

STUDIES ON POLIOMYELITIS VIRUS

II. THE IMMUNOGENIC POTENCY OF LIVE AND
INACTIVATED VIRUS IN GUINEA-PIGS

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INTRODUCTION

The purpose of the experiments reported here was to determine the immunizing power in guinea-pigs of live poliomyelitis virus and to determine what loss of antigenicity was involved in the inactivation of virus by formalin and by ultra-violet light. Much discussion has centred about the inactivation characteristics of poliomyelitis virus by formaldehyde in that, with increasing concentration of formaldehyde first the viability is destroyed and later the antigenicity.

The view has been expressed that with the low concentration of formaldehyde used in the manufacture of poliomyelitis vaccine the virus is not completely inactivated, but is so modified that it is capable of limited multiplication after injection into a susceptible animal. We have attempted to elucidate this problem by the use of virus preparations concentrated by the method previously described (Polson & Hampton, 1957). These concentrates were also used for preliminary studies of the effect of ultra-violet light on infectivity and antigenicity.

MATERIALS AND METHODS

(a) Virus suspensions

The concentrated virus suspensions prepared by the method previously described (Polson & Hampton, 1957) formed the material for these experiments. This material without further purification is referred to as 'crude concentrated virus'; it contained 4.4% protein which was almost entirely non-viral. To free the crude suspensions of extraneous protein they were subjected to two cycles of ultra-centrifugation at $66,000 \times g$. for 90 min. with resuspension in M/15 phosphate buffer of pH 7.0. The strains used were: Type 1, Brunhilde; Type 2, Collins; Type 3, Leon.

(b) Mixed virus

Aliquots of the three types were diluted with buffer and mixed in such a way that 1 ml. contained 10^9 T.C.I.D.₅₀ of each. This is referred to as mixed concentrated virus and was not further purified.

(c) Concentrated vaccine

This was prepared by the same method as that used for the live virus suspensions from a batch of commercially produced Salk-type vaccine which was rejected on account of incorrect filtration. The vaccine was concentrated a 1000-fold and used

without further purification, and without further testing for live virus beyond that which it had received in the course of its manufacture. The concentration of formaldehyde used in the inactivation of the virus was 1:10,000 and the reaction was allowed to proceed for 12 days at 37° C.

(d) *Inactivation by excess of formaldehyde*

A portion of purified concentrated virus was treated with formaldehyde at a final concentration of 0.2%. After 6 days at 37° C. the formaldehyde was neutralized with sodium bisulphite and the suspension used immediately.

(e) *Inactivation by ultra-violet light*

For these experiments a portion of mixed concentrated virus at 10^9 T.C.I.D.₅₀/ml. was irradiated in a rocking Petri dish situated 60 cm. from a naked 'Hanovia' high-pressure mercury lamp (The General Electric Co., Wembley, England). The depth of fluid was 1.91 mm. when the dish was stationary. Small aliquots were removed from the dish at intervals and titrated in tissue culture. A predicted complete inactivation could be determined from the graph plotted from these results, at 45 min. The rate of inactivation was found to be linear. Samples of the same material were then irradiated for periods of 45, 90 and 135 min. This amount of irradiation was sufficient to inactivate $10^{9.5}$, $10^{19.0}$ and $10^{28.5}$ T.C.I.D.₅₀/ml. of virus, respectively. The sample taken after 45 min. irradiation showed living virus in one out of three tissue-culture tubes receiving undiluted inoculum.

(f) *Antigenicity tests*

The method of Gard (see Gard, Wesslen, Fagraeus, Svedmyr & Olin, 1952) was adhered to, with the exception that larger animals of 300–400 g. weight were used. Five animals were used for each dose of virus tested in each experiment. The animals were bled by heart puncture. All sera were inactivated at 56° C. for 30 min. Most of the sera were tested for neutralizing antibody by mixing with an estimated 100 T.C.I.D.₅₀ of virus, and after incubation at 37° C. for 60 min. inoculated into roller-tube cultures of trypsinized monkey-kidney cells. The tubes were subsequently examined at regular intervals for the development of cytopathogenic effect. The remainder of the sera were tested by the colour-change method of Salk, Youngner & Ward (1954) against a test dose of virus which was accurately determined. The method used is stated in the tables of results. Most of the sera were tested twice, each time in triplicate.

(g) *Titrations*

All titrations of live virus were carried out by roller-tube tissue culture of trypsinized monkey-kidney cells.

RESULTS

Results are shown in Tables 1–3. In all cases the sera containing antibodies after immunization are given as a percentage of all sera tested in each group. After the initial tests were done it was realized that under the conditions of the experiments even small variations in the test dose of virus were significant. Ideally all sera

should have been tested at the same time with the same test virus. This, however, was impracticable and reliance had therefore to be placed on titration of the virus with each batch of tests.

Table 1. *A comparison of the antigenicity in guinea-pigs of live virus and of concentrated Salk-type vaccine*

(The strength of the test virus is shown in brackets.)

| | % of protective sera | | |
|---|----------------------------------|---------------------|--------------------------------------|
| | Type 1 | Type 2 | Type 3 |
| A. Mixed concentrated live virus | | | |
| Inoculum of $10^{8.5}$ T.C.I.D. ₅₀ /ml. | 80 (10^2) | 40 (10^2) | 62.5 ($10^{1.0}$)* |
| Inoculum of $10^{6.5}$ T.C.I.D. ₅₀ /ml. | 40 (10^2) | 100 ($10^{0.8}$)* | 80 ($10^{1.0}$)*, 0 ($10^{2.0}$) |
| Inoculum of $10^{4.5}$ T.C.I.D. ₅₀ /ml. | 0 (10^2), 55 ($10^{0.5}$)* | 0 (10^2) | 0 ($10^{2.0}$) |
| B. Concentrated vaccine | | | |
| Inoculum of $10^{8.5}$ T.C.I.D. ₅₀ /ml.† | 15 ($10^{2.0}$) | 31 ($10^{2.0}$) | 80 ($10^{1.0}$)*, 0 ($10^{2.0}$) |
| Inoculum of $10^{6.5}$ T.C.I.D. ₅₀ /ml. | 5 ($10^{2.0}$) | 35 ($10^{2.0}$) | 60 ($10^{1.0}$)*, 0 ($10^{2.0}$) |
| Inoculum of $10^{4.5}$ T.C.I.D. ₅₀ /ml. | 25 ($10^{1.5}$)* | 50 ($10^{0.8}$)* | 12.5 ($10^{1.3}$)* |

* Tests carried out by colour-change method, remainder by roller tube.

† Estimated.

Table 2. *The effect of inactivation by means of ultra-violet light ('Hanovia' high-pressure mercury lamp, unfiltered) on the antigenicity in guinea-pigs of live concentrated virus and of concentrated vaccine*

(The strength of test virus is shown in brackets.)

| | % of protective sera | | |
|--|----------------------|-------------------------|---------------------|
| | Type 1 | Type 2 | Type 3 |
| A. Irradiated crude concentrate of live virus | | | |
| (i) $10^{28.5}$ inactivation dose. Inoculum of $10^{8.5}$ T.C.I.D. ₅₀ /ml. | 0 ($10^{2.0}$) | 0 ($10^{2.0}$) | 0 ($10^{2.0}$) |
| (ii) 10^{19} inactivation dose. Inoculum of $10^{9.5}$ T.C.I.D. ₅₀ /ml.* | 0 ($10^{2.0}$) | — | — |
| (iii) $10^{9.5}$ inactivation dose. Inoculum of $10^{8.5}$ T.C.I.D. ₅₀ /ml.* | 0 ($10^{2.0}$) | Trace† 0 ($10^{2.0}$) | — |
| B. Irradiated vaccine concentrate | | | |
| $10^{9.5}$ inactivation dose. Inoculum of $10^{9.5}$ T.C.I.D. ₅₀ ‡ | 0 ($10^{2.0}$) | 100 ($10^{0.8}$)§ | 100 ($10^{1.3}$)§ |

* Sera remained negative after four inoculations.

† One out of ten sera gave a positive result when tested against very weak virus.

‡ Estimated.

§ Test carried out by colour-change method. Remainder by roller tube.

— Not done.

Table 3. *The effect of formaldehyde at 0.2 % upon the antigenicity of partially purified Type 1 virus in guinea-pigs*
(Strength of test virus = $10^{8.0}$.)

| | % of protective sera |
|---|----------------------|
| A. Partially purified live concentrated virus | |
| Inoculum of $10^{6.5}$ T.C.I.D. ₅₀ /ml. | 60 |
| Inoculum of $10^{4.5}$ T.C.I.D. ₅₀ /ml. | 0 |
| B. Partially purified virus inactivated with 0.2 % formaldehyde | |
| Inoculum of $10^{8.5}$ T.C.I.D. ₅₀ /ml. | 0 |
| Inoculum of $10^{8.5}$ T.C.I.D. ₅₀ /ml. | 0 |
| Inoculum of $10^{6.5}$ T.C.I.D. ₅₀ /ml. | 0 |

DISCUSSION

The non-susceptibility of the guinea-pig to poliomyelitis virus has been established in that an inoculum of less than $10^{4.5}$ T.C.I.D.₅₀/ml. of living virus produced little or no antibody. This has since been confirmed by Kelly & Dalldorf (1956). The antigenic potency of the strains of live virus used in these experiments was greatest with Type 1 which gave a 50 % neutralization of 100 T.C.I.D.₅₀ at $10^{7.05}$ T.C.I.D.₅₀/ml. of antigen, followed by Type 2 which gave less than 50 % neutralization of 100 T.C.I.D.₅₀ at a concentration of antigen of $10^{8.5}$, and finally by Type 3 which gave about 63 % neutralization of 10^1 T.C.I.D.₅₀/ml. test virus after an inoculum of $10^{8.5}$ live virus. This seemed a poor response and did not compare favourably with the results given by the author of this method. Similar results were obtained in other experiments not described here. The approximate weight of antigen can be calculated on the following assumptions: that the diameter of the virus is $30 \text{ m}\mu$, its density is 1.33, that no appreciable quantity of antigen other than such virus particles was present, and that 1 T.C.I.D.₅₀ = 5000 particles. On these assumptions two doses of 0.2 ml. each of a suspension containing 10^7 T.C.I.D.₅₀/ml. would contain about $4.0 \times 10^{-2} \mu\text{g.}$ of virus. Thus, for Type 1 virus in these experiments a 50 % response was obtained with about $0.4 \mu\text{g.}$ of antigen, for Type 2 with about $4.0 \mu\text{g.}$ and for Type 3 about $400 \mu\text{g.}$ would have been necessary. This is a very small quantity of antigen and amounts to about 1/40 of the amount of purified tetanus toxoid, for example, which will protect 50 % of mice against a challenge of 10 M.L.D. of toxin (1 Lf. = $1.41 \mu\text{g.}$) though the latter is given in one dose (Largier, personal communication).

The results obtained with concentrated formalinized vaccine (Table 1) compare very favourably with those obtained with live virus, only Type 1 virus having lost an appreciable amount of antigenicity during the formalinization. This may well vary with different strains. In our experience the Leon strain of Type 3 virus has often given anomalous results and we do not attach any significance to the differences shown in Table 1.

The results obtained with ultra-violet inactivated virus were singularly disappointing in view of more encouraging results obtained by other workers. The object of the experiments were, however, to determine the maximum dose of ultra-violet radiation that could be given without destruction of antigen. As a naked

lamp was used which was subsequently found to have a relatively weak emission in the 253.7 m μ region, and which was unable to inactivate virus when all other bands were filtered out by means of the method of Kasha (1948), it seems probable that the virus used in these experiments was inactivated by other wavelengths. The study of virus suspensions inactivated by monochromatic ultra-violet is being continued and will be reported later.

It will be seen from the results in Table 2 that concentrated vaccine given a similar inactivating dose of ultra-violet light to that received by the live virus was still antigenic. Whilst this may be a fortuitous finding the phenomenon has been encountered in subsequent experiments, and it seems of sufficient importance to warrant further investigation.

The object of the experiment, the results of which are set out in Table 3, was to study the effect of higher concentrations of formaldehyde and thereby perhaps help clarify the reason why low concentrations are required for effective vaccine manufacture. Formalized antigens of other kinds (toxoids, bacterial vaccines, etc.) are commonly prepared by treatment with 0.2% formaldehyde. There was present in our crude concentrated virus suspensions a thousand times as much virus as is usually present in vaccine. This was inactivated by only twenty times as much formaldehyde as is used in polio vaccine manufacture (1:4000 formalin). As such a concentration of formaldehyde completely destroyed the antigenicity it becomes obvious that successful inactivation of poliovirus is a function of concentration and not of relative amount of formaldehyde. Some light has been thrown on the nature of the groups reacting by Cartwright, Ritchie & Lauffer (1956), and Fraenkel-Conrat & Mecham (1949) in studies on the reaction of formaldehyde with tobacco mosaic virus. It would appear that the critical concentration of formaldehyde to ensure inactivation, but to just prevent destruction of antigen, is much higher than is commonly supposed, as Morgan, Howe & Bodian (1947) obtained negative results with 0.13% formaldehyde, while Dick, Schwerdt, Huber, Sharpless & Howe (1951) recommended the use of 0.1% formaldehyde (at 25° C. for 7 days). It would also appear that formaldehyde at low concentration (1:10,000) must combine with a virus group which is concerned with viability while at a higher concentration (1:500) different groups or centres are combined which are associated with antigenicity.

SUMMARY

The antigenic response of guinea-pigs to live poliomyelitis viruses of all three types has been studied and the loss of antigenicity involved in inactivation by formaldehyde and by ultra-violet light have been determined.

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