ASSESSMENT OF CORRELATION BETWEEN CERTAIN IN VITRO POLIOVIRUS MARKERS AND MONKEY NEUROVIRULENCE

BY

VICTOR J. CABASSO, Sc.D.; ERWIN L. JUNGHER, D.V.M.; SEYMOUR LEVINE, Ph.D.;
ARDEN W. MOYER, Ph.D.; MANUEL ROCA-GARCIA, M.D.; and HERALD R. COX, Sc.D.

From the Viral and Rickettsial Research Section, Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y.

The availability of proved and stable laboratory assays other than the monkey test for determining the degree of virulence of polioviruses would indeed be of great value. Search for such assays has uncovered in recent years certain *in-vitro* characteristics, the so-called d, t, and MS "markers," which have been proposed as criteria of acceptability of poliovirus strains intended as live oral vaccines (Advisory Committee to the Surgeon-General, 1959). But already evidence suggests that at least the d marker is not as well correlated with monkey neurovirulence as was originally thought, and information accumulated about the MS character is as yet insufficient. Consequently, until the value of these markers is definitely established, the residual neurovirulence of a poliovirus strain for monkeys remains its most reliable marker and the most acceptable measure of its attenuation.

It is the purpose of this communication to present results of monkey tests on recent lots of Lederle oral poliovirus vaccine, to examine the degree of correlation between the findings and the "marker" pattern of the vaccine strains, and to discuss the relative validity of these *in-vitro* characters.

Results of Recent Monkey Tests on Lederle Oral Poliovirus Vaccine

As all known poliovirus vaccine strains have some degree of neuroactivity in monkeys when inoculated intraspinally, the present discussion of Lederle virus strains will be limited to results of intracerebral inoculation. Detailed monkey-test results have been reported (Cabasso *et al.*, 1960) on 8 type I, 11 type II, and 11 type III vaccine lots. The histological examination, however, was limited in these tests to the cervical and lumbar enlargements of the cord, as brains were not removed from inoculated animals.

With recent vaccine lots, the animals were submitted to a more complete histological scrutiny. The brain stem was dissected into three portions at approximate levels of the red nucleus and the abducens and hypoglossus nerves; the thalamus was examined for inoculation trauma. Only animals with a definite track in the thalamus were considered valid. The three portions of the brain stem—the thalamus and pre- and post-central gyri—as well as the upper, middle, and lower segments of the cervical and lumbar enlargements, were embedded in paraffin, orientation being maintained by ink marks.

In all, 41 sections per monkey were stained by the galloxyanin method and examined. Lesions were estimated according to Melnick's system (Melnick and Brennan, 1959) on a scale of + to + + + +. Although this system permitted immediate appraisal of lesions regarding location, distribution, severity, and relation to needle track, a formula was still required to evaluate

relative neurovirulence among vaccine lots. Such a formula would allow detection of any undue changes when successive lots of vaccine are produced from the same or different seed virus. Accordingly, criteria were established which were based primarily upon spread or extension of lesions from site of inoculation. These were expressed in grades 1 to 4 as shown in Table I.

TABLE I.—Formula for Evaluation of Relative Monkey Neurovirulence, or Degrees Thereof, After Intracerebral Inoculation

Grade	Criteria
0	.. No lesions or track lesion with local inflammatory reaction
1	.. Significant inflammatory and neuronal changes homolateral to track
2	.. Lesions in brain stem
3	.. Lesions in brain stem and spread to cervical or lumbar cord
4	.. Spread to all portions of the C.N.S.

Grade 0 was assigned to no lesions or to track lesions with local inflammatory reactions; 1 to significant inflammatory and neuronal changes homolateral to the track; 2 to lesions in brain stem; 3 to spread to cervical or lumbar cord; and grade 4 to spread to all portions of the central nervous system.

In the final evaluation of results, grades 1 and 2, which symbolized little or no spread from the site of inoculation, were considered not significant, and the relative neurovirulence of a vaccine lot was expressed as the sum of the percentage of animals showing grades 3 and 4. By this grading system it was found that, in consecutive lots involving the examination of a total of 574 monkeys, the average incidence of combined grades 3 and 4 was 21% for type I, 57% for type II, and 16% for type III. Thus, a baseline of normally expected neurovirulence for each type of Lederle poliovirus was established as a reference for recognizing a vaccine lot with increased activity in monkeys.

As summarized in Table II, 4 out of 12 tests performed on 10 type I lots of vaccines, three of seven tests on

TABLE II.—Paralytic Rates and Degrees of Histological Lesions of Lederle Strains of Poliovirus Vaccine in Successive Lots

	Test No.												Av.
	1	2	3	4	5	6	7	8	9	10	11	12	
Type I:													
Paralytic rate (%) ..	13	0	13	0	0	0	44	0	0	0	0	15	8
Histol. lesions (%)	32	5	21	0	10	10	50	10	6	13	26	50	21
Type II:													
Paralytic rate (%) ..	0	0	0	0	0	5	13						2
Histol. lesions (%)	45	54	30	70	53	85	65						57
Type III:													
Paralytic rate (%) ..	0	0	0	0	27	0	10	0	29	0			6
Histol. lesions (%)	5	50	0	0	36	16	25	20	20	0			16

Bold figures denote pools showing above-average neurovirulence. Brackets indicate results of duplicate tests on the same vaccine lot.

six type II lots, and five of ten tests on five type III lots were above average. It must be emphasized, however, that the animals were inoculated with very large doses of virus (from over 10^3 to over 10^7 TCD₅₀); that most reactions occurred at 10^7 and 10^6 levels except for type II virus; and that the paralyses or weaknesses observed were non-progressive and non-fatal. In contrast, with much smaller doses of virulent polioviruses, sometimes less than 10 TCD₅₀, close to 100% of animals not only showed severe histopathological changes but also developed progressive paralysis and died of the disease 5 to 10 days after inoculation.

Some variation was observed between two consecutive tests with the same lot of vaccine (Table II). For example, type I tests Nos. 2 and 3 were obtained at two different times on the same lot of virus, as were tests Nos. 7 and 8 on another lot. Similarly, type II, Nos. 5 and 6, and type III, Nos. 1 and 2, results were also obtained in duplicate tests on the same materials. No ready explanation can be advanced for these seeming discrepancies except that the physical condition of the monkeys in the different tests varied substantially.

The type II Lederle vaccine strain has always shown greater intracerebral activity in monkeys than the other two types. It must be emphasized again, however, that this activity manifests itself only in histopathological changes and not in paralysis or other clinical signs in the animals. It might well be that this particular type II strain owes its neuronophilic characteristic to its long intracerebral-passage history in mice and suckling hamsters (Moyer *et al.*, 1952; Roca-Garcia *et al.*, 1952; Cabasso *et al.*, 1952, 1954), and that in this respect it may be compared to the 17 D yellow-fever vaccine strain (Strode, 1951).

Paradoxically, however, this same type II virus seems to possess most of the other laboratory characteristics currently attributed to avirulent polioviruses as well as the lowest infectivity for the human intestinal tract. Of the three Lederle vaccine strains it is, as is indicated later, the one with the most favourable "marker" picture. It is also the one which results in the lowest conversion rates from seronegative to seropositive (65 to 75% in most instances as compared with 85 to 95% for types I and III). Furthermore, it has consistently shown the least spread from vaccines to either contact siblings or the community and, unlike Lederle types I and III strains, type II virus recovered from either vaccinees or their contacts has caused no paralysis in monkeys after intracerebral inoculation of stool suspensions containing up to $10^{5.3}$ TCD₅₀/ml.

In-vitro Marker Characterization of Lederle Poliovirus Vaccine Strains

The d, t, and MS characters of Lederle poliovirus strains in relation to their residual monkey neurovirulence are shown in Table III. The MS character

TABLE III.—"Marker" Characterization of Lederle Poliovirus Vaccine Strains in Relation to Their Residual Monkey Neurovirulence

Virus Type	Marker			Degree of Intracerebral Neurovirulence (%)
	d	t	MS*	
I	+	—	±	21
II	+	—	—	57
III	+	—	+	16

* MS values were obtained by tube-titration method rather than efficiency of plating.

was obtained by the tube-titration method rather than by efficiency of plating, and ratings of the three markers were made by criteria described by Melnick *et al.* (1959) and Kanda and Melnick (1959). All three viruses possessed the t— character, but they differed in their d and MS markers; these were d+ MS± for type I, d± MS— for type II, and d+ MS+ for type III. Type II virus, which has consistently produced the most histological change in monkeys, has a marker pattern most like that suggested for avirulent polioviruses.

Pertinent questions at this time are how truly representative of avirulence these markers are, and how stable they may be in relation to monkey neurovirulence. Correlation between these characters and monkey neurovirulence was initially established by the use of known attenuated and virulent poliovirus strains, but whether these characteristics were inevitable accessories of avirulence in all polioviruses, or only characteristic of the particular attenuated strains studied, with incidental or no relation to avirulence, remains to be determined. Obviously this aspect of markers can be evaluated only through extensive investigation of a whole range of natural and laboratory-induced poliovirus variants.

A discrepancy connected with the d marker was already apparent in the original published report on this character (Vogt *et al.*, 1957); type I SM 90 strain, which was judged attenuated by the neurovirulence test, possessed the d+ character associated with the more virulent strains used in the same study. Similarly, Hsiung and Melnick (1958), working with natural poliovirus isolates, or with viruses recovered from persons fed avirulent virus, found several d+ strains which were classified as attenuated by the monkey test. Furthermore, Sabin (1959) has reported that a laboratory-induced d+ variant of his type I vaccine strain remained as avirulent for monkeys as was the original d variant. More recently Yoshioka *et al.* (1959), in a study of polioviruses excreted by children fed Sabin's type I vaccine strain, or those isolated from flies trapped in the trial area, found that the d marker was not always in agreement with results of the monkey virulence tests.

A systematic study of the effect that induced variation of the d and t characters may have on the monkey virulence of an attenuated d— t— poliovirus was undertaken in our laboratory with the original Ls-c type I virus, for which we are indebted to Dr. Morris Schaeffer. The history of the virus sample received is summarized in Table IV.

In Dr. Schaeffer's laboratory the virulent Mahoney strain was successively passed 14 times intracerebrally in monkeys, twice in monkey-testis-tissue culture, six times in monkey skin graft in chicken embryos, 34 times in monkey testis, 11 times in monkey

TABLE IV.—History of Type I Ls-c Virus Obtained from Dr. Schaeffer

	Mahoney strain (vir.)	→14 Monkey passages (Ic)→
	2 Monkey testis passages→	
	6 Monkey skin graft in chick embryos→	
Schaeffer's Lab.	34 Monkey testis passages→	→11 Monkey skin passages→
	13 Monkey kidney passages→	
	4 Consecutive plaquings in monkey kidney monolayers→	
Lederle Lab.	Pool 410 Ls-c progeny of 4th plaquing used in present study (d— t—)	

skin, and, finally, 13 times in monkey-kidney-tissue culture. Upon receipt in our laboratory the 13th monkey-kidney-tissue culture passage was plaqued four consecutive times on monkey-kidney monolayers by the Dulbecco method (Dulbecco and Vogt, 1954); pool 410 Ls-c, used in this study, represented the progeny of the fourth plaquing. This pool clearly possessed the d-t- characters. Starting with pool 410, variants of various marker combinations were induced as indicated in the Diagram. A d+t- pool

Induction of variants of various marker combinations from d-t- Schaeffer's Ls-c type I poliovirus.

was obtained after three plaquings in monkey-kidney-tissue culture under low bicarbonate overlay. Three terminal dilution passages in monkey-kidney-tube cultures at 39° C., followed by three similar passages at 40° C., yielded a d+t+ variant from the d+t- pool. Finally, the d-t+ variant came directly from the d-t- pool, after successive passages, twice at 39° C. and twice more at 40° C. by the terminal dilution method.

Table V summarizes the results obtained by virulence tests in monkeys with the four pools of virus as well as the effect which variation of the d and t markers had on the MS marker. It can be observed that, so long as the t- marker remained unchanged, varying d- to d+ did not induce any change in the virulence of the virus for monkeys; no paralysis followed intramuscular or intracerebral inoculation of either d-t- or d+t- variants, and histological lesions were only of grades 1 or 2 as defined earlier.

In contrast, changing the t- character to t+ was accompanied by reappearance of monkey virulence by both intramuscular and intracerebral routes. Similarly, histological lesions were more intense.

TABLE V.—Monkey Test Results and MS Character of Various d and t Variants of Schaeffer's d-t- Ls-c Type I Poliovirus

	Variant			
	d-t-	d+t-	d-t+	d+t+
TCD ₅₀ /ml. ..	7.5	7.5	6.5	7.5
MS character ..	MS-	MS-	MS-	MS-
Paralytic ratio:				
I.M., und. ..	0/4	0/4	3/4	5/5
I.C., und. ..	0/4	0/4	2/4	3/3
I.C. 10 ⁻¹ ..	0/4	0/4	2/4	4/4
Histol. ratio:				
I.M., und. ..	3/4*	1/4*	3/4	5/5
I.C., und. ..	0/4	2/4*	4/4	3/3
I.C. 10 ⁻¹ ..	0/4	3/4*	4/4	4/4

* Lumbar lesions only.

Variation of either the d or t characters had no effect on the MS marker, which remained unchanged in all three induced variants.

Thus, in confirmation of the findings of others, it appears that the presence of a d+ marker in a poliovirus strain is not necessarily synonymous with virulence for monkeys.

As regards the t marker, however, the finding by Lwoff and Lwoff (1959), that selection of t+ variants from an originally t- strain was accompanied by reappearance of monkey virulence, implied a closer correlation between the t marker and neurovirulence than is the case for the d character. Our results support this implication. It may be, as intimated by Lwoff and Lwoff, that this correlation is due to the relative multiplication rates of "cold" or "hot" virus variants within the central nervous system of the monkey, whose normal body temperature varies between 39° and 40° C. In this regard, it may be interesting to speculate on what the results might be if Pasteur's classic experimental method with anthrax in chickens were applied to an avirulent t- poliovirus strain in monkeys. Would the behaviour of the virus remain the same in an animal whose body temperature was lowered to that optimal for virus multiplication? Be that as it may, as yet insufficient investigation of field isolates has been reported to be sure that a t+ character is invariably associated with neurovirulence, and some exceptions have already been pointed out. For example, a mouse-selected derivative of type II MEF₁ virus which lost its neurovirulence for monkeys was shown by Sabin (1959) to be still capable of growing readily at 39° and 40° C., perhaps even better than a fully virulent type I Mahoney strain. Also, in at least one instance a d+t+ type I isolate from children fed Sabin's Ls-c strain was found by Yoshioka *et al.* (1959) to be still avirulent for monkeys by the intracerebral route. On the other hand, Melnick and Benyesh-Melnick (1960), studying viruses excreted by children vaccinated with Sabin's strain of virus, obtained evidence that excreted virus which became of increased neurovirulence for monkeys could still retain the t- character.

Information available on the MS marker is based primarily upon laboratory studies with a limited number of strains, and little can be said at this time about its correlation with monkey virulence. However, it should be pointed out that in the original study by Kanda and Melnick (1959) the correlation did not hold for a virulent type III strain (Saukett) which possessed an MS instead of the MS+ character.

It would be of interest to study induced MS+ variants from known attenuated MS strains to determine whether associated changes occur in their monkey virulence or other markers. Such an attempt is now in progress.

Discussion

No one questions the necessity for thorough laboratory testing of poliovirus strains intended for use in human immunization, by methods which genuinely portray the safety of these strains. But it should be stressed at this point that, in considering the acceptance of a vaccine strain, the laboratory criteria employed must be consonant with the behaviour of the strain in the ultimate host, and that laboratory and field findings supplement one another in the characterization of the agent. For example, 17 D yellow-fever virus has a long history of proved safety in man, although

still capable of paralysing and killing up to 20% of monkeys inoculated intracerebrally, and of provoking central-nervous-system changes in the rest.

No doubt the d, t, and MS markers do represent useful investigational tools of polioviruses. But it is important to recognize our ignorance concerning the basic mechanisms involved in neurovirulence and the degree to which these various markers reflect this mechanism. The correlation of these markers with neuroactivity is imperfect, indicating that other and unknown factors also play a part in neurovirulence. This is in all probability to be expected, since the growth of a virus within a cell is dependent not only upon the environmental conditions outside the cell, as pH and temperature, but also upon the presence of proper receptors on the cell surface and perhaps an entire battery of as yet unknown enzymes necessary for initiating and carrying out the complex operation of virus synthesis and release. It seems, therefore, that at this stage of our knowledge one is hardly justified in considering as indicators of avirulence unstable secondary characteristics of as yet unproved significance, when the direct method of assay in monkeys is readily available and accords well with the field behaviour of the strains in the human host.

Summary

Baselines of normally expected neuroactivity for each type of Lederle attenuated polioviruses in consecutive lots of vaccine were established by grading on a scale of 1 to 4 the spread of lesions from the site of inoculation in 574 monkeys inoculated intracerebrally with consecutive lots of vaccine. Relative neuroactivity of a vaccine lot was expressed as the sum of the percentage of animals showing grades 3 and 4.

The average incidence of combined grades 3 and 4 was 21% for type I, 57% for type II, and 16% for type III virus. The greater intracerebral activity of type II virus, which expresses itself in histopathological lesions and not in paralysis, is in marked contrast to its clinical behaviour in man, where it shows the least "aggressiveness" of the three poliovirus strains, and to its marker pattern, which conforms most closely to the one proposed for avirulence.

All three Lederle poliovirus strains possess the t-character, which appears to have the best correlation with monkey avirulence, but differ in their d and MS markers; type I was found to be d+MS±; type II was d±MS-; and type III was d+MS+. Some of the discrepancies in the correlation between markers and monkey virulence are discussed.

Since the value of d, t, and MS markers as criteria of attenuation for polio vaccine viruses has not been definitely established, residual neurovirulence for monkeys remains the most acceptable measure of strain attenuation.

REFERENCES

- Advisory Committee to the Surgeon-General (1959). Public Health Service. 2nd Report of the Committee on Live Poliovirus Vaccine.
- Cabasso, V. J., Jervis, G. A., Moyer, A. W., Roca-Garcia, M., Orsi, E. V., and Cox, H. R. (1960). *Brit. med. J.*, **1**, 373.
- Stebbins, M. R., and Cox, H. R. (1954). *Proc. Soc. exp. Biol. (N.Y.)*, **85**, 167.
- Dutcher, R. M., Moyer, A. W., and Cox, H. R. (1952). *Ibid.*, **81**, 525.
- Dulbecco, R., and Vogt, M. (1954). *J. exp. Med.*, **99**, 167.
- Hsiung, G. D., and Melnick, J. L. (1958). *J. Immunol.*, **80**, 282.
- Kanda, Y., and Melnick, J. L. (1959). *J. exp. Med.*, **109**, 9.
- Lwoff, A., and Lwoff, M. (1959). *C.R. Acad. Sci. (Paris)*, **248**, 154.
- Melnick, J. L., and Benyesh-Melnick, M. (1960). *Fed. Proc.*, **19**, 407.
- and Brennan, J. C. (1959). *J. Amer. med. Ass.*, **171**, 1165.
- and Brennan, J. C. (1959). First International Conference on Live Poliovirus Vaccines, June 22-26, 1959, pp. 65-101. Pan American Sanitary Bureau Sci. Pub., No. 44.
- Moyer, A. W., Accorti, C., and Cox, H. R. (1952). *Proc. Soc. exp. Biol. (N.Y.)*, **81**, 513.
- Roca-Garcia, M., Moyer, A. W., and Cox, H. R. (1952). *Ibid.*, **81**, 519.
- Sabin, A. B. (1959). First International Conference on Live Poliovirus Vaccines, June 22-26, 1959, pp. 14-33. Pan American Sanitary Bureau Sci. Pub., No. 44.
- Strode, G. K. (1951). *Yellow Fever*. McGraw-Hill, New York.
- Vogt, M., Dulbecco, R., and Wenner, H. A. (1957). *Virology*, **4**, 141.
- Yoshioka, I., Riordan, J. T., and Horstmann, D. M. (1959). *Proc. Soc. exp. Biol. (N.Y.)*, **102**, 342.

VENTRICULAR FIBRILLATION DURING LEFT ATRIAL PUNCTURE RECOVERY AFTER EMERGENCY MITRAL VALVOTOMY

BY

C. BISHOP, M.B., B.S., D.A.

M. HONEY, M.B., M.R.C.P.

AND

D. G. TAYLOR, M.B., F.R.C.S.

From the Anaesthetic, Cardiac, and Thoracic Surgical
Departments, Guy's Hospital, London

The technique of percutaneous left atrial puncture introduced by Björk *et al.* (1953) has been widely used in the investigation of mitral valve disease and is a routine method at this hospital (Hancock, 1959a). The case reported here developed ventricular fibrillation during the puncture. Immediate cardiac massage was followed by mitral valvotomy and the patient ultimately made a complete recovery.

Case Report

A married woman aged 37, who gave no rheumatic history, complained of increasing breathlessness on exertion since her first pregnancy five years ago, when mitral stenosis was diagnosed. She had had attacks of bronchitis during the previous three winters, with haemoptyses on the last occasion.

On examination the pulse was regular and there were the physical signs of mitral stenosis, with a soft early diastolic murmur at the lower left sternal border, attributed to slight aortic incompetence. The blood-pressure was 110/60. There was no evidence of systemic or pulmonary venous congestion.

A chest radiograph showed no cardiac enlargement, pulmonary venous congestion, or interlobular septal lines. Screening showed slight left atrial and right ventricular enlargement. An electrocardiogram showed P mitrale, slight right axis deviation, and right ventricular hypertrophy.

Left atrial puncture was undertaken to assess the severity of the mitral stenosis, as doubts existed whether the symptoms were due to the valve lesion or to pulmonary disease.

Left Atrial Puncture

Premedication: papaveretum $\frac{1}{2}$ gr. (20 mg.), hyoscine 1/150 gr. (0.43 mg.). Local analgesia with 2% lignocaine (without adrenaline). A No. 16 thin-walled cannula with