Reports

Minute Plaque Mutant of Type 2 Poliovirus

A mutation of polioviruses (type 1 and type 2) to resistance to neutralizing substances—most probably inhibitors—in normal bovine serums (NBS) has been previously described (*1*). Since the mutation is characterized by altered sensitivity to normal bovine serum inhibitors, it will be designated by the letter *i* (for inhibitor). It was shown that *i* mutants of both type 1 and type 2 viruses produce definitely larger plaques on plates overlayered with agar containing 10 to 20 percent inhibitory NBS than the respective wild type (i^+) viruses (*1*). Usually MEF-1 *i* mutants were readily obtained through several serial transfers of i^+ virus in tissue cultures in the presence of inhibitory NBS.

In the course of the studies, however, it was observed that in several series of serial passages of plaque-purified MEF-1 i^+ virus in HeLa cell cultures in the presence of inhibitory NBS, *i* mutants failed to appear even after more than ten passages. In these series of serial passages, virus titers of the harvested culture fluids, assayed on HeLa cell plates with noninhibitory horse serum agar overlay, decreased markedly after several passages, in spite of the fact that the cellular destruction of the cultures appeared as rapidly as was expected; the reasons for this rapid reduction in i^+ virus titers remained unexplained.

Later, however, upon closer examination of the plates that had been seeded with lower dilutions of the culture fluids, it was found quite unexpectedly that numerous very minute plaques, sometimes barely visible to the naked eyes (less than 0.5 mm in diameter), had been produced, besides the plaques of usual sizes. The virus, forming minute plaques, was purified either by plaque isolation or by the limiting-dilution technique, and it was shown to be neutralized specifically by anti-type-2 monkey serum in HeLa cell tube-neutralization tests. Accordingly it may reasonably be concluded that an MEF-1 mutant forming very minute plaques was isolated, which will be designated *m* (minute). A brief summary of the study on the *m* mutants is given in this report (*2*).

The strain of HeLa cells employed, the preparation of parental MEF-1 virus stock, and the method of detection of inhibitory NBS were described previously (*1*). The results of a typical series (E-399) of such serial passages are shown in Fig. 1. A bottle culture of HeLa cells (8×10^6 cells) with 9 ml of medium YLA (Earle, 0.5 percent lactalbumin hydrolyzate and 0.1 percent Difco yeast extract), supplemented with 20-percent inhibitory NBS-137, was inoculated with 1 ml of undiluted MEF-1 stock (10^8 pfu/ml) and examined daily for cytopathogenic action.

As soon as cellular destruction was complete, culture fluid was harvested, and 1 ml of the harvested fluid was inoculated again into a new bottle culture of HeLa cells containing 40 percent NBS-137. The harvested culture fluids were assayed for plaques on HeLa cell plates, and a marked decrease of the titers of parental MEF-1 in successive harvested culture fluids was observed in the course of several serial transfers (Fig. 1). Furthermore it was found that very minute plaques (less than 0.5 mm in diameter) appeared after three passages; approximate titers of these *m* mutants in the culture fluid were 10^7 pfu/ml (Figs. 1 and 2). Similar titers (10^7 to $10^{7.5}$ $TCID_{50}$/ml) were obtained by titrations in HeLa cell tube cultures. Plaques produced by the *m* mutant were as small on monkey kidney cell plates as on HeLa cell plates. In another experiment (E-222), the *m* mutant was also obtained through serial transfers in HeLa cell cultures with medium YLA containing 2 percent noninhibitory horse serum.

It should be emphasized that, in the majority of such serial passages in the presence of inhibitory NBS, *i* mutants were selected, as previously described (*1*). Moreover, no *m* mutants of Mahoney strain have thus far been obtained, in spite of the large number (more than 25) of series of serial passages of this virus, and in spite of the sometimes apparently identical conditions under which they were carried out. Therefore, the factor, or factors, responsible for the selection of MEF-1 *m* mutants remains thus far unknown.

It is known that differences in plaque size exist among strains of polioviruses (*3*). However, none of the several other type 2 polioviruses maintained in our laboratory produce plaques of equally small size. It seems worthy of note that *m* mutants are indistinguishable from m^+ virus in HeLa cell cultures in growth rate, maximum titer attained, and cytopathogenic effect. Dulbecco (*4*) described a *d* (delayed) mutant of poliovirus type 1 (Brunhilde). The plaques of this line are indistinguishable from those of the wild type at *p*H 7.4, but they appear 2 days later at *p*H 6.8. Similar findings were also reported by Sabin (*5*).

In the case of *m* mutants, however, plaque formation is apparently independent of the *p*H of the agar overlay employed. Besides, the plaques of *m* mutants do not increase in size in the course of several days. Slow Mahoney, forming small plaques (2.37 to 2.66 mm) on monkey kidney cell monolayers, was obtained by Dubes (*3*) as a mutant of Fast Mahoney in HeLa cell culture. The similarity of this mutant to *m* mutant remains to be demonstrated. We have assumed that a substance, or substances, in the agar overlay inhibits in one way or another the formation of plaques of usual

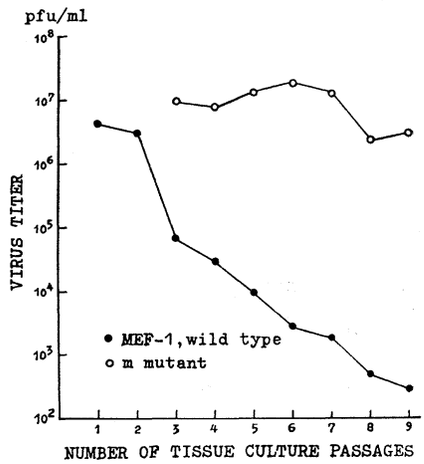

Fig. 1. Marked decrease of parental MEF-1 virus and appearance of *m* mutants in culture fluids during serial transfers of plaque-purified parental MEF-1 virus in HeLa cell cultures with medium YLA containing 20 to 40 percent inhibitory NBS-137.

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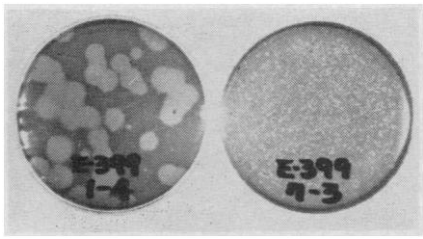

Fig. 2. Selection of MEF-1 *m* mutants in the course of the serial passage (E-399) of wild-type MEF-1 virus in HeLa cell cultures with medium YLA plus 20 to 40 percent NBS-137. Plate 1-4 was seeded with 10^{-4} dilution of first-passage fluid plaques of the MEF-1 wild-type virus. Plate 7-3 was seeded with 10^{-3} dilution of seventh-passage fluid plaques of MEF-1 *m* mutants. Both platings were made on the same lot of HeLa cell monolayers (55-mm petri dishes) with 10 percent horse serum agar overlay. The photograph was taken 5 days after the plates were seeded.

size by this particular mutant. This possibility is now being extensively studied, and the evidence thus far obtained seems to indicate clearly that *m* mutants have a unique sensitivity to the viral inhibitory activity of extract of agar overlay.

The *m* mutants have been compared with m^+ virus in virulence for mice by the intracerebral route of inoculation and have been found to be slightly less virulent than m^+ virus. The *m* mutants were stable through ten rapid serial passages in tissue cultures of both HeLa and monkey kidney cells. However it was found that a few larger plaques sometimes appeared on plates seeded with low dilutions (10^0 to 10^{-3}) of *m* mutants. Since the *m* mutants had been previously purified repeatedly by the limiting-dilution technique, it seems clear that the *m* virus gives rise to mutants of the m^+ type—that is, it reverts to wild type with respect to plaque size. Furthermore, the back mutant m^+ virus, after having been plaque-purified, was shown to have *i* character—that is, it is resistant to the inhibitory NBS used in the medium for serial transfers, and it produces large plaques on inhibitory NBS plates. Back mutations of other poliovirus mutants have also been noted by other investigators (*3, 4*).

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References and Notes

1. N. Takemori *et al.*, *Science* 125, 1196 (1957); *Virology*, in press.
2. This work was aided by a grant from the Asahi Shinbun. A full description of the details of these studies is in preparation.
3. G. R. Dubes, *Virology* 2, 284 (1956).
4. R. Dulbecco, *Ciba Foundation Symposium on The Nature of Viruses.* (Churchill, London, 1957), p. 147.
5. A. B. Sabin, *Bull. N.Y. Acad. Med.* 33, 17 (1957); *J. Am. Med. Assoc.* 162, 1589 (1956).

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Concentration of Ceruloplasmin in Plasma of Schizophrenic Patients

Akerfeldt has recently reported that N,N-dimethyl-*p*-phenylenediamine (DPP) is oxidized to red products more rapidly by serum from schizophrenic patients than by serum from healthy control subjects (*1, 2*). The oxidation of DPP is accelerated by ceruloplasmin (*1–4*), the plasma copper-protein, and inhibited by other substances in serum, including ascorbic acid (*1, 2*). Since measurement of the oxidation of DPP by serum may possibly have diagnostic value in mental disease (*1, 2*), and since ceruloplasmin is of prime importance in this oxidation, it is of interest to measure directly the concentration of ceruloplasmin in the serum, or plasma, of schizophrenic and healthy subjects. This report presents the results of such determinations in 20 schizophrenic and 21 control subjects.

The concentration of ceruloplasmin was measured spectrophotometrically (*3, 5, 6*). Blood was drawn from a fasting subject, and coagulation was prevented by the addition of 1.5 ml of 2.5-percent sodium citrate solution to each 10 ml of blood. The plasma was separated and clarified at 24,000 *g* in an International PR-2 centrifuge. After the optical density (O.D.) of the plasma had been measured at 610 mμ in a cuvette of 5-cm path length, the ceruloplasmin was decolorized by the addition of 0.1 ml of a 10-percent solution of sodium cyanide to the cuvette, which contained 2.7 ml of plasma. One hour later the optical density was again measured at 610 mμ. The difference in optical density

$$\Delta(\text{O.D.})_{610\ m\mu}^{5\ cm}$$

was divided by 0.0034 to obtain the ceruloplasmin concentration in milligrams per 100 milliliters (*3*). Measurement of the recovery of added ceruloplasmin to plasma has shown that this method is accurate to about ±4 percent (*6*).

All the schizophrenic patients studied were free from significant somatic disease and were hospitalized in either the New York State Psychiatric Institute or the Bronx Municipal Hospital Center. The diagnosis of schizophrenia was made by staff psychiatrists without knowledge of the results of this study. Some of the patients were undergoing insulin-coma therapy at the time blood was drawn, as shown in Table 1. The control subjects were healthy medical students, house officers, and other medical school and hospital personnel.

The results of the determinations in the 20 schizophrenic patients and the 21 control subjects are shown in Table 1. The distributions of the plasma ceruloplasmin concentrations in the two groups show a considerable overlap. There is a range of 19 to 37 mg/100 ml in the schizophrenic patients, with a mean value of 27.8 mg/100 ml, and a range of 16 to 33 mg/100 ml in the control subjects, with a mean value of 23.7 mg/100 ml. Only three of the patients have concentrations greater than the highest value shown by a control subject. Four of the 20 patients have ceruloplasmin concentrations below the mean value of the control group, and five of the 21 control subjects have values above the mean of the schizophrenic group.

These findings indicate that it would be hazardous to classify an individual into one of the two groups on the basis of his ceruloplasmin concentration. Furthermore, it is relevant to emphasize that plasma ceruloplasmin concentrations which are significantly higher than normal by the *t* test occur in such apparently unrelated clinical conditions as a variety of neurological diseases (*6*), liver disease (*6*), and pregnancy (*7*), and marked increases in the concentration of this protein have been reported in infection (*8*) and myocardial infarction (*9*). Therefore, although the slight difference in the mean value of plasma ceruloplasmin in the schizophrenic and healthy groups, 27.8 and 23.7 mg/100 ml, respectively, is statistically significant (*10*), we be-

Table 1. Concentration of ceruloplasmin in the plasma of 20 schizophrenic patients and 21 control subjects.

Schizophrenic patients		Control subjects	
Sex and age	Plasma ceruloplasmin (mg/100 ml)	Sex and age	Plasma ceruloplasmin (mg/100 ml)
F, 19	25	M, 33	33
M, 25	27*	F, 22	25
M, 42	34*	M, 34	31
F, 42	28*	M, 32	27
M, 34	27	F, 40	23
M, 19	19*	F, 22	29
M, 23	21	M, 32	16
M, 22	24*	F, 32	18
M, 38	31*	F, 22	24
M, 18	36*	F, 27	20
F, 30	30*	M, 28	29
M, 19	22	M, 35	25
M, 29	30*	M, 23	17
F, 41	29*	M, 22	32
M, 22	28	M, 25	18
F, 41	30	M, 24	25
F, 27	30	M, 23	23
F, 56	37	M, 22	24
F, 27	29	M, 27	16
M, 15	20	M, 23	26
		M, 25	16
Mean	27.8	Mean	23.7

* These patients were in insulin coma when blood was drawn.