Preparation of Formaldehyde-inactivated Poliomyelitis Virus and Its Use as an Immunizing Agent in Cotton Rats[1]

HUBERT S. LORING, C. E. SCHWERDT, NANCY LAWRENCE, and JANE COLLIER ANDERSON

Department of Chemistry and *School of Medicine, Stanford University, California*

The inactivation of relatively crude preparations of poliomyelitis virus by formaldehyde treatment and by ultraviolet irradiation has resulted in samples capable of producing a significant immunity against an intracerebral challenge of active virus. The extent to which such immunity has depended on the presence of humoral antibodies or of virus-resistant nervous tissue is not known, but the presence of neutralizing antibodies in the blood after immunization has been demonstrated (*1*). The vaccines used in such experiments have consisted of extracts of finely-ground brain and spinal cord of infected monkeys or mice and contained relatively small quantities of virus and large quantities of normal tissue components.

The use of such crude vaccines is objectionable for several reasons. It is likely that higher concentrations of formaldehyde would be required to produce complete inactivation because of the protective colloid action of the normal proteins on virus and because of competitive reactions between the two for formaldehyde. As formaldehyde concentration is increased, it is also likely that not only virus activity but antigenicity would be lost. A further objection to the use of crude preparations is the large mass of tissue proteins which must be injected in order to provide a sufficient quantity of inactive virus to stimulate an appreciable antibody response. If an inactive-virus antigen free from the normal tissue components were available, it is possible that a much greater response could be elicited and a higher degree of immunity achieved.

The development of a method of preparation of concentrated Lansing virus in moderate quantities (*2*) has made possible the study of the inactivation of virus relatively free from normal tissue constituents and the use of such a material for immunization against active virus. Lansing virus produced in cotton rats and purified by two cycles of differential ultracentrifugation was treated with formaldehyde in concentrations from 0.02 to 0.1 per cent by volume. The concentration of virus used varied from 3×10^{-4} to 4×10^{-4} grams of nitrogen/ml. The mixture of virus and formaldehyde in M/15 phosphate buffer at pH 7 was stored in the refrigerator and its infectivity in comparison with the original untreated virus determined after different time intervals. Infectivity of each preparation was measured by the intracerebral injection of six 10-fold dilutions in six groups of five young cotton rats each. A volume of 0.05 ml. was used for each animal, and the specific infectivity was calculated as the 50 per cent infective dose, or ID_{50} in grams of virus nitrogen/ml. (*2, 3*). The results of five experiments with three different formaldehyde concentrations are shown in Table 1.

The results show that at refrigerator temperatures the Lansing virus is completely inactivated by formaldehyde only when a concentration of 0.1 per cent is used and the virus and formaldehyde mixtures are allowed to stand for somewhat longer than 12 days. After four days of contact with 0.1 per cent formaldehyde, one treated virus sample was completely inactive, and after 12 days another showed only a trace of activity (less than 0.0002 of one per cent). In the latter experiment no activity could be detected when the mixture had stood for an additional 38 days. In contrast to concentrated influenza virus, the Lansing virus shows an appreciably greater resistance to inactivation by formaldehyde (*4*), a result in agreement with its recognized greater stability.

TABLE 1

ACTIVITIES OF ORIGINAL VIRUS AND OF VIRUS FORMALDEHYDE MIXTURES AFTER STANDING IN REFRIGERATOR FOR VARYING LENGTHS OF TIME

Expt. No.	Concentration of formaldehyde (%)	Specific activity as grams nitrogen/ml.* after: 4 days		12 days		50 days
		Original virus	Treated virus	Original virus	Treated virus	Treated virus
86	0.02	$10^{-9.7}$	$10^{-8.7}$	$10^{-9.0}$	$10^{-4.9}$	
88	0.05	$10^{-8.9}$	$10^{-7.1}$			
89-90	0.05			$10^{-9.7}$	$10^{-7.0}$	
87	0.10	$10^{-8.3}$	Completely inactive at $10^{-3.7}$			
91-2-4	0.10			$10^{-11.7}$	$10^{-4.0}$†	Completely inactive at $10^{-3.4}$

* Calculated by the method of Reed and Muench (*3*).

† At the highest concentration used, $10^{-3.7}$ grams nitrogen/ml., two out of three rats developed poliomyelitis. No animals showed positive signs at lower concentrations.

In the early experiments on the ability of the formaldehyde-inactivated virus to immunize cotton rats, the intraperitoneal route of injection was used. In one such experiment, in which 32 young cotton rats were immunized by two intraperitoneal injections containing a total of 3×10^{-6} grams of completely inactive virus nitrogen (Experiment 87, 0.1 per cent formaldehyde after 4 days), 18 per cent of the test animals survived a challenge of 100 ID_{50} doses given four days after the last injection. When 9 control animals were challenged with the same dose of virus, 1 was found dead after four days without previously recognized signs of the disease and 8 developed typical poliomyelitis.

More significant results were found when the rats were im-

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munized by three injections given at weekly intervals by a combination of the intradermal, intramuscular, and subcutaneous routes. A total of 10^{-5} grams of formalized virus nitrogen (Experiment 91-2-4 after 12 days) was administered to each of 30 rats in three doses as follows: $10^{-5.6}$ grams of nitrogen intradermally, $10^{-5.3}$ grams of nitrogen intramuscularly a week later, and $10^{-5.6}$ grams of nitrogen subcutaneously at the end of another week. A similar group of animals was immunized by the same method with 10^{-5} grams of nitrogen of the same vaccine in a petroleum oil suspension.

The degree of immunity present in the two groups was estimated by finding the titer of active virus in each in comparison with that of normal control animals of the same age. Concentrations of virus were prepared in steps of 10 from 2×10^{-10} to 2×10^{-5} grams of nitrogen/ml. Each concentration was then injected intracerebrally in a volume of 0.05 ml. into each of five animals from the two immunized groups one week after the last vaccine injection. The control animals were tested similarly with concentrations of virus ranging from 2×10^{-12} to 2×10^{-7} grams of nitrogen/ml. The titer of virus used was calculated from the control animals and, expressed as the ID_{50} dose, was found to be $10^{-11.7}$ grams of nitrogen/ml. Similar titers calculated for the two immune groups were $10^{-6.2}$ for the first mentioned and $10^{-6.5}$ for the group injected with vaccine in oil emulsion.

It is evident from these titers that over 300,000 times as much virus was required to infect 50 per cent of the first group of vaccinated animals as was found necessary for the normal controls. Similarly, in the second group, 150,000 times as much virus was required.

The survivors from both groups were pooled and rechallenged by the same method $10\frac{1}{2}$ weeks after the first challenge. The titer found with the immunized animals as compared with that of their normal controls showed that about 500 times as much virus was now required to infect 50 per cent of the immunized group.

It is possible that the high degree of immunity produced in the experiment cited above was due to the presence of the trace of active virus found in this preparation. Further immunization experiments with the vaccine which showed no virus activity were carried out to test this question. Twenty-four rats immunized with the same amount of completely inactive vaccine by the three routes previously used were each challenged intracerebrally one week after the last injection with about 1,000 ID_{50} doses of active virus. One of the group developed paralysis but recovered, while the remaining 23 showed no signs of the disease. In a similar number of normal control animals receiving the same dose of virus 83 per cent developed poliomyelitis. It is likely, therefore, that the extent of immunity produced by the vaccine after 12 days of exposure to formaldehyde was due to the inactive virus rather than to the traces of activity present.

The 24 survivors from the last-mentioned experiment were rechallenged with 10,000 ID_{50} doses of virus three weeks after the first challenge. A high degree of immunity was still present, as shown by the fact that 67 per cent of the animals survived without showing signs of poliomyelitis as compared to 92 per cent of a similar group of normal controls which developed the disease when injected with the same amount of virus.

The results presented show that cocenntrated Lansing virus from cotton rats can be completely inactivated by formaldehyde and that such inactive virus can be used to produce a high degree of immunity toward the homologous virus. The data show that infectivity as such is not a prerequisite for a vaccine which, in amounts of 10^{-5} grams of concentrated virus nitrogen, produces a highly significant protection against the intracerebral injection of active virus. Whether protection is obtained by neutralization of virus at the site of injection by humoral antibodies or by some other mechanism is not known. The presence of neutralizing antibodies in high titers in the blood of the immunized rats has been demonstrated both by neutralization tests and by the complement fixation test. These results will be presented in an early publication.

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Filaricidal Activity of Substituted Phenyl Arsenoxides[1]

G. F. Otto and T. H. Maren[2]

Department of Parasitology,
School of Hygiene and Public Health,
The Johns Hopkins University, Baltimore, Maryland

In the treatment of both canine and human filariasis antimony-containing compounds have been used to the exclusion of almost all other types of therapy. Accordingly, early in our search for an improved form of therapy considerable attention was given to new or hitherto unexplored antimony compounds. The results were disappointing in that we failed to obtain evidence that antimony in therapeutically feasible doses would ever regularly kill the adult filaria, *Litomosoides carinii*, of cotton rats or *Dirofilaria immitis* of dogs. We therefore intensified our search for an entirely different type of therapeutic agent. Consideration was given to the possible advantages of nonmetallic compounds, but in so far as our investigations were carried no such compound was found which appeared to offer any advantages over the antimonials.[3] However, preliminary screening revealed that Mapharsen had an *in vitro* microfilaricidal activity far greater than anything hitherto reported. This led to a study not only of Mapharsen itself but of the whole group of available substituted phenyl arsenoxides.

The attempt to use arsenic in the treatment of filariasis is not new. Among the more recent advocates of its possible value in the therapy of filariasis are King (*4*) and Van der Sar and Hartz (*8*), who obtained evidence of clinical improvement without destruction of the microfilaria following the use of

[1] This work was carried on initially under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and The Johns Hopkins University; it was continued under a contract between the Office of the Surgeon General, U.S.A., and the same university.

[2] Now in the Department of Pharmacology and Experimental Therapeutics, School of Medicine, The Johns Hopkins University.

[3] Welch, *et al.* (*9*) have since reported favorable results with cyanines in laboratory animals.