Comparison of Neutralization Rate and Agar Diffusion Methods for Intratypic Sero-Differentiation of Polioviruses.* (27805)

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With the extensive use of the live poliovirus vaccines there has been a great deal of interest in the intratypic antigenic analysis of the polioviruses. It was hoped that by this means it might be possible to distinguish between "wild" and vaccine strains. In addition, antigenicity has been employed as a marker in genetic studies with these viruses.

Three methods have been described for detecting antigenic differences within serotypes. (a) Wecker(1) employs monolayers of cells which are infected with virus before being overlaid with agar containing antiserum. Neutralization of the progeny of the infecting virus is measured by this test, and antigenic differences are reflected in the relative plaque sizes of the various strains. (b) In the method of McBride(2) the rate of neutralization of virus by a strain specific antiserum is measured. (c) The method devised by Melnick(3) measures the neutralization of virus during migration through an antiserum-agar overlay. Virus which has not been neutralized infects the cell monolayer beneath the overlay.

Each method has certain advantages. However, since the tests depend upon different types of interaction of virus and antiserum, it cannot be assumed that they give results that are interchangeable. Nakano and Gelfand(4) have compared a modification of the method of Wecker with that of McBride and have found similar results with the two tests. In this communication data are presented which indicate that the results obtained by the neutralization rate test (McBride) and the agar diffusion test (Melnick) are also comparable.

Materials and methods. The viruses examined were type 1 polioviruses of the following strains: Mahoney, received in this laboratory in 1954 from Connaught Laboratories of Toronto and subsequently passed numerous times in monkey kidney (MK) cells; LSc-2ab, received from Dr. A. B. Sabin and passed once in MK cells in this laboratory; 3 strains recovered from the feces of infant Jo on the 6th, 13th, and 44th days after oral administration of LSc-2ab. The Mahoney and LSc-2ab strains underwent 2 consecutive plaque passages immediately before preparation of stock suspensions for use in the study. Isolated plaques appearing on monolayer cultures inoculated with 10% fecal suspension from infant Jo were removed with capillary pipettes; the first tissue culture (TC) passage of these plaque isolated viruses was used for antigenic analysis.

Strain specific sera were produced in 250 g guinea pigs by the method of Gard(5). Mahoney and LSc-2ab plaque-purified viruses were used to inoculate groups of 12 guinea pigs each. Sera were obtained by cardiac puncture 2 weeks after completion of the immunization course of 3 inoculations of 1 ml undiluted tissue culture fluid (TCF) at 2-week intervals. The specificity of the individual sera was determined by the agar diffusion test; all sera obtained were found to be both strain specific and of high titer by this method. The antiMahoney sera were pooled in order to have sufficient quantity. AntiLSc-2ab sera from 3 guinea pigs formed the other specific serum pool.

All assays were performed by plaque technics in monolayer MK cell cultures in plastic 25-ml TC flasks. The basic agar overlay consisted of 2 ml of medium 199 (0.15% $NaHCO_3$) with 2% calf serum in 1% Noble agar (Difco). Plaques were visualized after 72 hours at 37°C by adding 2 ml of this nutrient agar containing 0.001% neutral red; further incubation of 1-2 hours at 37°C was sufficient to allow staining of the viable cells.

Neutralization rate determinations were carried out according to the method of Mc-

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Bride(2). All sera were inactivated at 56°C for 30 minutes. Five-tenths ml of virus suspension containing approximately 10^5 plaque forming units (PFU)/ml were added to 0.5 ml of an appropriate serum dilution, and the mixtures incubated at 37°C in a water bath. One-tenth ml samples were removed at appropriate intervals and immediately mixed with 100 ml of neutral Hanks' balanced salt solution at 4°C. The samples were titrated by the plaque technic employing 0.05 ml inocula. The log of the surviving virus was plotted against time of neutralization, and the slope of the "best straight line" through these points and the "K" value of the serum† were calculated.

MK cultures were prepared for the agar diffusion test as follows. Appropriate dilutions of the strain specific serum pools (inactivated at 56°C for 30 minutes) in 2x medium 199-4% calf serum were added to equal portions of 2% agar in distilled water at 45°C. Two ml of the serum-agar mixtures were overlaid on MK cultures. As soon as the agar had solidified, 0.05 ml of TCF containing approximately 10^6, 10^5, or 10^4 PFU of virus was dropped onto the surface of the agar, so that a box serum titration resulted. Controls overlaid with nutrient agar without guinea pig serum were inoculated similarly; serum toxicity controls were also included. A plaque titration of the virus suspension was included in each experiment employing 3 cultures per dilution. The highest serum dilution resulting in 0-10 plaques per culture was regarded as the neutralization endpoint at each virus concentration. Homologous serum-virus combinations were included as references in each test. A reproducible drop in endpoint of at least one dilution with any serum-virus mixture from that of the homologous strain was regarded as indicating a change in antigenicity.

Results. Neutralization rate test. Each serum-virus combination was examined in at least 2 experiments; reproducible neutralization rates were obtained as long as the serum concentration was not changed; however, the "K" values employed by McBride(2) to compare virus strains using different serum dilutions were not constant if serum dilutions were changed from test to test, or even in the same experiment. Therefore, comparisons of virus strains were made using only the same specific serum dilution for all viruses examined. The "K" value of LSc-2ab virus with antiMahoney serum and of Mahoney virus with antiLSc-2ab serum was markedly different from those observed with the homologous serum and virus, confirming the strain specificity of the sera (Table I). The viruses recovered from Jo on days 13 and 44 were no longer antigenically related to the LSc-2ab vaccine strain administered to the infant. Indeed, the virus isolated on day 44 appears more closely related to the Mahoney strain.

Agar diffusion test. Data from representative experiments are presented in Table I. Plaque sizes were not significantly decreased under serum-containing agar medium; therefore, neutralization of progeny viruses by serum present in the medium did not inhibit virus spread sufficiently to interfere with plaque formation. The titers of all viruses under 2% calf serum overlay were comparable to titers obtained in tube cultures maintained with serum-free medium, indicating that the calf serum was not inhibitory. When a countable number of plaques developed, the plaques were confined to the central area where the virus drop had been placed, indicating that virus did not run down between the edge of the agar and wall of the vessel.

The neutralization patterns of these viruses also revealed an antigenic shift in the viruses isolated 13 and 44 days after feeding. The 6-day virus was antigenically identical to LSc-2ab by the neutralization rate test. However, the serum diffusion test does appear to indicate a slight difference in antigenicity of this strain from the vaccine virus. The 44-day virus again shows a closer relation to the Mahoney strain than to the LSc-2ab virus.

The serum diffusion test appears to be slightly more sensitive and provides an additional advantage in that it required only 24

† "K" values were calculated according to McBride(2) by the formula $K = \frac{D}{t} 2.3 \log \frac{Vo}{Vt}$ where Vo and Vt = active virus at time 0 and t, respectively, and $D = \frac{1}{C}$ = dilution of antiserum.

TABLE I. Comparison of Serum Diffusion and Neutralization Rate Technics in Antigenic Analysis of Selected Type I Polioviruses.

Virus	Serum diffusion neutralization pattern						Neutralization rate constant*	
	AntiMahoney serum			AntiLSc serum			AntiMahoney serum	AntiLSc serum
	Log PFU† added			Log PFU† added				
	6	5	4	6	5	4		
LSc	500‡	750	1250	800	3200	3200	21.2	90.2
Jo—day 6§	500	1000	1000	800	800	800	21.2	75.4
" " 13§	500	1000	1000	200	200	200		17.2
" " 44§	1000	1250	1250	200	200	800	61.8	
Mahoney	1250	1250	1250	200	200	800	78.0	18.8

* "K" value. † Plaque forming units of virus. ‡ Reciprocal of serum dilution at neutralization endpoint (0-10 plaques). § Jo viruses were isolated from feces of an infant vaccinated with LSc virus.

cultures to characterize a virus with both strain specific sera, as opposed to approximately 72 cultures for the neutralization rate technic. This allows the screening for changes in antigenicity of aproximately 3 times as many viruses with a given number of cultures.

Discussion and summary. Although these data are derived from examination of only 5 strains of type 1 poliovirus they are representative of the results of a larger number of similar observations(6). It is evident that when antigenic differences were detected by the agar diffusion method they were confirmed by the neutralization rate test. Thus, the results obtained with these 2 methods of determining intratypic antigenic differences would appear to be highly comparable. When these findings are considered with those of Nakano and Gelfand(4), who showed that the neutralization rate test and their modification of the method of Wecker gave similar results, it can be concluded that in spite of the potential differences, the 3 technics are in remarkable agreement.

Our results are at variance with those of McBride(2) in that a clear difference could be demonstrated between the Mahoney and LSc-2ab strains, the latter being derived from the former, whereas he found no such distinction, but employed only antiserum prepared against Mahoney. Nakano and Gelfand(4) did find the 2 viruses to be antigenically distinct, but this was most evident in tests with the antiLSc serum. The discrepancy in results in the 3 laboratories might be explained by differences in the viruses tested or more likely by differences in specificity of the antisera employed. That the latter is the probable explanation is demonstrated by the results of tests in our laboratory with anti-Mahoney serum kindly supplied by Dr. Gelfand, which were in agreement with those of Nakano and Gelfand(4) and failed to differentiate clearly LSc and Mahoney viruses.

However, with suitable technics not only can the related Mahoney and LSc strains be distinguished one from the other, but alterations which occur during multiplication in the human intestine can be detected. Changes during passage in the human intestine are shown by the few data given here and further documented by more extensive observations in our laboratory(6).‡ Nakano and Gelfand (4) indicate that they too have demonstrated antigenic differences between the viruses excreted by vaccinated persons and the vaccine virus. Similar observations have been made by Wassermann and Fox(7). Thus, it is apparent that the antigenicity of polioviruses is not such a stable trait as had been hoped.

‡ Detailed report to be published later.

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Runt Disease Produced in Thymectomized F1 Hybrid Mice Injected with Parental Strain Lymphoid Cells.* (27806)

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It has recently been shown that in mammals the thymus gland plays in early life an important role in development of immunological capacity. Guinea pigs(1), rabbits(2) and mice(3) thymectomized in early life show a decreased ability to produce circulating antibodies and thymectomy performed in mice during the neonatal period interferes with the capacity to reject either skin(4,5) or tumor (6) homografts. In addition, our recent reports seem to demonstrate that spleen cells derived from adult mice thymectomized shortly after birth fail to elicit graft *vs* host reactions, as determined by the Simonsen's assay of histocompatability(7). These results and the observation that mice of several inbred strains thymectomized shortly after birth fail to grow normally and regularly develop disease and wasting during the second or third month of life prompted us to study the development and course of the homologous disease induced by injection of normal lymphoid cells into mice previously submitted to complete thymectomy. For this purpose the production of immunological "runt disease" was attempted in young adult F1 hybrids by injection of lymphoid cells taken from normal adult donors isologous with one of the parent strains, and the effect of previous thymectomy performed in the F1 hybrid either immediately after birth or at 40 days of age upon the development and severity of the graft *vs* host reaction was studied. These experiments show that early thymectomy of the recipient F1 hybrid mice facilitates and increases the severity of the immunological disease produced by administration of normal splenic cells. Indeed, it is demonstrated that thymectomy performed as late as 40 days after birth appears to favor the development of homologous immunological disease.

Method. Mice of the A and C3H strains and F1 hybrids resulting from the cross between A and C3H and between A and C57Bl strains were used. Three experiments were performed and the following groups of F1 hybrid mice were prepared for each experiment: (a) sham-thymectomized, to be injected with parental spleen cells, (b) thymectomized, to be injected with same parental cells as group "a", and (c) thymectomized, to be kept as non-injected controls.

In a first experiment (A × C3H)F1 hybrid mice were operated upon during their first day of life and 35 days later groups "a" and "b" were injected intraperitoneally with approximately 200 million viable A strain spleen cells taken from 10- to 12-month-old donors. Mice of all groups were of approximately the same weight at time of injection. In a second experiment (A × C3H)F1 hybrid mice were operated upon at 40 days of age and 10 days later groups "a" and "b" were injected intravenously with approximately 200 million spleen cells donated by 6-month-old C3H mice. In a final experiment (A × C57Bl)F1 hybrid mice were submitted to surgery at 40 days of age and 10 days later groups "a" and "b" received intraperitoneally 150 million spleen cells obtained from 10- to 12-month-old A strain

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