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Laboratory Studies in Connection with the Evaluation of Poliomyelitis Vaccine, Toronto, 1955[1]

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IT has been realized since the original field trial in 1954 that estimates of the prophylatic efficacy of formalinized poliomyelitis vaccine must be based on the results of virological studies of patients. Many virus diseases closely simulate poliomyelitis, especially the nonparalytic variety. In our experience in Toronto over the past several years, recently reviewed by Rhodes and Beale (6), virus studies suggested that the aetiology of 96 cases of aseptic meningitis ("nonparalytic poliomyelitis") was as follows: poliomyelitis, 19%; Coxsackie B infection, 24%; Echo virus infection, 11%. No aetiological agent was discovered in 46%. In contrast, laboratory studies on patients with paralysis diagnosed on clinical grounds as poliomyelitis confirm this diagnosis in about 90% of cases. It is possible that Echo and other viruses as yet undiscovered may be responsible for some of the paralytic illnesses resembling poliomyelitis, but which do not yield evidence of infection with poliomyelitis virus.

Clearly, therefore, any organization which sponsors a poliomyelitis vaccination program must be prepared to provide laboratory facilities for the study by virological techniques of those cases of illness which may subsequently develop in the vaccinated population. An evaluation of a vaccination campaign based only on clinical diagnoses is likely to be fallacious.

During the spring of 1955, a trial of formalinized poliomyelitis vaccine (Salk) prepared by the Connaught Medical Research Laboratories, University of Toronto, was undertaken by the Ontario Provincial Department of Health (3).

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The vaccine was administered to children in Grades 1, 2, and 3 of elementary schools. Illnesses occurring in all children aged 5–12 attending public and separate elementary schools throughout Ontario were reported to the Ontario Department of Health. The total study population in this age group comprised approximately 309,000 vaccinated and 362,000 unvaccinated children.

The authors of the present report were privileged to play a part in the evaluation of this program, for approximately half of those in the trial group who became sick with illnesses suggesting a diagnosis of poliomyelitis during the summer and fall of 1955 were admitted to The Hospital for Sick Children, Toronto.

This paper reports virological studies on 27 patients falling within the trial group. Approximately half of these had been vaccinated in the spring of 1955. Brief reference is also made to studies on 13 paralytic patients. These children, who had not been vaccinated prior to the onset of illness, were not in the trial group, either because they were not in the age group 5–12 or because they attended private schools.

Methods

Preparation of Specimens. Specimens of stool, cerebrospinal fluid, and blood serum were obtained at or about the time of admission to hospital. A further specimen of blood serum was obtained in the convalescent period.

Stools were prepared for inoculation in tissue cultures as follows:

The stool was thawed and about 2 grams were added to 20 ml. of Hanks' balanced salt solution in a 250 ml. centrifuge tube containing glass beads. The tube was shaken by hand at room temperature to suspend the faeces. This material was filtered through gauze and centrifuged at 15,000 r.p.m. for 30 minutes in a "Spinco" ultracentrifuge; the supernatant fluid was then used to inoculate tissue cultures. If no virus was isolated, the supernatant was later centrifuged at 40,000 r.p.m. for one hour, and the deposit resuspended in the lower 1.5 to 2 ml. of fluid in the tube. This ultracentrifuged deposit was also inoculated in cultures.

Cerebrospinal fluid was stored at −40° C. and inoculated into cultures without treatment.

Serum was separated from clotted blood and stored at −40° C.; it was used unheated, in neutralization tests.

Tissue Cultures. Trypsinized monkey kidney cells were prepared at first by the method described by Youngner (9). Later, the continuous trypsinization flask described by Rappaport (5) was used. At first, Medium 199 (4) was employed as nutrient; 2% horse serum was added during the period of growth of the cell sheet, but no serum was used after inoculation of the cultures. Later, 0.5% lactalbumen hydrolysate in Hanks' solution with 5% horse serum was used for growth; before infection, this fluid was replaced by Earle's solution with 0.5% lactalbumen and 0.1% yeast extract. Human amnion cells were prepared as described by Beale *et al.* (1). Pieces of amnion tissue were trypsinized in a Rappaport flask. The cells were grown in 20% human or horse serum and 0.5% lactalbumen hydrolysate in Hanks' solution. Before infection, the cultures were washed three times in Hanks' solution; finally, they were maintained in Earle's solution with 0.5% lactalbumen hydrolysate with 0.1% yeast extract and 5% horse

serum. Five hundred units of penicillin and 250 micrograms of streptomycin were added to each ml. of all media.

Isolation of Viruses from Pathological Specimens. Specimens were inoculated in 0.1–0.5 ml. amounts into groups of two or more cultures. These were observed daily for one week. If no cytopathogenic change occurred, one blind passage of culture fluid was made. In fact, this procedure did not result in any more isolations of virus. In some cases, toxic degeneration of the cells occurred; this effect was not usually noted when one additional passage was made in fresh cultures.

Fluids from cultures showing a cytopathogenic effect were tested against a pool of high titre type specific poliomyelitis monkey kidney antisera. Agents that were neutralized by this mixture were then tested with the individual Type 1, Type 2 and Type 3 poliomyelitis antisera.

Further investigation of those agents that were not neutralized by poliomyelitis antisera was carried out as follows:

1. The viruses were independently isolated in human amnion cells to exclude the possibility that they were derived from the monkey kidney tissue.
2. Suckling mice under 24 hours of age were inoculated with the tissue culture fluids intracerebrally. They were observed daily for four weeks and were then killed for histological examination. All animals that died or sickened were examined histologically.
3. Ether sensitivity was performed by adding an equal amount of ether to the tissue culture fluid and leaving overnight at 4° C. Ether was then removed, and the fluid tested for cytopathogenic effect in tissue culture.

Serological Tests. Acute and convalescent sera as well as gamma globulin were titrated against the homologous virus isolate (100 CPD_{50} of virus). Virus and serum dilutions were mixed together, and after one hour at room temperature were inoculated in 0.1 ml. amounts into tissue cultures. Five culture tubes were used at each serial tenfold dilution. The 50% endpoint was calculated by the Kärber method, in terms of the final serum dilution.

Tests for poliomyelitis antibody were performed by the pH inhibition method of Salk, Youngner, and Ward (8). Disposable transparent plastic trays were used (6) instead of test tubes. Cups were sealed with mineral oil (Drakeol) as described by Youngner (10). The results were read after 7–10 days, by observing colour changes. Control titrations of cells, virus, and gamma globulin were done with each test. Each serum was first tested in three tenfold dilutions (1/10, 1/100, and 1/1000) and later over the appropriate range in six twofold dilution steps. Only one cup was used for each dilution. The highest dilution (initial) of serum giving complete protection of the cells was taken as the endpoint.

Results

Virus Isolations. A total of 27 patients aged 5–12 and falling into the trial group were finally accepted on clinical grounds as suffering from poliomyelitis. Only one of these patients had paralysis. The distribution was as follows:

	Vaccinated	*Unvaccinated*
Nonparalytic illness	12	14
Paralytic illness	1	0
Total	13	14

In addition, thirteen cases of paralytic poliomyelitis not falling into the trial group were studied. These patients were unvaccinated at the time of falling sick. Poliomyelitis virus was recovered from the stools of 12 of these 13 patients.

The results of inoculating stools from the 27 cases in the trial into monkey kidney and human amnion cells are shown in Table 1. It will be seen that a single strain of poliomyelitis virus, one strain of Coxsackie B2 virus and 13 strains of Echo virus were recovered. Twelve patients yielded no virus in stools.

The 13 strains designated as belonging to the Echo group had the following properties: they were not pathogenic for adult or suckling mice; they were resistant to treatment with ether; they were not neutralized by antisera to those members of the Coxsackie family that cause cytopathogenic changes in tissue cultures (A9, B1–4); all of the seven strains so tested were neutralized by gamma globulin (Connaught Medical Research Laboratories), the titres ranging from $10^{-0.5}$ to $10^{-2.5}$.

Specimens of cerebrospinal fluid from all cases were likewise inoculated in tissue cultures, but no viruses were isolated.

TABLE I—Poliomyelitis Vaccine Trial, Toronto, 1955
Results of inoculating stools of patients in tissue cultures.

Vaccination History	Type of Illness	Number of Cases	Poliomyelitis Virus Isolated	Coxsackie B2 Isolated	Echo Virus Isolated	No Virus Isolated
Nonvaccinated	Paralytic	0	0	0	0	0
	Nonparalytic	14	1	0	9	4
Vaccinated	Paralytic	1	0	0	0	1
	Nonparalytic	12	0	1	4	7
Total		27	1	1	13	12

Serological Tests. The acute and convalescent sera of the patient excreting Coxsackie B2 virus, and seven of those excreting Echo viruses, were tested for neutralizing antibodies to the homologous viruses by inoculation in groups of monkey kidney cultures. The results are shown in Table 2.

It will be seen that the patient excreting Cocksackie B2 developed a substantial increase in virus neutralizing antibody in convalescence. In one of the seven patients excreting Echo virus (JR) there was very little antibody present, and the results do not suggest that the agent isolated from the stool was the causal agent of the aseptic meningitis. In four of the remaining six patients excreting Echo viruses, there was a well marked increase in antibody. In another patient (FH) antibody was high in both specimens, suggesting current infection.

Tests for poliomyelitis antibody were carried out by the pH inhibition technique. This was done mainly because of the very low rate of isolation of poliomyelitis virus (1 strain from 27 patients) in the trial group.

For comparison, poliomyelitis antibody titrations were done with the sera of the 13 patients suffering from paralytic poliomyelitis, but who were not in the trial group, and were not vaccinated. The antibody response to the

type of virus isolated from the stool was substantial. Titres in the convalescent phase sera ranged from 1:256 to 1:8192. Many patients already showed substantial amounts of antibody in the acute phase serum, as is usual in poliomyelitis, but five of the ten patients from whom paired sera were available showed an increase in antibody. In most patients, antibody levels to the heterologous types of poliomyelitis virus were very low. This is of interest, as it suggests little previous contact with poliomyelitis viruses in Ontario children under the age of eight. One patient is of interest. Although the clinical diagonsis was one of paralytic poliomyelitis, no virus was recovered from the stool, and no antibody to any of the three types of poliomyelitis virus was present in the acute or convalescent sample of serum.

TABLE 2—POLIOMYELITIS VACCINE TRIAL, TORONTO, 1955
Serum antibodies for homologous Coxsackie or Echo viruses excreted in stool.

Patient*	Virus Isolated From Stool	Neutralizing Antibody Titre for Homologous Virus†	
		Acute	Convalescent
L.W.	Coxsackie B2	0.5	2.5
M.E.	Echo	1.5	1.5
D.R.	Echo	0.5	1.5
T.W.	Echo	1.3	2.9
F.H.	Echo	2.5	1.9
J.R.	Echo	0.5	0.5
G.G.	Echo	0.5	1.5
W.C.	Echo	0.5	2.3

*All patients suffered from aseptic meningitis.
†Titre expressed as negative logarithm of the dilution of serum inhibiting 100 CPD of virus in half the cultures.

It is of interest to compare these results with those obtained in the 13 vaccinated patients in the trial group. All of these had some antibody to all three types. The antibody levels fell into two groups. In the first group, the levels were 1:128 or below, presumably the result of the vaccination given some weeks before in persons with no earlier exposure to poliomyelitis virus. In the second group, the levels were much higher, the result of vaccination acting as a "booster" in persons previously exposed to virus. In none of the 13 vaccinated persons did the poliomyelitis antibody results suggest that the illness was in fact poliomyelitis.

Of the 14 unvaccinated patients, all of whom had aseptic meningitis (Table 1), four had no antibody to any type of poliomyelitis virus. These patients were all aged 6. It will be recalled that one patient excreted Type 1 poliomyelitis. This child showed an increase in antibody titre to Type 1 virus. In none of the other cases did the serologic results suggest the presence of poliomyelitis infection.

DISCUSSION

This report has been made at this time to illustrate some of the difficulties inherent in the evaluation of the prophylactic efficacy of poliomyelitis vaccine. A group of 27 children was admitted to a paediatric hospital with a diagnosis of poliomyelitis, 26 nonparalytic, and one paralytic. Thirteen of these had been vaccinated earlier in the season. Virus studies showed that only a single one

of these 27 patients, a patient suffering from a nonparalytic illness, was in fact infected with poliomyelitis virus. One of the remainder was infected with Coxsackie B2 virus. Echo viruses were recovered from 13 of the group, and were probably the cause of the meningitis in many instances.

It is of interest that in 1955 there was an outbreak of Echo aseptic meningitis in nearby parts of the State of New York; Type 6 virus was identified (2).

Our experience in the following year (1956), to be separately reported, confirmed the important part played by Echo viruses, for there was an extensive epidemic in Ontario of aseptic meningitis, often with a rash. Echo viruses were isolated from many patients.

Our data do not allow us to say whether or not the vaccine used in 1955 was demonstrated to have a prophylactic effect. However, from the practical viewpoint, of 13 isolations of poliomyelitis virus made in the laboratory in the summer and fall of that year, all came from unvaccinated children.

Summary

1. This paper describes virological studies carried out on a group of 27 children aged 5–12 admitted to a paediatric hospital in the summer and fall of 1955 with a clinical diagnosis of "poliomyelitis".

2. Thirteen of these children had been vaccinated with formalinized poliomyelitis vaccine in the spring of 1955. One of these developed a paralytic illness later in the year, and 12 a nonparalytic illness. None excreted poliomyelitis virus, but four isolations of Echo viruses, and one of Coxsackie B2 virus were made. The aetiology of the illnesses in the others has not been determined.

3. Fourteen unvaccinated children in the same age group were studied for comparison. All of these had a nonparalytic illness. One isolation of poliomyelitis virus (Type 1) and nine isolations of Echo viruses were made from the stools of the children.

4. The tissue culture methods used were highly sensitive to the presence of poliomyelitis virus, for this agent was recovered from the stools of 12 out of 13 paralyzed patients, all unvaccinated, and not forming, for various reasons, part of the trial group above mentioned.

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