2 also on BMMC. The buffy coat of heparinized peripheral or bone marrow blood was fixed in 1.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3 for 2h (4 °C) and postfixed in 1% Osmium tetroxide in the same buffer (4 °C). The samples were then dehydrated through a graded series of ethanol and embedded in Epon 812. Thin sections were double stained with uranyl acetate and lead citrate and examined with a Philips EM 300.

Results

Laboratory investigations

Case 1. The patient's PBMC response to polyclonal mitogens was poor at the time of diagnosis and at subsequent controls during the follow-up.

The cell immunophenotyping performed on PBMC (WBC count $4.7 \times 10^9/l$, lymphocyte count $1.6 \times 10^9/l$) is reported in Table 1. Reactivity of B cell-associated (anti DR, B2, B7, sIg), T cell-associated, monocyte-associated and granulocyte/monocyte-associated MoAb with PBMC was determined. PBMC of Case 1 was reactive, in relatively high proportion for HCL, with T-associated antigens, while they reacted in relatively low proportion with the pan B anti DR MoAb: the B2 antigen, which appears after the pre-B cell stage and is lost when pre-secretory Ig appears, was even lower than DR, as sIg, while the B7 antigen, which appears at the later stages of maturation, is higher. The granulocyte/monocyte-restricted M1 antigen is high, while the monocyte-restricted M5 is low, as well as the myeloid-restricted My9. The PCA-1 antigen, characteristic of plasma cells, was weakly expressed in our case, whereas it is frequently strongly expressed on the HCs. Also the Tac antigen, although higher than in the normal population, is lower than that reported in HCL [13, 24].

Table 1

Immunologic phenotyping of peripheral blood mononuclear cells (PBMC) and of bone marrow mononuclear cells (BMBC) of two cases of HCL, performed with monoclonal antibodies

	Case 1	Case 2	
	Lymph. count ($1.6 \times 10^9/l$)	Lymph count ($9.4 \times 10^9/l$)	
	% of PBMC	% of PBMC	% of BMBC
OKT3 ⁺	48.9	14	17
OKT4 ⁺	35.5	8.7	8.6
OKT8 ⁺	12.1	4.1	10.6
OKT11 ⁺	66.1	15.2	18.3
OKT6 ⁺	0.1	0	0.6
OKT9 ⁺	1.1	4.6	7.2
OKT10 ⁺	1.5	3.9	16.3
OKB2 ⁺	10.2	55.2	66.1
OKB7 ⁺	16.4	15.0	60.9
sIgtot ⁺	9.3	3.5	3.6
sIgD ⁺	9.7	2.4	1.2
sIgM ⁺	6.1	0.9	1.3
Kappa ⁺	4.9	1.3	—
Lambda ⁺	3.8	0.5	—
DR ⁺	13.7	73.3	73.2
OKT1(CLL) ⁺	53.9	80.7	80.4
OKT26a ⁺ (anti-Tac)	7.6	6.8	2.1
OKB-cALLa ⁺	6.7	1	23.1
OKM1 ⁺	33.5	48.1	63.9
OKM5 ⁺	20.7	0.9	12.3
Leu 7 ⁺	14	5.6	9.7
Leu 11a ⁺	14.9	6.2	6.2
PCA-1 ⁺	4.2	2.8	—
My9 ⁺	1	0.3	—

Case 2. The patient's PBMC response to polyclonal mitogens was below the normal range, whereas the response of non-PHA-activated PBMC to IL 2 was higher than normal and the response of PHA-activated PBMC to IL 2 was significantly higher than the response to PHA alone. This may indicate a defective production of endogenous IL 2 in HCL or, alternatively, the possibility that the IL 2 produced by the T cell population may be readily utilized by the neoplastic B cells, like in B-CLL: the observation that HCs and B-CLL cells may express the IL 2 receptor at high levels seems to confirm this possibility [25]. Cell immunophenotyping performed both on PBMC (WBC count $18 \times 10^9/l$, lymphocyte count $9.4 \times 10^9/l$) and on BMNC is reported in Table 1. PBMC showed a dramatic reduction of total T cells and of their subsets, while the number of OKT1⁺ cells representing B cells of HCL was particularly high. In this case the pan B DR antigen is high as well as the B2 antigen, as in Case 1. The PCA-1 and Tac antigens were markedly lower than that reported in HCL [26]. Cell immunophenotyping on BMNC showed a feature substantially similar to that of peripheral blood.

TEM investigations

Case 1. The TEM morphology of cells examined is shown in Fig. 1 to 3.

Figure 1 shows the characteristics of the cytoplasm of HCs: many villous projections, either thin and delicate or large and irregular, different in size and variously arranged spatially, either straight or with bizarre foldings are observable. The characteristics of the nucleus and of cytoplasmic organelles are typical for

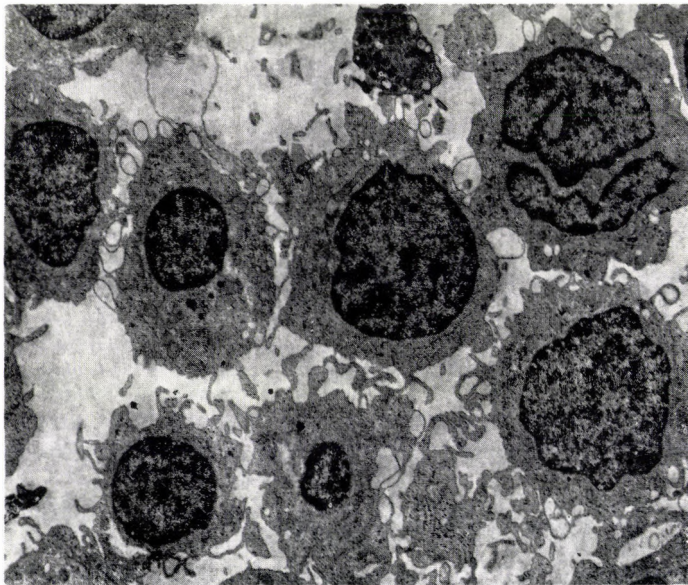


Fig. 1. Case 1. Hairy cells of peripheral blood: the typical hairy-like cytoplasmic projections ($\times 7,500$)

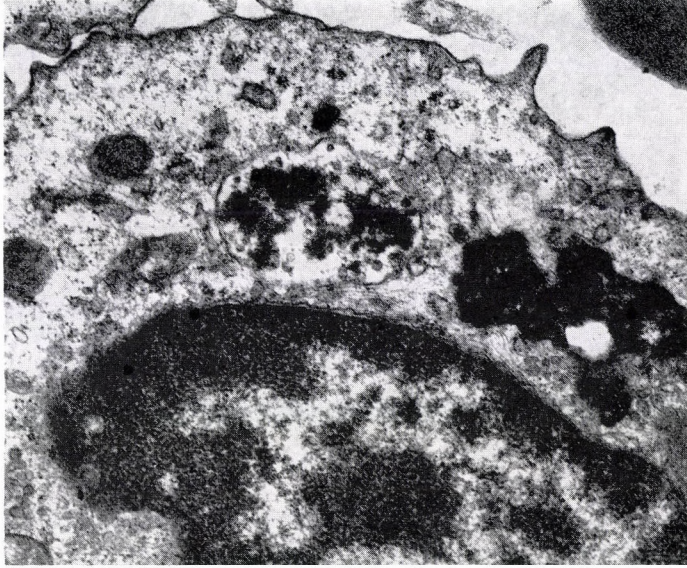


Fig. 2. Case 1. Hairy cell: cytoplasmic "tubuloreticular inclusions" are visible ($\times 48,000$)

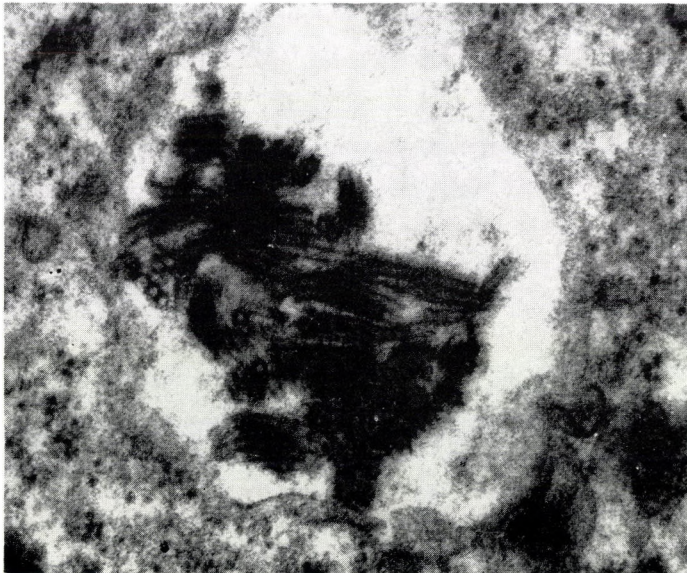


Fig. 3. Case 1. The "tubuloreticular inclusions" at higher magnification, showing the characteristics of these structures ($\times 135,000$)

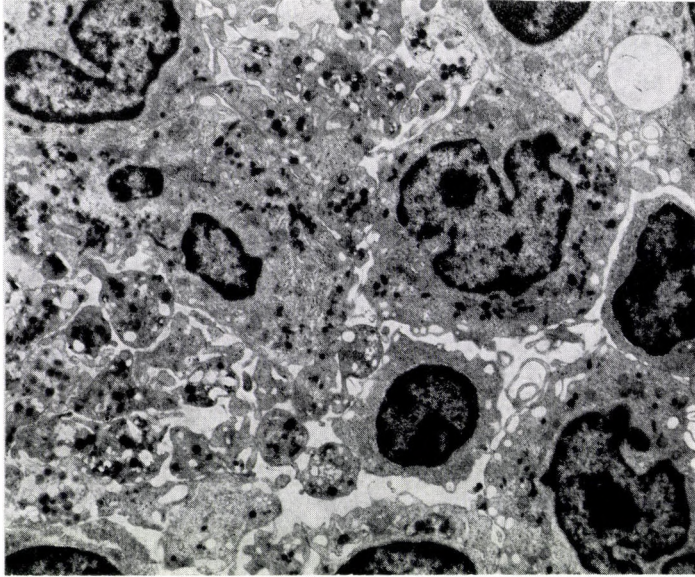


Fig. 4. Case 2. Hairy cells of peripheral blood ($\times 7,500$)

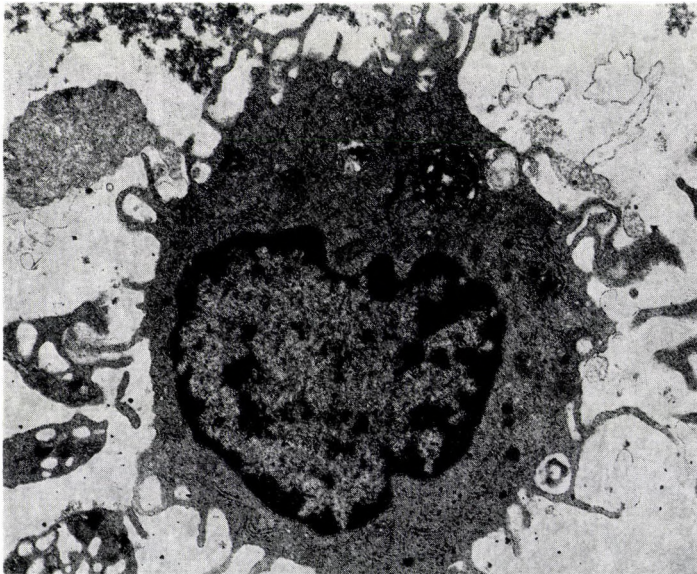


Fig. 5. Case 2. Hairy cell of bone marrow: the typical hairy-like projections are evident. The nucleus is cleaved and the cytoplasmic organelles are visible ($\times 14,200$)

HCs. The most striking feature was that in approximately 20% of the HCs examined abnormal subcellular organelles (Figs 2 and 3) were present consisting of intracytoplasmic structures which seem to be identical with "tubuloreticular inclusions" described by Grimley et al. in peripheral mononuclear cells [27, 28]. These structures consist of nuclease and RNase resistant complexes of lipid-rich membranes and proteins [29, 30]. They are formed in the cytosecretory apparatus and consist of fine tubular meshworks that distend the endoplasmic reticulum. The structures within HCs are located close to the nucleus, surrounded by a thin rim and identical with the tubuloreticular inclusions described in the cytoplasm of PBMC of adults and an infant with AIDS and of homosexuals with Lymphadenopathy Syndrome (LAS) [31–34].

Case 2. HCs of peripheral blood and bone marrow were studied. The morphology of peripheral blood HCs is substantially similar to that of the above reported Case 1, and is shown in Fig. 4. The HCs of bone marrow have many hairy-like cytoplasmic projections, very typical of such cells (Fig. 5). The nuclei were relatively large, seldom round or oval, more often irregular and cleaved: this feature is distinctive, unlike HCs of Case 1. The chromatin was rather thin, often with "spongy" pattern, but especially condensed at the nuclear periphery. One (or rarely two) nucleolus is sometimes visible in the cells, it is more often large than small. The cytoplasm, rather poor in organelles, showed a moderate amount of mitochondria often piled and then with "cristae" swelling, condensed matrix and dense granules. A single or multiple centrioli were frequently seen, and the Golgi apparatus was generally well developed or even hypertrophic. Lysosome granules, frequently grouped and more or less electron-dense, were also present, and there was evidence of phagocytic vesicles and of pynocytic activity. A smooth endoplasmic reticulum was present in many cells together with numerous free ribosomes in the cytoplasm. Many of these morphological findings have been already described in our previous work [35].

Discussion

Beyond the interesting finding of the very unusual ultrastructural pattern found in Case 1, i.e. the "tubuloreticular inclusions" reported for the first time in HCs, the main interest of our study was focused on the exceptional incidence of two cases of HCL, a very rare disease in itself, in two close relatives (mother and daughter).

To the best of our knowledge, this is the third report of such an association and the first involving mother and daughter.

The possibility that genetic factors might play a role in the etiology of leukaemia in man is an ancient suggestion already made more than a century ago by Biermer and others who reported the occurrence of multiple cases of leukaemia in some families. Interest increased when Dameshek in 1929 first reported the classical cases of twin brothers affected by CLL and, twenty-five years later,

the case of a son of one of the twins who developed CLL, too [36]. One of the most important studies of hereditary factors in leukaemia, published in 1969 by Gunz and Veale [37], was focused on CLL. In all series familial CLL tends to occur in closely related persons, siblings, parents, children, but otherwise there was no clearcut pattern of inheritance. Most of the patients with familial leukaemia, however, belonging to the elder age group, had not shared a common environment with other affected members of the family for many years [38].

Although the report of Wylin et al. [16], which described HCL affecting three siblings with a common haplotype, strongly suggests a genetic linkage, a control series of ten other unrelated patients with HCL who were typed by the same author showed no evidence of a common HLA phenotype: this is in agreement with other reports.

On the other hand, the identical HLA types (A1, 3 and B8, 14) in HCL of father and son [17] seem to postulate a genetic mechanism causing increased susceptibility to this disease.

Unfortunately the HLA typing of the whole family of our two patients was not possible: the typing of the three members studied seems simply to suggest a weak association of HCL with DQw3, as has already been reported previously [39]. Indeed both the father and mother had a DQw3 allele which was inherited by both the daughter affected by HCL and her healthy sister.

Explanations for the fact that only a few persons with a specific HLA type actually develop the disease have been summarized by Bodmer [40]: 1) environmental factors, 2) other genetic factors not related to HLA, 3) the hidden locus occurring less frequently than the HLA locus and 4) the heterogeneity of the disease being studied have been suggested. Similarly to other HLA-associated diseases it seems likely that it is not the haplotype itself, but perhaps a closely linked and as yet unidentified gene that is responsible for the increased disease susceptibility [16].

Besides the above discussed HLA linkage, the problem of the relationship between human oncogenes and B cell malignancies has arisen: of the two putative oncogenes involved (*bcl-1* and *bcl-2*) *bcl-1* seems to be associated with CLL of the B cell type carrying a t(11:14)(q13; q32) translocation [41]. Moreover, a trisomy of chromosome 12 was described as characteristic for HCL [42]. The possible role of oncogenes and of chromosomal abnormalities in the development of HCL remains, however, to be established.

Most, although inconclusive, suggestions support that a genetic factor may play a role in the etiology of HCL: in our two cases cytogenetic investigations did not prove this factor, whereas the HLA typing revealed a non-significant association of HCL with DQw3 allele.

Alternatively, environmental factors may also be considered in the etiology of HCL. It must be stressed that our two patients, although relatives, had not lived together for over twenty years, i.e. from the time of the daughter's marriage. Both patients were housewives and had never been exposed to medical risks nor to radiation or chemical hazards.

An interesting fact to be considered is the above described ultrastructural pattern of "tubuloreticular inclusions" (TRI) seen in the HCs of Case 1. Although the pathophysiologic function of these inclusions is still unknown, the discovery of TRI in patients with herpes virus encephalitis and especially in those with AIDS and LAS (see above) clearly suggests a relationship to virus infection [43, 44]. Thus an etiologic association between a virus infection and the outbreak of HCL may be assumed. The virus involved may be a HTLV retrovirus identical with the etiological agent of AIDS, although HTLV-III antibodies were not detected in either case. An association between AIDS and HCL has been previously reported [45].

An alternative hypothesis might be that the virus infection had been an occasional opportunistic one and therefore it did not play any role in the etiology of HCL. In any case, the long time (over 6 years) which elapsed between the occurrence of HCL in the daughter and in the mother, and the fact that the two patients had not lived together in this period seem to exclude or minimize the likelihood of a viral transmission. An interesting fact is that also in the report of Ramseur et al. [17] the time of onset of the father's disease was within five years of the proband's.

A striking defect in cellular immune function was found in both of our patients: unfortunately we could not study all members of the family to detect the possible subclinical immune defects in unaffected members.

Finally, another interesting factor is the immunological pattern of HCL cells. Our data on immunological phenotyping are partly in agreement with the above reported findings [25]: they differ in regard to Tac and PCA-1 antigens. The panel of B cell-restricted and -associated antigens used were not complete. It should be emphasized, however, that an exhausting immunophenotyping was not our main purpose.

Several data support the suggestion that HCL is a neoplasm of B cell origin. The presence of the B cell-associated antigens and of the B cell-restricted determinants on HCL cells strongly suggests that these tumors are derived from B cells [8]. The recent report that heavy and light chain gene rearrangements were found in 8/8 patients with HCL is a further support for this hypothesis [13].

Most recent studies [25] suggests that HCL is not derived from an early- or mid-stage B cell, but is a tumor of presecretory B cells: moreover, their unique morphology and cytochemistry indicate that the HCL is derived from a phenotypically unique subset of presecretory B cells. Whether a subset of normal B cells with these properties exists is still unknown. However, the unique biologic and clinical behaviour of this disease suggests that it is derived from a small subset of normal cells that invade the bone marrow and spleen.

Acknowledgement

This work was supported in part by the CNR (Italian National Research Council) Applied Project "Oncology" (Contract No. 86.00389.44).

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Protective Effects of Vitamins E and C on Erythrocytes in Blood Preserved in ACD Solution and Stored at 4 °C

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(Received 24 August, 1987; accepted 15 October, 1987)

Vitamins E and C were used for the enrichment of the fluid for blood preservation. Whole blood with added ACD solution and vitamins E and C was stored at 4 °C for 21 days. The performed determinations included: haematocrite, osmotic fragility of the erythrocytes, ATP and 2,3-DPG content in the erythrocytes, and echinocyte count.

A protective effect of these vitamins on erythrocytes was manifested by the decreased osmotic fragility, the steep fall of 2,3-DPG compared with the blood preserved in the vitamin-free ACD solution.

Keywords: stored erythrocytes, vitamins E and C

Introduction

Structural and functional changes of erythrocytes with reduced osmotic fragility and cell membrane integrity as well as deformability of the whole cell is shown in preserved blood [1, 2, 3]. A loss of cell membrane fragments is caused by the separation of microvesicles [4, 5], and peroxidation processes change the molecular structure of this membrane [6].

By modifying the composition of solutions used for blood preservation it was possible to improve the vitality and osmotic fragility of erythrocytes, to reduce the degree of haemolysis [7], to reverse processes of cell deformation [8, 9], and to maintain the level of metabolically highly active compounds such as ATP and 2,3-DPG [10, 11]. The damage to cell membrane structure in stored erythrocytes resembles the processes that take place during cell aging, when peroxidation processes become prevalent [12]. Consequently, one might assume that antioxidants could prevent the adverse effects of peroxidation processes and improve erythrocyte survival. Vitamin E [12, 13] and vitamin C [14, 15, 16] are well-known physiological antioxidants.

This work aimed at studying the effects of vitamins E and C on the erythrocytes of blood preserved in ACD solution at 4 °C, and at comparing the results obtained with those of similar blood samples preserved without these vitamins.

The marked parameters were: osmotic fragility of the erythrocyte, echinocyte count, adenosine triphosphate (ATP) and 2,3-diphosphoglycerol (2,3-DPG) levels in the erythrocytes.

Materials and Methods

Investigations were carried out on blood samples obtained from five healthy subjects. Vitamins E and C were added to ACD solution before it was mixed with whole blood, or to the whole blood before it was mixed with ACD. The blood was kept in glass bottles at 4 °C for 21 days. Various amounts of vitamins E and C were added, then haematocrite values, erythrocyte osmotic fragility (according to the method of Seeman and Weinstein [17]) and the echinocyte count were compared [8, 18]. The osmotic fragility of the erythrocytes was expressed as the degree of haemolysis in a buffered hypotonic solution of NaCl (60 mM NaCl at 37 °C for 1 h), and was calculated as a per cent of total haemolysis of the same erythrocyte volume in water. In estimating the number of deformed erythrocytes in the preparation of blood smears, echinocyte grades I and II were counted jointly [19]. The ATP and 2,3-DPG levels in the erythrocytes were determined in three blood samples taken from healthy subjects with vitamin E 60 µg/ml and vitamin C 2 µg/ml using standard enzymatic methods (Sigma test). Similar determinations were carried out in blood samples which lacked vitamins. The results obtained were subjected to Student's *t* test statistical analysis.

Results

Haematocrite index

During 21 days of blood storage (with ACD), the haematocrite index remained unchanged (from 30 to 40%, depending on the blood sample) in the samples with and without vitamins.

Erythrocyte osmotic fragility

The addition of vitamins E and C caused a slight reduction of the osmotic fragility of the erythrocytes at several concentrations expressed as the grade of haemolysis in a hypotonic solution in comparison to a control sample (Table 1). The addition of vitamin E 60 µg/ml and vitamin C 2 µg/ml to the ACD solution before mixing it with blood caused a much more evident reduction of haemolysis degree both in the early ($p \sim 0.1$) and in the late period of blood storage (on day 21), when the osmotic fragility was 25% lower than in the control sample ($p < 0.05$), (Table 2, Fig. 1a).

Table 1

Effect of various concentrations of vitamin E and C on the erythrocytes of blood preserved in ACD at 4 °C and stored for 21 days

Determination	Vitamin concentration in 1 ml of blood together with ACD	Duration of blood storage (days)				
		0	3	7	14	21
Erythrocyte osmotic fragility (% of hemolysis)	control	17.2	15.0	37.7	50.2	65.9
	Vit. E – 30 µg/ml		18.6	31.4	49.3	63.9
	Vit. E – 60 µg/ml		16.7	28.5	50.2	64.6
	Vit. C – 1.5 µg/ml		19.8	34.8	49.6	67.9
	Vit. C – 2.0 µg/ml + vit. E – 60 µg/ml		19.8	31.9	46.8	64.7
echinocytes (%)	control	1.3	370.0	164.0	200.0	280.0
	Vit. E – 30 µg/ml		90.0	100.0	145.0	260.0
	Vit. E – 60 µg/ml		35.0	160.0	188.0	83.0
	Vit. C – 1.5 µg/ml		80.0	96.0	82.0	100.0
	Vit. C – 2.0 µg/ml + vit. E – 60 µg/ml		6.0	120.0	124.0	140.0

* Vitamins added to whole blood before mixing it with ACD
 Attention: The values show means of samples from two blood donors

Echinocyte count

Samples with added vitamins E and C showed lower echinocyte counts during the 21 days of observation than the vitamin-free samples, although fluctuations did occur (Table 1). The best results were obtained when vitamins E and C were added to ACD at a concentration of 60 µg/ml and 2 µg/ml, respectively. On day 21, the reduction of the echinocyte count in these samples was statistically significant as compared to that of vitamin-free blood samples (Table 2, Fig. 1b).

ATP and 2,3-DPG levels in the erythrocytes

ATP concentration in the erythrocytes in blood preserved with 60 µg/ml E and 2 µg/ml C vitamin did not differ from that in control samples over the whole 21-day period of investigation, and the degree of decrease in the erythrocytes was nearly identical (Table 2, Fig. 2a).

The level of 2,3-DPG in the erythrocytes decreased with equal rapidity in samples with and without vitamins, although a slight delay was noted in the steep fall of 2,3-DPG concentration on day 7; by that time this concentration was about 30% higher than in the control samples (Table 2, Fig. 2b).

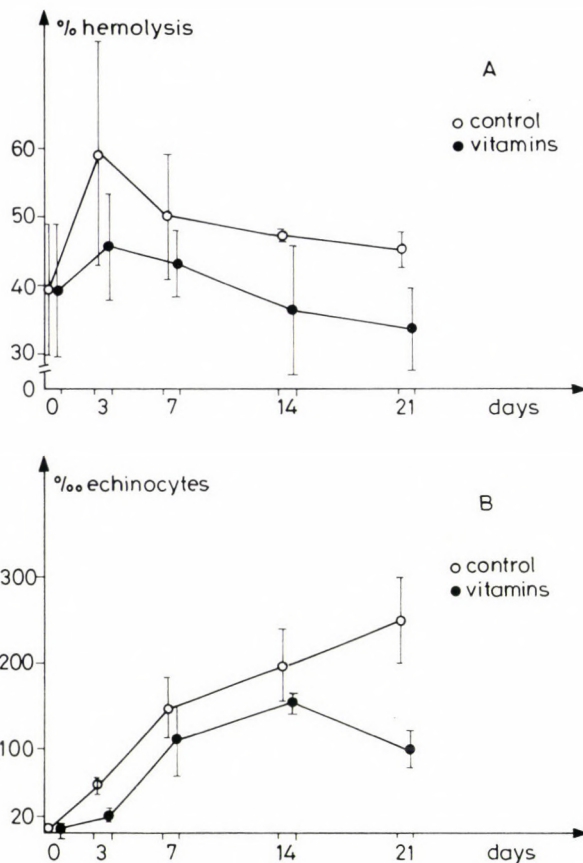


Fig. 1a. Osmotic fragility of erythrocytes preserved in ACD with added vitamin E (60 $\mu\text{g}/\text{ml}$) and vitamin C (2 $\mu\text{g}/\text{ml}$) stored at 4 $^{\circ}\text{C}$. The fragility expressed as percent of haemolysis
 Fig. 1b. Number of echinocytes and blood preserved in ACD with added vitamin E (60 $\mu\text{g}/\text{ml}$) and vitamin C (2 $\mu\text{g}/\text{ml}$) stored at 4 $^{\circ}\text{C}$

Discussion

Various trial tests were performed for the selection of an optimal composition of solution to be used for blood preservation. Some aimed at the correction of ATP loss from the erythrocytes by addition of adenine [2] resulting in a transformation of echinocytes into erythrocytes of normal appearance [8]. The addition of mannitol resulted in a decrease of spontaneous haemolysis [7, 10], and a reduction of the number of deformed erythrocytes [10]. Other purines and pyruvates also have a favourable effect on the survival of the stored erythrocytes [11].

Table 2

The effect of joint addition of vitamin E (60 µg/ml) and C (2 µg/ml) added to ACD on erythrocytes preserved in ACD at 4 °C stored for 21 days

Determination	Sample	Storage time (days)				
		0	3	7	14	21
$\bar{x} \pm SD$ Erythrocyte osmotic fragility (% hemolysis)	C		59.2 ± 16.1	50.0 ± 9.0	47.1 ± 0.3	45.1 ± 2.6
	V	39.3 ± 9.6	45.6 ± 7.8	43.1 ± 4.9	36.4 ± 9.3	33.7 ± 5.9*
$\bar{x} \pm SD$ Echinocytes (%)	C		58.3 ± 3.5	148.0 ± 36.6	198.3 ± 41.6	250.3 ± 50.0
	V	10.1 ± 0.0	21.6 ± 9.7*	110.0 ± 42.3	155.6 ± 68.6	100.3 ± 21.2*
$\bar{x} \pm SD$ 2,3-DPG in erythrocytes (µM/g Hb)	C		9.27 ± 1.2	2.0 ± 0.0	0.79 ± 0.2	0.0 ± 0.0
	V	12.5 ± 1.6	8.72 ± 2.3	2.9 ± 0.1	0.68 ± 0.2	0.0 ± 0.0
$\bar{x} \pm SD$ ATP in erythrocytes (µM/g Hb)	C		3.8 ± 0.1	3.2 ± 0.2	2.5 ± 0.3	1.6 ± 0.1
	V	3.5 ± 0.1	3.8 ± 0.1	2.9 ± 0.2	2.3 ± 0.3	1.3 ± 0.1

\bar{x} = mean of samples from 3 donors

* – statistically significant differences in relation to control

C – control sample

V – sample with vitamins

Vitamin C in a final concentration of 5 mM was added to the ACD solution with adenine by Wood and Beutler [20], who obtained a higher 2,3-DPG level in the erythrocytes stored for 28 days than in those stored without this vitamin. In our experiments the concentration of vitamin C was about 500 times lower, and a transient, slight delay of the steep fall of the 2,3-DPG level was observed. High concentration of vitamin C may cause spontaneous haemolysis of erythrocytes [21] and its antioxidative effect is observed at a relatively low concentration [15]. Moreover, vitamin C may co-act with vitamin E which has extraordinary antioxidative properties [13] that intensify the effect of these vitamins [14, 15, 16].

In our investigations the use of both vitamins permitted to achieve an increase of the osmotic resistance of erythrocytes (14–25%) during blood storage for 21 days as well as a decrease in the degree of erythrocyte transformation into echinocytes (20–60%) in that time period. The analysis of the effects of various concentrations of vitamins E and C on the decrease of the osmotic fragility of the erythro-

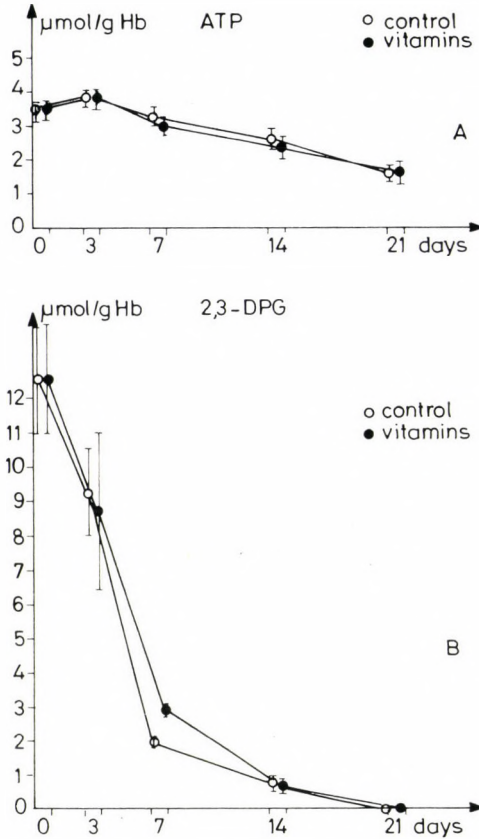


Fig. 2. ATP (A) and 2,3-DPG (B) levels in the erythrocytes preserved with ACD at 4 °C with added vitamin E (60 µg/ml) and vitamin C (2 µg/ml)

cytes and on the decrease of echinocyte count suggests that the combination of both vitamins at the concentrations of 60 µg/ml and 2 µg/ml, respectively, added to ACD solution before mixing it with blood, gives the best results as compared to various other concentrations. Further investigations may help to establish the optimal concentration of these vitamins in order to improve the effect of erythrocyte viability maintenance, since no positive effect of both vitamins on ATP level in the erythrocytes has been obtained yet.

The results obtained so far suggest that vitamins E and C, due to their antioxidant ability [13, 14, 15, 16] reduce the intensity of peroxidative processes in erythrocyte membranes and protect their integrity. Some authors, however, claim that the loss of microvesicles plays a more important role in the disintegration of the erythrocyte membrane than peroxidation [22].

Free peroxides attacking the membrane lipids seem to have, however, an effect on the viability of stored erythrocytes [23].

The results of Webster and Toothill [24] also indicate the role of the anti-oxidative system in the red cell damage during storage. They observed a significant decrease of superoxide dismutase activity and reduced glutathione during the 10-day storage period.

In the light of these preliminary results it seems that they may be helpful in further investigation on the optimization of media for blood preservation. The enrichment of ACD solution with appropriately selected concentrations of vitamins E and C may help in the protection of stored erythrocytes.

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Serum and Leukocyte Lactate Dehydrogenase Activity in Leukaemias

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(Received 23 June, 1987; accepted 22 October, 1987)

Lactate dehydrogenase (LHD) content of serum and leukocytes was examined in 42 haematologically normal healthy volunteers and in 34 patients suffering from various types of leukaemia. All patients were studied at the time of presentation and before any therapeutic intervention. Serum LDH was elevated in all types of leukaemia. In acute myeloid leukaemia (AML) a significant elevation of leukocyte LDH activity ($p < 0.005$) was noted. In acute lymphoblastic leukaemia leukocyte (ALL), LDH was significantly elevated when compared to normal lymphocyte LDH ($p < 0.01$) levels, but not when compared to total normal leukocyte LDH levels. In chronic leukaemias, leukocyte LDH levels were not significantly different from the normal. Comparison of LDH isoenzyme pattern in peripheral blood cells with that of serum, both in normal and in leukaemia cases showed more "M" type enzyme in the cells than in the serum. However, the "M" type enzyme was significantly elevated only in AML cases ($p < 0.005$).

Serum LDH and peripheral blood leukocyte count compared in normal subjects and in leukaemia cases showed no correlation.

Keywords: lactate dehydrogenase, intracellular enzyme, leukaemia

Introduction

Serum or plasma LDH (Lactate dehydrogenase E.C.1.1.1.27) activity has been found to be increased in some types of leukaemias [1–4]. LDH catalyses the reversible oxidation of pyruvic acid to lactic acid. Hence it is one of the key enzymes involved in anaerobic glycolysis. It was noted by Warburg [5] that many tumour tissues show a high rate of anaerobic glycolysis. Therefore elevated LDH levels in leukaemias were not surprising. Serum LDH levels may be elevated in several ways: 1) Each leukaemic cell might be producing a higher quantity of LDH than its normal counterpart. 2) Each leukaemic cell might be producing a normal amount of LDH, but as a result of vast increase in cell burden the serum LDH level may rise. 3) Release of LDH from normal tissue infiltrated and damaged by leukaemic cells. 4) A combination of any of aforementioned mechanisms may

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be responsible. Very few studies [3, 4] have estimated both the cellular and serum level of LDH in leukaemia, and to our knowledge only one study is available in English literature comparing the isoenzyme pattern of LDH in serum and leukaemic cells in three cases of ALL [6]. Such isoenzyme studies might explain the biochemical differentiation of cells in various leukaemias as it has been done for thymocytes [7].

Materials and Methods

42 healthy volunteers between the ages of 20–38 years, staff members of the institute were used as controls. 34 patients (14 with AML, 7 with ALL, 9 with chronic myeloid leukaemia (CML) and 4 with chronic lymphocytic leukaemia (CLL), attending the haematology laboratory, were included in the present study. Tests were performed prior to therapeutic intervention. Diagnosis was established by using standard clinical and laboratory protocol [8]. All cases of acute leukaemia were classified according to FAB classification [9].

For investigation of serum and cellular LDH activity, 5 ml blood was collected in a clean dry test tube, and 15 ml of blood was collected in heparinised BSS solution. Serum was separated from 5 ml clotted blood after 2 hours, and cells were separated from heparinized blood according to the methods of Dioguardi et al. [10]. The cells were lysed by repeated freezing and thawing, both cells and serum were assayed for LDH activity according to the method of Wootton [11]. Isoenzyme pattern of LDH from cells and sera was also studied on the same day by the agar gel electrophoresis technique [12]. Isoenzymes were designated 1–5 starting from the positive end. The electrophoresis strip was quantitated in a Carl Zeiss densitometer. Quantitation of “H” and “M” type LDH protein involved the assumption that LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5 are tetramers with H_4 , H_3M_1 , H_2M_2 , H_1M_3 and M_4 compositions, respectively. Serum concentration of the enzyme was expressed as I.U./L, and cell concentration as milli international units/ 10^6 cells ($ml\mu/10^6$ cells). Lymphocytes from 8 healthy volunteers were separated on Ficoll-paque (Pharmacia, Sweden), and total LDH levels in lymphocytes were measured as above. Results were analysed by Students' *t* test.

Results

Total LDH activity of serum and peripheral blood leukocytes are presented in Table 1. Serum LDH activity was found to be significantly increased in all types of leukaemia ($p < 0.05$).

There was no correlation between serum LDH and peripheral blood leukocyte count in normals or in leukaemia patients. The increase in serum LDH activity was the highest in ALL and the lowest in CLL. Cellular and serum LDH levels did not correlate with clinical parameters such as liver size, spleen size or mediastinal mass.

Table 1

Serum and leukocyte LDH levels in peripheral blood of normal subjects and leukaemia patients at the time of diagnosis

Cases (Nos.)	Total leukocyte count $\times 10^9/L$ (Range)	Serum LDH (IU/L) Mean \pm I S.d.	Leukocyte LDH (m.I.U./ 10^6 /cells) Mean \pm I S.d.
Normal (42)	4.2–9.6	62.83 \pm 30.10	12.92 \pm 7.46
Normal lymphocytes (8)	—	—	7.1 \pm 4.8
Acute myeloid leukaemia (14)	9.0–100.0	501.0 \pm 113.0	24.0 \pm 5.4
Acute lymphatic leukaemia (7)	4.4–450.0	677.0 \pm 203.6	14.2 \pm 3.12
Chronic myeloid leukaemia (9)	55.0–600.0	184.0 \pm 41.4	10.36 \pm 2.8
Chronic lymphatic leukaemia (4)	8.0–105.6	101.7 \pm 74.18	9.02 \pm 2.76

Cellular LDH activity showed a striking increase in AML cases ($p < 0.005$), whereas in other types of leukaemia cellular levels of LDH were within the normal range ($p > 0.05$). When the LDH content of leukocytes in ALL was compared to that of normal lymphocytes, significant elevation was noted ($p < 0.01$).

In most patients, no LDH-5 isoenzyme was detected. Cellular isoenzyme pattern showed a cathodal shift compared to its counterpart in serum (Table 2). The rise of cellular LDH activity in AML cases was mainly due to the rise of the "M" fraction of the enzyme ($p < 0.005$). In other types of leukaemia, the elevation of cellular LDH was due to the rise of both H and M fractions.

Discussion

The present study confirmed the findings of Kornberg et al. [2] that marked elevation of serum LDH occurred in ALL. This study also showed the lack of correlation between the levels of serum LDH and peripheral leukocyte count in both patients and healthy controls, a finding not difficult to explain because LDH is synthesised by almost all cells of the body and peripheral leukocyte count is a poor indicator of body leukocyte burden. One of the recent studies in childhood ALL showed fair correlation between leukocyte count and serum LDH levels in a large series of patients [13]. This good correlation may be due to the fact that a homogenous group of ALL patients was selected for this study.

Serum or cellular LDH levels in our study did not correlate with age, sex, hepatosplenomegaly, which is in agreement with other reports [14]. The present study did not explore the prognostic value of serum or cellular LDH levels or the isoenzyme pattern in cases of leukaemia. Hiçsönmez et al. [15] pointed out that high risk ALL patients at the time of initial diagnosis had higher serum LDH levels than patients at standard risk, and LDH-3 was higher than LDH-2 in high risk cases, a finding which other authors have disputed [16]. Serum LDH levels

2. táblázat

LDH isoenzyme patterns in serum and peripheral blood leukocytes in normal subjects and in patients with leukaemia (Mean \pm I.S. d.)

LDH Iso enzymes	Normal (42)		Acute myeloid leukaemias (14)		Acute lymphatic leukaemias (7)		Chronic myeloid leukaemia (9)		Chronic lymphatic leukaemia (4)	
	Serum LDH (I.U./L)	Cell LDH (m.I.U./10 ⁶ cells)	Serum LDH	Cell LDH (m.I.U./10 ⁶ cells)	Serum LDH (I.U./L)	Cell LDH (m.I.U./10 ⁶ cells)	Serum LDH (I.U./L)	Cell LDH (m.I.U./10 ⁶ cells)	Serum LDH (I.U./L)	Cell LDH (m.I.U./10 ⁶ cells)
LDH-1 (H ₄)	20.20 \pm 11.58	3.67 \pm 2.68	122.0 \pm 26.4	2.12 \pm 0.5	212.3 \pm 74.5	1.09 \pm 0.25	64.4 \pm 13.5	1.59 \pm 0.39	51.3 \pm 35.6	2.64 \pm 0.75
LDH-2 (H ₃ M ₁)	24.39 \pm 14.45	4.28 \pm 3.74	171.0 \pm 52	6.82 \pm 1.78	215.8 \pm 62.5	4.23 \pm 1.10	76.7 \pm 20.0	2.40 \pm 0.46	48.6 \pm 33.4	3.92 \pm 1.13
LDH-3 (H ₂ M ₂)	21.64 \pm 10.08	3.58 \pm 2.27	98.0 \pm 27.0	7.55 \pm 1.76	178.7 \pm 88.8	5.24 \pm 1.44	37.6 \pm 16.2	3.78 \pm 0.9	1.6 \pm 1.6	2.17 \pm 1.18
LDH-4 (H ₁ M ₃)	8.05 \pm 5.73	1.72 \pm 1.76	50.0 \pm 14.0	8.79 \pm 1.79	66.3 \pm 31.9	3.37 \pm 0.82	5.2 \pm 3.7	3.02 \pm 0.89	—	0.23 \pm 0.2
LDH-5 (M ₄)	3.24 \pm 0.97	2.63 \pm 3.64	—	—	—	—	—	—	—	—
LDH-H	46.47 \pm 22.55	4.10 \pm 5.62	313.0 \pm 66.8	2.9 \pm 2.9	503.0 \pm 167.0	7.86 \pm 1.7	141.9 \pm 30.0	5.92 \pm 1.79	88.9 \pm 64.5	6.75 \pm 1.84
LDH-M	16.28 \pm 9.13	3.78 \pm 2.48	109.0 \pm 21.3	11.14 \pm 2.6	193.0 \pm 83.0	6.3 \pm 1.47	42.2 \pm 12.7	4.71 \pm 1.69	13.19 \pm 9.66	2.28 \pm 0.91

in cases of non-Hodgkin's lymphoma appear to be telling a different story: a high level of serum LDH seems to be an early marker of the disease, preceding clinical manifestation [17]. Serum LDH level was found to have a prognostic value independent of other parameters, like histology and staging of the tumour [18].

The absence of the LDH-5 band in most of our cases of leukaemia is surprising. It is unlikely that this is due to an error of method, because in normal cases LDH 5 fractions were regularly obtained using the same methodology. There are two possibilities explaining this finding: either band-4 and -5 got fused in these cases, or fraction was so small that it was not detected as a separate band. In our opinion, the second possibility seems to be more likely, as other workers [15, 16] have detected LDH-5 in sera from ALL patients in extremely small quantities ($1.2 \pm 2.1\%$ in high risk ALL and $1.1 \pm 2\%$ in standard risk ALL). Though serum LDH activity was increased in all types of leukaemia, cellular levels of this enzyme were increased only in acute leukaemias. This might mean that in acute leukaemia the rise in serum LDH was due not only to an expanding cell mass, but also to higher production of LDH by each cell. High cellular levels of LDH in AML cases are unlikely to be due merely to large size and larger volume of cytoplasm of the leukaemic cells because these cells contained a qualitatively different enzyme with high M : H ($p < 0.005$). AML cells contained approximately five times more "M" fraction of LDH than "H" fraction, whereas in other types of leukaemia either the proportion of the two fractions were the same, or a slightly higher amount of "H" fraction was noted. Ho et al. [3] demonstrated that high LDH/Phosphohexose isomerase ratio characterizes the lymphoblasts, high LDH "M"/LDH "H" ratio seems to characterize leukocytes of AML in peripheral blood.

It was demonstrated by Bloom et al. [19] that LDH isoenzyme pattern in lymphocytes depends on the stage of cell cycle. If the cell is in mitosis then the LDH pattern becomes cathodal, i.e. more "M" type enzyme is produced, and the pattern reverts back to normal when the mitosis is over. We do not know whether higher "M" fraction of LDH in AML cases simply denotes the higher growth fraction of the leukaemic cells. The finding of higher "M" fraction in AML cells is interesting from the point of substrate requirements of various isoenzyme fractions of LDH. Km value of "M" type LDH is much lower than that of "H" type LDH [20, 21], which means that this enzyme is more efficient in converting pyruvate into lactate. Hence high "M" type LDH in a cell might mean a biochemical adaptation where the leukaemic cells produce a higher amount of pyruvate. The higher amount of pyruvate may be due to high rates of glycolysis and high activity of phosphohexose isomerase, a finding which is in agreement with previously published reports [3].

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Screening of IgA Deficiency by ELISA: Population Frequency in Hungary

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(Received 2 November, 1987; accepted 1 December, 1987)

The probability of the development of an anaphylactic shock in IgA deficient patients following repeated blood transfusion or the administration of any other IgA containing preparation is high. Since these individuals usually show mild, if any, clinical symptoms, the detection of deficiency and/or sensitisation is very important. We have developed an ELISA system suitable for both the rapid mass screening of IgA deficiency and for the detection of anti-IgA already present in the serum. Applying these methods for screening of serum IgA deficiency in healthy Hungarian population resulted in a similar pattern to that reported for other countries. Interestingly, the prevalence of IgA deficiency among Gipsies living in Hungary was significantly higher. We could detect anti-IgA only in one of our IgA deficient patients.

Keywords: IgA deficiency, mass screening, anti-IgA

Introduction

The significance of serum IgA is less than that of IgG and IgM in the systemic humoral immune response. Secretory IgA (in the saliva, colostrum, intestinal fluid), however, plays an important role in the defence of the mucosa against antigen penetration. In newborns, IgA cannot be detected by nephelometry, while in children the normal IgA level varies according to age. In healthy adults the normal level of IgA fluctuates between 1.3 and 3.4 g/l. IgA has two subclasses: IgA1 and IgA2. The latter comprises in average only about 10% (6–35%) of total serum IgA (Vaerman et al., 1968, Grey et al., 1968, Morell et al., 1973; Conley et al., 1983). In newborns the number of IgA1 and IgA2 secreting cells is equal. They are probably immature cells and produce both IgM and IgD. In the 3–5 month old infant, IgA1 synthesizing cells become dominant. In the peripheral blood of adults over 80% of IgA secreting B cells produce IgA1 (Conley et al., 1980).

Selective IgA deficiency is one of the most frequent immunodeficiencies. Its definition according to Amman and Hong (1971) is as follows:

1. less than 50 mg/l serum IgA concentration
2. other immunoglobulins (IgG, IgM, IgD, IgE) are present
3. normal cellular immunity
4. normal humoral antibody production.

Total serum IgA deficiency is almost without symptoms in children, except for a sometimes higher incidence of upper respiratory tract infections. IgG, given very frequently to these children, almost always contains IgA as contaminant. Therefore, these children may become sensitized and, depending on their individual response, develop anti-IgA resulting in a very severe anaphylactic reaction at a repeated exposition to IgA (Pineda and Taswell, 1975). Thus, IgA deficient children (Koistinen et al., 1978) and IgA deficient Rh-negative mothers receiving anti-D after giving birth (Pai et al., 1974) need special care. Multiple blood transfusions may confer a similar danger. IgA deficiency can even be transferred by otherwise healthy donors by bone marrow transplantation (Hammarström et al., 1985).

We developed a rapid assay suitable for mass screening for IgA deficiency as well as for detecting the presence of anti-IgA. The utility of the method is demonstrated by a pilot population study.

Materials and Methods

Serum samples

Blood samples of healthy individuals, blood donors, people from Hungarian and Gipsy settlements as well as of suspected IgA deficient patients were tested. Screening was performed either within two days of sampling, or sera were stored at -30°C . The ELISA method was developed by modifying the procedure of Hunt et al. (1985), using Dynatech M129A plates.

Covering of the plates

Human IgA was purified according to Heide and Schwick (1978), and $100\ \mu\text{l}$ of a $10\ \mu\text{g}/\text{ml}$ solution (in bicarbonate buffer pH 9.6) was applied in each well. After incubation in humidity chamber overnight at 37°C , unbound IgA was removed by washing three times with PBS-Tween. Plates were stored at $+4^{\circ}\text{C}$. Identically covered plates can be used for both IgA screening and anti-IgA detection. In the former method, a competitive ELISA assay was applied when plate bound IgA and free IgA in the serum compete for the peroxidase labeled antibody. In the anti-IgA assay serum anti-IgA competes for the possible binding sites with peroxidase labeled anti-IgA.

Screening for IgA deficiency

$50\ \mu\text{l}$ undiluted serum, immediately followed by $50\ \mu\text{l}$ of peroxidase conjugated anti-human IgA (Human Institute) diluted 1 to 200 by PBS-Tween gelatine was pipetted to each well. Plates were incubated for two hours in a humidity chamber at 37°C and washed three times with PBS-Tween.

Detection of anti-IgA

A serial dilution (10 dilutions) was prepared from both the sera and a control anti-human IgA (Hyland). 100–100 μ l of each were pipetted into the wells, and incubated in a humidity chamber for two hours at 37 °C. After three washings with PBS-Tween, 100–100 μ l of diluted peroxidase conjugated anti-human IgA (400 fold dilution, Human Institute) were added, and incubation was continued in a humidity chamber at 37 °C for another two hours. Finally, the unbound conjugate was removed by washing with PBS-Tween three times.

Development of the plates

Both ELISA systems were developed by the same method. Bound peroxidase conjugate gives a characteristic colour reaction with ortho-phenylene-diamine (OPD), in a reaction mixture containing 0.025 M citric acid, 0.05 M disodium phosphate, 34 mg % OPD and 6 mg % hydrogen peroxide. 200 μ l reagent per well was used. After 30 minutes the colour reaction was stopped by 50 μ l 4 N sulfuric acid. Thus the colour became stabilized for at least two hours.

Evaluation of the plates

For screening of IgA deficiency, the plates can be evaluated visually. The deep purple colour of totally IgA deficient sera can easily be distinguished from the faint red colour of the partially IgA deficient samples.

Quantitative evaluation can be performed by spectrophotometry using any type of ELISA photometer with 490 nm filter. As for the detection of anti-IgA, if the serum contains anti-IgA, an increase of extinction with dilution will be obtained, while in the absence of the antibody a constant value corresponding to the maximally bound antibody will be detected.

A Beckman-type nephelometer was used to control our results. IgG and IgM concentration in sera with a suspected IgA deficiency were also determined. As normal values 7.8–15.4 g/l for IgG and 0.4–2.3 g/l for IgM were used as assessed from our previous statistical evaluations (data not shown). For the healthy IgA-deficient patients the nephelometric method gave a < 11.1 mg/l cut-off level.

Results

After preparation the human IgA covered plates were stored at 4 °C and controlled monthly both for screening and anti-IgA detection during a half year period. Dilution series for plotting calibration curves were prepared from the Hyland anti-human IgA and from mixed normal human sera. The results were well reproducible over half a year.

Table 1

Nephelometrically determined immunoglobulin levels in sera of patients found to be IgA deficient by the ELISA method

	IgG g/l	IgA g/l	IgM g/l
1. L. P.	<0.011	<0.011	<0.011
2. J. I.	2.17	<0.011	0.15
3. É. J.	12.04	<0.011	0.25
4. V. A.	14.00	<0.011	1.85
5. B. CS.	11.80	<0.011	0.79
6. V. E.	4.75	<0.011	3.80
7. P. L.	26.19	<0.011	0.98
8. S. J.	16.97	<0.011	17.39
9. F. M.	8.08	<0.011	4.59
10. N. G.	15.99	<0.011	2.91
11. V. L. (2.5)*	6.70	<0.011	0.64
12. F. V. (8)	16.35	<0.011	0.92
13. T. M. (9)	18.23	<0.011	6.09
14. L. M. (12)	0.79	<0.011	10.20
15. A. G. (3)	13.11	<0.011	1.08
16. A. B. (7)	11.87	<0.011	1.37
17. A. L. S.	18.02	<0.011	0.59

* Numbers in parantheses are ages (years) of the children

Among the sera of patients with suspected IgA deficiency, 17 totally IgA deficient patients were found (Table 1). Patients 1. and 2. had absolute hypogammaglobulinaemia, while 3, 4, and 5 had normal IgG and IgM levels. IgG and/or IgM values in patients 6–14 were elevated as compared to the normal values. Patients 15 and 16 were children of the patient 17th (mother). Immunoglobulin levels in the brother of 17 and his children were normal. Parents of the mother had already died.

The blood of approximately 2800 healthy volunteers was tested by ELISA. Three totally IgA deficient donors were found. Nephelometric control could not detect IgA due to the already mentioned insensitivity of the method (11.1 mg/l IgA).

In further population studies, sera of 479 Hungarian Gypsies were assayed. Samples were taken in six villages and included the blood of full families (except children under the age of 10). We found altogether 4 IgA deficient persons. These four were from three villages and they were not relatives. As a control, a total of 490 samples, obtained from eight villages inhabited by non-Gypsy Hungarians, were assayed likewise. No IgA deficient persons were found among them.

Besides the full IgA deficient patients and donors, 6 donors had all the mentioned criteria of selective IgA deficiency, but their deficiency was not complete: 8–20 mg/l IgA was still present in their sera. We were only able to obtain

a saliva sample from one IgA deficient child, where the presence of secretory IgA was demonstrated.

Anti-IgA was detected only in one of our IgA deficient patients. This is not surprising since, as mentioned earlier, the occurrence of anti-IgA is greatly dependent on the individual sensitivity.

Discussion

The ELISA method developed in this study is suitable for mass screening of IgA deficiency as well as for the detection of anti-IgA. Its advantage is that IgA covered plates remain stable for at least a half year period. This means that for the long term application, e.g. for mass screening, a higher quantity of plates can be prepared and stored safely. In addition, in case of an emergency the results can be obtained within 3 hours instead of two days. Table 2 shows a comparison

Table 2
Frequency of IgA deficiency in blood donors

References	Number of samples	Method	Frequency
Ropars (1982)	108.000	RIA*	1/2.571
Vyas (1975)	73.569	PHI**	1/886
Koistinen (1975)	64.588	PHI	1/507
Koistinen (1975)	64.588	RIA	1/821
Holt (1977)	29.745	PHI	1/875
Hunt (1985)	41.851	PHI+ELISA	1/721
Present work			
donors	2.800	ELISA	1/933
Non-Gipsy Hungarians	480	ELISA	0/480
Gipsies	479	ELISA	1/120

* RIA = Radioimmunoassay

** PHI = Passive Haemagglutination Inhibition

of our results with those reported in the literature for healthy donors, Hungarian and Gipsy population. The number of patients is lower in our work than the respective data of other authors. However, our results indicate that while in Hungarians the incidence of IgA deficiency roughly corresponds to the data in the literature, it is, apparently, much higher in Hungarian Gipsies. Further studies are required to verify this assumption.

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Abstracts

Changes in both calcium pool size and morphology of human platelets incubated in various concentrations of calcium ion. Calcium-specific bleb formation on platelet-membrane surface. W. J. Tsai, J. C. Chen and C. T. Wang (Institute of Life Science, National Tsing Hua University, Hsingchu, Taiwan). *Biochim. Biophys. Acta* 940, 105 (1988).

In this study, the response of gel-filtered human platelets to extracellular Ca^{2+} at Ca^{2+} concentrations $[\text{Ca}^{2+}]_0$ of 1–10 mM was investigated. The distribution of Ca^{2+} among various pools was studied using: (1) quin2, to estimate the cytosolic free Ca^{2+} concentration $[\text{Ca}^{2+}]_i$; and (2) $^{45}\text{CaCl}_2$ plus EGTA, to quantitate the sizes of the EGTA-releasable, EGTA-nonreleasable and surface-bound Ca^{2+} pools. The morphological changes were revealed by scanning electron-microscopy (scanning EM), and the effect on thrombin-stimulated aggregation was examined using an aggregometer. Platelets continuously sequestered Ca^{2+} into both EGTA-releasable and EGTA-nonreleasable pools to maintain a low $[\text{Ca}^{2+}]_i$ level. The rate of sequestration to the EGTA-releasable pool was independent of $[\text{Ca}^{2+}]_0$, while that of the EGTA-nonreleasable pool exhibited first-order kinetics. The cell morphology changed gradually from discoid to the tadpole-like type, and finally to irregular forms. This morphological change correlated with the gradual increase in $[\text{Ca}^{2+}]_i$. The EGTA-nonreleasable pool saturated at about 3000 pmol/ 10^8 cells. This saturation resulted in a drastic increase in the EGTA-releasable pool size, and the cell was lysed

concomitantly. The maximum safety capacity of the EGTA-releasable pool was estimated to be 1100 pmol/ 10^8 cells. The contribution of the cellular compartments to these two pool sizes is extensively discussed. The surface-bound pool size also increased continuously. When two different capacities were reached, i.e. 160 and 600 pmol/ 10^8 cells, the binding rate increased above the initial rate by 7- and 11-fold, respectively. Hence, the surface-binding capacity might be a critical factor which alters the membrane structure and exposes more binding sites. The cell surface appeared to have blebs, after the binding size had reached more than 600 pmol/ 10^8 cells. Bleb formation resulted in the inhibition of platelet function. Divalent cations, such as Mg^{2+} , Sr^{2+} and Ba^{2+} did not cause bleb formation, which could mean that this formation is a Ca^{2+} -specific phenomenon.

Ilma Szász

Mechanism of improved maintenance of 2,3-diphosphoglycerate in stored blood by the xanthone compound 2-(2-hydroxyethoxy)-6-(1-H-tetrazole-5-yl)xanthen-9-one (BW A440C). E. Beutler, L. Forman, C. West and T. Gelbart (Research Institute of Scripps Clinic, La Jolla, CA, USA). *Biochem. Pharmacol.* 37, 1057 (1988).

The effect of the xanthone derivative 2-(2-hydroxyethoxy)-6-(1-H-tetrazole-5-yl)-xanthen-9-one (BW A440C) on red cells was studied. When added to stored red cells at a concentration of 6 mM, greatly improved

preservation of 2,3-diphosphoglycerate (2,3-DPG) was observed. There was no effect on internal pH of the erythrocyte. At a concentration 0.500 mM, many red cell enzyme activities were inhibited completely. At a 0.050 mM concentration, however, inhibition of pyruvate kinase and diphosphoglycerate phosphatase was most striking. Inhibition of either of these enzymes could result in elevation of 2,3-DPG levels. BW A440C in concentrations which elevated 2,3-DPG levels in human caused a decrease in 2,3-DPG levels in rabbits and markedly impaired the viability of 21-day stored rabbit erythrocytes.

B. Sarkadi

Red blood cell sodium and potassium fluxes in psoriatic patients. A. Semplicini, M. G. Mozzato, E. Rigon, O. Parolin, B. Sama, S. Padovan, P. Degan, A. Peserico and A. C. Pessina (Clinica Medica I and Clinica Dermatologica, University of Padova, Italy). *Eur. J. Clin. Invest.* 18, 47 (1988).

Psoriasis might be a widespread membrane disorder. Therefore, the red blood cell sodium, potassium and lithium outward fluxes (through Na-K-ATPase, Na-K-Cl co-transport, Li-Na countertransport and passive permeability), as well as the Na and K content, were studied in 31 psoriatic patients and 23 normal controls. A significant increase in intracellular potassium content, in the maximal velocity of the Na-K-ATPase and of Na-K-Cl co-transport as well as in the outward passive permeability for Na were found in the psoriatic patients compared with controls. On the contrary, no differences were observed in sodium content, Li-Na counter-transport and passive potassium permeability between the two groups. These results are compatible with a selective increase in inward, as well as outward, membrane permeability to sodium, which is compensated for by increased activity of the Na-K pump, and of the outward Na-K-Cl co-transport with a secondarily increased erythrocyte potassium content. They indicate that the red blood cell might be a useful model for the study of membrane transport in psoriatics.

G. Gárdos

Dependence of the red blood cell calcium pump on the membrane potential. B. Gassner, S. Luterbacher, H. J. Schatzmann and A. Wüthrich (Department of Veterinary Pharmacology, University of Bern, Switzerland). *Cell Calcium* 9, 95 (1988).

It is shown that the rate of calcium extrusion from intact human red cells is faster at a membrane potential of $\sim +50$ mV (inside) than at ~ -50 mV. The positive potential applied was the chloride potential of KCl cells in a K-gluconate medium when the Ca^{2+} sensitive K^+ channel was blocked by 0.3 mM quinidine. The negative potential resulted from the high K^+ permeability in Ca^{2+} loaded cells (the cells were loaded to a Ca^{2+} activity in the cell water of about $50 \mu\text{M}$). It is further demonstrated that the Ca^{2+} affinity of the pump ATPase is decreased both at the internal (high affinity) and external (low affinity) site by increasing the proton concentration. Acidification thus inhibits internally and stimulates externally. An indirect effect of the membrane potential on the pump activity via the accompanying pH shifts on either side of the membrane could be ruled out by choosing Ca^{2+} concentrations which are fully activating at the internal Ca^{2+} binding site at pH 6.5 and not yet inhibitory at the external Ca^{2+} binding site at pH 8. The result is compatible with the assumption that the human red cell Ca-pump is exchanging Ca^{2+} for protons, yet is electrogenic by virtue of a stoichiometry of $1\text{H}^+ : 1\text{Ca}^{2+}$ for this exchange.

G. Gárdos

Effects of a monoclonal anti-calpain antibody on responses of stimulated human neutrophils. Evidence for a role for proteolytically modified protein kinase C. S. Pontremoli, E. Melloni, G. Damiani, F. Salamino, B. Sparatore, M. Michetti, and B. L. Horecker (Institute of Biological Chemistry, University of Genoa, Genoa, Italy). *J. Biol. Chem.* 263, 1915 (1988).

A monoclonal antibody directed against the Ca^{2+} -requiring proteinase (calpain) of human neutrophils was employed to assess the role of this proteinase in mediating the

responses to stimuli such as phorbol 12-myristate 13-acetate or fMet-Leu-Phe. In the presence of either phorbol 12-myristate 13-acetate or fMet-Leu-Phe the antibody is taken up by the neutrophils, and a marked inhibition of intracellular calpain is observed. The decreased calpain activity is accompanied by (a) a significant decrease in the proteolytic conversion of native protein kinase C (Ca^{2+} /phospholipid-dependent enzyme) to the soluble form that does not require Ca^{2+} or phospholipids for activity; (b) a marked increase in the production of superoxide anion; and (c) a decrease in the exocytosis of granule contents. The increase in superoxide production can be attributed to a more prolonged association of native protein kinase C with the plasma membrane, thus enhancing the phosphorylation of membrane proteins that precedes O_2^- production. The decreased exocytosis can be attributed to a decreased phosphorylation of certain cytoskeletal proteins, catalyzed by the soluble form of protein kinase C; the subsequent reorganization of the cytoskeleton appears to be related to degranulation. These effects of the monoclonal anti-calpain provide direct evidence for an essential role for calpain in the activation of human neutrophils.

G. Gárdos

Red blood cell deformability and venous blood P_{O_2} in diabetics. Y. Kikuchi, T. Koyama, N. Ohshima and K. Oda (Department of Biomedical Engineering, Institute of Basic Medical Sciences, University of Tsukuba, Ibaraki 305, Japan). *Clin. Hemorheology* 8, 171 (1988).

Red cell deformability (filterability) was studied in 17 diabetic patients and 8 healthy subjects by a modified Nuclepore filtration method. Under a pressure difference of 10 cm H_2O , diabetic red cells suspended in autologous plasma took longer (1.75 ± 0.44 msec; mean \pm S.D.) than normal red cells (1.23 ± 0.07 msec) to pass through 5 μM filter pores. The indicated decreases of red cell filterability in the diabetics showed a correlation with increases in fasting blood glucose level (correlation coefficient 0.74) and also a correlation with increases in

HbA₁ content but with a lower correlation coefficient. In accord with these results, measurements of oxygen partial pressure in venous blood, PvO_2 , suggested increased capillary transit times in these patients; values obtained in 8 of the diabetic patients were 35.1 ± 8.6 mmHg, significantly lower than 44.6 ± 2.1 mmHg in the 8 healthy subjects. Effect of pentoxifylline on whole blood filterability was studied in 10 of the patients, in 8 of whom positive changes of blood passage time were obtained.

Ilma Szász

Role of Na^+/H^+ exchange in thrombin- and arachidonic acid-induced Ca^{2+} influx in platelets. D. Ghigo, S. Treves, F. Turrini, A. Pannocchia, G. Pescarmona, and A. Bosia (Cattedra di Chimica e Propedeutica Biochimica, Dipartimento di Genetica, Biologia e Chimica Medica, Turin, Italy). *Biochim. Biophys. Acta* 940, 141 (1988).

Platelet activation is accompanied by an increase of cytosolic free Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, (due to both extracellular Ca^{2+} influx and Ca^{2+} movements from the dense tubular system) and an Na^+ influx associated with H^+ extrusion. The latter event is attributable to the activation of Na^+/H^+ exchange, which requires Na^+ in the extracellular medium and is inhibited by amiloride and its analogs. The present study was carried out to determine whether a link exists between Ca^{2+} transients (measured by quin2 method and the $^{45}\text{CaCl}_2$ technique) and Na^+/H^+ exchange activation (studied with the pH-sensitive intracellular probe, 6-carboxyfluorescein) during platelet stimulation. Washed human platelets, stimulated with thrombin and arachidonic acid, showed: (1) a large and rapid $[\text{Ca}^{2+}]_i$ rise, mostly due to a Ca^{2+} influx through the plasma membrane; (2) a marked intracellular alkalization. Both phenomena were markedly inhibited in the absence of extracellular Na^+ or in the presence of an amiloride analog (EIPA). Monensin, a cation exchanger which elicits Na^+ influx and alkalization, and NH_4Cl , which induces alkalization only, were able to evoke an increase in $[\text{Ca}^{2+}]_i$, mostly as an influx from the extra-

cellular medium. The results suggest that Ca^{2+} influx induced by thrombin and arachidonic acid in human platelets is strictly dependent on Na^+/H^+ -exchange activation.

Ilma Szász

Thrombin-induced inositol trisphosphate production by rabbit platelets is inhibited by ethanol. M. L. Rand, J. D. Vickers, R. L. Kinlough-Rathbone, M. A. Packham and J. F. Mustard (Department of Biochemistry, University of Toronto, Ontario, Canada). *Biochem. J.* 251, 279 (1988).

Ethanol has an inhibitory effect on some platelet functions, but the mechanisms by which it exerts this effect are not known. Using suspensions of washed platelets, ethanol (1–9 mg/ml) did not affect the aggregation of rabbit platelets stimulated with ADP (0.5–10 μM). When platelets were prelabelled with 5-hydroxyl [^{14}C] tryptamine, aggregation and secretion of granule contents in response to thrombin (0.01–0.10 unit/ml) were not inhibited by ethanol, but these responses to thrombin at lower concentrations (< 0.01 unit/ml) were inhibited

by ethanol (2–4 mg/ml). Platelets were prelabelled with [^3H] inositol so that increases in inositol phosphates upon stimulation could be assessed by measuring the amount of label in these compounds. ADP-induced increases in IP (inositol phosphate) and IP_2 (inositol bisphosphate) were not affected by ethanol. IP_3 (inositol trisphosphate) was not changed by ADP or ethanol. Although ethanol did not affect the increases in IP, IP_2 and IP_3 caused by stimulation of platelets with thrombin at concentrations greater than 0.01 unit/ml, ethanol did inhibit the increases observed at 2 and 3 min in these inositol phosphates caused by lower concentrations of thrombin (< 0.01 unit/ml). Since ADP did not cause formation of IP_3 in rabbit platelets, and since no thromboxane B_2 was detected in platelets stimulated with the lower concentrations of thrombin, it is unlikely that the inhibitory effect of ethanol in IP_3 formation was due to effects on further stimulation of platelets by released ADP or by thromboxane A_2 . Ethanol may inhibit platelet responses to thrombin by inhibiting the production of the second messenger, IP_3 .

B. Sarkadi

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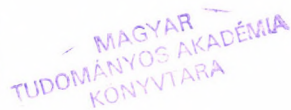
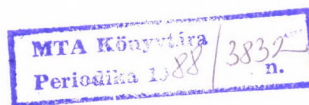
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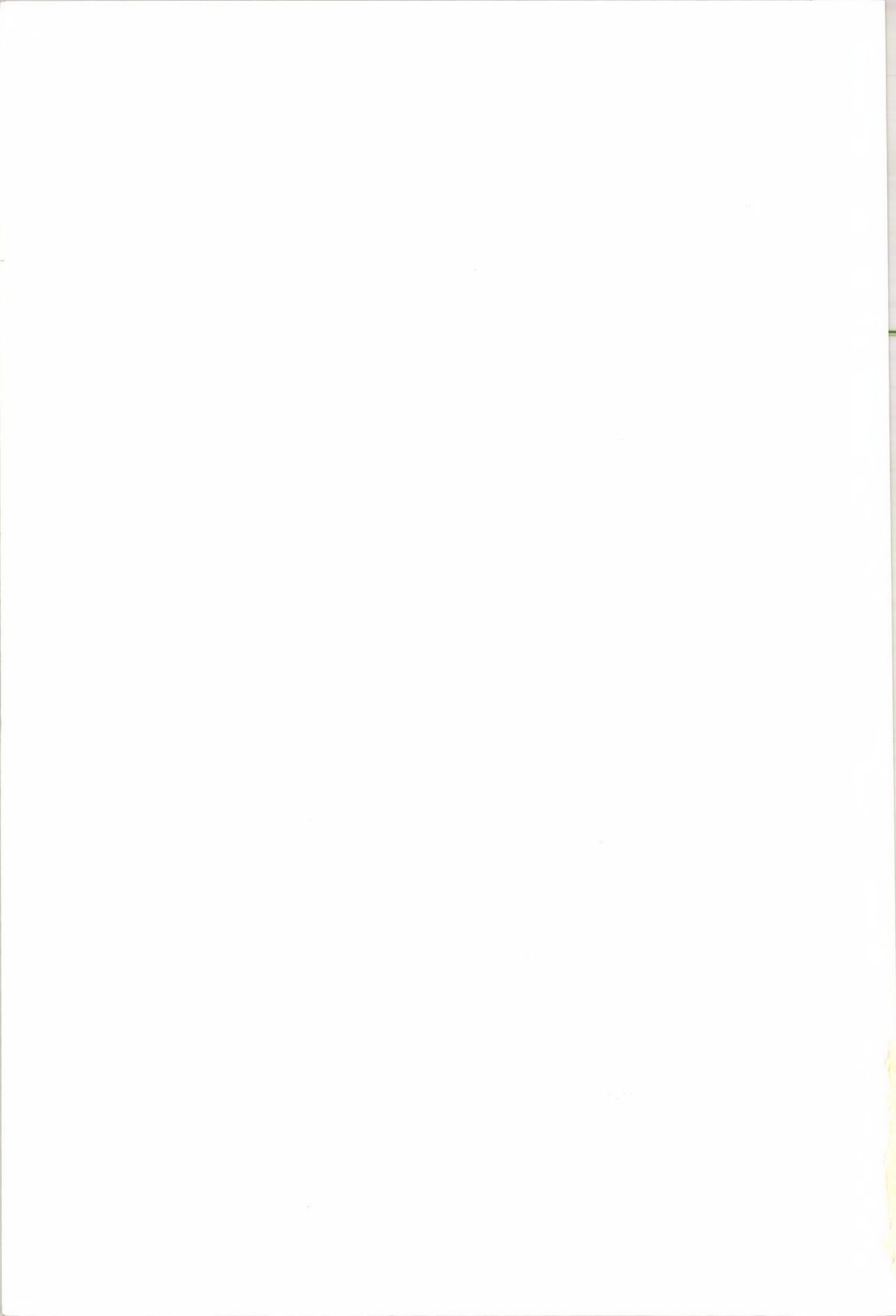
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Printed in Hungary

Akadémiai Kiadó és Nyomda Vállalat, Budapest



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