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Immunological Strain Specificity within Type 1 Poliovirus *

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The demonstration of immunological differences between poliovirus strains of any one type is a valuable procedure in epidemiological research as it may allow a virus strain to be identified as derived from or unrelated to a given possible source of infection. It is obviously of particular importance in connexion with live poliovirus vaccination campaigns. Both kinetic tests and conventional neutralization and complement-fixation techniques have been used to this end, the former involving a more complicated test procedure and the latter demanding greater nicety in the pre-standardization of reagents. The present paper reports on attempts to establish a simplified technique.

Neutralization titres of sera obtained by immunization of guinea-pigs with three strains of type 1 poliovirus (including one isolated from a patient in the 1958-59 epidemic in Léopoldville described in the two preceding papers) indicated a degree of strain specificity sufficient to permit the design of a simple screening method for the purpose of a rough immunological classification.

Preliminary observations on isolates from persons fed attenuated virus indicate that antigenic changes may occur in the course of multiplication of the virus in the human intestinal tract.

McBride (1959) recently described certain immunological differences between poliovirus strains belonging to the same immunological type. According to this author differences are not readily demonstrable with the conventional neutralization technique but become manifest in kinetic studies, a given antiserum neutralizing its homologous strain faster than it does heterologous strains. In this sense, most of the strains of all three types studied by McBride appeared to be immunologically distinct. However, a number of derivatives of the Mahoney strain behaved as homologous strains or as very closely related to the parent strain, and a group of several strains isolated in the same geographical region in the course of an epidemic likewise appeared to be closely related.

McBride is not the first worker in this field. In 1956 Wenner and his associates had published results of a systematic study of immunological strain differences within poliovirus type 2 (Wenner et al., 1956), and recently similar reports by this group on types 1 and 3 have appeared (Wenner et al., 1959; Dubes et al., 1959). Unlike McBride, Wenner and his associates have not resorted to kinetic tests, but used conventional neutralization titration methods as well as complement fixation with largely the same net results. Thus, they reported in 1956 distinct differences within type 2 and observed that strains isolated in the same geographical region seemed to be more closely related than strains from different areas. Using the same approach as McBride they also found that a number of " mutants " of the type 1 Akron strain were not significantly different from the parent virus, although some other biological properties had changed considerably. A similar study of type 3 Leon variants gave somewhat less clear-cut results, a certain evidence for minor intra-strain variations being presented. The authors were, however, inclined to regard the antigenic structure as a comparatively stable genetic marker.

These observations, if confirmed, indicate the possibility of identification of a given strain of virus

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as either derived from or unrelated to a certain source of infection. This would furnish a valuable tool for epidemiological research, particularly important in connexion with live virus vaccination campaigns.

The present paper is a preliminary report on attempts to establish a simplified technique for application of the findings of Wenner et al. and of McBride on the practical problems mentioned.

MATERIAL AND METHODS

Strains of virus

Three strains of type 1 were chosen as prototypes in the present study: (1) The attenuated CHAT strain [1] (Wistar Institute (WI) seed lot, pool 19); (2) Mahoney (WI pool 5); (3) strain Liku,[2] isolated in 1959 from a patient with paralytic poliomyelitis in Léopoldville, Belgian Congo (see the article by Plotkin et al. on page 215 of this issue) in its third passage in monkey kidney tissue culture. In one experiment the Parker strain was used.

In exploratory screening tests an additional number of strains were employed, to be briefly described in the text.

Stock virus suspensions were prepared by inoculation of monkey kidney tissue cultures at a multiplicity of about 10, adsorption for three hours, washing and re-feeding, and incubation for 24 hours at 37°C. The cultures were then frozen and thawed three times, centrifuged at 2000 r.p.m. for 20 minutes, distributed in 1-ml portions in rubber-stoppered tubes and stored in a deep-freeze refrigerator.

Immune sera

Groups of ten or more guinea-pigs were inoculated subcutaneously with 1.0-ml doses of undiluted stock virus. A second inoculation of the same dose was given 3-4 weeks later. Bleeding by heart puncture was carried out seven days after the second inoculation. Sera were inactivated for 30 minutes at 56°C and titrated against their homologous strain of virus. Sera with comparable titres were pooled, the highest titring pools being used in most of the experiments.

Tissue cultures

For these experiments monkey kidney tissue cultures were purchased from Microbiological Associates, Inc. Upon arrival in the laboratory the tubes were re-fed with 1 ml of bovine amniotic fluid (BAF), containing 0.02% phenol red and 100 units each of penicillin and streptomycin. The cultures were maintained without any further change of medium. The pH of the medium remained virtually constant at about 7.4 throughout the observation period, usually 12 days. Spontaneous degeneration of uninoculated cultures was usually not observed until after about three weeks of incubation. Foamy degeneration was not uncommon but seemed to have little effect upon the results of the experiments.

Virus titrations

Activity of stock viruses was carefully determined in repeated titrations. Tenfold serial dilutions in BAF were prepared and 0.1-ml amounts of each dilution inoculated into each of eight tissue culture tubes. Cultures were incubated stationary at 37°C. Readings were taken daily for 10 or 12 days. Results were recorded as: + = one or more distinct foci of typical degeneration; ++ = generalized degeneration, normal-looking cells still present; +++ = no normal cells left. Time of transition from + to +++ seldom exceeded 48 hours, provided that pH was maintained around 7.4. Only occasionally did specific degeneration appear after the sixth day of incubation. Titres were computed according to the Reed & Muench method and expressed in terms of logarithms of ID_{50}/ml.

Neutralization tests

The immuno-inactivation technique (Gard, 1957) was employed throughout. On the basis of repeated titrations viruses to be used were standardized at a concentration of 10^4 $TCID_{50}$ per ml. Sera were diluted serially in fivefold steps. Tests were set up with 0.8 ml of BAF, 0.1 ml of serum dilution and 0.1 ml of standard virus in Kahn tubes, ingredients being added in the order mentioned. The rubber-stoppered tubes were thoroughly shaken, placed in a water-bath at 37°C for six hours and kept overnight in the refrigerator. The reaction mixture was then diluted 1 : 10, and 0.1-ml amounts of this dilution inoculated into each of four tissue culture tubes. Incubation and readings were carried out as above. Each test included virus controls of 0.9 ml BAF and 0.1 ml standard virus, incubated

[1] Described by T. L. Nelson & H. Koprowski in a paper presented at the meeting of the Western Society for Pediatrics Research, San Francisco, Calif., 28-29 October 1957.

[2] This strain is designated No. 81 in Table 1 of the paper by Koprowski et al. on page 244 of this issue.

along with the tests. Virus controls were tested in tissue cultures in dilutions 1 : 10, 1 : 100, and 1 : 1000, four tubes per dilution. BAF was used as diluent throughout.

In this procedure the dose of virus added to the reaction mixture amounts to 100 ID_{50} per 0.1 ml, whereas the test for residual activity is performed at a level corresponding to an initial amount of 10 ID_{50} per inoculum of 0.1 ml. The result was considered acceptable if the virus control showed specific degeneration in all tubes inoculated with dilution 1 : 10 and in not more than three or not less than one of the eight tubes inoculated with dilutions of 1 : 100 and 1 : 1000. In qualitative tests a result was considered positive if at least two of the four tissue culture tubes remained normal, corresponding to a neutralization of at least 90% of the original activity. Serum titres were calculated according to the Reed & Muench technique as 50% end-points, and expressed as logarithms of the *final* dilutions in the reaction mixture.

EXPERIMENTAL

Twelve guinea-pigs were immunized with the CHAT strain as described above. When screened for neutralizing capacity at a final dilution of 1 : 1000 two sera failed to neutralize and were discarded. The others were pooled, and the pool was titrated against the homologous virus, giving an end-point of 4.9. In tests with virus types 2 and 3 no neutralization was obtained with the same serum in a final concentration of 1 : 10.

With this serum pool a number of titrations were performed against homologous and heterologous virus strains. As shown in Table 1 the results seemed to be reproducible and showed an obvious trend, the homologous titre being consistently about 1 log unit higher than the heterologous.

An anti-Mahoney serum pool similarly produced showed much less strain specificity (Table 1); however, the difference between anti-CHAT and anti-Mahoney titres, although slight, seemed to be reproducible and significant.

An anti-Liku serum again showed a clear-cut difference between homologous and CHAT titres; strain Mahoney was neutralized to the same extent as the homologous virus (Table 1).

A certain degree of strain specificity was thus apparent. However, the narrow margin between homologous and heterologous titres necessitated exact standardization of the test system; small deviations in test virus concentrations could distort the whole picture. With the aim of improving the conditions of the test an attempt was made to remove heterologous antibodies by means of absorption.

Freshly harvested Mahoney culture fluid was treated with formaldehyde 1 : 4000 at 37°C for four days. An equivalent amount of bisulfite was then added; tests for residual virus activity and residual formaldehyde were negative. Anti-CHAT serum and formol-treated Mahoney virus were mixed in the proportion 1 : 9 and kept at 37°C for six hours. Titration for residual neutralizing activity showed a reduction in heterologous titre from 3.5 to 2.3, at the same time the homologous titre was reduced from 4.7 to 3.0. The margin was thus not widened by the absorption but had actually shrunk from 1.2 to 0.7 log units.

In a second experiment another CHAT serum pool was absorbed with a formol-treated, purified, and concentrated Parker virus material made available by courtesy of Dr Maurice Hilleman (Merck, Sharp and Dohme Research Division). Absorption was carried out in two steps. The original titres were: homologous 5.7, heterologous 5.3; after one absorption 4.1 and 3.8; after two absorptions 3.1 and 3.1, respectively. In this case the difference between homologous and heterologous titres was thus completely erased by absorption.

The animals given CHAT virus were bled also after the first inoculation. Furthermore, immunization was continued with third and fourth doses

TABLE 1

RESULTS OF CROSS-NEUTRALIZATION TITRATIONS OF POOLED SERA AGAINST THREE STRAINS OF POLIOVIRUS TYPE 1

Serum	Test strain [a]		
	CHAT	Mahoney	Liku
CHAT	4.9	—	—
	4.6	3.4	—
	4.6	3.5	3.7
	4.8	—	3.5
Mahoney	2.7	3.1	2.9
Liku	3.4	4.1	4.1

[a] Titres expressed as logarithms of dilution end-points.

TABLE 2

HOMOLOGOUS AND HETEROLOGOUS NEUTRALIZATION TITRES OF GUINEA-PIG SERA DRAWN ONE WEEK AFTER FOUR CONSECUTIVE IMMUNIZING INOCULATIONS ABOUT ONE MONTH APART

Bleeding No.[a]	Test strain		Difference
	CHAT (homologous)	Liku (heterologous)	
1	3.8	1.7	1.1
2	4.7	3.6	1.1
3	5.75	5.0	0.75
4 a	4.5	≤3.5	≥1.0
4 b	4.5	≤3.5	≥1.0
4 c	5.3	4.3	1.0
4 d	4.5	4.5	0
4 e	4.3	4.0	0.3
4 f	4.0	3.3	0.7
4 g	4.3	3.5	0.8
4 h	4.5	4.3	0.2
4 i	4.7	4.5	0.2
4 k	4.7	4.2	0.5
4 (calculated mean)	4.7	4.2	0.5

[a] First three bleedings tested as pools only: fourth-bleeding sera titrated individually.

after intervals of about one month each. The three first bleedings were titrated as pools only, but after the fourth bleeding individual titrations were carried out. The results shown in Table 2 indicate that the capacity to respond with formation of strain specific antibodies varies from one animal to the next. On this account conclusions based on a limited number of observations cannot be considered definitive. In particular, quantitative estimates based on results obtained with serum pools must be regarded as unreliable. It must therefore be left an open question whether the decrease in specificity with increasing numbers of inoculations, suggested by the figures in Table 2, represents a general trend.

The CHAT serum, second bleeding, was used in a series of experiments aimed at the establishment of a simple technique by which large numbers of strains could be screened for the purpose of immunological classification. To this end an attempt was made to find a concentration of serum that would regularly neutralize the standard dose of homologous virus but leave heterologous strains virtually intact. Not unexpectedly it was found that the concentration $10^{-4.0}$, being halfway between the homologous and heterologous end-points, seemed best to fulfil this requirement. Tests were accordingly set up with a final serum concentration of 10^{-4}, the standard virus concentration of 10^3 ID_{50}/ml, incubation at 37°C for six hours, and storage overnight in the refrigerator. Virus controls were included for each strain tested. Tests and controls were diluted 1 : 10, and 0.1-ml amounts inoculated in groups of four monkey kidney tissue cultures.

Table 3 gives an example of the kind of results obtainable with this technique. In this case complete protection against the homologous strain was observed and almost complete protection against strain B 156, isolated from an infant fed the CHAT strain. The heterologous Liku broke through in all four tubes, with only a slight delay as compared with the control. The same is true of strains 99 and 94, both isolated from paralytic cases in the Belgian Congo and presumably related to Liku.

In Table 4 some results from a reciprocal experiment are presented. In this case the anti-Liku serum was used in a concentration of $10^{-3.7}$, again about halfway between homologous and heterologous end-points. With this system as well protection was afforded against the homologous virus, while the heterologous broke through.

With the aid of this technique a number of strains, presumably derived from the CHAT virus, were screened. These strains had been isolated either from children fed the CHAT strain or from contacts of such children. So far, acceptable tests have been completed on ten American and five Swedish isolates. In addition, tests were set up with seven strains isolated from paralytic patients in Léopoldville (see the article by Plotkin et al. on page 215 of this issue); and with the Sickle strain, which together with Mahoney made up the mixed virus from which SM, the supposed parent strain of the CHAT virus, was initiated (Koprowski et al., 1954).

In general, as shown in Table 5, results obtained with this method have not been clearly distinctive. Some strains showed apparently complete identity with the homologous strain. Usually a certain difference was discernible, however, varying in degree from insignificant or slight to that of a clearly heterologous virus. Under such conditions a strict classification is hardly possible. Tentatively a rough division into three groups was adopted: (*a*) " homo-

TABLE 3

DAILY READINGS RECORDED IN SCREENING TEST OF 5 TYPE 1 STRAINS WITH ANTI-CHAT SERUM IN CONCENTRATION 10^{-4}

Strain	Test								Control							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
CHAT	−	−	−	−		−	−	−	−	−	−	++		+++		
	−	−	−	−		−	−	−	−	+	++	+++				
	−	−	−	−		−	−	−	−	−	++	+++				
	−	−	−	−		−	−	−	−	+	+	+++				
B 156	−	−	−	−		−	−	−	−	++	+++					
	−	−	−	−		−	−	−	−	+	++	+++				
	−	−	−	−		−	++	+++	−	++	+++					
	−	−	−	−		−	−	−	−	−	+++					
Liku	−	−	+	+++					−	++	+++					
	−	−	++	+++					−	++	+++					
	−	−	++	+++					−	++	+++					
	−	+	++	+++					−	+	+++					
99	−	+	+++						−	++	+++					
	−	+	++	+++					−	++	+++					
	−	−	−	+		+++			−	+	+++					
	−	+	++	+++					−	+	+++					
94	−	+	++	+++					−	−	+++					
	−	−	−	++		+++			−	−	++	+++				
	−	−	++	+++					−	+	++	+++				
	−	+	++	+++					−	+	+++					

TABLE 4

DAILY READINGS IN SCREENING OF STRAINS CHAT AND LIKU AGAINST ANTI-LIKU SERUM IN CONCENTRATION $10^{-3.7}$

Strain	Test								Control							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
CHAT	−	−	+	+		+++			−	−	+	++		+++		
	−	−	−	−		+++			−	+	+	++		+++		
	−	−	−	−		+++			−	−	+	++		+++		
	−	−	+	++		+++			−	+	++	+++		+++		
Liku	−	−	−	−		−	−	−	−	+	++	+++				
	−	−	−	−		−	−	−	−	−	++	+++				
	−	−	−	−		−	−	−	−	+	++	+++				
	−	−	−	−		−	−	−	−	−	+	+++				

TABLE 5
RESULTS OF SCREENING OF TYPE 1 POLIOVIRUS STRAINS AGAINST ANTI-CHAT AND ANTI-LIKU SERUM

Group	Strain	Serum	
		CHAT	Likubukidi (No. 81) [a]
Wild [a] or virulent	81 [b]	Heterologous	Homologous
	89 [b]	,,	,,
	93 [b]	,,	Intermediate
	99 [b]	,,	
	4	Intermediate	Intermediate
	90	Heterologous	
	94	,,	
	Mahoney	,,	Homologous
	Sickle	Intermediate	
CHAT and its passages [c] through the human intestinal tract in USA	CHAT	Homologous	Heterologous
	B 117	,,	,,
	B 119	,,	
	B 148	,,	
	B 156	,,	
	C 1	,,	
	D 1	,,	
	L 1	,,	
	O 5	,,	
	Q 1	,,	
	Q 2	,,	
CHAT passages through human intestinal tract in Sweden	AD	Heterologous	
	CL	Homologous	
	MN	,,	
	JO	Heterologous	
	PO	Homologous	
Naturally attenuated	W 1063	Heterologous	

[a] Origin of these Léopoldville strains is shown in Table 1 of the paper by Koprowski et al. (p. 244).
[b] Fed CHAT virus during vaccination campaign.
[c] Origin of these strains is shown in Table 2 of the paper by Koprowski et al. (p. 245).

logous" reactions, with complete protection or occasionally with a late break-through in one out of four tubes; (*b*) "intermediate", with clearly delayed break-through in two or three tubes; and (*c*) "heterologous", when degeneration appeared consistently in all tubes.

In such terms the results of the screening tests may be summarized as follows. The wild Léopoldville strains fell into two groups: six gave a clearly heterologous reaction in relation to anti-CHAT serum, and two of the three tested behaved as homologous strains to the Liku virus, whereas one (No. 93) gave an intermediate reaction. One of the Léopoldville strains (No. 4), isolated from a child who had never received CHAT virus and who became sick at the beginning of the epidemic (see the preceding article), gave an intermediate reaction in the test against anti-CHAT and anti-Liku serum.

The Sickle strain gave on one occasion an intermediate reaction and in a repeat test a delayed heterologous reaction.

The ten American isolates all gave homologous reactions, the majority of them with a late break-through in one tube.

The Swedish strains were chosen on the basis of their bicarbonate sensitivity; three of them were clearly of the *d*+, large-plaque type; one had maintained the *d* character of the parent CHAT, but produced large plaques; the fifth was a typical *d* variant. Two of these strains, one *d*+ and the *d*, large-plaque variant, gave clearly heterologous reactions; the others behaved as homologous strains.

DISCUSSION

According to McBride, conventional neutralization techniques are not sufficiently sensitive to "uniquely characterize individual strains", which, however, should be feasible with the kinetic method. As a measure of the relationship between two strains McBride has chosen the "normalized K values", i.e., the ratio of the neutralization rate constants (heterologous/homologous). The values thus obtained range between 0.05 and 0.5, the average being about 0.25. Expressed as logarithms the differences recorded amount to between 1.3 and 0.3 units, average 0.6.

In comparison, Wenner's group using conventional methods finds titre ratios ("R" values—Archetti & Horsfall, 1950) of from 0.06 upwards for type 2, or up to 1.2 log units, similar values for type 1 and somewhat smaller differences within type 3. In the material here presented the titre differences in the heterologous pair CHAT-Liku were 1.2 and 0.7.

Apparently, then, there are hardly any differences in sensitivity between the various methods, and most probably they measure the same qualities. Which

method to prefer is mainly a matter of taste. The kinetic test is less exacting as far as pre-standardization of the various reagents is concerned, but the test itself is more complicated; the reverse is true of the titration methods.

The true nature of the strain differences is still obscure. The fact that absorption did not serve to improve the specificity might indicate that quantitative rather than qualitative differences in the antigen mosaic are responsible. It should be pointed out, however, that formaldehyde treatment of the virus tends to broaden its range of antigenic reactivity (Black & Melnick, 1955; Le Bouvier, 1955), and until absorption experiments with active virus have been undertaken, no definite conclusions can be drawn.

As regards the technique, the following facts must be emphasized. In neutralization titrations the antigen concentration is of primary importance. If the immuno-inactivation technique is used, the serum titres recorded will be inversely proportional to the antigen dose. In this connexion it has to be remembered that " spontaneously " inactivated virus has the same antibody-combining capacity as active virus (Melén, 1959). Therefore, a test in which partially inactivated virus is used and the dose of virus is determined on the basis of residual virus activity will give a lower titre value than a test on the same serum with 100% active virus to the same ID_{50} value. For this reason it is important that the virus used in the test be produced under such conditions as to ensure that spontaneous inactivation is reduced to a minimum. Furthermore, a stock virus showing a drop in activity upon storage has to be discarded. A simple adjustment of the test dose to correct for the drop in activity will introduce a serious error in the test.

The variation in the capacity of the experimental animals to respond specifically to immunization indicates that selected individual sera rather than pools should be used in studies of this nature. It also calls for great caution in attempts to evaluate relationships between strains on the basis of quantitative calculations. For the time being only immunological identity established in reciprocal tests with several individual sera can be considered to carry sufficient weight to justify the conclusion that two strains may be derived from the same source.

From a practical as well as a theoretical point of view the question of the stability of the antigenic character is of central interest. McBride concludes that " the antigenic character of a polio virus is highly stable under both selective and nonselective conditions ". Wenner and co-workers are a little more cautious. Regarding type 1 they state that " it would not seem to be easy in the laboratory to change the antigenic character of polio viruses ". Studying two groups of variants of certain type 3 strains they found " some evidence of small intra-strain antigenic variation ".

The preliminary results here reported of tests on attenuated strains after passage through the human intestinal tract seem to indicate that the antigenic stability might be less than assumed by the authors quoted above. For the time being, however, it is necessary to leave the question open; the test methods are probably not yet definitive; much larger numbers of strains have to be studied; reciprocal tests have to be performed, etc. In the present situation a homologous reaction may be taken to indicate a common origin, whereas in the case of intermediate or heterologous reactions no opinion on the derivation of the strains can be expressed.

RÉSUMÉ

La mise en évidence de différences sérologiques entre les souches appartenant au même type de virus poliomyélitique, est d'une importance primordiale dans les recherches épidémiologiques, en particulier celles qui ont trait à la vaccination par le virus vivant. Des méthodes grâce auxquelles on peut distinguer des variétés sérologiques au sein d'un même type permettent de déterminer si les virus excrétés appartiennent au même groupe sérologique que ceux qui ont été ingérés ou si des variations antigéniques sont survenues. Plusieurs méthodes sont appliquées dans ce but: le test cinétique et les méthodes habituelles de neutralisation et de fixation du complément — la première plus compliquée, la dernière exigeant une standardisation préalable minutieuse des réactifs.

Dans cet article, l'auteur donne les premiers résultats d'une technique simplifiée de la méthode de neutralisation par le sérum de cobayes immunisés, qui paraît satisfaisante pour le premier classement des virus isolés de sujets vaccinés. L'étude a porté sur des virus de type 1. La technique est décrite, et divers points soulignés dont dépend la précision de l'épreuve.

On ignore encore en quoi les souches diffèrent les unes des autres. Le fait que l'absorption n'augmente pas la sensibilité du test semble indiquer qu'il s'agit de diffé-

rences plus quantitatives que qualitatives. Toutefois, il faut signaler que le traitement par la formaldéhyde étend la gamme de réactivité antigénique du virus. Les animaux s'immunisent plus ou moins facilement. Il y a donc lieu de choisir des sérums individuels plutôt que des mélanges provenant de plusieurs animaux.

La question de la stabilité antigénique des virus est primordiale, pour l'étude des vaccins vivants. Les auteurs qui l'ont étudiée jusqu'ici n'ont fait état que de légères variations au sein des souches. L'auteur estime, d'après ses premiers résultats, que la stabilité antigénique est peut-être moindre qu'on ne le pensait, mais il n'est pas possible dans l'état actuel des recherches, de tirer des conclusions plus précises.

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