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VOLUME III

Edited
by
J. G. WEISZFEILER

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FOREWORD

The present year-book of the Microbiological Research Group contains a comprehensive paper on the hemagglutination of adenoviruses written by Anna Lengyel and I. Nász. Hereby we publish the material of a round-table conference on atypic mycobacteria, especially the results achieved in the study of *Mycobacterium simiae*. The participants of the Conference, held in April, 1968, in Budapest, discussed the experimental results of German, Danish, Soviet, Swiss, French and Hungarian research workers. In conclusion, decisions were brought in some questions of importance. The paper of F. Szelényi and G. Berencsi contains results of isolation and study of mycobacteria from soil. Furthermore, the volume includes the achievements of the comparative investigations carried out by T. B. Yablokova, T. P. Kozhevnikova and D. T. Levi on the W 115 attenuated M. tuberculosis strain suggested for vaccination against tuberculosis and a short summary of the work about the same question accomplished by T. K. Kocherbaev.

Prof. J. G. Weiszfeiler

Director of the Microbiological
Research Group of the Hungarian
Academy of Sciences

CONTENTS

STUDIES ON THE HEMAGGLUTINATION OF ADENOVIRUSES

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The direct hemagglutinating activity of adenoviruses was shown first by Rosen (1958). He demonstrated that certain types of human adenoviruses agglutinate the rhesus and rat erythrocytes, and on the basis of these characteristics they can be divided into subgroups. This grouping is essentially still valid, furthermore, it appears as if similarity existed also among the members of certain subgroups from other points of view, such as oncogenicity.

According to this grouping the members of subgroup I are the types which agglutinate the erythrocytes of *Macacus, rhesus* members of subgroup II agglutinate completely the rat erythrocytes, while types belonging to subgroup III cause only the partial agglutination of rat erythrocytes, but this can be increased and made complete by adding to the system immune sera prepared against another type of the same subgroup. Subgroup IV comprises types at first considered as not being able to perform hemagglutination (Rosen 1960), however later they proved to possess such capacity in appropriate concentration (Schmidt et al. 1965).

Rosen (1960) demonstrated subsequently that the hemagglutination by adenoviruses can be inhibited with type-specific immune sera, thus it can be applied successfully both for typing and in the diagnostics besides the neutralization test.

Further examinations indicated that adenoviruses contain different kinds of hemagglutinins (Zuschek 1961, Bauer and Wigand 1962, Pereira and De Figueiredo 1962, Wigand and Bauer 1962). Ultrastructural examinations carried out in recent years provided us with a thorough knowledge of this problem, by revealing the structure of adenoviruses. Concerning the details of the morphology of adenoviruses we refer to relevant literature (Nász et al. 1967), and only those data will be described here which are important to elucidate the mechanism of the hemagglutination.

The virion of adenoviruses is cubical, its diameter is 70 mμ as an average, it has an icosahedral shape and consists of 252 capsomers. Each vertex of the icosahedron bears a projection, formed by a rod that ends in a knob. These projections have been named *fiber* (Ginsberg et al. 1966). The fiber with the vertex capsomer form the so-called *penton*, which is constructed of

the trypsin-sensitive group-specific vertex capsomer or penton base, the subgroup-specific rod and the type-specific knob on its end. The remaining 240 capsomers — the *hexons* — are different from the vertex capsomers, they build the adenovirus capsid with the exception of the vertices. Each hexon has six similar neighbours, they are group-specific, although some data indicate that they have type-specific parts too (Köhler 1965, Norrby 1968b).

Antigens are synthetized during the virus multiplication separately. Significant quantities of surplus are produced from certain antigens which explains the phenomenon of the regular independent shapes consisting of 12 penton antigens appearing occasionally during the multiplication of certain adenovirus types. They seem to enclose a central mass not yet defined precisely (Norrby 1966). This structure has been named *dodecon* (Gelderblom et al. 1967).

Most probably the knob of the fiber attaches directly to the receptors of the erythrocytes, thus, it is obvious that both the virion and the dodecon attached simultaneously to the receptors of several erythrocytes behave like complete hemagglutinins. The isolated fibers and pentons represent monovalent hemagglutinins and so — being attached by only one pole to the erythrocyte — they fail to cause hemagglutination by themselves — thus they are called *incomplete hemagglutinins*. Otherwise, when two components representing incomplete hemagglutinins get somehow connected with each other, a regular *complete hemagglutinin* may develop. Such connections occur sometimes spontaneously with certain types (Norrby and Skaaret 1968), but it can also be performed under experimental conditions, by adding adequate heterologous immune sera to the system. The antibody attaching to the penton bases or to the stem of the fiber connects the incomplete hemagglutinins, creating thus complete hemagglutinins. This is the mechanism of the hemagglutination of subgroup III. Types belonging to this subgroup have large quantities of incomplete hemagglutinins, they absorb a part of the erythrocyte receptors, thus only the remaining erythrocytes being left to be agglutinated by the complete hemagglutinins, the system shows the pattern of a partial hemagglutination till heterologous immune serum is added. This hemagglutination enhancing effect of the heterologous sera has been named in the literature *hemagglutination enhancement* (HE).

In the present publication we summarize our examinations concerning the hemagglutination of adenoviruses. The experiments were carried out at the Institute of Microbiology of the University of Medicine, Budapest; at the National Institute for Medical Research, London; at the Institute of Microbiology and Public Health of the University of Homburg; in the De-

partment of Virology, School of Medicine, Karolinska Institutet, Stockholm; and in the Microbiological Research Group of the Hungarian Academy of Sciences, Budapest. We wish to express our thanks for the valuable collaboration of Ida Cserba, P. Dán (Budapest), H. Gelderblom (Homburg), Gizella Kulcsár (Budapest), E. Norrby (Stockholm), H. G. Pereira (London), Katalin Rózsa (Budapest), W. A. K. Schmidt and R. Wigand (Homburg). We endeavour to give an account of our results in chronological order, thus demonstrating simultaneously the progress made in this field of research, provided by the rapid and intensive development of experimental methods.

Materials and methods

Viruses. Human adenovirus prototype strains 1—30, and strains isolated from patients were used in the experiments; the latter are designated by the names or numbers given by the laboratories where their isolation was performed (Nász et al. 1962). The virus strains were propagated in HeLa, Detroit-6, MAS-A, HEp-2 and in continuous and primer human amniotic cell cultures. Virus materials were used either after repeated freezing-thawing and low speed centrifugation, or in concentrated form, partly purified by fluorocarbon.

Immune sera. Immune sera were prepared in rabbits and guinea-pigs. Materials used for i.v. or i.m. administration were prepared as described above, or separated and purified by chromatographic and ultracentrifugation procedures, in some cases adding Freund adjuvant to the preparations.

Hemagglutination (HA) and *hemagglutination inhibition* (HI). Experiments were performed in Takátsy's microtitrators, in WHO plastic plates or in tubes. To prevent spontaneous agglutination 0.1—1.0% normal rabbit serum was given to the erythrocyte suspension when using rat erythrocytes. HI reaction was carried out with 4—8 HA units. Immune sera used in the tests were absorbed with the erythrocytes of the corresponding species and occasionally treated with caolin.

Hemagglutination enhancement (HE). In the case of the hemagglutination of types belonging to subgroup III or of incomplete hemagglutinins, heterologous adenovirus immune serum was mixed into the erythrocyte suspension in a ratio of 1 : 100, or into the diluent of the antigens. The HE titer value of the heterologous sera was determined by chessboard titration and the double amount of the end titer was used. Diluting antigens in solution containing heterologous serum, they were incubated at room temperature for 1 hour before the erythrocyte suspension was added.

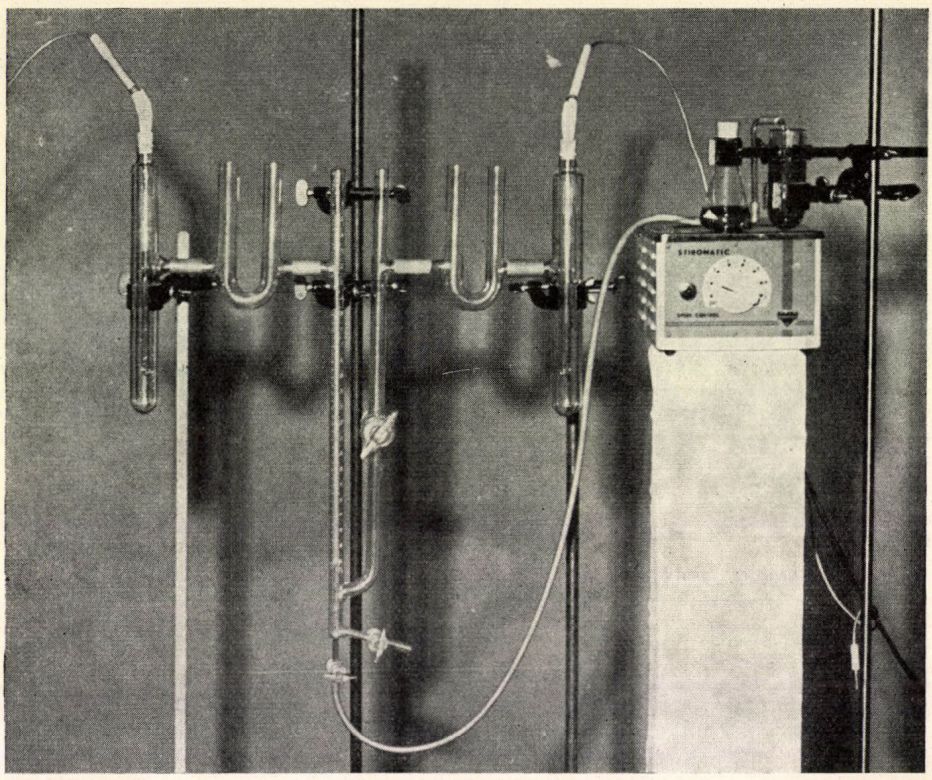

FIG. 1

Complement fixing (CF) reaction. Tests were performed in tubes or by drop method as described earlier (Svedmyr et al. 1952, Pereira 1956, Wigand 1956).

Separation methods. Virus antigens were separated by zonal centrifugation, in continuous or preformed sucrose gradient and in CsCl gradient by equilibrium centrifugation. DEAE Sephadex A-25 or A-50 and DEAE cellulose columns were used in the anion exchange chromatography. 0.01 M phosphate buffer pH 5.2 or pH 7 containing rising concentration of NaCl, or 0.04 M Tris-HCl buffer pH 8.4 was used as eluent. Elution was carried out either stepwise or by introducing a sodium chloride gradient. Fractions were collected with LKB fraction collector and the absorption was registered by Uvicord absorptiometer or by Unicam SP800 spectrophotometer.

Zonal electrophoresis. These experiments were performed in continuous sucrose gradient with an instrument demonstrated in Fig. 1 (Schmidt 1967). Experiments were carried out at 17 °C, in veronal-acetate buffer pH 8.4 (Michaelis), ionic strength 0.12, the current used was 400

V.D.C. and the field intensity 3.5 V/cm. Each experiment lasted 15 hours, thereafter 0.5 ml fractions were collected and examined. At least three or even more experiments were performed with each adenovirus strain.

Immune electrophoresis and immune osmophoresis. These experiments were performed on slides, 2.5 centimeters wide and 7.5 centimeters long, by pouring on them a 3-millimeter thick agar-agar layer (Nász et al. 1967). Concentrated and purified complete virus material or concentrated virus antigens separated by column chromatography were used and rabbit sera prepared against complete virus or against purified antigens were applied as immune sera.

Results

Studies on the hemagglutination spectra of human adenoviruses and on certain factors influencing the hemagglutinating activity

The hemagglutination spectra of forty-two strains belonging to adenovirus types 1 to 16 have been tested (Nász et al. 1962). Erythrocytes of 97 different birds and mammals of the Municipal Zoological and Botanical Gardens, Budapest, and those of laboratory animals and human erythrocytes belonging to different blood groups were used in the experiments.

Erythrocytes of 26 bird species out of 47 were agglutinated by one or more adenovirus strains, the titer varied between 1 : 16 and 1 : 128. Erythrocytes of only 7 out of 33 mammals of the Zoological Garden were agglutinated by one or two strains at low titers. Experiments with the erythrocytes of 17 birds and mammals of the Zoological Garden could not be evaluated for various reasons.

No hemagglutination could be observed with the erythrocytes of 10 rabbits, a few strains agglutinated the erythrocytes of guinea-pigs and white mice at titers of 1 : 16 to 1 : 32. Considerably high titers — 1 : 128 to — 1 : 512 — were reached in 38 cases with a part of strains belonging to the serotypes 9, 10, 13 and 15, when rat erythrocytes were used. In general, 1 : 16 to 1 : 64 titres were obtained in 56 cases of human erythrocytes tested with strains belonging to types 9, 10, 13, and in a single case with type 15. No correlation was found between the blood group and the agglutinability by adenoviruses. None of the erythrocytes tested was agglutinable by every strain under study. Six strains out of 42 were found to agglutinate the erythrocytes of more than five bird species or mammals. Individual quantitative differences were found in the agglutinability of human and rat erythrocytes. It should be noted that our strains type 8 and 12 used in these

experiments did not show the specific characteristics of these types when checked after some years.

From the hemagglutination influencing factors the effect of temperature and H-ion concentration have been studied on the hemagglutination activity of types 9, 10 and 13 belonging to subgroup II (Lengyel et al. 1963). Results obtained from a considerable number of experiments suggested that the highest HA titers with the above-mentioned types were attained by using rather low temperatures for incubation (+ 4 °C and room temperature), while incubation on air and in water bath at 37 °C and 40 °C respectively, were less favourable, as shown in Table 1, presenting the geometric means of the titers.

TABLE 1

HA titers of adenovirus types at 5 different temperatures

Adenovirus type	HA titers* at temperatures					Average	
	+4 °C	room temperature (r.t.)	37 °C		40°C	+4° C and r.t.	37 °C and 40° C
			incubator	water bath			
9	124	113	93	96	86	118	91
10	256	278	215	215	183	267	204
13	32	30	24	18	19	31	20

* Reciprocals.

Studying the influence of H-ion concentration between the pH ranges 6.0 and 10.0, the alkaline reaction was found to have the most favourable effect on the titers. The differences observed at various pH values were too slight to have any definite influence on hemagglutination. The H-ion concentration left the HI reaction unaffected between the pH ranges mensioned.

Hemagglutination inhibition tests with human adenoviruses

The HI test with adenoviruses proved to be useful in the laboratory diagnostics (Nász et al. 1963a, Augustin et al. 1964), though cross reactions exist among certain types. These cross reactions have been studied in our experiments in the sera of immunized animals, in the sera of patients recovered from adenovirus infections and of healthy persons (Nász et al. 1963b, Lengyel and Nász 1964, Lengyel et al. 1965). Results obtained from the sera of rabbits immunized with different types of adenoviruses are presented in Table 2

TABLE 2

HI titers to adenovirus prototype strains of adenovirus sera prepared in rabbits

Type serum	HI titers* to adenovirus type									
	3	6	7	8	9	10	11	13	15	16
1	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—
3	256	—	—	—	—	—	—	—	—	—
4	—	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—
6	—	2048	—	—	—	—	—	—	—	—
7	—	—	512	—	—	—	128	—	—	—
8	—	—	—	4096	1024	32	—	—	—	—
9	—	—	—	1024	4096	128	—	—	—	—
10	—	—	—	—	64	16384	—	—	—	—
11	—	—	1024	—	—	—	16384	—	—	—
13	—	—	—	—	—	—	—	16384	—	—
14	—	—	128	—	—	—	1024	—	—	—
15	—	—	—	—	—	—	—	—	1024	—
16	—	—	—	—	—	—	—	—	—	512

* Reciprocals.
— Negative at 1 : 8 dilution.

Cross reactions were found among types 7, 11 and 14, this phenomenon as well as the cross reaction between types 8 and 9 was already known (Rosen 1960, Dawson et al. 1960), our experiments revealed heterotypic reactions among types 8, 9 and 10, too. To investigate this cross reaction more closely, further animals were immunized with the three types in ques-

TABLE 3

Percentage distribution of the HI titers to the three adenovirus types and the degree of the increase in these titers in the paired sera of 40 patients with EKC

Adenovirus type	Serum	HI titer*			Increase		
		<4	4—8	≧16	no	twofold	fourfold and over
8	acute	75.0	7.5	17.5	—	5***	95
	convalescent**	—	7.5	92.5			
9	acute	72.5	12.5	15.0	—	17.5	82.5
	convalescent	—	12.5	87.5			
10	acute	85.0	5.0	10.0	37.5	12.5	50.0
	convalescent	37.5	12.5	50.0			

* Reciprocals.
** Taken 4—6 weeks after onset of EKC.
*** To another type 8 strain these, too, showed a fourfold rise.

tion as well as sera of healthy persons and those of patients recovered from adenovirus type 8 infections were studied. Table 3 shows the results with the paired sera of the patients.

The antibody level against type 9 rose in each patient suffering from adenovirus type 8 infection and in 60 per cent of the cases against type 10, too. Relations could be detected in the occurrence of antibodies against these three types also in the sera of healthy persons.

Further 17 rabbits and 6 guinea-pigs were immunized against the three types studied, thus it enabled us to evaluate quantitatively the rate of the cross reaction. The strongest cross reaction has been found between types 8 and 9, followed by that between types 9 and 10, the reaction between types 8 and 10 proved to be the weakest.

The antibody level of the immunized rabbits has been recorded for 210 days. Heterotypic antibodies were detectable half a year after the immunization of rabbits; their level changed parallel with the homologous antibodies.

Homologous and heterotypic antibodies passed through diaplacental and colostral ways into the offspring of the immunized sucklers and could be demonstrated for as long as 60 days.

Studies on the electrophoretic mobility of certain adenovirus components with density gradient electrophoresis

Experiments were carried out with six adenovirus strains belonging to subgroup II: prototype strains 9, 15, 19 and 23, type 9 strain "French" and the intermedier wild strain 9—15 (Lengyel and Schmidt 1968).

Viruses for zonal electrophoresis were concentrated with polyphosphoric acid (Schmidt and Wigand 1966). Three experiments were performed with each of the six strains and the calculated geometric means of the results are presented in Fig. 2.

The infectivity and complement fixing antigen of the fractions were examined parallel with the hemagglutinating activity, thus the rate of migration of the virion and that of the group-specific hexon antigen could also be determined. The migration rate of the strains 9 French, 9—15 and type 15 appeared to be similar in the electric field, their maximums were in the same fractions, type 19 migrated slower, while hemagglutination maximums obtained with prototype strains 23 and 9 indicated a faster migration. Virus particles of the 9—15 strain — its behaviour in hemagglutination inhibition corresponding to type 9 and in neutralization to type 15 — migrate faster and type 23 virions slower than those of the other four strains, likewise localized in fractions 17—22. The hexon antigens of all the six strains dis-

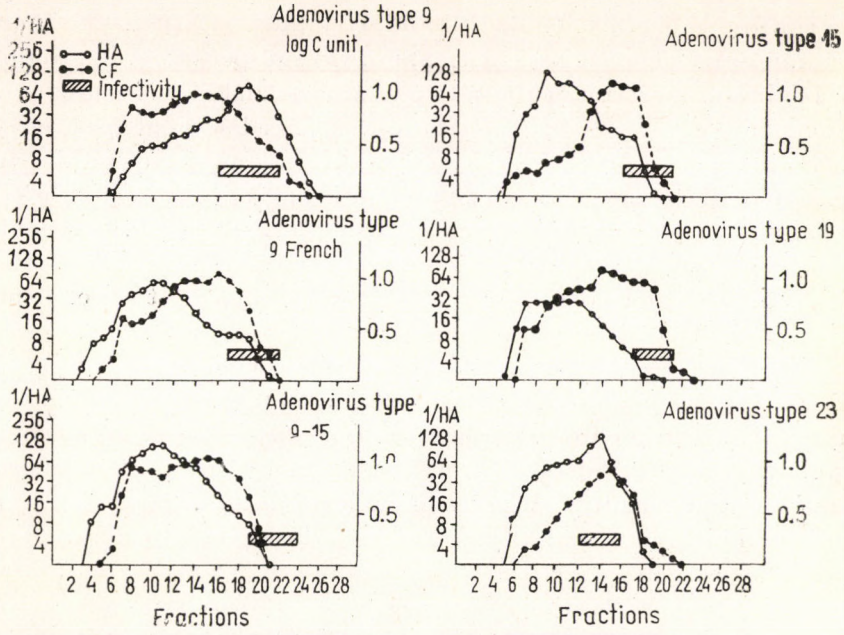

FIG. 2. Zonal electrophoresis with adenoviruses

played identical velocity, their peaks being in the fractions 14—16, in most cases with some samller peaks in fractions 7—8.

Examinations on the electrophoretic mobility of type 5 adenovirus hemagglutinins in immune electrophoresis and immune osmophoresis

From the soluble antigens of adenovirus type 5, the group-specific hexon antigen migrated toward the anode, the type-specific fiber antigen toward the cathode, while the trypsin-sensitive complete hemagglutinin, the penton, showed intermediate electrophoretic mobility in experiments performed with homologous rabbit immune sera (Fig. 3) (Nász et al. 1967, Pereira et al. 1959).

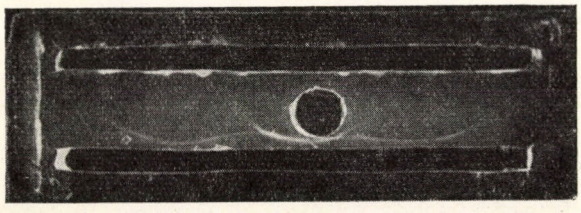

FIG. 3. Immuno-electrophoresis of adenovirus type 5 for 7 hours. Precipitin lines with homologous rabbit immune serum

The traditional method of Ragetli and Weintraub (1964) was applied in the immuno-osmophoretic experiments, i.e. virus suspension was introduced in one of the two reservoirs cut in a thin agar layer and homologous immune serum in the opposite one; the system was placed thereafter in electric field, the immune serum was put on the side of the positive and the virus suspension on the negative pole. Two precipitin lines appeared within 15—60 minutes, and prolonging the time of exposure to 2—4 hours, further lines were obtained. The precipitin line appearing earliest and localized closest to the serum reservoir corresponds to the hexon antigen. It is well demonstrable with both homologous and heterologous sera and with immune serum prepared against purified hexon antigen. This component migrates thus anodically just as in immune electrophoresis, its electrophoretic mobility being relatively the greatest. Precipitin line appearing closer to the antigen reservoir amounts to the complete hemagglutinin (penton), that migrates under given experimental conditions likewise anodically, though its electrophoretic mobility is less than that of the hexon. This precipitin line could not be obtained using trypsin-treated material.

Prolonging further the time of exposure, a new precipitin line appeared, strongly arched and in some cases doubled, and 2—3 lines became visible in the position of the hexon line. The former arched line localized closest to the negative pole corresponded to the incomplete hemagglutinin (fiber). Trypsin treatment made the line appear stronger, while the penton line disappeared. As compared with the immune electrophoresis, the migrating direction of the fiber changed, thus becoming anodical, though its electrophoretic mobility was far less than those of the other two components as shown in Fig. 4.

The elaboration and introduction of new methods provided useful means to demonstrate antigens migrating cathodically in immuno-osmophoresis

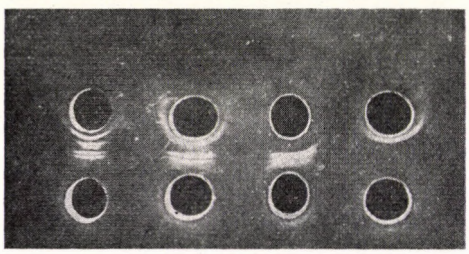

FIG. 4. Immuno-osmophoresis of adenovirus type 5 for 3 hours. Immune serum against type 5 in the four lower reservoirs. The upper reservoirs contain the following virus materials form left to right: unfractionated adenovirus-5 antigens; trypsin treated unfractionated adenovirus-5 antigens; purified hexon of adenovirus type 5; purified fiber of adenovirus type 5

and it could be stated that the hexon and fiber antigens migrate with similar rate in both directions.

Studies on the hemagglutinins of an oncogenic adenovirus type 7 strain

Our experiments were carried out with the Pinckney strain of adenovirus type 7 (henceforth 7P) which induces tumours in newborn hamsters (Girardi et al. 1964). The purified and concentrated virus material was fractionated by anion exchange chromatography or by CsCl equilibrium centrifugation, and the separated virus antigens were examined (Nász and Pereira 1965).

With these methods we succeeded in separating a soluble complete hemagglutinin that agglutinated the rhesus erythrocytes. This hemagglutinin was eluted from the DEAE-Sephadex column by 0.25—0.35 M sodium chloride concentration, but certain elution could be observed at about 0.075 concentration, too.

The soluble hemagglutinin was eluted after the group-specific hexon antigen and before the type-specific fiber antigen in column chromatography, and could be demonstrated by CsCl equilibrium centrifugation, separated from the infective virion, in the fractions localized in the upper part of the

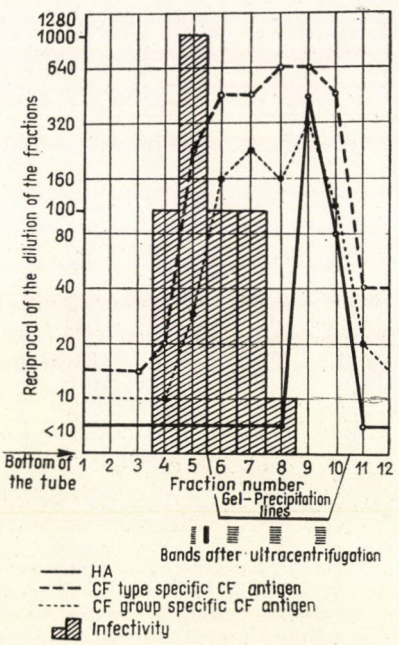

FIG. 5. Fractionation of adenovirus type 7P strain by CsCl equilibrium centrifugation

tubes. Neither intact nor disintegrated virus particles could be discovered by electron microscopic examinations in these fractions, on the other hand, they contained complement fixing hexon antigen in high titers (Fig. 5).

The soluble hemagglutinin became absorbed completely to the rhesus erythrocytes at 37 °C and was eluted from them at + 4 °C. In appropriate concentration the hemagglutinin formed a precipitin line with homologous rabbit and guinea-pig immune sera when tested with the agar-gel diffusion method. Low titers were obtained in complement fixing reaction with homologous and still lower with heterologous immune sera. The hemagglutinin proved to be heat labile being inactivated at 45 °C within 10 minutes to a considerable degree and completely at 56 °C within the same time. Trypsin treatment inactivated the soluble hemagglutinin as well.

Characteristics of the hemagglutinins of adenovirus type 19

Concentrated prototype strain 19 was separated by DEAE cellulose column chromatography in 0.01 M phosphate buffer pH 7 and pH 5.2, using a 0—0.65 M sodium chloride gradient (Gelderblom et al. 1968). Antigens were purified subsequently with CsCl equilibrium and sucrose gradient ultracentrifugation and by erythrocyte absorption. The following components were obtained by the appropriate combination of these methods: infective virus particles, disintegrated virions, soluble complete hemagglutinins (dodecon) (Gelderblom et al. 1967), type-specific fiber antigen and group-specific hexon antigen. The first three of the listed components showed the characteristics of complete hemagglutinins, thus agglutinating the rat erythrocytes, the fiber antigen gave only partial agglutination in the presence of corresponding heterologous immune serum, whereas the hexon antigen showed no hemagglutinating activity at all.

To determine the specificity of the fiber antigen acting as incomplete hemagglutinin, the sera of 66 rabbits immunized against the first 30 adenovirus serotypes were tested for HE, CF and HI effects.

Negative results were obtained in the cases of twelve sera belonging to the hemagglutination group I (Rosen 1960), ten belonging to subgroup III, and four belonging to subgroup IV. Twenty-six immune sera of subgroup II gave likewise negative results. Results obtained from the remaining sera of subgroup II are presented in Table 4. On the basis of these data, type 17 immune serum was applied as heterologous serum in the hemagglutination of the fiber antigen.

The fiber antigen failed to become attached to the column in our chromatographic experiments, thus it was the first to be eluted, before introducing

TABLE 4

Reactivity of immun e sera belonging to subgroup II with adenovirus type 19

Type of rabbit immune serum	CF	HI titers*		HE effect with the fiber
		Dodecon	Fiber	
9	±	20		+
	—**	—***	—	—
10	+	640	80	
	+	160	40	
17	+	20		+
	+	—		+
	+	—		+
	—	40	—	—
19	+	5120	640	
	+	2560	320	
	+	2560	80	
	+	2560	160	
25	+	80	80	—
	—	—	—	—

* Reciprocals.
** No reaction in serum dilution 1 : 10.
*** No reaction in serum dilution 1 : 20.

the sodium chloride gradient; it was followed by the soluble complete hemagglutinin (dodecon), the hexon antigen and the infective virus. The latter three components overlapped each other, their separation could be performed only by ultracentrifugation and erythrocyte absorption (Fig. 6).

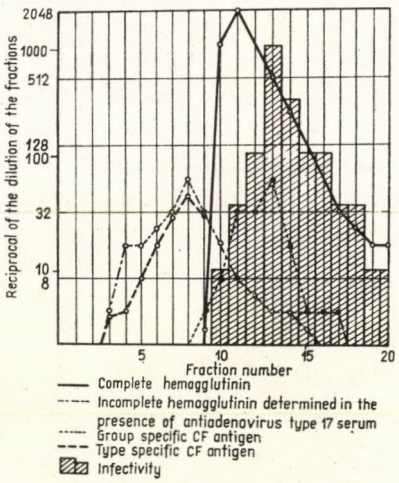

FIG. 6 Anion exchange chromatography of adenovirus type 19 on DEAE cellulose column in phosphate buffer 0.01 M, pH 5.2, elution by increasing molarity of NaCl (0 to 0.65 M) from fractions 9 to 20

Rabbits were immunized with purified components obtained by the methods listed above, and the reactivity of the immune sera was tested by CF and HI reactions and by neutralization test. Tables 5 and 6 present the results.

TABLE 5

Cross reactivity among adenovirus type 19 components. Complement fixation

Antigen	Antiserum prepared against				
	Hexon	Fiber	Dodecon	Virion	Crude virus
Hexon	160	< 5	10	20	160
Fiber	< 5	80	< 5	40	20
Dodecon	< 5	10	10	80	160
Virion	20	80	80	20	80
Crude virus	160	160	20	80	80

TABLE 6

Cross reactivity among adenovirus type 19 components. HA-inhibition and neutralization

Antigen	Antiserum prepared against				
	Hexon	Fiber	Dodecon	Virion	Crude virus
Fiber*	< 5	10	10	640	640
Dodecon	20	40	80	2560	2560
Virion	< 5	< 5	160	640	1280
Crude virus	20	40	160	2560	5120
Neutralization	160	2	40	320	640

* Incomplete hemagglutinin.

According to these results, the immune serum against purified hexon antigen gives no serologic reaction with the soluble complete and incomplete hemagglutinin and with purified virus, or only a very slight one, however, it reacts in very high titer with crude virus material. Immune serum against fiber antigen fails to react with hexon antigen in neutralization, however, it shows serologic reactivity with the fiber antigen, the dodecon and the crude virus. Purified virus and fiber immune serum react with each other only in complement fixation. Immune serum against dodecon gives no, or a very minimal reaction with the fiber and reveals a reactivity of very low titer with hexon antigen. Positive serologic reactions were obtained in neutralization and with virion preparations. Finally sera prepared against both

crude and purified virus materials contained antibodies against each component.

Studying the characteristics of the dodecon antigen it has been found that this component becomes easily adsorbed both to rat and human erythrocytes and shows positive CF reaction with type-specific immune sera; the CF titer being 100 times lower than the HA titer. The dodecon is heat labile, when kept at 55 or 60 °C for 1 hour, it loses capacity to induce complete hemagglutination, whereas the heat-treated material showed hemagglutination in the presence of a heterologous serum, indicating that the fiber got released during the breakage of the dodecon.

The fiber antigen is heat stable at the temperatures mentioned above, and it is type-specific. The positive reaction with type 10 as shown in Table 4 is the effect of the cross reaction already known from the literature (Bell et al. 1960).

Studies on the hemagglutinins of adenovirus type 9

These examinations were performed with the prototype strain 9 belonging to subgroup II (Norrby et al. 1967). Virions and empty capsids were removed by ultracentrifugation, and the soluble components were subsequently separated by DEAE-Sephadex A-25 column chromatography and by sucrose gradient zonal ultracentrifugation. The incomplete hemagglutinin eluted first in anion exchange chromatography and in further experiments agglutinated both rat and human erythrocytes in the presence of heterologous sera of subgroups II and III (Norrby 1968a). The next component was another incomplete hemagglutinin which agglutinated in the presence of heterologous sera belonging to any of the subgroups. This component was followed by the group-specific complement fixing hexon antigen, while the soluble complete hemagglutinin eluted last.

The incomplete hemagglutinin eluting first sedimented in ultracentrifugation slower than the other one. This component was not sensitive to trypsin treatment and absorbed the hemagglutination inhibiting antibodies of the homologous immune serum. The component gave positive CF reaction with immune serum prepared against the complete soluble hemagglutinin. These data suggest that this component is the fiber antigen, as compared with the results of other adenovirus types (Norrby and Skaaret 1967).

The incomplete hemagglutinin eluting second showed a faster sedimentation rate in sucrose gradient, its hemagglutination could be enhanced by any immune serum of the four subgroups, and was trypsin-sensitive. On the basis of these results this component proved to be the penton antigen.

The soluble complete hemagglutinin corresponds to the star pattern consisting of 12 penton antigens as observed by the electron microscope (Fig. 7), similarly to those demonstrated with other types (Norrby 1966, Norrby and Wadell 1967, Gelderblom et al. 1967). Trypsin treatment trans-

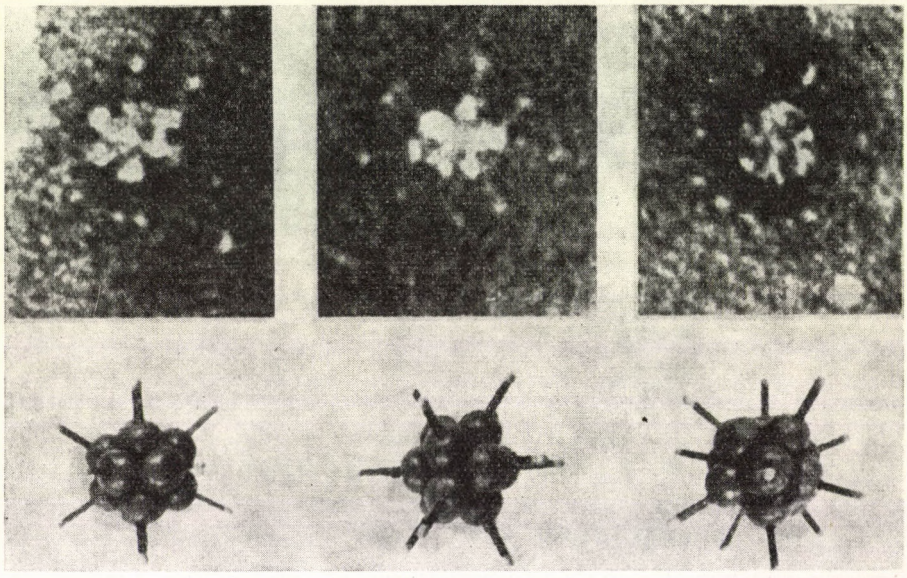

FIG. 7. Electron microscopic appearance of adenovirus type 9 purified complete hemagglutinin. Three particles in positions corresponding to three different symmetry axes, as demonstrated on the models

TABLE 7

Effect of three subsequent erythrocyte absorptions on the biological activities of adenovirus type 9 components

No. of absorption	Complete HA (units/0.4 ml)	Incomplete HA (units/0.4 ml)*	Group-specific CF (units/0.02 ml)**
0	12.000	—***	256
1	128	1.024	256
2	<4	1.024	256
3	<4	1.024	256
Eluate from cells of absorption 1	12.000	—***	<4

* Determined in the presence of an antiadenovirus type 15 serum.
** CF tests with antiadenovirus type 5 serum.
*** Not measurable due to the presence of high concentrations of complete hemagglutinin.

formed the dodecon into an incomplete hemagglutinin corresponding to the fiber antigen. The dodecon got easily absorbed to the human and rat erythrocytes and could be eluted from human erythrocytes with receptor destroying enzyme (RDE). No similar good results were obtained with rat

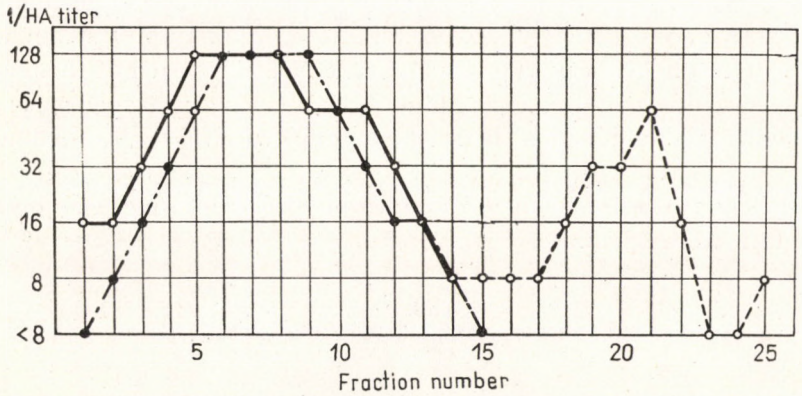

FIG. 8. Zonal centrifugation of adenovirus type 9 dodecon-containing material before absorption with, and after elution from rat erythrocytes.
○———○ HA before absorption,
○--------○ HA in the presence of heterologous serum before absorption,
●—·—● HA after elution with and without heterologous serum.

erythrocytes or by using KIO_4 because of the hemolysis, due to the fragility of the erythrocytes. An absorption elution experiment is presented in Table 7 which shows that the complete hemagglutinin can be recovered quantitatively by this method, thus the incomplete hemagglutinin remains practically unchanged in the solution, similarly to the hexon antigen, whereas the eluate contains only dodecons (Fig. 8).

Similar components were separated from a type 8 adenovirus strain isolated during an epidemic, the sequence of elution being likewise fiber–penton–hexon–dodecon. The penton and the dodecon were trypsin-sensitive and transformed after treatment into incomplete hemagglutinins, corresponding to fiber antigen (Lengyel et al. 1969).

Discussion

Studying the hemagglutination spectra of adenoviruses, no species has been found among the mammalian and avian species examined, whose erythrocytes were well agglutinable by every type of adenovirus used in the experiments. Fourteen of the 42 strains showed no hemagglutinating capacity. Another group of strains agglutinated the erythrocytes of only one, or of a few mammals or birds in considerably low titers, though it should be

noted that we insisted on unusually severe criteria in judging HA. Not fully typical sedimentation and partial hemagglutination were rejected as positive. Had all these been accepted, the registered titers would have been higher and many negative reactions should have been considered positive.

Out of the 42 virus strains tested only 6 were found to agglutinate the erythrocytes of more than five species. These were: type 9 strain which agglutinated 24 specimens, type 10 and type 15 strains each of them agglutinated 7 specimens, and types 10, 11 and 13 agglutinating 6 specimens each.

Surveying the behaviour of the four subgroups with rhesus, rat and human erythrocytes (Table 8) it can be stated that five of the six strains listed above belong to the members of subgroup II displaying the broadest hemagglutination spectra.

TABLE 8

Hemagglutination characteristics of adenoviruses

Rosen's subgroup	Type	Species of the erythrocytes agglutinated		
		Rhesus	Rat	Human
I	3, 7, 11, 14, 16, 20, 21, 25, 28	+		
II	8, 10, 19, 26, 27		+	+
	9, 13	+	+	+
	15, 23	+	+	
	22, 29, 30		+	
III	1, 2, 4, 5, 6		+	
IV	12, 18, 31		+?*	

* Hemagglutination has been described so far only with type 12, this group being characterized originally by he lack of hemagglutinating capacity.

Another noteworthy relatedness can be observed also in the HA of the adenovirus group. As it was shown in our earlier studies (Nász and Tóth 1958), the cytopathic changes caused by two groups of adenoviruses are well distinguishable from each other in human amnion cell cultures. The changes caused by the group including adenovirus type 3 have been termed "type-3" degeneration, those characteristic of the other group "type-5" degeneration. Out of the 42 strains tested in the study 21 showed "type-3" and 21 "type-5" degeneration. The latter types were less active in HA than the former, which agglutinated the erythrocytes of far more mammals and birds. Five of the six strains described to be the most active belonged to the group causing "type-3" degeneration and one to the "type-5" group. Virus strains agglutinating all the rat erythrocyte samples tested belonged to "type-3"

degeneration, whereas only a few strains showing "type-5" degeneration agglutinated rat erythrocytes and even those which did so were agglutinating not more than a few of the specimens tested. Of the non-hemagglutinating strains, on the other hand, only four belonged to the "type-3" group and ten to the other. Finally, every strain agglutinating human erythrocytes belonged to the "type-3" group.

Our results indicate that HA with adenoviruses is specific and can be inhibited by immune sera, nevertheless, it appears to be a complex phenomenon. Erythrocytes which are well agglutinable by some strains are agglutinated often only in very low titers or not at all by others. The same erythrocytes are well agglutinated by certain strains and sparsely or not at all by other strains belonging to the same serotype, under identical experimental conditions. Several authors pointed out (Rosen 1960, Simon 1962) that the same virus strains may agglutinate the erythrocytes of different rhesus monkeys to different extent. Similar phenomenon was observed in our experiments with human and rat erythrocytes. This phenomenon is not related either to the blood-group factors or to a previous adenovirus infection of the monkey (Simon 1962), nor did our observations reveal any relation between the blood group and the occurrence or titer of the hemagglutination with human erythrocytes.

As shown by our experiments the temperature and H-ion concentration are not likely to play an important role in this phenomenon, at least not in the HA of strains representing the most populous subgroup II.

Several investigators have reported cross reactions among certain serotypes of adenoviruses using immune sera of human origin, and sera prepared in laboratory animals. The titres of these cross reactions were low. Cross reactions were demonstrated in neutralization tests between types 8 and 9 using rabbit immune sera (Grayston et al. 1956, Uchida et al. 1959), and in human sera from EKC cases (Dawson et al. 1960). Minimal cross reaction was observed in the experiments of Grayston et al. (1956) between type 9 virus and type 10 immune serum. Rosen (1960) has found with the HI test cross reactions between types 7 and 7/a, 11 and 14, 8 and 9, 10 and 19.

No direct data were found concerning the heterotypic reaction among types 8, 9 and 10 observed by us in the HI test. The cross inhibition between types 8 and 9 is known, but only one reference concerning type 10 was found in the literature (Rosen et al. 1962): type 10 virus was inhibited besides the homologous serum by anti-9 rabbit immune serum, too, in a low dilution, but this phenomenon could not be demonstrated conversely. As for human sera, no literary data were found concerning the heterologous reaction observed in our experiments among these three types.

Data summarized in Table 2 confirm Rosen's observations on the cross reaction among types 7, 11 and 14, and our findings about the type 8 virus being inhibited only by type 9 heterologous serum, correspond to Rosen's results as well. Disagreeing with them are the observations with immune sera types 8, 9 and 10. These reacted both with type 9 and type 10 viruses in our experiments, the titer of the homologous serum being in all cases considerably higher than those of the heterologous types.

In the examinations of paired sera of patients with EKC antibody titer rise was observed in the convalescent sera, against the etiologic agent adenovirus type 8 as well as against type 9. The rise was significant (at least fourfold) in 95 and 82%, respectively. In 25 paired sera (62.5%) titer rise was also shown against type 10, and it was in 20 cases significant. Taking into consideration: (i) results obtained with type specific immune sera, (ii) that virus isolation experiments carried out simultaneously failed to reveal the presence of type 9 or type 10 adenoviruses in the conjunctival washings of the patients (Nász et al. 1963a, 1963b), (iii) that no differences were found with type 11 and type 16 between the acute and the convalescent sera (Lengyel and Nász 1964), thus, the titer rise mentioned against type 9 and type 10 must be interpreted as heterotypic reaction against these types. However, type 8 adenovirus shows HI cross reaction not only with type 9 but also with type 10, the latter being less frequent and less marked in quantitative respects. This might explain why this phenomenon has not been discovered before.

Data obtained from a considerable number of animal experiments confirm these observations and suggest a certain quantitative sequence, too. According to these, the cross reaction between types 8 and 9 appears to be the strongest, the degree between types 8 and 10 is only half of the former and that of 9 and 10 is in between the two values mentioned.

Heterotypic HI antibodies formed during immunization showed no difference in their stability as compared with the homotypic antibodies, their level changing parallel with those, only they disappeared from the serum somewhat earlier, their level being originally lower than that of the homotypic antibodies. Heterotypic antibodies could be demonstrated both in immunized rabbits and guinea-pigs.

Both homo- and heterotypic antibodies could be detected in the newborn offspring of immunized rabbits pointing thus to their transportability through diaplacental ways. The same antibodies could be detected in the colostrum and could be traced in the sera of the animals as long as for 40—60 days.

All these results refer to the observation that the appearance of heterotypic antibodies is not due to an aspecific immune reaction, but proves the

existence of a relationship of antigen structure among the types mentioned. Such relationships were reported by other researchers, too (Kasel et al. 1965) and a more thorough knowledge on the compound of the individual antigen components may elucidate these problems satisfactorily.

As the zonal electrophoresis method appeared to be useful in previous experiments (Schmidt 1967) for the separation of the soluble hemagglutinins of two different adenovirus prototype strains, we also applied it to examine the behaviour of a considerable number of adenovirus strains.

As indicated by the results, the complement fixing antigens of different strains show identical behaviour, whereas that of the soluble hemagglutinin and infective virus particle is closely dependent on the individual strain.

The soluble hemagglutinins of the prototypes 9 and 23 revealed identical migration rate, while in the cases of the four other strains (another strain of type 9 among them) the soluble hemagglutinin and the infective virion appeared in separate fractions. Thus, the zonal electrophoresis seems to be suited in the case of these latter strains — together with other methods — for separating the virion from the dodecon, though, this property does not seem to be characteristic for the adenovirus types. Therefore, it cannot be used for typing and has no advantage over the zonal ultracentrifugation.

Results obtained with immune osmophoresis suggest that this method will be a useful diagnostic procedure for adenoviruses, and with the elaboration of a suitable method diagnosis may be established as soon as within 1/2—1 hour. No such rapid method has been available until now for demonstrating adenovirus infections.

The trypsin-sensitivity of penton — one of the components acting like hemagglutinin — is well known (Pereira and de Figueiredo 1962), thus trypsin treatment transforms it into fiber. The precipitin line of the fiber becoming more intensive simultaneously with the disappearance of the penton line can be interpreted as being due to the trypsin treatment.

The fiber displays in some cases double precipitin line which may be explained by its composition, consisting of a rod and a knob on its end. On the other hand, it seems to be possible that certain other factors (such as quantity or concentration, diffusion, host cell antigen) induce this phenomenon. As the fiber together with the vertex capsomer forms the penton, it is understandable that the sera of animals immunized against the fiber react with both components.

Our experiments with adenovirus type 7P strain showed that this strain has a soluble complete hemagglutinin which can be separated from the virus particle and is type-specific. The virions of the type 7 strains failed to show hemagglutinating activity; this observation as well as their heat- and

trypsin-sensitivity have been confirmed by other researchers, too (Bauer and Wigand 1967).

The characteristics of the soluble hemagglutinins observed in our experiments and the morphological examinations on the ultrastructure of adenoviruses (Valentine and Pereira 1965, Norrby 1966, Gelderblom et al. 1967) indicate that the soluble hemagglutinin of type 7P — similarly to the complete hemagglutinins of the types belonging to subgroups I and II — consists of 12 penton antigens (dodecon). The proximal ends (vertex capsomers) of the penton are situated according to the icosahedral symmetry, in the center of the starlike pattern. This hypothesis is supported by the demonstration of the dodecon of prototype strain 7 (Neurath and Rubin 1968).

The composition of the penton antigen — type-specific fiber and group-specific trypsin-sensitive vertex capsomer — might be the explanation for the low titers obtained in CF reactions with heterologous immune sera belonging to other subgroups, besides the stronger type-specific serologic reactivities.

It is noteworthy that the elution sequence of the type 7P soluble antigens was found to be similar in DEAE column chromatography to that of oncogenic type 12 (Huebner et al. 1964), but was reversed as compared with types 2 and 5 (Klemperer and Pereira 1959), or with the sequence observed by us with types 8 and 9, although these types do not actually belong to the same subgroup as type 7. Some publications on types 3 and 7 (Haruna et al. 1961, Norrby and Skaaret 1967, Zhdanov and Mekler 1962) do not present data concerning the elution sequence of every soluble antigen component. Recent data on the soluble components of subgroup I are found in several publications of Norrby and his coworkers, but type 7 is not included. The fiber of types 3, 11 and 16 tested elutes always before the complete hemagglutinin in column chromatography (Norrby 1968b, Norrby and Skaaret 1968), whereas the type-specific fiber antigen of oncogenic type 12 is the last among the soluble components (Huebner et al. 1964, Norrby and Ankerst 1969).

With the knowledge of recent data we may find an explanation as to why a small quantity of complete hemagglutinin elutes at a low concentration of NaCl isolated from the bulk of the soluble hemagglutinins, which elute later by introducing a sodium chloride solution of higher molarity. Recently, certain adenovirus types, e.g. type 15, have been shown to possess also another type of complete hemagglutinin, which is produced by the aggregation of a few, possibly of two fiber components. Such product may exist in the case of our type 7P strain tested. Both its situation in the elution diagram and the fact that the addition of heterologous adenovirus immune sera failed to alter the hemagglutination titers refer to this phenomenon.

The neutralizing capacity of the immune serum prepared against type 19 hexon antigen contradicts group-specificity, but agrees with similar findings of other authors on type 5 adenovirus (Wilcox and Ginsberg 1963a). This suggests that the hexon antigen must be endowed with both group- and type-specific determinants (Norrby 1968b).

No serologic relationship was found between type 19 hexon and fiber antigens (Tables 5 and 6) and sera against the fiber failed to reveal neutralizing activity. No cross reactivity was found in precipitation with similar components of type 5 (Wilcox and Ginsberg 1963b, Valentine and Pereira 1965) and type 2 (Köhler 1965) either. The lack of neutralization contradicts the observations obtained on type 5 (Wilcox and Ginsberg 1963a), which might be explained only by the low antibody titer of the serum.

The complete soluble hemagglutinin, dodecon, exhibited in CF reaction mainly group specificity, but showed reaction in a small degree with the hexon serum, too (Table 6). This finding is similar to the results obtained with type 7P, and can be explained likewise by the group-specificity of the vertex capsomers building the dodecon. The neutralizing activity of the dodecon serum refers to the fact that the projections of the virion vertices may play an important role in the adsorption of the virus onto the cell, thus in virus infections, as the attachment to the erythrocyte receptors doubtlessly occur by them (Valentine and Pereira 1965, Norrby 1966). It is known also that the purified projections of type 5 protect, so to say, the cells from the adenovirus infection (Levine and Ginsberg 1967).

The relationship between the fiber and the dodecon is clearly shown by the fact that heat treatment decomposes the dodecon, thus releasing the fiber, which reacts as incomplete hemagglutinin in the presence of a heterologous immune serum within the subgroup. The relationship between the two components is demonstrated in Tables 5 and 6. Only the CF test fails to show this relation, which is due to the low antibody titer of the dodecon serum even against homologous antigen. Similar conclusions may be derived from the results presented in Table 4. Studying the dodecon–fiber relationship as compared with other types, the HA of the fiber was inhibited only by a type 25 heterologous serum, besides the type 10 sera, whereas the HA of the dodecon was inhibited by type 9 and type 17 sera as well. The fiber represents thus the type-specific determinant of the dodecon, while the complete hemagglutinin shows wider relations within the subgroup, as confirmed by the data of other investigators (Kasel et al. 1965).

Purified virus particles reacted in CF test with hexon and fiber sera, although high antigen concentration was needed to the reaction with the latter. This phenomenon is easily understood since the adenovirus capsid

contains 240 hexon capsomers and only 12 fiber elements. Similar observations were made with type 5 adenovirus (Wilcox and Ginsberg 1963b).

Besides the soluble hemagglutinin, rat erythrocytes were equally agglutinated by the purified complete virion, partially destroyed virions and empty capsids of type 19, whereas in the case of type 7P only the soluble hemagglutinin reacted with the appropriate sensitive erythrocytes.

Adenovirus type 9 was likewise found to have complete soluble hemagglutinin formed by 12 penton antigens, named dodecon (Fig. 7), similarly to type 3 (Norrby 1966), type 4 (Wadell et al. 1967), type 11 (Norrby 1968c) and to types 13, 15 and 19 belonging to subgroup II (Gelderblom et al. 1967). This structure seems to contain — in the cases of the types mentioned — an internal component, too, but its nature is still unknown. The vertex capsomers aggregate around this internal component. This supposition is based on the intermediate sedimentation ratio of type 9 dodecon, between those of type 3 and type 11, in spite of the fact that the projections of type 9 are longer than those of the other two types. This phenomenon can be interpreted as being due to the difference in the mass of the internal component. The diameter of the complete hemagglutinin of type 9 together with the projections is 48—58 mμ, depending on the chosen symmetry axis, from whose direction the structure of dodecahedral symmetry is looked upon (Fig. 7). The diameter of the central part, thus containing 12 vertex capsomers without projections (and within them the postulated internal component) is 24—29 mμ, whereas the length of the projections, that is of the fiber antigens, is 12—15 mμ.

The attachment of type 9 complete hemagglutinin to the rat and group 0 human erythrocytes appears to be irreversible, though a quantitative elution from the latter ones could be attained with the receptor destroying enzyme, produced by the *vibrio cholerae*. The same procedure was successful with type 8 adenovirus as well. This would suggest that it reacts with the same erythrocyte receptors as the myxoviruses. Still there seems to be a difference among the receptors reacting with the two kinds of viruses, as the KIO_4 treatment was less successful and the results obtained with formalin-treated erythrocytes were found to be different (Buckland 1959). The fact that no elution was attained with RDE in the case of rat erythrocytes confirms this divergence as well.

The elution sequence of the soluble components of type 8 and type 9 was found to be fiber–penton–hexon–dodecon in anion exchange chromatography. Thes equence of the latter two components was in accordance with the results obtained with the members of subgroup II (Gelderblom et al. 1965).

The incomplete hemagglutinin being eluted first from the column showed

hemagglutination in the presence of a heterologous serum belonging to subgroups II or III, but failed to do so with sera of subgroup I. This component absorbed the HI antibodies of the homologous immune serum, was trypsin resistant and proved to be type-specific in CF reaction, thus corresponding to the fiber.

Another incomplete hemagglutinin was eluted second, agglutinating in the presence of sera belonging to any of the four subgroups, it absorbed the HE antibodies of the immune sera, and proved to be trypsin-sensitive. Trypsin treatment limited its agglutination activity exclusively to the sera of subgroups II and III, indicating its transformation into fiber. This component failed to become absorbed onto the erythrocytes, thus it could be separated from the dodecon by absorption and by elution with RDE (Fig. 8). This is the penton, its proximal end being formed by the group-specific trypsin-sensitive vertex capsomer. The penton of certain types exhibits toxic effect on the cell cultures and is responsible for the "early cytopathic effect" (Pereira 1958). The same effect could be revealed with type 8 (Lengyel et al. 1969), but failed to be demonstrable with type 9.

The complement fixing antigen was the third in the elution diagram, showing no hemagglutinating capacity, it was trypsin-resistant, could not be adsorbed to the erythrocytes, so it corresponded to the hexon antigen.

The soluble complete hemagglutinin, the dodecon, eluted last from the column, and being destroyed by trypsin treatment in both cases of type 8 and type 9 strains, the fiber antigen content increased.

On the basis of the results presented here, and the literary data given, five complete adenovirus hemagglutinins were identified in the preparations of the types tested: 1. complete virions, 2. destroyed virions, empty capsids, 3. dodecon, 4. penton dimer formed by the aggregation of penton antigens, and 5. fiber dimer formed in a similar way. Two components may act like incomplete hemagglutinins: the penton and the fiber antigen.

Differences can be found in the hemagglutination characteristics of the individual components, depending on the type of the virus and the subgroup into which they belong, moreover, it appears to be uncertain whether viruses of the same type show always identical characteristics from this point of view. Hemagglutination characteristics of the soluble components and virions tested until now are summarized in Table 9.

Summarizing our present knowledge on the hemagglutination of adenoviruses it can be stated that the hemagglutination is useful for demonstrating the individual antigen components, though, the capsomers failed to show hemagglutinating activity from the known components. Still vertex capsomers can be demonstrated indirectly with the HA method by their

TABLE 9

The hemagglutinins of human adenoviruses

HA subgroup	Complete HA					Incomplete HA	
	Type	Virion, empty capsid	Dodecon	Penton dimer	Fiber dimer	Penton	Fiber
I	3, 11	+	+			+	—
	7	—	+			+	—
	16	+	—	+	+	+	—
	3—16	+	—	+		+	—
II	8, 9, 9—15	+	+			+	+
	13	—	+				+
	15	+	+		+	+	+
	19	+	+				+
III	4	± (partial)	+			+	+
	1, 2, 5, 6	+	—	+	+	+	+
IV	12	+	—		+	+	+

ability to absorb the HE antibodies (Norrby and Skaaret 1967). The fiber antigens of subgroup I can be likewise detected by their capacity of absorbing the HI antibodies. The only component for which the methods presented fail to be applicable is the hexon antigen, although, this is just the component that attaches to the tannic acid treated sheep erythrocytes in passive hemagglutination with adenoviruses (Friedman and Bennett 1957).

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ROUND-TABLE CONFERENCE ON ATYPICAL MYCOBACTERIA, ESPECIALLY *M. SIMIAE*

held on 28—30 April, 1969

Organized by the Microbiological Research Group of the Hungarian Academy of Sciences

J. G. WEISZFEILER

The Microbiological Research Group of the Hungarian Academy of Sciences organized a round-table conference to discuss the results of the investigations in the field of atypical mycobacteria isolated from monkeys. The results of the Weiszfeiler group and those achieved by Dr. Schröder (Borstel), Dr. Magnusson (Copenhagen), Dr. Sakellarides (Lausanne), Dr. Makarevich (Moscow), Professor Tacquet (Lille) and Dr. Käppler (Berlin-Buch) were appraised. Besides, the investigation on mycobacteria isolated from soil samples, a work accomplished by Dr. F. Szelényi and Professor G. Berencsi, was included in the material of the Conference.

The Conference was attended by Dr. Ter-Zakharian (Erevan, USSR), Dr. Sonja Budić (Ljubljana, Yugoslavia), Dr. Schröder (G.F.R.), Professor G. Berencsi (Szeged, Hungary) and from the following Hungarian institutes and research groups: Microbiological Research Group, National Institue for Tuberculosis "Korányi", Research Institute for Veterinary Medicine, Pneumological Clinic of the University Medical School, Budapest, and County's Dispensary for Tuberculous Patients, Szolnok. The lectures were followed by a discussion, in which several research workers including Dr. I. Földes, Dr. I. Szabó, Dr. E. Medveczky and Dr. S. Tuboly took part.

As the result of the round-table conference the following conclusions were drawn. Some of the facultatively pathogenic mycobacteria isolated from monkeys obviously represent a new species which is named *Mycobacterium simiae*. *M. simiae* may be divided into several subgroups or types on the basis of antigenic and cultural differences. The separation of the individual types from one another needs further research. It was moreover established that the enzymological characterization of one and the same strain may lead to divergent results in different laboratories. The divergencies may be explained partly by assuming that the populations of some strains are genetically heterogeneous, partly by the variability of the methods and reagents employed. It would be desirable to examine genetically homogeneous strains, and the methods should be standardized. The immunolo-

gical tests, viz. agglutination, gel-precipitation and immunoelectrophoresis, have proved to be important methods in this field.

The participants agreed in publishing the material of the round-table conference in the Proceedings of the Microbiological Research Group of the Hungarian Academy of Sciences.

STUDIES ON *MYCOBACTERIUM SIMIAE* AND SOME OTHER ATYPICAL MYCOBACTERIA ISOLATED FROM MONKEYS*

J. G. WEISZFEILER, V. KARASSEVA and E. KARCZAG

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We first drew attention in 1964 that monkeys may carry atypical mycobacteria. In order to estimate the frequency of the occurrence of such infections and to characterize the taxons of the mycobacteria occurring in monkeys wide-scale investigations were undertaken. Keeping monkeys in captivity either in zoos or in other places for a longer period of time they are able to contract mycobacterial infection, thus, we decided to examine monkeys sacrificed soon after their arrival in Europe.

Sixty-six *Macacus rhesus* monkeys imported from India and three *Cercopithecus aethiops* monkeys from Africa were examined. Mycobacterium strains were isolated from 33 animals, i.e. from 48% of the monkeys tested. The total number of strains was 50, strains were isolated from more than one organ of 8 animals.

The cultural, biochemical and pathogenic properties of the strains were investigated (1965). Many of the strains were of the slow-growing type. We attributed special importance to the observation that 22 of the strains induced pathological lesions when tested in mice; some of the mice died. The strains multiplied in the inoculated mice intensely and a great number of colonies were obtained even 90 days after inoculation. The strains were variable in pigment production, enzyme activity and sensitivity to antibacterial substances. Our greatest attention was attracted by some photochromogenic strains that proved to be different from both of *M. kansasii* and *M. marinum*. The strains have been considered to be representatives of a new, hitherto unknown species. We named this species *M. simiae*. It was also interesting to initiate experiments in order to reveal whether mycobacterial infections only occur in *Macacus rhesus* imported from India or also in African Cercopithecus monkeys. In a single case during our investigations in Budapest we succeeded in isolating a mycobacterium strain (No. 58) from a *Cercopithecus aethiops*. Further research

* This investigation received financial support from the World Health Organization.

was undertaken by Vichkanova in the Institute of Poliomyelitis and Viral Encephalitides of the Academy of Medical Sciences, Moscow. She has examined the organs of 22 Cercopithecus monkeys imported from Ethiopia. In order to identify the strains isolated by us, an evaluation of the investigations conducted by other research workers seemed to be desirable. Fortunately, some of the strains have been examined by a number of investigators, namely, Professor Hauduroy (Lausanne), Professor Meissner and Dr. Schröder (Borstel), Dr. Bönicke (Borstel), Professor Tacquet (Lille), Dr. Magnusson (Copenhagen), Dr. Šula (Prague), Dr. Makarevich (Moscow), Dr. Käppler (Berlin-Buch) and Dr. Runyon (Salt Lake City).

The results of our further investigations have been published in Weiszfeiler's monograph "Biologie und Variabilität des Tuberkelbakteriums und die atypischen Mycobakterien" (1969). We have revealed that neither the enzyme spectrum nor the antigenic structure of the dysgonic photochromogenic strains is homogeneous and so two or three subspecies could be distinguished within *M. simiae*. Several non-pigment-producing, slow-growing strains were excluded from the species. The pigment-producing strain No. 52 was examined by Dr. Šula, mainly in animal experiments. The strain was identified by us as *M. aquae*, which corresponding to the description of Boenicke agreed with Käppler's results. Among the fast-growing strains, strains No. 4 and No. 15 proved to be *M. abscessus* (Weiszfeiler et al. 1969), whereas strain 55a was identical with *M. phlei*. Vandra, who examined the phage sensitivity of 30 strains, found 26 to be resistant to all the 17 Mycobacterium phages employed, while three strains were sensitive to the phlei phage and one strain to the smegma phage. We also investigated the mouse pathogenicity of 20 slow-growing strains. Many of them caused severe lesions in the lungs, several strains had the same effect even in the kidneys and spleen. Five strains were examined for immunogenicity against tuberculosis infection. One of these, strain No. 59, was definitely immunogenic. The contagiosity of some strains for cage mates was also studied (Karasseva, Weiszfeiler and Karczag 1969).

The results of the experiments with 32 strains are summarized in Table 1. Based on their enzymatic activity they can be divided into two groups: urease-positive and urease-negative. Of the 13 strains in the urease-positive group 11 are niacine-positive, five are nicotinamidase- and pyrazinamidase-positive and one strain, No. 14, is succinamidase-positive. Part of both the urease-positive and urease-negative strains are photochromogenic, while the others produce no pigment. Many of the urease-negative strains are nicotinamidase- and pyrazinamidase-positive, properties characteristic of avium strains.

TABLE 1

	Strain No.	Runyon Group	Niacine	Nicotine-amidase	Pyrazine-amidase	Succine-amidase	Nitrate-reductase	Aryl-sulfatase	Lipase	Mouse-virulence	Immuno-genicity	Conta-giosity	Antigenic-structure
Urease +	5	I	−	+	+	−	+	+	±				
	14		−	+	+	+	+	+	−	+		+	+
	20		+	+	+	−	−	+	±	+			
	25		+	+	+	−	±	+	−	+			+
	29		+	+	+	−	−	+	−	+	−	+	+
	55i		+	−	−	−	−	+	−	++			
	58		+	−	−	−	−	+	−				+
	47	III	+	−	−	−	−	+	−				
	51		+	−	−	−	−	+	−	++		+	+
	53		+	−	−	−	−	+	−	+			+
	54i		+	−	−	−	−	+	−	±			
	57		+	−	−	−	−	+	−				
	59		+	−	−	−	−	+	−	++	+	+	+
Urease −	27a	I	−	−	−	−	−	+	−				
	61		−	−	−	−	−	++	+	±	−		+
	64a		−	−	−	−	−	++	+	++			
	32		−	−	−	−	−	++	+	±			+
	63		−	+	+	−	+	−	+	+			+
	60		−	+	+	−	−	+	−				
	68		−	+	+	−	+	−	+	+	−		+
	3	III	−	+	+	−	−	+	+	++			
	11		−	+	+	−	−	+	−				
	23		−	+	+	−	+	++	+	+			+
	27b		−	+	+	−	±	+	−				+
	33		−	+	+	−	±	++	−				
	46		−	+	+	−	−	+	−	++			+
	47		−	+	+	−	−	++	−	±			
	49		−	+	+	−	−	++	−				
	54		−	+	+	−	−	+	−				
	62		−	+	+	−	±	−	−				
	64b		−	+	+	−	+	++	−				+
	52	II	−	−	−	−	±	+	+	++	−+		

In our opinion, the photochromogenic strains which do not produce pigment belong to the new species *M. simiae*. Nor did the photochromogenic strains prove to be homogeneous as regards their enzyme spectrum; on this basis they can be divided into type 1 and type 2. The strains in type 1 are urease-positive, niacine-positive, lipase-negative and, partly, nicotinamidase- and pyrazinamidase-positive; the strains belonging to type 2 are urease-negative, lipase-positive and, partly, nicotinamidase- and pyrazinamidase-positive.

The photochromogenicity of the *M. simiae* strains is of special interest, because earlier, in genus *Mycobacterium*, only *M. kansasii* and *M. marinum*

were found to be photochromogenic. In the case of the *M. simiae* strains photochromogenicity shows some variation. Twenty-four hours after illumination, strains No. 61 and 32 show an orange-coloured pigment, while the colonies of the photochromogenic strains No. 29 and 14 are light yellow.

When subcultured several times *in vitro*, some of the strains appear to have lost their pigment-producing capacity which, however, reappears

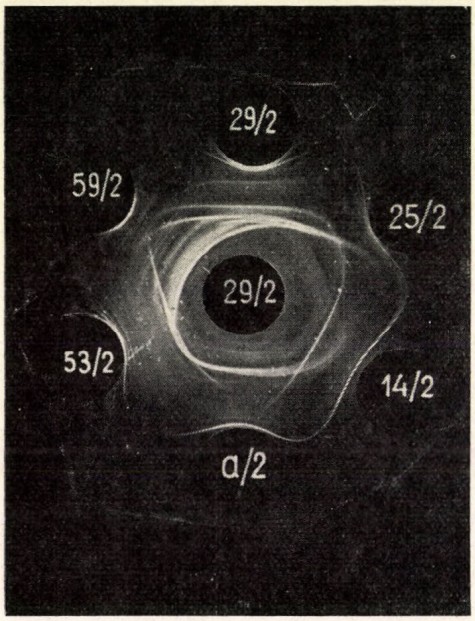

FIG. 1. Precipitation system of *M. simiae* 29 antiserum (central well) and antigens of *M. avium* (a), *M. simiae* Nos 14, 25, 29, 59 and 53. Denominators indicate the degree of dilution

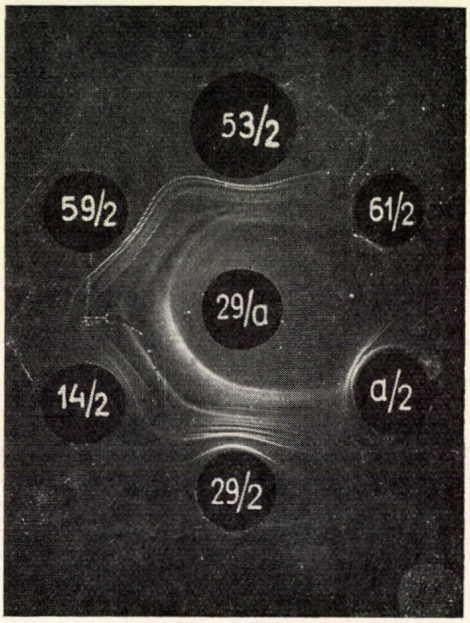

FIG. 2. Central well: *M. simiae* 29 antiserum absorbed by antigen of *M. avium* (denominator = a). Peripheral wells: antigens of *M. avium* (a) and different strains of *M. simiae*

during passages in animals. From several pigment-producing strains, e.g. No. 27 and No. 61, variants producing no pigment were isolated. It has been shown that for a mycobacterium, like other bacteria, a negative enzyme reaction does not indicate the lack of the corresponding enzyme; it may mean that the enzyme activity is just below the threshold of detectability, e.g., the urease-negative strain No. 61 exert a weak nicotinamidase and pyrazinamidase activity as it had been shown by Käppler (1968).

Examining the antigenic structure of *M. simiae* strains by the gel-precipitation technique, emphasized by us in a previous paper and our mono-

graph, enables us to establish with certainty the relation existing among them. The antigenic structure of 14 of our strains was examined by the Ouchterlony technique; three immune sera, namely, anti-simiae 29, anti-simiae 61 and anti-Mycobacterium sp. No. 64 were used. At the same time, using the same method, we compared our strains to *M. tuberculosis*, *M. avium*, *M. kansasii*, *M. intracellulare* and *M. marinum*. The antigenic relations were

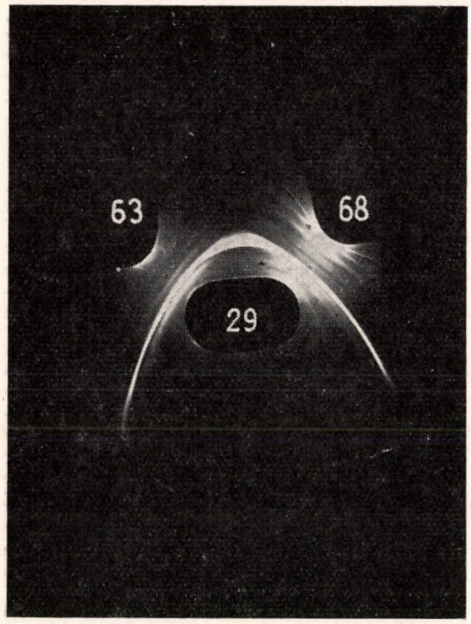

FIG. 3. M 29 = antiserum; antigens 63 and 68.

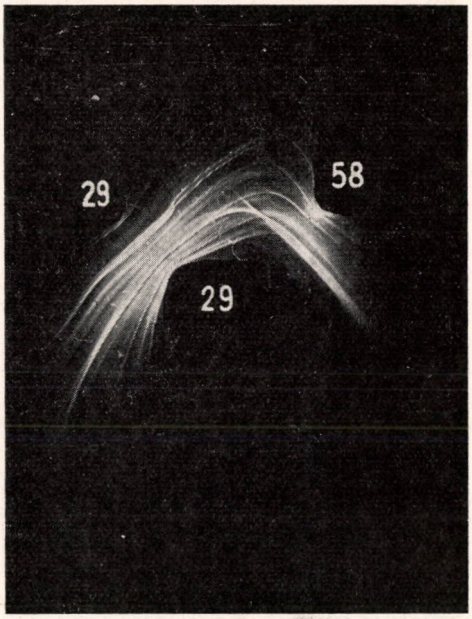

FIG. 4. M 29 = antigen; antigens 29 and 58

demonstrated by identifying the precipitation lines, and the common antigens were demonstrated by serum absorption. As seen in Fig. 1 with the serum anti-simiae 29 it was not possible to distinguish between strains No. 14, 25, 29, 59 and 53, and these strains appeared to be related to the avium strain. Figure 2 shows the same reaction except that before the reaction the antibody components reacting with *M. avium* had been removed from the serum. Thus the divergence from *M. avium* as well as the identity of strain No. 29 with strains No. 53, 59 and 14 are clearly seen. It is also clear that in antigenic structure strains No. 61 and 29 are far from each other. With the help of the serum anti-simiae 29 an antigenic relation was revealed between strains No. 63 and 68 and strain No. 29 (Fig. 3). A similar relation exists between strains No. 29 and No. 59 (Fig. 4). Figure 5 shows the common antigens of

strain No. 29 of *M. simiae*, on the one hand, and of *M. tuberculosis* H37, *M. avium*, *M. kansasii* and *M. balnei* and strain No. 64, on the other. For the reaction shown in Fig. 6 serum anti-simiae 29 had been absorbed by the antigen of strain 33. Although very little of the homologous antibodies had been removed, the absorbed serum only gave few precipitation lines with the antigens of strains No. 61, 64a, 33 and 60, while a close relationship with

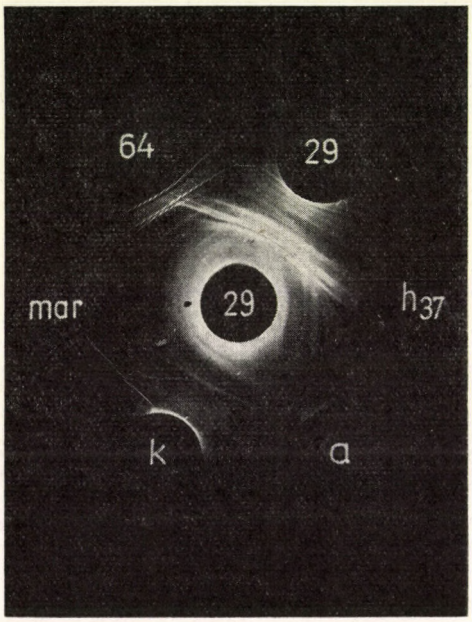

FIG. 5. Central well: antiserum 29; peripheral wells: antigens of *M. tuberculosis* H37Rv; *M. avium*, *M. kansasii* (k), *M. marinum* (mar); *M. Simiae* 64b and 29

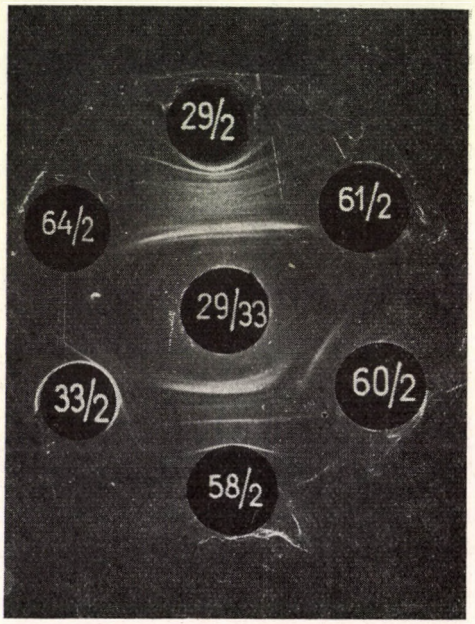

FIG. 6. Central well: antiserum 29 absorbed by antigen of strain 33. Peripheral wells: antigens of strains indicated by numbers

strain No. 58 is confirmed. The gel-precipitation test confirmed the species identity of strains No. 61 and 32 (Fig. 7), which had already been demonstrated by other techniques. The non-pigment-producing strain No. 64b does not belong to the group of. *M. simiae* (Fig. 8). The antiserum to this strain shows it antigenically identical with *M. intracellulare* and with strain No. 46, indicating that strain No. 64 belongs to this species; antigenically it is substantially different from strains No. 5, 3 and 23. The results of the gel-precipitation tests are summarized in Table 2. Accordingly, *M. simiae* may be divided into two serological types, one represented by strain No. 29, the other by strain No. 61. These two strains are related to each other as shown by the seven common precipitation lines. Of course, the investigations have

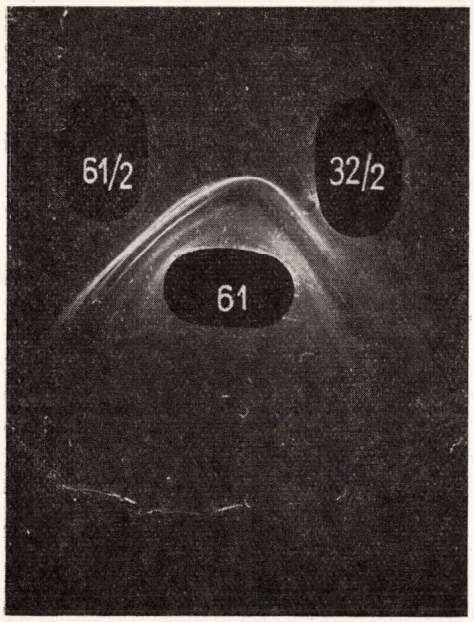

FIG. 7. M61 = antiserum; antigens 61/2 and 32/2 diluted 1 : 2

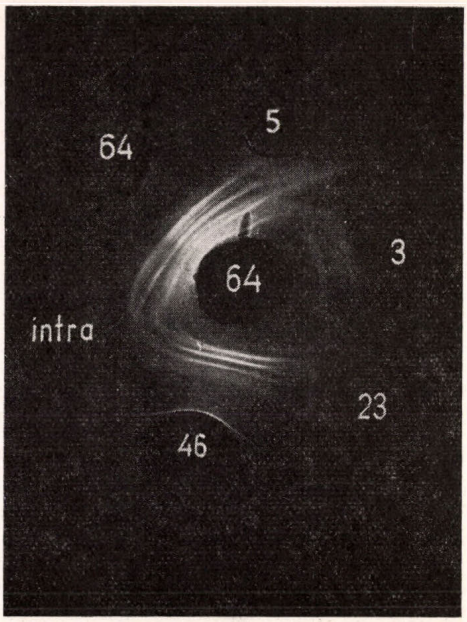

FIG. 8. Central well: antiserum against strain 64b. Peripheral wells: antigens of *M. intracellulare* (intra) and strains Nos 64b, 5, 3, 23, 46

not yet been completed, numerous strains have not yet been examined and the identification of the common antigens of the different strains is still under way.

Summary

The *Mycobacterium* species named by us *M. simiae* is inhomogeneous in its biochemical characteristics and antigenic structure. Within the species two serological types can be differentiated: strain No. 29, being the prototype strain of the first type, and strain No. 61, the prototype strain of the second type. Strains belonging to already known mycobacterial species were also isolated from monkeys. Strain No. 64 proved to be a representative of *M. intracellulare*, whereas strain No. 52 may be classified into species *M. aquae*.

Our type strains are deposited in the American Type Culture Collection under the numbers ATCC 25275 = 29, ATCC 25274 = 27a and ATCC 25276 = 61.

TABLE 2

Number of precipitation lines

Immune sera of strains	Antigens of strains No.																
	29	59	58	53	25	14	68	63	64b	61	32	27s	H37Rv	*avium* ATCC	*kans.* ATCC	*mar.* ATCC	*intracell.* ATCC
M. simiae 29	16	14	10	13	10	10	10	10	9	7	6	5	5	8	2	2	
M. 64b	8	6	5	6	5	5	4	4	9	4	4	3	5	8	4	4	9
M. simiae 61	3	3	4	3	1	1	2	1	5	15	12	8	3	4	7	4	4
H37 Rv	5	5	4	7	4	4	4	3	5	4	5	3	12	5	6	5	5
M. avium ATCC 15 769	8	8	3	7	5	5	4	5	8	4	2	2	5	10	4	4	8
M. kansasii ATCC 12 478	4	4	3	5	2	2	2	2	4	7	5	3	4	4	10	6	3
M. marinum ATCC 927	4	4	3	6	3	3	2	2	5	4	3	3	4	4	6	10	

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MYCOBACTERIUM SIMIAE

(PRELIMINARY REPORT)

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In addition to our previous study about photochromogenic Mycobacteria we have investigated also *M. simiae*. Karasseva and coworkers (1965) stated that these strains were sensitive to light-induced pigmentation.

We have received from Prof. Weiszfeiler 14 strains, named *M. simiae*. The methods used for classification are the same as for *M. kansasii* and *M. marinum* (Schröder and Magnusson 1968). The properties of the two species provided points for comparing them to those of *M. simiae*.

Results

1. *Cultural properties.* All strains of *M. simiae* grow more or less in dysgonic colonies. Growth is observed even at 41 °C with the exception of strain No. 27a (Table 1). In guinea-pigs after s.c. infection, reactions were observed only at the regional lymph nodes, but not in visceral organs. *M. simiae* is inhibited by Oleate, in Tween-Agar the reaction manifests itself in form of a zone of turbidity. Microscopical examinations in all cases reveal the cross-band phenomenon.

2. *Biochemical properties.* The results are summarized in Tables 1 and 2. Not all strains show photochromogenicity, in some cases the reaction is doubtful and some of our cultures seem to be mixtures with regard to the light-induced pigmentation.

We found 4 groups of amidase pattern:

(a) splitting of urea (amide 3)
(b) splitting of nicotinamide and pyrazinamide (amides 5 and 6)
(c) one strain splits urea + nicotinamide + pyrazinamide (3 + 5 + 6)
(d) 2 strains do not fermentate any of the amides.

Tween-hydrolysis is to be observed in groups (b) and (c), in group (a) only one strain is positive. The same strain has a great phosphatase activity — in contrast to the rest of this group — but in general this enzyme is present

TABLE 1

Strain No.		Character growth	Growth at °C						Pathogenicity for guinea-pig Index	Growth on oleate	Tween opacity	Iron uptake	Cross-band	Thiocalcide
Borstel	Budapest		22	31	37	39	41	45						
8987	14	dysgon.	+	+	+	+	+	0	0	+	+	0	+	R
8988	20	dysgon.	+	+	+	+	+	0	2.5	+	+	0	+	R
8989	24	dysgon.	+	+	+	+	+	0	0	+	+	0	+	R
8991	55	dysgon.	+	+	+	+	+	0	2.5	+	+	0	+	R
8992	58	dysgon.	+	+	+	+	+	0	3.5	+	+	0	+	R
3595	29	dysgon.	+	+	+	+	+	0	0	+	+	0	+	R
3596	52	dysgon.	+	+	+	+	+	0	3.5	+	+	0	+	R
3597	59	dysgon.	+	+	+	+	+	0	2.0	+	+	0	+	R
8990	27a	dysgon.	+	+	+	0	0	0	0	+	+	0	+	R
8994	68	dysgon.	+	+	+	+	+	0	0	+	+	0	+	R
3599	N3	dysgon.		+	+	+	+	0	0	+	+	0	+	R
8993	64	dysgon.	+	+	+	+	+	0	0	+	+	0	+	R
3598	61	dysgon.	+	+	+	+	+	0	0	+	+	0	+	R
8986	5	dysgon.	+	+	+	+	0	0	0	+	+	0	+	R
M. kansasii	—	eugon.	+	+	+	+	?	0	(+)	0	+	0	+	S
M. marinum	—	eugon.	++	+	+	0	0	0	0	0	+	0	+	R

in all other strains in different degrees of activity. The activity of the α- and β-esterase is different and none of the strains is able to react with putrescine.

3. *Behaviour against tuberculostatica.* The results are not identical; we observed no characteristic pattern (Table 3).

Attention must be drawn to a very important fact, namely to the absence of THC-sensitivity.

TABLE 2

Strain No.		Urease	Nicotine-amidase	Pyrazin-amidase	Tween hydro-lysis	Nitra-tase	Phos-pha-tase	Esterase		Putres-cine degra-dation
Borstel	Buda-pest							α	β	
8987	14	+	—	—	0	0	0	+	+	0
8988	20	+	—	—	0	0	0	+	+	0
8989	25	+	—	—	0	0	0	(+)	(+)	0
8991	55	+	—	—	0	0	0	+	+	0
8992	58	+	—	—	0	0	0	+	+	0
3595	29	+	—	—	0	0	0	+	+	0
3596	52	+	—	—	+	0	+	+	+	0
3597	59	+	—	—	0	0	0	0	0	0
8990	27	—	+	+	+	0	+	(+)	+	0
8994	68	—	+	+	+	0	+	0	+	0
3599	N3	—	+	+	+	0	(+)	(+)	+	0
8993	64	—	—	—	0	0	+	0	+	0
3598	61	—	—	—	0	0	+	+	+	0
8986	5	+	+	+	+	0	+	+	+	0
M. kansasii		—	+	+	—	+	+	+	0	0
M. marinum		—	+	+	+	+	0	+	0	0

Discussion

In some cases the received cultures seem to be mixtures. Therefore, for the time being, we are unable to give a clear description of the new species *M. simiae*, all the more, since the 14 strains sent to us are not homogenic.

This is why we think it impossible to classify them as members of *one* species.

Moreover, it is necessary to determine exactly which of the strains are *M. simiae*.

Furthermore the character of pigmentation is also doubtful in some strains. On the other hand, it is sure that *M. simiae* is different from *M.*

TABLE 3

Strain No.		INH		SM		PAS		TSC		CS	ETH	VM	KM	THC	PZA	CM	EMB	RAMP
Borstel	Budapest	0.2	1	5	10	1	10	1	10	20	20	20	10	50	50	16	0.5	4
8986	5	R	R	S	S	S	S	R	S	S	S	S	S	R	R	S	R	S
8987	14	R	R	R	R	R	S	R	R	S	S	R	R	R	R	R	R	R
8988	20	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
8991	55	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
8992	58	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
3595	29	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
3596	52	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
3597	59	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
8990	27	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
8994	68	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R
3599	3	R	R	O	O	R	R	R	R	S	S	R	R	R	R	R	R	S
8993	64	R	R	R	R	R	S	R	R	S	S	R	R	R	R	R	R	R
3598	61	R	R	S	S	S	S	R	R	S	S	S	S	R	R	S	S	R
8989	25	R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	R	R
M. kansasii		R	(R)	R	R+	R	R+	R+	S	S	S	R	R	S	R	R+	R	R+
M. marinum		R	R	R	R	R	R	R	R	S	S	R	R	R	R	R	2S 2R	2S 2R

(R) = 50% of the strains are resistant.
R+ = the majority of the strains are resistant.
Kons. in mcg.

kansasii and also from *M. marinum*. This opinion will be supported by the results of agglutination (Dr. Anz). *M. simiae* shows no reaction either with anti-kansasii serum or with anti-marinum serum.

The comparison of allergic (Magnusson), serological (Hauduroy and Sakellarides) and biochemical methods (Schröder) revealed that a few strains are identical, and on the ground of these results the examined strains could be regarded as type-strains of *M. simiae*. Consequently, the taxonomic position of the remainder must still be clarified.

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SPECIFICITY OF MYCOBACTERIAL SENSITINS. STUDY ON *MYCOBACTERIUM SIMIAE*

(Progress Report, April 1969*)

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Summary

Eleven cultures of mycobacteria isolated from monkeys, including eight labelled *Mycobacterium simiae* when received, have been studied by an immunological method based on the specificity of the delayed type skin reaction on guinea-pigs.

By this method four strains labelled *M. simiae* could be grouped together with another strain isolated from monkeys. The four other isolates labelled *M. simiae*, two of which could not be distinguished from each other, were clearly distinct from the first-mentioned group. Designation of a type culture of *M. simiae* is therefore required. The two remaining cultures could be identified as *Mycobacterium abscessus* and *Mycobacterium phlei*, respectively.

Further studies are in progress.

Introduction

Fifty strains of mycobacteria were isolated from monkeys and described by Valentina Karasseva, J. G. Weiszfeiler and E. Krasznay in 1965 (Karasseva et al. 1965). Nine of these strains were classified as a new species *Mycobacterium simiae*. No type culture was designated. Strains referred to this species were photochromogenic, showed dysgonic growth on Löwenstein–Jensen medium, and multiplied intensively in mice (Karasseva et al. 1965). The other strains varied in pigmentation, growth rate and enzymatic reactions.

The antigenic structure of one of the strains of *M. simiae* — the present # 839 — was investigated by immune diffusion by J. G. Weiszfeiler, I. Jókay, Erika Karczag, K. Almássy and P. Somos (1968). The strain showed the closest relationship to *M. kansasii* and *M. marinum* (syn. *M. balnei*), but

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cannot be regarded as a typical representative of *M. simiae* before being compared with other strains of *M. simiae* (Weiszfeiler et al. 1968).

An immunological method based on the specificity of the delayed type skin reactions in guinea-pigs has been useful for the classification of mycobacteria, and particularly for the identification of species within that genus (Magnusson 1961, 1965, 1967). The purpose of the present work was to classify, by means of that immunological method, some of the strains mentioned above, including eight strains of *M. simiae*.

Method

Organisms. Eleven strains received from Professor J. G. Weiszfeiler, Hungarian Academy of Sciences, Pihenő út 1, Budapest, Hungary, were included in the study, together with some laboratory reference strains of *Mycobacterium* and *Nocardia* (see Table 1).

TABLE 1

Strains studied

Laboratory No.	Name when received, source and culture collection numbers*
10	*Mycobacterium avium* Maren Cecilie. Originally isolated from a female Danish patient named Maren Cecilie, see Case 6 in Dragsted, Inger,: Lancet (1949), *257*, 103—105. Received from Dr. H. C Engbaek, Tuberculosis Department, Statens Seruminstitut, Copenhagen, Denmark. SSC 314.
14	*Mycobacterium* sp. A 3721. Originally isolated from sputum of an African patient by Dr. H. C. Engbaek. Received from Dr. Engbaek. SSC 263.
20	E 41167. Originally isolated from sputum of a Danish patient named P. J. Jensen by Dr. H. C. Engbaek. Received from Dr. Engbaek. Laboratory reference culture of *Mycobacterium fortuitum*. SSC 214.
23	V 22147. Originally isolated from gastric lavage of a Danish patient named Leo Jacobsen by Dr. H. C. Engbaek, see H. C. Engbaek, M. Magnusson and J. A. Nielsen: Acta tuberc. scand. (1959), *37*, 227—244. Received from Dr. Engbaek. Laboratory reference culture of *Mycobacterium intracellulare*. SSC 209.
29	R 4800. Originally isolated from a patient named Bent Folkersen by Dr. H. C. Engbaek. Received from Dr. Engbaek. Laboratory reference culture of *Mycobacterium abscessus*. SSC 210.
30	*Mycobacterium kansasii* Goss. Originally isolated from a British female patient named Goss, see Young, R. D.: Lancet (1955), *269*, 750—752. Received from Dr. H. C. Engbaek. SSC 225.

* ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; L, Centre International de distribution de souches et d'informations sur les types microbiens, Lausanne, Switzerland; SSC, Collection of Dr. H. C. Engbaek, Statens Seruminstitut, Copenhagen, Denmark.

Table 1 (*cont.*)

Laboratory No.	Name when received, source and culture collection numbers.*
40	*Mycobacterium* sp. R 4991. Originally isolated from a patient named M. N. Winther by Dr. H. C. Engbaek, see Case 7 in H. C. Engbaek, and M. Magnusson: Acta tuberc. scand. (1961), *40*, 1—34. Received from Dr. H. C. Engbaek. SSC 211.
84	598/51—52. Originally isolated from placenta of cow, see Case 2 in H. Fey, P. Holm and E. Teuscher: Schweiz. Arch. Tierheilk. (1954), *96*, 642—648. Received from Dr. P. Holm, Mycological Department, Statens Seruminstitut, Copenhagen. Laboratory reference culture of *Nocardia farcinica*.
95	*Mycobacterium scrofulaceum* 526. Originally isolated from a child with lymphadenitis, see F. H. Prissick and A. M. Masson: Canad. med. Ass. J. (1956), *75*, 798—803. Received from Dr. A. M. Masson, McGill University, Montreal, Canada. L 2235.
96	*Mycobacterium scrofulaceum* 1637. Originally isolated from a child with lymphadenitis, see F. H. Prissick and A. M. Masson: Canad. med. Ass. J. (1956), *75*, 798—803. Received from Dr. Masson. L 2239.
102	T 9340. Originally isolated from a 3-year-old Danish girl with lymphadenitis by Dr. H. C. Engbaek, see Case 20 in Engbaek, H. C.: Acta tuberc. pneumol. scand. (1964), *44*, 108—137. Received from Dr. Engbaek. SSC 321. ATCC 19076.
115	*Mycobacterium phlei* 5. Received from Dr. J. H. Hanks, Leonard Wood Memorial Laboratory, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachussetts, U.S.A. via Dr. Ruth E. Gordon, Institute of Microbiology, Rutgers University, New Brunswick, New Jersey, U.S.A.
127	1551. Originally isolated from sputum of a patient living at the Sentanie district, Dutch New Guinea. Received from Dr. G. Wijsmuller, Tuberculosis Research Section, Communicable Disease Center, Bethesda, Maryland, U.S.A.
140	3155. Originally isolated from sputum of a patient living at the Sentanie district, Dutch New Guinea. Received from Dr. G. Wijsmuller.
198	E 6707. Originally isolated from sputum of a Danish patient by Dr. H. C. Engbaek. Received from Dr. Engbaek.
545	P 36, Mycobacterial Culture Bank, Utah, U.S.A. Originally isolated at the Southwest Florida Tuberculosis Hospital, Tampa, Florida, U.S.A. from sputum and resected lung tissue of a patient named M. Robinson with pulmonary disease. Received from Dr. F. Dunbar, Tampa, Florida, U.S.A. via Dr. E. Runyon, Veterans Administration Hospital, Utah, U.S.A. and Dr. H. C. Engbaek.
578	280. Originally isolated from cattle in the U.S.A. Received from Professor D. Berman, Department of Veterinary Science, The University of Wisconsin, Madison, Wisconsin, U.S.A.
579	296. Originally isolated from cattle in the U.S.A. Received from Professor D. Berman.
606	*Nocardia caviae* 617. Originally isolated from a cow with mastitis. Received from Dr. E. N. Azarowicz, University of California, U.S.A., his No. 33, via Dr. Ruth E. Gordon. Laboratory reference strain of *Nocardia otitidis-caviarum*.

Table 1 (*cont.*)

Laboratory No.	Name when received, source and culture collection numbers*
631	TB 24. Received from Dr. E. Nassau, Department of Pathology, Harefield Hospital, Harefield, Middlesex, England, via Dr. Engbaek. Laboratory reference strain of *Mycobacterium xenopi*. SSC 991.
691	*Mycobacterium borstelense* SN 282. Originally isolated from gastric juice, see R. Bönicke: Zentr. Bakteriol. I. Abt. Orig. (1965), *196*, 535—538; and R. Bönicke and A. Ewoldt: Beitr. Klin. Tuberk. (1965), *130*, 149—154 and 210—222. Received from Dr. Gertrud Meissner, Forschungsinstitut Borstel, GFR, via Dr. R. Bönicke, Borstel, GFR. ATCC 19535.
762	*Mycobacterium gastri* W–417. Originally isolated from gastric lavage of a patient, see L. G. Wayne: Amer. Rev. resp. Dis. (1966), *93*, 919—928. Received from Dr. Lawrence G. Wayne, Veterans Administration Hospital, San Fernando, California, U.S.A., via Dr. Engbaek. Type strain. International Working Group on Mycobacterial Taxonomy (IWGMT) working culture. ATCC 15980—15754.
763	*Mycobacterium terrae* ATCC 15755. Received from Dr. S. Froman, Microbiological Research Laboratory, Olive View Hospital, Olive View, California, U.S.A., his No. F 628, via Dr. L. G. Wayne, his No. W–45, and Dr. Engbaek. Type strain. IWGMT working culture. ATCC 15981—15755.
815	W 338. Received from Dr. L. G. Wayne.
836	N 15. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler, Hungarian Academy of Sciences, Microbiological Research Group, Budapest, Hungary.
837	*Mycobacterium simiae* N 29. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.
838	N 29. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.
839	*Mycobacterium simiae* N 61. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282, and J. G. Weiszfeiler, I. Jókay, E. Karczag, K. Almássy and P. Somos: Acta microbiol. Acad. Sci. hung. (1968), *15*, 69—76. Received from Professor J. G. Weiszfeiler.
840	N 64. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.
861	*Mycobacterium kansasii* ATCC 12478. Originally isolated from a fatal case by Dr. Ann Pollak, her No. G 133 Bostrom. Received from Dr. Engbaek. Type strain, see E. F. Lessel: Intern. Bull. Bacteriol. Nomen. Taxon. (1962), *12*, 71—88. IWGMT working culture.
894	W–1344. Received from Dr. L. G. Wayne. ATCC 23284.
897	W–1406. Received from Dr. L. G. Wayne. ATCC 23430.
945	*Mycobacterium simiae* 5. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.

Table 1 (*cont.*)

Laboratory No.	Name when received, source and culture collection numbers*
946	*Mycobacterium simiae* 14. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.
947	*Mycobacterium simiae* 20. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.
948	*Mycobacterium simiae* 25. Originally isolated from monkey, see V. Karasseva, J. G. Weiszfeiler and E. Krasznay: Acta microbiol. Acad. Sci. hung. (1965), *12*, 275—282. Received from Professor J. G. Weiszfeiler.
949	*Mycobacterium simiae* 60. Originally isolated from monkey. Received from Professor J. G. Weiszfeiler.
950	*Mycobacterium simiae* 64ax. Originally isolated from monkey. Received from Professor J. G. Weiszfeiler.

Conditions for growth and harvest were the same as described previously (Magnusson and Mariat, 1968). The cultures were incubated for 4—7 weeks.

Immunological method. The procedure was the same as that described previously (Magnusson and Mariat, 1968), except for sensitization of the guinea-pigs with strains # 945, # 947, # 948, # 949 and # 950, where the immunogen consisted of heat-sterilized culture filtrate emulsified in incomplete Freund adjuvant (Freund 1956). The data presented here were drawn from a series of twelve studies performed on a total of 126 guinea-pigs.

Calculation of specificity differences (spd) was carried out as described previously (Magnusson and Mariat, 1968).

Results

The results of calculation of the specificity differences (spd values) of sensitins prepared from mycobacteria isolated from monkeys are shown in Table 2.

The spd-values between the sensitin prepared from *M. simiae* # 837 and the sensitins prepared from four other strains (# 838, # 946, # 947 and # 948) are < 5 mm. The three last-mentioned strains were labelled *M. simiae* when received. The spd-values between the sensitin of *M. simiae* # 837 and the sensitins of four other strains labelled *M. simiae* (# 839, # 945, # 949 and # 950) are > 4 mm. The spd-values between # 837 and the two other strains tested (# 836 and # 840) are also > 4 mm.

TABLE 2

Specificity differences of sensitins prepared from mycobacteria isolated from monkeys

The table shows the differences (in mm) between homologous and heterologous reactions to intradermal tests in guinea-pigs sensitized with the strains used for preparation or with culture filtrates of the strains

Species / Sensitin	cf 838 *Mycob.* sp.	cf 837 *M. simiae*	cf 946 *M. simiae*	cf 947 *M. simiae*	cf 948 *M. simiae*	cf 839 *M. simiae*	cf 950 *M. simiae*	cf 945 *M. simiae*	cf 949 *M. simiae*	cf 836 *Mycob.* sp.	cf 840 *Mycob.* sp.
Mycob. sp. cf 838	—	2				10				13	13
M. simiae cf 837	2	—	2	0	1	14	9	20	10	17	16
M. simiae cf 946		2	—	4	4		11				
M. simiae cf 947		0	4	—	4		9				
M. simiae cf 948		1	4	4	—		7				
M. simiae cf 839	10	14				—	—2	23	14	17	17
M. simiae cf 950		9	11	9	7	—2	—	18	9		
M. simiae cf 945		20				23	18	—	11		
M. simiae cf 949		10				14	9	11	—		
Mycob. sp. cf 836	13	17				17				—	18
Mycob. sp. cf 840	13	16				17				18	—

The spd-value between sensitins prepared from *M. simiae* # 839 and *M. simiae* # 950 is —2 mm.

The spd-values between sensitins prepared from mycobacteria isolated from monkeys and sensitins prepared from reference strains of species of mycobacteria are shown in Table 3.

The spd-value between the sensitin of # 836 and *M. abscessus* # 29 is 1 mm. The spd-values between the sensitin of # 836 and the other sensitins studied are > 4 mm.

The spd-values between the sensitins of # 837 and # 838 and the other sensitins studied are > 4 mm.

TABLE 3

Specificity differences of sensitins prepared from mycobacteria isolated from monkeys and from reference strains of mycobacteria. The table shows the differences (in mm) between homologous and heterologous reactions to intradermal tests in guinea-pigs sensitized with the strains used for preparation of the sensitins

Species / Sensitin*	RS 29 *M. abscessus*	RS 10 *M. avium*	cf 691 *M. bovis-tense*	RS 20 *M. fortuitum*	cf 762 *M. gastri*	RS 23 *M. intracellulare*	RS 30 *M. kansasii*	cf 861 *M. kansasii*	RS 95 *M. marianum*	RS 96 *M. marianum*	RS 115 *M. phlei*	cf 763 *M. terrae*	RS 631 *M. xenopi*	RS 14 *Mycob.* sp.	RS 40 *Mycob.* sp.	cf 102 *Mycob.* sp.	RS 127 *Mycob.* sp.	RS 140 *Mycob.* sp.	RS 198 *Mycob.* sp.	cf 545 *Mycob.* sp.	RS 578 *Mycob.* sp.	RS 579 *Mycob.* sp.	cf 815 *Mycob.* sp.	cf 894 *Mycob.* sp.	cf 897 *Mycob.* sp.	RS 84 *Nocardia farcinica*	RS 606 *N. otitidis-cariarum*
Mycob. sp. cf 836	1		5	8								10		13				14									
M. simiae cf 837							5								13					16	10						
Mycob. sp. cf 838		7				7	6						12		16				11	16	13						
M. simiae cf 839					7		12	10	11	9		13				9	3				12	9	13	12	13		
Mycob. sp. cf 840		16				14			14	13	1															14	15

* cf: culture filtrate.
RS: purified sensitin.

The spd-value between the sensitin of # 839 and that of *Mycobacterium* sp. # 127 is 3 mm. The other spd-values obtained with sensitin of # 839 are >4 mm.

The spd-value between the sensitin of # 840 and *M. phlei* # 115 is 1 mm. The other spd-values obtained with sensitin of # 840 are >4 mm.

Classification of the strains on the basis of sensitin specificity. On the basis of the spd-values of the sensitins, and with the criteria for their utilization in classification used earlier (Magnusson and Mariat 1968), the strains can be classified as follows: Strain 836 as *Mycobacterium abscessus*, strain 840 as *Mycobacterium phlei*, strains 837, 838, 946, 947 and 948 into one species, presumably *M. simiae*, and strains 839 and 950, presumably together with the isolate designated # 127, into another separate species. Strains 945 and 949 should be classified into two other separate species.

Discussion

The present results can be supported in some cases by investigations performed in other laboratories and by other methods.

Thus, the identification of # 836 as *Mycobacterium abscessus* is in agreement with observations made by Dr. Käppler, Berlin, using biochemical and physiological methods (Prof. J. G. Weiszfeiler: Personal communication, 1968).

As far as the present identification of # 840 as *Mycobacterium phlei* is concerned, it has been confirmed by conventional methods that the culture received was not pure, and that the contamination was due to *M. phlei* (J. G. Weiszfeiler: Personal communication, 1968).

In the present study, four of the strains labelled *Mycobacterium simiae* (# 837, # 946, # 947 and # 948) cannot be distinguished from each other or from *Mycobacterium* sp. # 838. However, these strains are clearly distinct from four other strains labelled *M. simiae* when received (# 839, # 945, # 949 and # 950).

Thus, according to the present results, all the strains classified as *Mycobacterium simiae* in the original report (Karasseva et al. 1965) are not similar. Designation of a type culture of the species *Mycobacterium simiae* is therefore required.

According to the present findings, the four strains (# 839, # 945, # 949 and # 950) which were previously classified as *M. simiae* (Karasseva et al. 1965) should be referred to another species, if one of the four strains (# 837, # 946, # 947 or # 948) is to be designated as type culture, and *vice versa*.

Continuation of investigations

Further studies of the relationship between strains of *M. simiae* and other isolates of mycobacteria from monkeys and strains of a number of other species of mycobacteria are in progress. These may contribute to our general knowledge of the relationship between species of mycobacteria.

Preparation of sensitin from *Mycobacterium simiae* # 839 by the same method as that used for the purified protein derivative of tuberculin is in progress. Comparative tests in man and animals with such a preparation and similar preparations from other species of mycobacteria may provide information concerning the distribution and pathogenic significance of similar isolates.

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BIOLOGICAL PROPERTIES OF SIMIAN MYCOBACTERIUM STRAINS

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The cultural, pathogenic and immunological properties of three Mycobacterium strains, namely, Nos 14, 25 and 52 were investigated. The strains, which had been isolated by Karasseva and Weiszfeiler from organs and lymph nodes of monkeys, were received in a lyophilized state.

Cultural properties. On Löwenstein medium, colonies of the strains appeared between the 20th and 28th days after inoculation. In subcultures incubated at 37 °C the colonies of strains No. 25 and 52 appeared as early as between the 10th and 12th days, those of strain 14 appeared later, on the 18th day. In contrast with the two other strains, strain No. 14 showed dysgonic growth. On broth agar at 37 °C or 24 °C, strains No. 14 and 25 displayed poor growth, even

TABLE 1

Strain No.	Growth at different temperatures after days				
	24 °C		37 °C		43 °C
	Löwenstein—Jensen	Agar	Löwenstein—Jensen	Agar	Löwenstein—Jensen
14	45	45	18	20	15
25	60	60	12	20	17
52	14	—	10	—	17

45 to 60 days after inoculation. Strain No. 52 failed to grow on simple agar medium (Table 1). In the liquid, semisynthetic Shkolnikova medium strains No. 14 and 25 showed a granular growth at the bottom of the tube, whereas the growth of strain No. 52 was homogeneous, and yellow in colour. At 43 °C the growth of all the three strains became apparent between the 15th and 17th days.

In the dark, strains No. 14 and 25 produced no pigment, but 24 hours after illumination with natural light or electric lamp they produced a yellow pigment. Thus, these strains should be regarded as photochromogenic.

Strain No. 52, producing pigment even in the dark, may be considered as scotochromogenic.

All the strains gave an intensive catalase reaction, and the catalase proved to be heat-resistant (Table 2). Strain No. 14 is peroxidase-positive, the two others are peroxidase-negative. All three strains are formamidase-negative. The niacine reaction is positive in strains No. 14 and 25, whereas strain No. 52 is niacine-negative.

TABLE 2

Strain No.	Niacine	Catalase 68 °C	Peroxydase	Formamidase
14	+	+	+	−
25	+	+	±	±
52	−	+	−	−

All three strains resist 5—10 μg/ml of streptomycin, INH, PAS, ryfamycin and thiosemicarbazone, 30—50 μg/ml ethionamid or viomycin; all are sensitive to 30 μg/ml cycloserine (Table 3). Strain No. 52 is more sensitive to kanamycin than the other strains.

TABLE

Growth at concentrations of chemotherapeutic

Strain No.	Streptomycin				INH				PAS			Cycloserine	
	2	5	10	50	0.2	1	5	25	0.5	1	5	30	50
14	+	+	+	−	+	+	+	±	+	+	±	−	−
25	+	+	+	−	+	+	±	−	+	+	+	−	−
52	+	+	±	−	+	+	+	+	+	+	+	−	−

Pathogenicity, immunogenicity and antigenic structure. The pathogenicity of the strains was tested on 36 guinea-pigs, 21 rabbits and 45 albino mice. Ten guinea-pigs (6 subcutaneously and 4 intratesticularly), 6 rabbits and 10 mice were inoculated with each strain. To check a possible increase in pathogenicity, the strains re-isolated from experimental animals were also tested, each on 4 guinea-pigs by intratesticular inoculation, 1 rabbit and 5 mice. Each animal was inoculated with 1 mg bacterium. Guinea-pigs were sacrificed 1, 2 and 3 months after inoculation, the mice after 20 days and 3 months and the rabbits after 1, 2 and 3 months.

The subcutaneously inoculated guinea-pigs, independent of the strain, developed local infiltration and abscess, which subsequently became

ulcerous; intratesticular inoculation caused focal necrosis. No macroscopic lesions developed in the internal organs. In the lungs of the inoculated rabbits peculiar foci were seen, which became most pronounced one month after inoculation. Then, these lesions stopped growing, suggesting that the pathogenicity of the strains was low. The strains are definitely pathogenic for mice; 20 days after inoculation the spleens of the mice were swollen, in the lungs light focal lesions were perceivable, which tended to grow in size and number by the end of the third month. In several animals focal lesions developed in the spleen as well as in the liver. The strains could be re-isolated from the organs of each inoculated animal. From the animals infected by strain No. 14 or No. 25 two types of colonies, a white and a yellow, were obtained, suggesting that the population of these strains was heterogeneous. The re-isolated strains caused in guinea-pigs, rabbits and mice lesions similar to those caused by the original strains, indicating that the properties of the strains are stable.

To investigate the immunizing properties of strain No. 14, 28 guinea-pigs were divided into four groups, each consisting of 6 to 8 animals. Group 1 was immunized with the BCG strain, group 2 with a strain of *M. kansasii*,

3

substances in mg/ml

Ethionamid			Kana-mycin		Rifamycin			Thiosemicarbazone			Viomycin	
20	30	50	30	50	1	5	10	0.5	1	5	30	50
+	+	−	+	±	+	±	−	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+
+	±	−	−	−	+	+	+	+	+	+	+	±

and group 3 with *M. simiae* No. 14. The guinea-pigs in group 4 were not immunized, they served as controls. The animals were immunized by subcutaneous inoculation, with a suspension of 1 mg bacterium each. One month after inoculation each animal was challenged with 0.0001 mg of the virulent strain H37Rv, subcutaneously. In addition, tuberculin-sensitivity was tested on several guinea-pigs, which had been inoculated with strain No. 14, but not challenged. The animals gave a more intensive reaction to the avian tuberculin than to the human one; their sensitized state did not change by the end of the sixth month, when the animals were sacrificed.

All the 28 guinea-pigs were sacrificed three months after challenge. The severity of the reaction was expressed in index numbers as suggested

by Weiszfeiler (Table 4). The index for the non-immunized animals, 21.,7 closely approximated the maximum value of the index (22.0). The lymph nodes of the animals were considerably swollen, with necrotic lesions; in the organs a large number of focal lesions was present. In the guinea-pigs immunized with the strain *M. simiae* No. 14 the local ulcers and infiltrations were small in size, the lymph nodes were moderately swollen and a small

TABLE 4

Immunized by strain	No. of animals	Tuberculous index
BCG	6	7.0
M. kansasii	6	13.5
M. simiae No. 14	8	9.3
— (Control)	8	21.7

number of focal lesions was seen in the organs. For this group the tuberculous index was 9.3, while for the BCG-immunized group the index was 7.0, i.e., the degree of tuberculous lesions in the last two groups was approximately identical. In the animals immunized with *M. kansasii* the tuberculous lesions were more severe (index 13.5). The results of these experiments suggest that in its immunizing power strain No. 14 is similar to the BCG strain.

The antigenic structure of the strains was investigated by the Ouchterlony technique. In these tests anti-simiae and anti-avium immune sera were used. Strains No. 14 and No. 25 developed three precipitation lines, while strain No. 52 developed two lines with the anti-simiae 29 serum and no line with the anti-avium serum.

Summary

Strains No. 14 and 25 of *Mycobacterium simiae* proved to be photochromogenic, strain No. 52 is scotochromogenic. Strains No. 14 and 25 are niacine-positive, No. 52 is niacine-negative. All the three are catalase-positive and formamidase-negative. They grow well at 43 °C. They are definitely resistant to streptomycin, PAS and isoniazide and sensitive to cycloserine. All multiply intensively in guinea-pigs, albino mice and rabbits and can be re-isolated from the organs of the infected animals. They are virulent for albino mice, causing focal lesions in the lungs, occasionally also in the livers and spleens of these animals. The strains are avirulent for the guinea-pig and the rabbit,

they produce in these animals local abscess when administered subcutaneously or intratesticularly and regressive focal lesions in rabbit when given intravenously. Strain No. 14 is more immunogenic than *M. kansasii*.

The strains under study differ in their properties from the known photochromogenic mycobacteria — from *M. kansasii* and *M. marinum* — represent a new species. The antigenic structure of the strains needs further thorough investigation. The fact, that atypical mycobacteria were isolated from monkeys captured in the jungle supplies some information on the origin of atypical mycobacteria and their reservoir in nature.

SOME QUESTIONS OF IDENTIFICATION OF ATYPICAL MYCOBACTERIA AND THE RESULTS OF THE STUDY ON SIMIAN MYCOBACTERIAL STRAINS*

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(Director: Professor V. Bonifas)

It is often difficult to determine the species of bacterium to which atypical mycobacteria belong, and the problem of classification encounters many difficulties in such cases.

In order to determine a given strain, we study the following properties in the Microbiological Institute of Lausanne:

1. Growth and colony-morphology of the strain in various culture media (Löwenstein's, Dubos', bouillon, agar), at different temperatures, i.e. +20, +37, +45 °C.
2. Pigmentation has long been regarded as an easily variable property. Today no great importance is attached to this property.
3. Biochemical properties: amidases, lipase, iron reaction.
4. Serological properties.
5. Pathogenic properties.

When an investigated strain shows the same properties as a type strain, we conclude to species identity; but our task is often made difficult by variability: after several inoculations the properties of the strain change, and the question arises whether the strain forming in this way is actually the original one, or is a mutant, or is perhaps a completely different strain which had been inherent in the original strain, but has become observable only later.

Consequently — although we consider the comparison of an unknown strain with the reference strain to be of fundamental importance — we must take into account variability, adaptation, possible mutation, which become manifest during inoculations. I should like to mention a few observations made in this respect:

(a) We have compared the properties of a large number of atypical strains with the properties of reference strains; the results have led to the conclusion that part of the properties are variable, and this is why we must distinguish between characteristic and subordinate properties. For example, the cultivation and biochemical properties of strain No. 1217 agree with

* This work was initiated under the direction of late Prof. P. Hauduroy. (Red.)

TABLE 1

Strain No.		Photo-chromo-genicity	Catalase	Peroxy-dase	Acetami-dase	Urease	Isonico-tinami-dase	Nicotin-amidase	Pyrazin-amidase	Salicyl-amidase	Succin-amidase	Leucinoamidopeptidase			Lipase	Iron-ammon citrate	Agglu-tination with Schäf-fer's sera
Lau-sanne	Buda-pest											2^h	4^h	7^h			
674	27	+	+	−	−	−	−	−	−	−	−	+	++	+++	+	−	0
675	5	+	+	−	+	−	−	−	−	−	−	0	0	+	+	−	0
676	20	+	+	−	−	−	−	−	−	−	−	0	0	+	−	−	0
677	25	+	+	−	−	+	−	+	+	−	−	0	+	+	−	−	0
678	68	+	+	−	−	−	−	+	+	−	−	0	+	+	+	−	0
679	59	+	+	−	−	+	−	−	−	−	−	0	0	+	−	−	0
173	14	+	+	−	−	+	−	−	+	−	+	0	+	+	−	−	0
153	29	+	+	−	−	+	−	+	+	−	−	+	+++	++++	−	−	0
507	52	+			−	−	−	−	−	−	−				−	−	
20	61a	+			+	+	+	−	+	+	−				+	−	0

those of *M. kansasii*, but the strain does not agglutinate with specific anti-kansasii serum. Can this negative property be disregarded? Did this strain develop through the changes of a "capsular" antigen, or does it belong to another bacterium species even if its other properties resemble those of *M. kansasii*?

(b) We studied 6 strains with identical amidase spectra with respect to other properties. We found that their other enzyme activities were not so similar; 2 were lipase-positive, 4 lipase-negative. Nitrate reductase was only found in a few strains.

The next problem is the comparability of the results of various researchers. For instance, there are differences among the methods of preparation of the culture media, and other working methods; in certain cases researchers only study lyophilized strains, while others only study strains that had not been lyophilized.

Also the testing methods vary in accordance with the personal views of researchers. I think that the problem of the classification of atypical mycobacteria can only be solved by joint, coordinated studies; I recommend the following restrictions to this end:

(a) Standardization of test methods, which would make possible the comparison of results. For example, when amidase activity is studied, some employ incubation of 15 hours, others of 20, even 24 hours, although there is no doubt that the same strain yields different results with different incubation times;

(b) An obligatory minium of properties should be accepted for every species, and without these properties no strain should be classified as belonging to this species;

(c) The highest number of properties characteristic of a species should be accepted, and this number should not be raised until and unless our knowledge has been enriched.

TABLE 2

Agglutination titres with specific immune sera

Antisera	Agglutination titres of strains No.						
	61	29	5	20	25	68	59
29	—	1 : 160	—	1 : 320	1 : 640	—	—
61	—	—	—	—	—	—	—
64a	—	—	—	—	—	—	1 : 40

We have studied 8 mycobacterium strains cultivated from monkeys by Weiszfeiler and Karasseva. The results of the tests are summarized up in Table 1. Apart from strain No. 52, which was an R variant, and as such, showed autoagglutination, none of the other strains gave agglutination with any immune sera, of which we had 24 from Schäffer (anti-kansasii, anti-scrofulaceum, anti-avium, etc.). By the same method of agglutination 7 strains were examined with specific sera anti 29, anti 64 and anti 61, obtained from Weiszfeiler. The results are summarized in Table 2.

I believe that the strains we have studied do not belong to any strain known and identified so far, neither on the basis of their amidase spectrum, nor on other biochemical properties. The amidase spectrum of some strains might warrant the conclusion for *M. aquae*, *M. ureolyticum*, *M. non-ureolyticum*, *M. scrofulaceum*, but they did not agglutinate with the proper specific sera. We think that the different strains do not constitute a common, specific unity.

OCCURRENCE OF MYCOBACTERIA IN *CERCOPITHECUS AETHIOPS* MONKEYS

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Recently it has been established that various mycobacteria may cause infection in man and lower animals. The question has thus arisen whether free-living animals may spontaneously be infected by such micro-organisms. Since Karasseva, Weiszfeiler and Krasznay isolated numerous hitherto unknown mycobacteria from *Macacus rhesus* monkeys imported from India, it has been justified to search for spontaneous mycobacterial infection in monkeys received from other continents, like Africa. African *Cercopithecus aethiops* monkeys have become accessible to us recently, these animals have been used in the poliomyelitis vaccine production instead of the rhesus monkeys which often had proved to be infected with the monkey virus SV40. Thus, we were able to initiate investigations in the Institute for Poliomyelitis and Viral Encephalitis, Moscow, into the question of a possible spontaneous mycobacterial infection of newly-imported Cercopithecus monkeys.

Materials and methods

The kidneys of twenty-two Cercopithecus monkeys were used immediately after removal for tissue-culture purposes under sterile conditions. The monkeys were 1 $^1/_2$—2 years old and their body weight ranged from 1600 g to 3200 g. Pieces weighing 10 to 20 g each were excised from the lungs, the liver, the spleen, and were also taken from the submandibular, tracheobronchial, mesenteric and inguinal lymph nodes under sterile conditions. The organs were homogenized with scissors and subsequently in a mortar, treated with 4% sulphuric acid and inoculated onto Löwenstein—Jensen medium and a medium containing hemolyzed blood. The cultures were observed for three months, from the colonies, if appeared, smears were stained with Ziehl—Neelsen method and examined under the microscope. The cultures were examined for cultural characteristics, growth at different temperatures,

and for biochemical properties. Niacine reaction and the tests for catalase, peroxidase, amidases, arylsulphatase, lipase and nitrate-reductase were carried out as described by Meissner. Pathogenicity was tested by inoculating 1 mg of culture in Balb/c albino mice intravenously and in guinea-pigs by subcutaneous route. The animals were sacrificed 30—90 days after inoculation. The antigen structure of the strains was examined by the gel-precipitation test as modified by Weiszfeiler and collaborators.

Results

Microscopic growth was observed on media in 5 cases out of 22. In two cases, however, we failed to obtain subcultures of the bacteria. Thus, only 3 mycobacteria strains could be examined. Strain No. 574 was isolated from a mesenteric lymph node, strain No. 768 from a submandibular lymph node, and strain No. 234 from a tracheobronchial lymph node. All three

TABLE 1

Properties of the atypical Mycobacteria strains

	Strains		
	234	768	574
Colony morphology	S	S	R
Microscopical shape	short	short	slim
Pigmentation in dark	white	white	white
Photochromogenicity	—	—	—
Growth at 24 °C	±	±	—
Growth at 37 °C	+	+	+
Growth at 42 °C	+	+	—
Growth at 45 °C	—	—	—
Niacine	—	—	+
Catalase	+	+	+
Peroxidase	—	—	+
Nitrate reductase	—	—	+
Lipase	—	—	—
Arylsulphatase after 3 days	—	—	—
Arylsulphatase after 7 days	+	—	—
Arylsulphatase after 14 days	+	+	—
Tween opacity after 7 days	—	—	—
Tween opacity after 28 days	+	+	+
Urease	+	+	+
Nicotinamidase	±	±	+
Pyrazinamidase	+	+	+
CFA reduction	—	—	—
Virulence mice	—	—	—
Virulence guinea-pigs			—

strains grew slowly, two to three weeks at 37 °C; at room temperature or at 42 °C, strains No. 234 and 768 required 4 to 6 weeks, while strain No. 574 failed to grow. They produced no pigment. Strain No. 574 was R variant, whereas strains 234 and 768 S variants. The findings are shown in Table 1. Strain No. 574 is niacine-positive, his cultural and biochemical characteristics completely agree with those of *Mycobacterium tuberculosis hominis*. As regards their cultural properties, strains No. 234 and 768 resembled *M. avium*, but both were urease-positive.

The albino mice were sacrificed 40 to 90 days after inoculation, none of the mice died spontaneously. In the mice inoculated with strain No. 574 the spleen was hypertrophied, the lungs and the liver were without macroscopic alterations. Of the mice inoculated with strain No. 234 or No. 768 only those sacrificed 90 days after inoculation showed focal lesions in the lungs. With strain No. 574 four guinea-pigs were inoculated. They were sacrificed after 30, 45, 90 and 120 days, after inoculation. No focal lesions were observable in the spleens, but in the lungs of the animal examined 120 days after inoculation a few grey foci occurred. At the site of inoculation the animal developed an abscess, which subsequently ulcerated, but later healed up.

In the gel-precipitation test strain No. 574 gave nine lines with the anti-H37Rv serum, while it reacted weaker with other reference sera. Strain No. 234 showed the highest number of precipitation lines with serum anti-M 64b; previous studies had shown that strain M 64 is closely related to *M. intracellulare*. Strain No. 768 elicited the most intensive reaction with the anti-*M. simiae* 29 serum, indicating that it is different from strain No. 234. These investigations are as yet unfinished.

Discussion

The present studies with African Cercopithecus monkeys, though small in number, have shown that *Macacus rhesus* is not the only monkey species that may be spontaneously infected by mycobacteria. Of the Mycobacterium strains isolated from five animals only three could be subcultured, allowing for the supposition that for some strains to which the method was employed in the routine work are not quite favourable. Among the subcultured strains No. 574 is identical with the human tubercle bacterium as indicated by its cultural and biochemical properties. However, the strain proved te be of attenuated virulence for the guinea-pig. This is a further evidence, in addition to our previous one, for the existence of attenuated virulence of *Mycobacterium tuberculosis* in the monkeys. The remaining two

strains, No. 234 and No. 768 failed to produce pigment, in cultural characteristics they are related to *M. avium* and *M. intracellulare*, respectively. Their urease-positivity is striking.

Summary

Mycobacterium strains were isolated from 5 out of 22 African *Cercopithecus aethiops* monkeys. All are slow-growing and non-pigment-producing strains. One of them is a *M. tuberculosis* strain of attenuated virulence, two are related to the avian group, but are urease-positive. Two strains failed to grow in subcultures.

IDENTIFICATION OF ATYCIPAL MYCOBACTERIA FROM MONKEYS

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Seven mycobacterial strains isolated from monkeys, received from J. G. Weiszfeiler, have been investigated. The results obtained are given in the subsequent table.

	Strains						
	61 h	52 m	59 h	29 ny	3 ny	4	15
Bacterial morphology	thin	short	short and long	short	short	thin	thin
Growth at 25 °C 4 days	—	—	—	—	—	+	+
Growth at 25 °C 20 days	+	+	+	+	+	+	+
Growth at 37 °C 4 days	—	—	—	—	—	+	+
Growth at 37 °C 20 days	+	+	+	+	+	+	+
Growth at 45 °C 20 days	—	—	—	—	—	—	—
Cultural character	eugon	eugon	eugon	eugon	eugon	eugon	eugon
Type of colony	S	S	S	S	S	S	S
Pigmentation in dark	creme	orange	creme	creme	creme	creme	creme
Pigmentation after light	orange	orange	creme	creme	creme	creme	creme
Growth on toluidin-blue	+	+	+	+	+	+	+
Niacine	—	—	+	+	—	—	—
Catalase	+	+	+	+	+	+	+
Peroxidase	—	—	—	—	—	—	—
Nitrate reductase	—	—	—	—	—	—	—
Phosphatase	+	+	—	—	—	—	—
α-Esterase	+	—	+	—	—	—	—
β-Esterase	+	+	+	—	+	+	+
Lipase	+	+	—	±	+	—	—
Sulphatase 3 days	—	—	—	—	—	+	+
Sulphatase 7 days	±	±	±	±	+	+	+
Acetamidase	—	—	—	—	—	+	+
Urease	—	—	+	+	—	+	+
Nicotinamidase	±	—	—	+	+	+	+
Pyrazinamidase	+	—	—	+	+	+	+
Pathogenicity for guinea-pig	—	—	±	±	—	—	—

Further eight presumed *M. simiae* strains are under investigation. Results will be published later.

Conclusions

1. Strain No. 61 h corresponds to the new species of *M. simiae*.
2. Strain No. 52 m has been identified as *M. aquae*.
3. Strains No. 4 and 15 have been identified as *M. runyonii-abscessus*.
4. Strains No. 3 ny, 59 h and 29 ny belong to the Runyon III group of atypical mycobacteria. More exact identification was not possible.

RESULTS OF THE STUDY OF SOME M. SIMIAE STRAINS

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Three strains presumed to represent *M. simiae* have been investigated. The results are presented in the following two tables.

M. Simiae strain No.	Colony morphology	Microscopical shape	Pigmentation in dark	Photoinduction	Niacine	Catalase	Peroxidase	Thiophencarbonic acid hydrazide	Iron-ammonium-citrate	Nitrate	Nitrite	Urease	Nicotineamidase	Pyrazinamidase
29	smoth	very short, fine	—	+	++++	++++	+++	+	0	0	0	+	+	±
59	smooth	small	orange	—	++++	++++	+++	+	0	0	0	+	0	0
61	smooth	small	white-rose	0	0	++++	+++	0	0	0	0	0	0	0

M. simiae strain No.	Sensibility to antibacterial compounds (concentration in γ per ml)													
	Streptomycin		INH		PAS		Ethionamide 1314		Cycloserine		Viomycin		Kanamycin	
	S	R	S	R	S	R	S	R	S	R	S	R	S	R
29	10	4	10	5	—	10	—	40	20	10% 10	—	40	30	50% 10 30% 20
59	10	2 50% 4	5	0.2 50% 1	—	1 20% 10	20	50% 10	10	—	—	40	—	10 50% 20 50% 30 50% 40
61	10	2 10% 4	5	1	1	0.25 10% 0.5	10	—	20	10	20	10	10	—

SOIL AS THE RESERVOIR OF MYCOBACTERIA

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The authors have studied the kinds of mycobacteria which can be cultivated from the soil of arable land. The study of 205 prime cultures, 168 cultures free from contaminants, and 61 analysed strains, revealed that a relatively rich and varied mycobacterial flora is present in the soil. The authors call attention to possible medical, veterinary and pedological importance of this fact.

In recent years we have reported several times (Szelényi et al. 1965, 1967, 1968, 1969) on the results of our numerous and extensive studies and tests relating to the isolation of mycobacteria inhabiting the soil, their cultivation free from contaminants, and the determination of their typical morphological and physiological characteristics. Thus, it has been ascertained that there is a relatively rich and varied mycobacterial flora in the various soil types of arable land.

Method

We have carried out a large number of comparative tests using internationally known culture media. The culture medium free from ovalbumin, which has been described by Krebs as "Eigelbsubstrat modifiziert nach Gottsacker", proved to be the best; in the following, this medium will be marked G (Krebs 1964).

The great advantage of this culture medium is that besides organic and inorganic nutrients of p. a. purity it contains only yolk as the sole heterogeneous component. This provides better conditions for comparative physiological studies than if the culture medium contained some other organic substance of unknown composition in addition to ovalbumin which is otherwise not favourable for the mycobacteria. Using this culture medium, we developed the agar variety suitable for plate cultures (GA). This proved to be the most efficient for the isolation of prime cultures and for the cultivation

of mycobacteria in the Petri dish from soil samples (Szelényi et al. 1968, 1969). The solution of this task was substantially facilitated by keeping the Petri dishes in plastic bags within the thermostate. In this way, evaporation and drying up were practically excluded, even when cultivation lasted for 4 weeks. This method was suitable to overcome the difficulty arising as the result of the long period of cultivation of mycobacteria in Petri dishes at the beginning of our experiments. Concerning colony isolation, we used for decontamination NaOH in several varieties and with several modifications. Sodium hypochlorite was added as complement, neutralization was effected by H_2SO_4, the end concentration of the dilution of soil suspension was usually 1 : 500 and 1 : 1000, and cultivation was carried out at 30 and 37 °C in GA culture medium.

Unfortunately, the properties of the mycobacteria isolated from these soils could not be determined conclusively in the prime culture. Therefore, we had to elaborate a method by which we started from the 4-week culture of the second subculture with the help of the G culture medium; this yielded pure strains suitable for the determination of morphological and physiological characteristics. To ensure air supply for the cultures we placed a sterile silk thread mark 3 between the wall of the test tube and the rubber plug. So far we obtained 168 non-contaminated cultures from 205 prime cultures of soil. Detailed comparative analyses to determine colony-morphological and certain physiological characteristics were made with 61 strains obtained from them. We studied the growth of these strains at 22, 30, 37 and 45 °C, on the 5th, 8th, 14th, 21st and 28th day. One series of each culture was exposed to light for several hours on the 8th, 14th and 21st day in order to study photochromogenicity.

The drug sensitivity of the strains, their nutrient requirements, their nitrate and nitrite reduction, niacine, TTC testing were studied in the suspensions of 4-week cultures grown at 37 °C. We developed a simple and reliable method for studying nitrate and nitrite reductase. 0.5 ml $NaNO_3$ and $NaNO_2$ solution was added in agglutination tubes to 0.5 ml 1/15 M phosphate buffer solution of pH 7. The concentration of the former solutions was 0.2 mg/ml NO_3—N and 0.2 mg/ml NO_2—N. 0.5 mg of moist bacteria were added by means of a platinum wire to the sterile solution prepared in this way, and nitrite was determined with the Gries–Ilosvay method, ammonia with Russel's phenol-hypochlorite method after 24 hours at 37 °C.

Results

Our results are summed up in the table below. The data reveal that the strains studied in detail so far were placed in 11 dissimilar groups within 5 classes according to the temperature requirement, growth vigour and pigment formation. Bönicke's biochemical test are under way. A report on these investigations will be given at a later date.

Classification of strains*	Number of strains
I. Strains not developing at 22 and 45 °C and growing slowly	
1. Yellowish grey, scotochromogenic	3
2. Yellow, scotochromogenic	3
II. Strains not developing at 45 °C, growing slowly	
1. Yellowish grey, scotochromogenic	11
III. Strains not developing at 45 °C, growing fast	
1. Colourless, non-photochromogenic	7
2. Yellowish grey, scotochromogenic	17
3. Yellow, scotochromogenic	2
4. Light coral-coloured, scotochromogenic	4
IV. Strains not developing at 22 °C, growing fast	
1. Yellow, scotochromogenic	2
V. Strains developing at 22, 30, 37 and 45 °C, growing fast	
1. Colourless, non-photochromogenic	2
2. Yellowish grey, scotochromogenic	6
3. Yellow, scotochromogenic	4

* Additionally, we studied the drug sensitivity of the strains, their nutrient requirements, nitrate and nitrite reduction, niacine and TTC test, the Ziehl—Neelsen colouring quality and the macro- (colony) and micromorphological properties of the different strains. On this ground, further differentiation and classification of the materital included in the table are possible.

Discussion

We are aware of the fact that our classification based on our strain determinations is provisional, and cannot be regarded as final in any sense. Yet the results obtained so far would seem to indicate beyond doubt that the morphological and physiological characteristics of mycobacteria living in the soil differ substantially from one another. We believe that special attention must still be devoted to those species which in respect to colony-morphology bear a striking resemblance to the colony-morphological picture reminding of the *M. tbc. humanum* as seen in routine diagnostics. Two problems present themselves at once in connection with this conclusion:

1. Are there any human-pathogenic mycobacteria in the soil, or some which may become pathogenic to humans under special circumstances?

2. In what numbers, and with what frequency can a diagnostic error result from the circumstance on someone inhaling soil dust, subsequently discharging it with the secretion of the respiratory tract, and this secretion is then subjected to bacteriological analysis. Colonies will evidently develop, and may give the impression of Koch-positiveness in case of routine methods employed at present. The possibility described may not be a rare case in agricultural areas. It often happens that persons reporting in an ambulatorium for the analysis of their respiratory secretion have been previously in an environment where they inhaled soil dust containing mycobacteria.

In our opinion, the more than 10 000 analyses made so far have confirmed our original assumption, namely, that soil is rich in mycobacteria and that there is a variety of different species. We believe that further profound studies will lead to a more precise classification, and to a better understanding of the medical, veterinary and agrobiological importance of the mycobacteria inhabiting the soil.

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COMPARATIVE EXPERIMENTAL STUDIES ON ATTENUATED MYCOBACTERIUM STRAINS TO REVEAL SPECIFIC IMMUNIZATION AGAINST TUBERCULOSIS

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This is a follow up of our research work which has been conducted in recent years in collaboration with the Microbiological Research Group of the Hungarian Academy of Sciences.

At a conference held in Budapest in 1966* it was decided to continue the comparative study of the attenuated strains BCG and W 115 in coded experiments. The object of this work has been a thorough investigation of Weiszfeiler's strain No. 115 with respect to an occasional substitution of the BCG strain in practice if necessary.

The Scientific Council of the Hungarian Ministry of Health requested us to lyophilize the strains W 115, and BCG Paris 1012. The ampoules containing one of the strains were labelled with "K", while those containing the other with "O". The code was registered by the Scientific Committee of the Hungarian Ministry of Health.** The strains "O" and "K" as well as the lyophilized strain H37 Rv, produced for standard virulent infection, were cultured in our Institute and re-lyophilized for further use. The strains "O" and "K" were examined for the following properties: (1) capacity to sensitize guinea-pigs; (2) immunogenicity in the albino mouse and (3) residual virulence in the chick embryo.

Sensitizing capacity

Thirty guinea-pigs weighing 300 to 350 g each were inoculated with the "O" strain and other 30 with the "K" strain. For inoculum 0.01 mg of 14-day cultures were used. Tuberculin sensitivity was examined by inoculating the animals with 100 units of old-tuberculin on the 10th, 20th, 30th and

* Proceedings of the Microbiological Research Group of the Hungarian Academy of Sciences, Vol I, pp. 51—101 (1966).

** After receiving these results the ampoules were decoded: K = strain W 115, O = BCG Paris.

TABLE 1

Tuberculin-sensitivity of vaccinated guinea-pigs

Vaccinated by strain	No. of animals	No. of positive reactors with 100 TU at days after vaccination							
		10		20		30		60	
		No.	%	No.	%	No.	%	No.	%
"O"	27	13	48	26	96.2	27	100	27	100
"K"	26	10	38.4	18	69.2	25	96.1	26	100

60th day after infection. Table 1 shows the numbers and percentages of the tuberculin-positive animals. On the 10th and 20th day the rate of positive animals was higher in the group infected with the "O" strain than in those infected with strain "K". However, the difference on the 10th day was not significant; for the 20th day the significance is at the level $P < 0.05$. By the 30th and 60th day the percentage of tuberculin-sensitive animals reached 96 to 100%, and no statistical significance appeared.

The degree of tuberculin-sensitivity was expressed in mm of the diameter of the dermal reaction (Table 2). No difference occurred between the two groups.

TABLE 2

Diameters of tuberculin reactions to 100 TU in vaccinated guinea-pigs

Vaccinated by strain	No. of animals	Average diameters in mm at days after vaccination				
		10	20	30	60	significant difference
"O"	27	5.4	8.6	12.2	13.0	$P < 0.45$
"K"	26	5.0	8.7	12.1	13.5	

Immunogenicity

The immunogenicity of the two strains was examined in albino mice 14—91 g in weight. The inocula contained 0.1 mg of 13-day cultures. Each group included 40 animals. Twenty-one days after inoculation all these and 35 non-immunized animals were challenged by a 19-day culture of the H37Rv strain, intravenously. The following indices were registered.

1. The number of survivors, at the time when more than half of the control mice had died of tuberculosis (Table 3). The difference in the mortality of each of the inoculated groups and the control group is highly signif

TABLE 3

Mortality of superinfected and control mice

Vaccinated by strain	Dose of vaccine		No. of animals	Died from tuberculosis	
	mg	viable units		No.	%
"O"	0.1	4.07×10^5	37	11	29.7
"K"	0.1	5.33×10^5	33	7	21.2
Control	—	—	35	22	62.8

icant ($P < 0.01$). In this respect, there is no significant difference between groups "O" and "K" (mortality rates 29.7 and 21.2).

2. Severity of the pulmonary tuberculous lesions. All the spontaneously dying animals and those sacrificed at the end of the experiment were autopsied and the extent of the tuberculous lesions in their lungs registered in three groups according to a scheme introduced in our laboratory (Table 4). In the control group both the spontaneously dying and the control animals showed

TABLE 4

Tuberculous alterations of the lungs in mice

Vaccinated with strain	No. of mice		No. of mice with degrees of tuberculosis						
			severe		moderate		slight		no lesions
	died	sacrificed	died	sacrificed	died	sacrificed	died	sacrificed	
"O"	11	26	11	7	—	2	—	10	7
"K"	7	26	7	8	—	4	—	10	4
Control	22	13	22	7	—	6	—	—	0

focal lesions over the whole or the greater part of their lungs. In the two immunized groups the animals having spontaneously died after challenge also had lesions over their whole lungs, whereas those sacrificed at the end of the experiment showed only scattered or no lesions at all. Considering the numerical results, a highly significant difference ($P < 0.01$) occurred between the immunized groups and the non-immunized control group with regard to tuberculous lesions; while no significant difference was observed in this respect between the two immunized groups.

3. We examined the proliferation of virulent bacteria in the spleens and lungs of the immunized and control animals. Smears from the organs were stained according to the Ziehl–Neelsen method and the acid-fast bacteria were counted under the microscope. Five animals were sacrificed on the 7th, 14th and 21st day and 10 smears were prepared from each organ. The acid-fast bacteria were counted in 100 fields (Table 5). The bacterium multiplied

TABLE 5

Vaccinated with strain	Average number of acid-fast rods in the smears per field after days					
	spleen			lungs		
	7	14	21	7	14	21
"O"	66	60	208	95	363	1510
"K"	62	108	277	81	438	1660
Control	185	460	928	105	1370	4120

much more intensely in the non-immunized control animals than in the immunized ones. Contrary to this, there was no significant difference between the immunized groups in this respect either.

4. The degree of relative immunity to tuberculosis was estimated on the basis of the survival time. Two groups of 50 albino mice each were immunized by injecting 0.1 mg of the "O" and "K" strain, respectively. Twenty-one days later, the immunized animals as well as 50 non-immunized controls were challenged by a suspension of 0.5 mg of the H37 Rv strain. The infected animals were observed until their spontaneous death (Table 6). While most

TABLE 6

Vaccinated with strain	No. of mice	Death of vaccinated mice (days after infection)			
		20	25	30	35 and more
"O"	43	0	7	19	17
"K"	42	0	13	19	10
Control	49	4	28	11	6

of the control animals had died by the 25th day following challenge, the majority of the immunized animals died after 30 to 35 days. The difference between the immunized and control animals is significant in the range between $P < 0.1$ and $P < 0.01$. There was no statistically significant difference between the two immunized groups.

Residual virulence

The residual virulence of strains "O" and "K" was examined in chick embryos. Nine-day-old embryos were infected with suspensions of 0.5 mg of bacteria taken from 11-day cultures of the strains. After re-incubation for 10 days at 38 °C the embryos were examined. Those which had been inocu-

lated with the strain "K" showed 1 to 20 pocks, while on the chorioallantoic membrane of the embryos inoculated with strain "O" the lesion was diffuse. No significant quantitative difference was found between the two groups.

Summary

1. In the frame of co-ordinated, co-operative investigations two attenuated mycobacterium strains, W 115 and BCG Paris, were examined. One of the strains was coded by "K", the other by "O".

2. The two strains were examined for sensitizing capacity in guinea-pigs, for immunogenicity in albino mice and for residual virulence in chick embryos.

3. The indices for the two strains showed no significant difference.

4. Before deciding whether strain W 115 can be regarded as a vaccine strain, experiments should be carried out in monkeys, and a limited administration to humans would also be necessary.

CHRONICLE

The attenuated *Mycobacterium tuberculosis* strain No. 115 has been studied by T. K. KOCHERBAEV at the Kirgiz Medical University in Frunze, USSR. The results were presented in 1969 as a dissertation for the degree of candidate of Medical Sciences with the title: *Experimental study of the immunogenic properties of the strain Mycobacterium 115.*

Hereby the summary of the dissertation and 2 tables from it are given.

1. The biological and immunological characteristics of the strain Mycobacterium 115 were studied.

2. The strain Mycobacterium 115 is growing in cords; after adding 4—8—10 per cent glycerine to the egg-culture media it grows more rapidly. The strain has a low catalase-activity.

3. Strain Mycobacterium 115 is innocuous for guinea-pigs. 20 mg of the bacteria given subcutaneously does not induce a tuberculous process during 10 months of observation.

4. After giving of 0.05 mg of strain Mycobacterium 115 intracutaneously or subcutaneously the bacteria are living in the organisms of guinea-pigs, albino rats and mice for a long time (378 days). More bacteria can be cultured from the lymph nodes of the experimental animals receiving strain 115 than after injecting the same quantity of BCG-1 strain (Table 1).

TABLE 1

Multiplication of the bacilli of strains 115 and BCG-1 in mice, rats and guinea-pigs (Results of 5 experiments)

Strain	Animals	No. of animals	No. of colonies grown from organs					
			Regional lymph nodes	Other lymph nodes	Spleen	Liver	Lungs	Total
115	mice	44	1891	916	163	26	91	3087
	rats	47	1295	451	40	44	24	1854
	guinea pigs	32	665	362	137	5	12	1181
BCG-1	mice	44	1364	508	34	21	2	1929
	rats	47	779	283	20	1	2	1085
	guinea pigs	32	315	168	57	—	—	540

5. Strain Mycobacterium 115 has a stronger and more durable allergenic effect than that of BCG-1 strain. This can be demonstrated by 0.1—1.0—10.0 mg tuberculine tests.

6. The residual virulence of strain Mycobacterium 115 is somewhat stronger than that of BCG-1 strain.

7. The immunogenic activity of strain Mycobacterium 115 is stronger than that of BCG-1 strain. This was demonstrated in 6 experiments on guinea-pigs, using the Ravenel strain for challenge infection 2, 4 ½, 8, 9, 11 and 12 months after vaccination and assessing the severity of the tuberculous process. The average index proved to be: 1.3 after vaccination by strain 115, and 2.7 after vaccination by strain BCG-1 (Table 2). The index of effectivity proved to be 82.3 per cent after vaccination by strain 115, and 70.4 after vaccination by strain BCG-1, respectively.

TABLE 2

Statistical analysis of the results of immunization of guinea-pigs with strains 115 and BCG in 6 experiments

No. of experiments	Immunized with strain	No. of animals	M*	m	t	P
I	BCG-1	18	1.53	±0.26	2.66	< 0.01
	115	21	0.73	±0.15		< 0.001
II	BCG-1	16	2	±0.21	3.28	< 0.01
	115	15	1.1	±0.22		< 0.001
III	BCG-1	10	2.1	±0.21	2.41	< 0.05
	115	13	1.2	±0.25		< 0.02
IV	BCG-1	13	2.2	±0.17	3.3	< 0.01
	115	16	1.3	±0.21		< 0.001
V	BCG-1	15	2.2	±0.18	3.1	< 0.01
	115	8	1.1	±0.32		< 0.001
VI	BCG-1	23	3.4	±0.16	3.1	< 0.01
	115	17	2.5	±0.24		< 0.001

*M = Tuberculosis index with maximal number of 10 in severe generalized tuberculosis.

8. On the basis of the experimental results concerning in vivo multiplication, innocuity, sensibilizing and immunizing properties of the Mycobacterium 115, author emphasizes that it has some advantages to use vaccines prepared from strain Mycobacterium 115 in the fight against tuberculosis.