STUDIES ON PREPARATION OF TYPING SERA FOR POLIOMYELITIS VIRUSES TYPES 1, 2, AND 3 BY VACCINATION OF RABBITS

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BERNARD FRANKLIN TAYLOR

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INTRODUCTION

Following reports (1, 2, 3, 4) that there were at least 3 immunological types of poliovirus, the Committee on Typing of the National Foundation for Infantile Paralysis proposed that standard antisera should be prepared for the 3 known types. Wenner, Miller, Kanitsuka and Wilson (5) prepared standard polio antisera in monkeys to be used as reference in comparative studies with related viruses and antisera.

Until 1954, the monkey was used exclusively for the preparation of anti polio typing serum. This animal served the researcher well in the area of poliomyelitis studies, but many difficulties were encountered limiting the expansion of this important field of endeavor. The monkey is an expensive animal. Care and maintenance of this animal presented further problems. Handling of the monkey requires experience, even so it is at times laborious. With these difficulties amassing, the researcher experimented with other animals as possible replacement for the monkeys.

One laboratory animal that has been used more recently to further research in virology, is the rabbit. The rabbit is generally considered to be non susceptible to poliovirus, however, Blanc (6) reported that poliovirus does produce an ailment in rabbits that is transmissible serially, but does

not produce paralysis or histological lesions in the spinal cord. The only sympton associated with this sickness is a rise in temperature followed, sometimes, by death of the animal.

Levinson et al. (7), Ramos-Alvarez and Sabin (8), and Mountain (9) showed that the rabbit can be used with success for the preparation of anti polio sera. The rabbit, unlike the monkey, obviously can not be used for isolation of poliovirus.

The purpose of this study is to compare the methods described and to find the most satisfactory method for the production of anti polio typing sera in rabbits, using live virus (cell culture fluid) an antigen without adjuvants, and further, to compare the prepared sera with standard typing sera prepared by the immunization of monkeys.

Three methods of immunization were used in this study. Two of the methods are essentially those of Levinson et al. (7), while the third was described and used by Ramos-Alvarez and Sabin (8).

I. TISSUE AND CELL CULTURE TECHNICS

Tissue and cell culture methods are becoming more and more valuable in virological work. The presence of virus is usually indicated by its cytopathogenic effect on the cells. This does not indicate, however, the nature of the virus. To identify a virus in tissue culture, antisera are necessary. In the case of poliovirus, three type-specific antisera are required.

Today, tissue culture inoculation has replaced the reproduction of the experimental disease in the monkey as the preferred method for the direct isolation and immunologic identification of poliomyelitis viruses.

Tissue culture technics were being described around the turn of the century. Viruses were first propagated in tissue culture as early as 1907 by Harrison (10, 11). Levaditi (12, 13) showed that infected spinal ganglia of monkeys maintained during 31 days their capacity to produce paralysis after repeated transfer in a plasma medium. Not until Flexner and Noguchi (14, 15) inoculated pieces of normal rabbit kidney suspension in human ascitic fluid with infected monkey cords was anyone able to produce paralysis in monkeys with tissue cultivated for longer period of time.

Parker and Nye (16) showed that viruses definitely

multiply in tissue culture. In 1930, Long, Olitsky and Rhoads (17), using the same technique as described by Flexner and Noguchi (14, 15), could sometimes produce paralysis in monkeys with 10th passage material. One of the simplest and most effective methods of cultivating viruses in living cells *in vitro* was devised by Li and Rivers (18) in 1930, employing the plasma-drop culture technique.

Gildemeister (19) in 1933, propagated poliomyelitis virus through 18 tissue culture passages, using chicken embryo brain tissue and a balanced salt solution (Tyrode) containing normal monkey serum, and reported that he was able to produce polio in monkeys from the 18th subculture. However other investigators failed in their efforts to confirm these observations.

In 1944, Sanders and Huang (20) suggested the use of tissue culture for virus investigations in the field. Evans and Green (21) showed that poliovirus would grow in extra-neural tissue. This work was confirmed by Smith *et al.* (22). In 1949, Weller, Robbins and Enders (23) gave an account of the cultivation of the Lansing strain of poliomyelitis virus in suspended tissue cultures of human foreskin and embryonic tissue, and noted certain degenerative changes in cells, possibly caused by the pathogenicity of the virus. These cytopathic changes induced by poliovirus in cultures have since been observed by many investigators: Milzer *et al.* (24), Robbins, Enders and Weller (25, 26), Enders (27), Scherer and

Syverton (28, 29), Syverton and Scherer (30), Pollard and Bussel (31), Hsu (32), and Thicke (33).

In 1953, Gey *et al.* (34) was successful in developing a new strain of malignant human epithelial cells (strain HeLa). It proved to be about as susceptible to the three types of poliomyelitis virus as monkey kidney cells. Syverton and Scherer (35) and Gey, Bang and Gey (36), demonstrated further that HeLa cells can readily be used for the quantitation of virus, for the measurement of type specific antibody to poliovirus by the cell culture neutralization techniques and for the mass production of virus.

The HeLa cell line is one of the most widely used cell lines. It is easy to maintain and can be cultured serially with satisfactory results. All of the polioviruses used in this study were propagated in this cell line.

II. MATERIAL AND METHODS

The methods for the production of antisera used in this study were designated as follows: Schedule A, Schedule B, and Schedule C. The first two methods were essentially those of Levinson *et al.* (7), while the third was described and used by Ramos-Alvarez and Sabin (8).

All the viruses used for vaccination were live viruses grown in cell culture (HeLa strain of epithelial cells). No adjuvants were used in this study. Strains of poliovirus used as antigen were: Mahoney strain (type 1), MEF_1 strain (type 2) and Saukett strain (type 3). Bottles of HeLa cell cultures, having confluent "sheets", were inoculated with suspensions of poliovirus; after all cells on the glass wall were affected, the liquid phase of the cultures together with what was left of the cells were frozen and thawed three times before centrifugation. After centrifugation the preparation was stored at -40°C. A sample was plated on blood agar plates before use. The titer of the virus used for vaccination was not determined.

The rabbits were bled before vaccination. Cardiac puncture was used exclusively for bleedings. All intravenous injections were administered via the marginal vein of the ear and all intramuscular injections were administered in the hind legs. Sera obtained from bleedings were removed after

clotting and centrifuged at 2,000 RPM for 15 minutes. After centrifugation the serum was removed with a pipette and stored at -40°C.

A total of nine rabbits was used for each method or 3 rabbits per method per type of poliovirus. All antibody titrations were done by neutralization tests in FL cell cultures, using standardized amounts of virus as expressed in $TCID_{50}$ (50 per cent tissue culture infective doses). $TCID_{50}$ is the highest concentration of virus in which 50 per cent or more of the inoculated cell cultures show cytopathogenic effect (CPE).

Prevaccinal sera were tested for antibody against the 3 strains of poliovirus using 1:20, 1:100, 1:500, 1:2,500, 1:12,500 and 1:62,500 dilutions of serum against 100 $TCID_{50}$ of virus (final dilution). Titration of prevaccinal sera showed no evidence of polio antibodies.

Sera from each polio type were pooled and titrated three times, then an average of the three determinations was taken as representative of the antibody level. A sample of all sera used in this study were inoculated on blood agar plates and inactivated at 56°C. for 30 minutes before use.

The antibody titer was expressed as 50% serum dilution end points (against 100 $TCID_{50}$) per 0.1 ml., calculated by the method of Reed and Muench (37).

The vaccination schedules used in this comparative study for the production of polio typing sera in rabbits, for clarification, appear in the results.

III. THE FL CELL LINE

Cell cultures were used throughout this study for propagation of poliovirus, as well as for virus titrations and titrations of antibody in the prepared sera by neutralization tests. HeLa cells were used for the preparation of a stock of virus used in the vaccination of rabbits. In the meantime, Mountain (38) published a paper describing the cytopathogenic effect of antiserum to human malignant epithelial cells (strain HeLa) on HeLa cell culture. Her findings were confirmed in this laboratory, although CPE was not demonstrated at serum dilutions greater than 1:50 (final dilution).

To by-pass this difficulty, Doctor Lensen obtained from Fogh and Lund their cell line derived from normal human amnion (FL cells). Fogh and Lund (39) were able to cultivate these cells continuously for 38 passages. Takemoto and Lerner (40) reported that these cells were comparable to HeLa cells in susceptibility to poliovirus. All of their data concerning this FL cell line were confirmed in this laboratory. The only noticeable difference is the CPE observed in poliovirus infected cells. HeLa cells infected with poliovirus become rounded and are distributed singly on the glass wall, whereas infected FL cells become rounded but remain in clusters. Parallel titrations of poliovirus in HeLa and FL cells showed no appreciable difference in titers.

All efforts to produce cytopathogenic effect of antiserum to HeLa cells on FL cell cultures were unsuccessful. The FL cell line, like the HeLa cell line, can be maintained and satisfactorily cultured serially.

All titrations of virus and serum for this study were done in these cells.

IV. PROPAGATION OF POLIOVIRUS IN CELL CULTURE

Propagation of the polioviruses in HeLa cell cultures was carried out as follows:

1. Cultures with confluent "sheet" were chosen discriminately using microscopic examination.

2. The cultures were next washed 3 times with Hanks' balanced salt solution.

3. The culture bottles were inoculated with 1.0 ml. of a 10^{-1} dilution of each poliovirus, Mahoney strain (type 1), MEF_1 strain (type 2) and Saukett strain (type 3).

The culture bottles were immediately incubated at 35°-37°C. for 1 hour.

4. After the hour of adsorption, 9 ml. of maintenance medium (95% Eagle's Basal Medium and 5% inactivated calf serum) were added to the bottles.

5. A control bottle was washed as previously stated and given 10 ml. maintenance medium (no virus added).

All bottles were incubated at 35°-37°C. and observed twice daily for cytopathogenic effect. When all cells on the glass wall were affected (rounded and/or leaving the glass wall), the liquid phase was removed and frozen and thawed 3 times before centrifugation. It was then centrifuged at 3,000 RPM for 20 minutes. After this procedure was completed, the fluid was removed and frozen at -40° to -50°C. for storage.

A sample of all stock viruses were plated on blood plates as a sterility test before use.

V. POLIOVIRUS NEUTRALIZATION TEST IN FL CELL CULTURE*

1. Determine titer of virus in cell culture system to be used for neutralization test as follows:

   a. Prepare series of tubes containing 1.8 ml. Scherer's Maintenance Solution (M.S.).

   b. To tube #1, add 0.2 ml. virus--mix 7 times with 1 ml. pipette. Transfer 0.2 ml. to tube #2. With fresh pipette mix 7 times. Using separate pipettes throughout, repeat procedure to last tube for serial 10-fold dilutions.

   c. Prepare 3 cultures per dilution by washing each culture 3 with Scherer's M.S.

   d. Add 0.1 ml. of virus dilution (to each of 3 tubes) directly on cells.

   e. Incubate for 30 minutes at room temperature for adsorption.

   f. Add 1.0 ml. Ginsberg's modification of Scherer's M.S., as used in our laboratory.

GINSBERG'S METHOD		MICROBIOLOGICAL ASSOCIATES' MODIFICATION OF GINSBERG'S METHOD	
Scherer's M.S.	67.0%		70.0%
Tryptose Phosphate Broth	25.0%		25.0%
Chicken Serum (inactivated)	7.5%		5.0%

* Modification from Diagnostic Procedures for Virus and Rickettsia Diseases, 2nd Ed., 1956. Published by American Public Health Association

GINSBERG'S MODIFICATION OF SCHERER'S M.S. AS USED IN OUR LABORATORY

Scherer's M.S.....70.0%

Tryptose Phosphate Broth.....25.0%

Calf Serum (inactivated)..... 5.0%

Incubate at 35°-37°C.

g. Wash 10 control tubes culture with Scherer's M.S. (no virus is added). Incubate at 35°-37°C.

h. Observe daily for CPE.

Observe and record for 7 days, unless controls show degeneration before this time.

i. Calculate $TCID_{50}$ per 0.1 ml. Accordingly, prepare proper dilution containing 200 $TCID_{50}$/0.1 ml. for neutralization tests.

2. Preparation of serum. Before use heat at 56°C for 30 minutes.

a. Prepare a series of tubes containing Scherer's M.S.; 0.9 ml. in 1st tube, 0.8 ml. in the remainder.

b. Using separate pipettes throughout, add 0.1 ml. serum to the 1st tube, 0.2 ml. to the remainder for serial 5-fold dilutions. Discard last 0.2 ml. In this way set up serum dilutions 1:10, 1:50, 1:250, 1:1,250, 1:6,250 and 1:31,250, each tube now contains 0.8 ml.

3. For neutralization test.

a. To each serum dilution tube add 0.8 ml. of virus (200 $TCID_{50}$ per 0.1 ml.). Shake in rack to mix. The final mixture now contain 100 $TCID_{50}$/0.1 ml. of virus

and serum dilutions of 1:20, 1:100, 1:500, 1:2,500, 1:12,500 and 1:62,500.

b. Incubate at room temperature for 1 hour.

c. Wash tube cultures as previously described.

d. Using 3 cultures per serum dilution, add 0.1 ml. of serum-virus mixture to cell layer.

e. Allow 30 minutes for adsorption.

f. Add 1.0 ml. maintenance medium and incubate at 35°-37°C.

4. For Cross Reaction Test.

a. Prepare a series of tubes containing Scherer's M.S.; 0.8 ml. in 1st tube and 2nd tube.

b. Using separate pipettes throughout, add 0.2 ml. serum to first tube; mix 7 times. Transfer 0.2 ml. to the second tube for serial 5-fold dilution. In this way set up serum dilutions 1:5 and 1:25. Discard last 0.2 ml.

c. To each dilution tube add 0.8 ml. of virus 200 $TCID_{50}$/0.1 ml.). Shake in rack to mix. The final mixture now contains 100 $TCID_{50}$ per 0.1 ml. of virus and 1:10 and 1:50 dilutions of serum.

d. Incubate at room temperature for 1 hour.

e. Wash tube cultures as mentioned earlier.

f. Using 3 cultures per serum dilution, add 0.1 ml. of serum-virus mixture to cell layer.

g. Allow 30 minutes for adsorption.

h. Add 1.0 ml. maintenance medium.

Cross Reaction Test (Set-up)

Type 1 serum: 1:10 and 1:50 (final dilutions) against MEF_1 strain (type 2) and Saukett strain (type 3) of polioviruses (100 $TCID_{50}$/0.1 ml., final dilution).

Type 2 serum: 1:10 and 1:50 (final dilutions) against Mahoney strain (type 1) and Saukett strain (type 3) of polioviruses (100 $TCID_{50}$/0.1 ml., final dilution).

Type 3 serum: 1:10 and 1:50 (final dilution against Mahoney strain (type 1) and MEF_1 strain (type 2) of polioviruses (100 $TCID_{50}$/0.1 ml., final dilution)

Neutralization Controls: With each series of neutralization tests, virus control tests were made (5 tubes), the final concentration being the highest dose (100 $TCID_{50}$) used in the serum-virus mixture. Also tested was the highest concentration of each serum used in FL cell culture tubes (5 tubes per serum type) for non-specific degeneration.

Ten tubes of FL cell cultures were washed 3 times with Scherer's M.S. and then maintained in Ginsberg's modification of Scherer's M.S. as used in our laboratory, but left uninoculated to serve as cell controls.

Observation: The tubes were observed and recorded daily. Observation was made for 7 days unless the conditions of the cells in the control tubes necessitate earlier termination. After the tubes were discarded, the 50% end points serum titers were calculated.

A complete virus titration was included in each neutralization test, the titers obtained were used to calculate the virus dilutions to be used for the next neutralization test.

VI. RESULTS

Table 1. Shows a work sheet of a typical poliovirus titration as used in this study. The dilutions were 10-fold, there were 7 dilutions per virus type (in other cases 6 dilutions were used) and three tubes per dilution. A total of 73 tubes were used to titrate the polioviruses (21 tubes per virus type and 10 control tubes). The tubes were observed microscopically daily for 7 days before discarding.

The data presented in table 1 show that the FL cells were degenerating after 2 days with the higher concentration of poliovirus (10^{-3}). With the 10^{-4} and 10^{-5} dilutions, CPE was observed on the 3rd day following infection. On the 4th day, all cells of the 10^{-6} dilution and some of the cells of one tube of the 10^{-7} dilution were affected. On the next day (5th day subsequent to infection), all cells in the one tube of the 10^{-7} dilution were affected, while the cells in the two remaining infected tubes of 10^{-7}, 10^{-8} and 10^{-9} remained normal throughout the observation period (7 days).

The cell controls showed no degenerative changes throughout the observation period thereby giving validity to the titration.

The $TCID_{50}$ titer obtained in this titration was $10^{-6.75}$ - (1:5,623,000). For example: for the initial dilution of $200TCID_{50}$ the poliovirus was diluted 1:28,165, giving a final dilution of $100TCID_{50}$ when equal amounts of the virus dilution and of the serum dilutions were mixed.

Table 1
Titration of poliovirus in cell culture

SCHEDULE B

Titration in FL Cells — Date February 19, 1958

Virus MAHONEY — Passage 2nd in FL Cells

0.1 ml. of virus dilution incubated on cells at room temperature for ½ hour. Dose 1.0 ml. of Maintenance Medium

	Infected Tubes							
Date	2/19		2/21		2/23		2/25	
Days	0	1	2	3	4	5	6	7
	√	.	+					
	√	.	+					
10^{-3}	√	.	+/−	+				
	√	.	.	+				
	√	.	.	+				
10^{-4}	√	.	.	+				
	√	.	.	+				
	√	.	.	+				
10^{-5}	√	.	.	+				
	√	.	.	.	+			
	√	.	.	.	+			
10^{-6}	√	.	.	.	+			
	√	.	.	.	.	.	.	.
	√	.	.	.	.	.	.	.
10^{-7}	√	.	.	.	+/−	+		
	√	.	.	.	.	.	.	.
	√	.	.	.	.	.	.	.
10^{-8}	√	.	.	.	.	.	.	.
	√	.	.	.	.	.	.	.
	√	.	.	.	.	.	.	.
10^{-9}	√	.	.	.	.	.	.	.

Table 1 -- Continued

Control tubes

2/19		2/21		2/23		2/25	
0	1	2	3	4	5	6	7
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•
✓	•	•	•	•	•	•	•

Legend: (•) denotes normal cells

(±) indicates that some cells on the glass wall are affected

(+) indicates that all cells on the glass wall are affected

Table 2. This was a work sheet of a typical neutralization test and cross reaction test. The serum dilutions were 5-fold, there were 5 dilutions per serum type (in other cases 6 dilutions were used) and 3 tubes per dilution. In the cross reaction test, again the serum dilutions were 5-fold with 2 dilutions per serum type (1:10 and 1:50, final dilutions) against heterotypic polioviruses (containing 100 $TCID_{50}$, final dilution) 81 tubes were used for a complete neutralization and cross reaction test. At the end of the observation period (7 days), the tubes were discarded.

This table presents the outcome of a neutralization test using poliovirus (100$TCID_{50}$, final dilution) and 5-fold dilutions of polio antisera. The left half of the table shows the neutralization test with homotypic poliovirus, while the right half of it shows the test for cross reaction with heterotypic polioviruses.

It can be seen from this table that there was no neutralization with the highest dilution of the serum (1:62,500) and only partial neutralization with the dilution 1:12,500 (2 out of 3 tubes showed CPE). At all lower dilutions starting with 1:2,500 there was complete neutralization during the observation period of 7 days.

In the cross reaction test, 1:10 and 1:50 final dilutions of serum were tested against heterotypic polioviruses (again in a concentration of 100$TCID_{50}$, final dilution). This table shows that there was no cross neutralization at all with heterotypic polioviruses.

The homotypic antibody level of the Mahoney antiserum was: 1:6,100 (as expressed in 50% endpoints).

Table 2
Neutralization and cross reaction tests in cell culture

SCHEDULE B

$TCID_{50}$ 1:5,620,000
$200TCID_{50}$ 1:28,100

Neutralization and cross reaction tests in FL Cells

Date February 19, 1958

Mahoney Serum

0.1 ml. of serum-virus mixture incubated at room temperature for 1 hour, then on cells for 30 minutes at room temperature.

Dose 1.0 ml. Maintenance Medium

Date	2/19		2/21		2/23		2/25	
Days	0	1	2	3	4	5	6	7
	✓	•	•	•	•	•	•	•
	✓	•	•	•	•	•	•	•
1:100	✓	•	•	•	•	•	•	•
	✓	•	•	•	•	•	•	•
	✓	•	•	•	•	•	•	•
1:500	✓	•	•	•	•	•	•	•
	✓	•	•	•	•	•	•	•
	✓	•	•	•	•	•	•	•
1:2,500	✓	•	•	•	•	•	•	•
	✓	•	•	•	•	•	±	+
	✓	•	•	•	•	•	•	+
1:12,500	✓	•	•	•	•	•	•	•
	✓	•	•	•	+			
	✓	•	•	•	+			
1:62,500	✓	•	•	•	+			

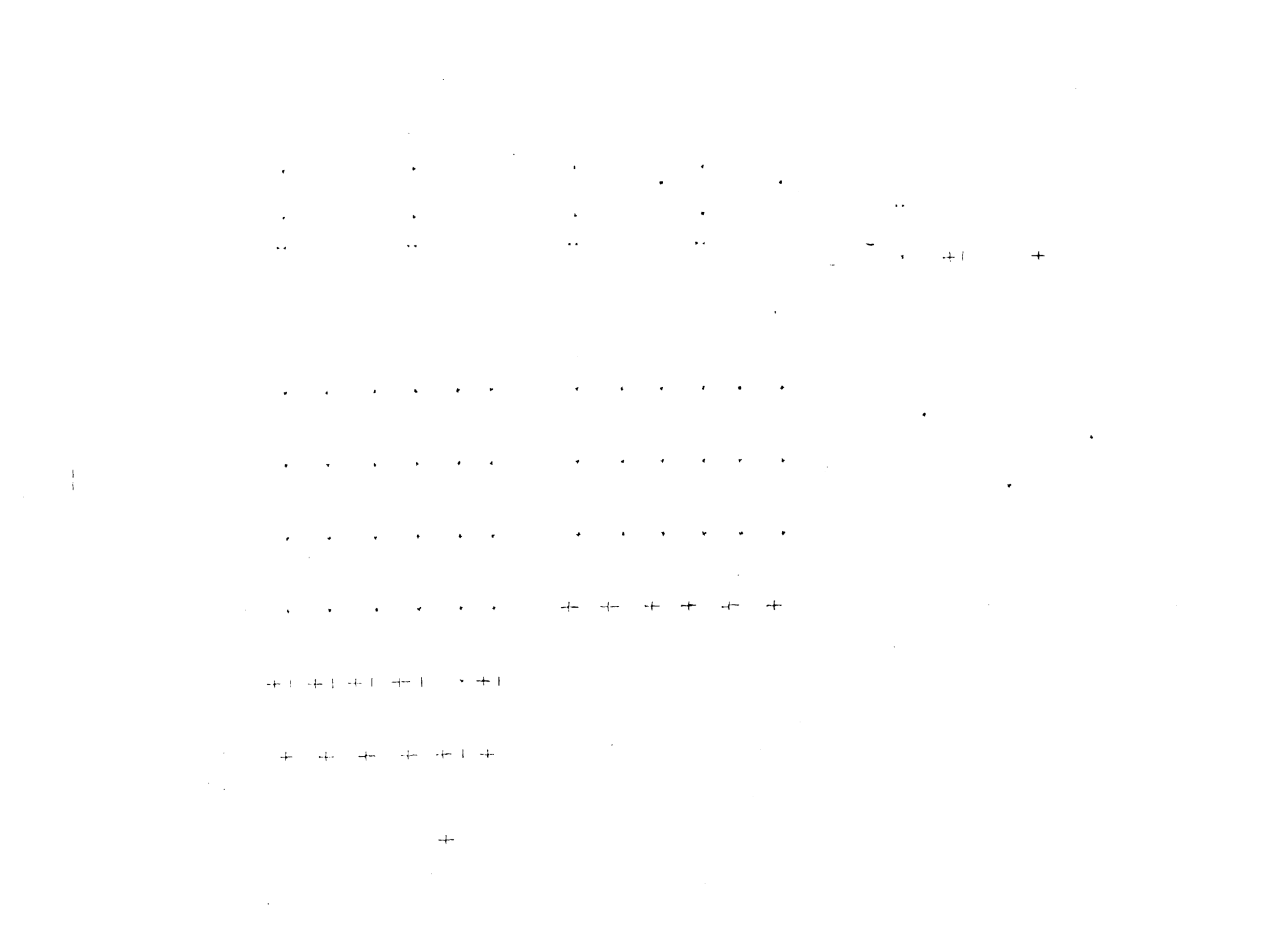
Table 2 -- Continued

	2/19		2/21		2/23		2/25	
	0	1	2	3	4	5	6	7
Mah. Ser. 1:10	✓	.	.	.	.	±	+	
MEF_1 Virus	✓	.	.	.	.	±	+	
	✓	.	.	.	.	±	+	
Mah. Ser. 1:50	✓	.	.	.	.	±	+	
	✓	.	.	.	.	.	±	+
MEF_1 Virus	✓	.	.	.	.	±	+	
Mah. Ser. 1:10	✓	.	.	.	+			
	✓	.	.	.	+			
Sauk. Virus	✓	.	.	.	+			
Mah. Ser. 1:50	✓	.	.	.	+			
	✓	.	.	.	+			
Sauk. Virus	✓	.	.	.	+			

Legend: (.) denotes neutralization of the virus (no CPE observed).

(±) denotes partial neutralization of the virus (normal and degenerative cells remaining on the glass wall).

(+) denotes absence of neutralization of the virus by the serum (no normal cells remaining on the glass wall).



SCHEDULES FOR VACCINATION

I. Schedule A.

The method of vaccination used in this schedule is esentially that of Levinson et al. (7) The rabbits used with this schedule were of mixed sexes. Their weight ranged from 4 1/2 to 5 3/4 pounds. A total of 21 ml. of virus (no adjuvants were used in this study) per rabbit (or 63 ml. per type) was injected during the course of vaccination which was completed in 77 days.

All rabbits were bled prior to being put on a vaccination schedule.

On the first day the rabbits were given 5 ml. of virus (tissue culture fluid) intramuscularly (2.5 ml. per hind leg) and 5 ml. intravenously (marginal vein of the ear). Twenty days later the rabbits were bled. The sera were processed as previously stated.

Twenty-nine days following the first injections, 10 ml. of virus was injected intravenously. Twenty-seven days after the second injection and/or 56 days after the first injections, the rabbits were bled a second time during the course of vaccination.

Sixty-two days after the first injections, the rabbits

were injected for the third and last time. This time the inoculum was 1 ml. of virus injected intravenously.

Fifteen days later or 77 days after the first injections, the rabbits were exsanguinated.

During the vaccination schedule, the rabbits were observed twice daily. No rabbits showed any central nervous system involvement. No rabbits died during the course of vaccination.

The rabbits were bled three times during the course of vaccination. The first blood sample was taken 20 days after the first injection, the second blood sample was taken 56 days after the first injection and 27 days after the second injection and the third blood sample was taken 77 days after the first injection and 15 days after the third and/or last injection. The rabbits were exsanguinated at the time of the third bleeding.

The average serum yield was 57 ml. per rabbit. The sera were processed as stated previously.

The specificity, as well as the content of serum antibody in rabbits vaccinated with types 1, 2 and 3 poliovirus, was determined by cell culture methods. Serum pools obtained at each bleeding were tested for neutralizing antibodies. The

results obtained in the tests appear in table 3.

Table 3

Tests for Homotypic Titers* and Cross Reaction**
Antibody titers of polio antisera
prepared by using schedule A.

Serum	VIRUS TYPE								
	MAHONEY			MEF$_1$			SAUKETT		
	Bleedings			Bleedings			Bleedings		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Type 1	215	1,260	5,720	0	10	10	0	0	0
Type 2	0	0	0	0	1,980	5,720	0	0	0
Type 3	0	0	0	0	0	10	0	840	6,400

*Homotypic titers - 50% end points (average 3 titrations)
**For testing for cross reaction with heterotypic poliovirus the dilutions 1:10 and 1:50 were used. Zeros indicate that no cross reaction was observed even at 1:10.

There was a progressive increase in antibody following each vaccination. The sera were type specific with a very slight degree of heterotypic activity. Type 2 antiserum shows no cross reaction with type 1 or type 3 poliovirus, wherein, types 1 and 3 antisera show cross reaction with only type 2 poliovirus.

II. Schedule B.

This method of vaccination, like schedule A, was described by Levinson et al. The rabbits vaccinated by this method were of mixed sexes and ranged in weight from 5 3/4 to 8 pounds.

Twenty ml. of virus (without adjuvants) per rabbit (or 60 ml. per type) were injected intramuscularly. The full course of vaccination was completed in 54 days.

All rabbits were bled before vaccination.

On the first day the rabbits were given 5 ml. of virus intramuscularly (2.5 ml. per hind leg). Eight days later this was repeated, and again on the 12th day. Thirty days after the third injection and 42 days subsequent to the first injection, blood samples were taken. The three samples per type were pooled and later titrated.

Four days following this bleeding and 46 days after the first injection, the rabbits were again injected (last injection) with 5 ml. of virus as described above.

Eight days after the last injection or 54 days after the first injection, the rabbits were exsanguinated.

Each rabbit received 5 ml. of virus at each of 4 vaccinations.

The rabbits were bled two times during the course of vaccination. The first bleeding was 42 days after the first injection and 30 days after the third injection. At the time of the 2nd bleeding (54 days after the first injection or 8 days after the 4th injection), the rabbits were exsanguinated.

The average yield of serum was 64.5 ml. per rabbit.

Testing of the sera, taken prior vaccination, was negative for antibodies.

Cell culture methods were used to determine the specificity and serum antibody level of the rabbits vaccinated with types 1, 2 and 3 poliovirus. Table 4, below, shows the results of these tests.

Table 4

Tests for Homotypic Titers* and Cross Reaction**
Antibody titers of polio antisera
prepared by using schedule B.

	VIRUS TYPE					
	MAHONEY		MEF1		SAUKETT	
	Bleedings		Bleedings		Bleedings	
Serum	1st	2nd	1st	2nd	1st	2nd
Type 1	2,910	3,410	0	0	0	0
Type 2	0	0	4,990	5,860	10	10
Type 3	0	0	10	0	1,370	1,290

* Homotypic titers - 50% end points (average of 3 titration)
** For testing for cross reaction with heterotypic poliovirus the dilutions 1:10 and 1:50 were used. Zeros indicate that no neutralization of poliovirus by homotypic or heterotypic serum was observed even at 1:10

There was progressive increase in antibody following the vaccinations with types 1 and 2 poliovirus, however, the antibody level of type 3 serum did not rise after the third injection.

The sera were type specific, but there was some degree of cross reaction between type 2 antiserum and type 3 poliovirus; while type 3 antiserum with type 2 poliovirus also showed very slight heterotypic activity.

III. Schedule C.

Ramos-Alvarez and Sabin (8) used and described the method of vaccination in this schedule. As before, rabbits of mixed sexes were used, ranging in weight from 5 3/4 to 7 1/4 pounds.

A total of 10.5 ml. of virus per rabbit (or 31.5 ml. per type) was used. It took 65 days to complete this schedule.

All rabbits were bled before being put on vaccination schedule.

On the first day, the rabbits were given 1 ml. of virus intravenously and 1 ml. intracutaneously (injected at 5 sites on the back). This was followed by two intravenous injections of 1 ml. daily, then a rest period of 4-5 days, again two additional series of 3 daily intravenous injections separated by 4-5 day rest periods. Twenty-nine days after the third series of injections, blood samples were taken from each rabbit by means of a cardiac puncture. The sera were processed as mentioned earlier.

Thirty-five days after the third series of injections, a booster injection of 0.5 ml. was administered intravenously. The rabbits were bled out 65 days after the first injection. No rabbits were sick or died during the course of vaccination.

The rabbits were vaccinated 11 times during the course of this schedule. The rabbits were bled two times during the course of vaccination. The first bleeding was 46 days after the first injection and/or 29 days after the 10th injection. The rabbits were exsanguinated at the time of the 2nd bleeding (65 days after the first injection and 13 days after the 11th and last injection).

The average yield of serum was 50 ml. per rabbit. The sera were processed and frozen.

Prevaccination sera were titrated and there was no evidence of antibodies observed in any serum.

Table 5

Tests for Homotypic Titers* and Cross Reaction**
Antibody titers of polio antisera
prepared by using Schedule C.

	VIRUS TYPE					
	MAHONEY		MEF_1		SAUKETT	
	Bleedings		Bleedings		Bleedings	
Serum	1st	2nd	1st	2nd	1st	2nd
Type 1	9,320	6,240	0	0	0	0
Type 2	0	0	10,880	11,700	0	0
Type 3	0	0	10	10	3,500	3,850

* Homotypic titers - 50% end points (average of 3 titration)
** For testing for cross reaction with heterotypic poliovirus 1:10 and 1:50 were used. Zeros indicate that no neutralization of poliovirus by homotypic or heterotypic serum was observed even at 1:10.

The specificity and serum antibody content of rabbits vaccinated with types 1, 2 and 3 poliovirus was determined using cell culture methods. There was progressive increase in antibody following vaccinations with type 2 poliovirus. There was no significant rise in antibody content following the last injection (booster dose) of type 3 poliovirus. Type 1 polio antiserum, however, showed no increase in antibody level following the last injection, actually the antibody content declined.

The antisera were type specific. The only heterotypic activity observed was type 3 polio antiserum with type 2 poliovirus, however, cross reaction was not observed at a serum dilution greater than 1:10.

D. ANTIBODY TITERS — POLIO ANTISERA

Antibody titers of sera obtained after the completion of each of the three schedules used for the production of polio antisera in rabbits with types 1, 2, and 3 poliovirus are shown in table 6. The titers of antisera are repeated in the composite table for the purpose of comparison of these titers.

Table 6

Tests for Homotypic Titers* and Cross Reaction**
Antibody titers of polio antisera
prepared by using schedules A, B and C.

	VIRUS TYPE								
	MAHONEY			MEF_1			SAUKETT		
	Schedules			Schedules			Schedules		
Serum	A	B	C	A	B	C	A	B	C
Type 1	5,720	3,410	6,240	10	0	0	0	0	0
Type 2	0	0	0	5,720	5,860	11,700	0	0	0
Type 3	0	0	0	10	10	10	6,400	1,290	3,850

* Homotypic titers - 50% end points (average 3 titrations)
** For testing for cross reaction with heterotypic poliovirus the dilutions 1:10 and 1:50 were used. Zeros indicate that no neutralization of poliovirus by homotypic or heterotypic serum was observed even at 1:10.

The above table shows that MEF_1 strain of poliovirus gave the highest antibody response in rabbits, while the Saukett strain gave the lowest. Mahoney strain showed slightly less antigenic potency than did MEF_1 strain of

poliovirus. The data are inconclusive in view of the fact that the concentration of the poliovirus suspensions used in these vaccination methods were undetermined.

VII. TITRATION OF POLIO ANTISERA PREPARED BY AND RECEIVED FROM DR. WENNER*

In 1954, Wenner *et al.* (5) prepared standard polio antisera in monkeys for the three known types. This work was proposed by the Committee on Typing of the National Foundation for Infantile Paralysis. These sera are used as references in comparative studies with related viruses and antisera.

The method of vaccination of monkeys used by Wenner *et al.* (5) was as follows:

1. Each monkey received 4 ml. of vaccine (10-20% virus-adjuvant emulsions) at each of 3 vaccinations given at bi-weekly intervals; 2 ml. were inoculated intramuscularly into each calf or thigh region.

2. Three weeks after the last vaccination, the monkeys were bled.

The sera were tested for presence of bacteria and fungi by blood agar plates and thioglycollate broth.

Wenner *et al.* (5) reported that there was a progressive increase in antibody following each vaccination. These sera were type specific and vaccination did not increase the specificity of type 2 serum pool.

*Chief, the Department of Pediatrics and the Hixon Memorial Laboratory, University of Kansas School of Medicine, Kansas City, Kansas

This laboratory received from Dr. Wenner three polio antisera, type 1 (Brunhilde), 2 (Lansing), and 3 (Leon). These monkey polio typing sera were lyophilized. After reconstitution, the sera were titrated using the same technics as for other sera used in this study.

Table 7 shows the antibody content of the monkey polio typing sera prepared by Wenner et al. (5) and used as the reference sera in this comparative study.

Table 7

Test for Homotypic Titers* and Cross Reaction**
Antibody content of polio typing sera
prepared by Dr. Wenner et al. (5)

Serum	VIRUS TYPE		
	MAHONEY	MEF$_1$	SAUKETT
Brunhilde (type 1)	4,370	0	0
Lansing (type 2)	0	1,120	0
Leon (type 3)	0	0	5,590

* Homotypic titers - 50% end points (average of 3 titrations)
** For testing for cross reaction with heterotypic poliovirus the dilutions 1:10 and 1:50 were used. Zeros indicate that no neutralization of poliovirus by homotypic or heterotypic serum was observed even at 1:10.

As demonstrated in table 7, there was no cross reaction

in neutralization tests in cell culture with any of the typing sera against types 1, 2 and 3 poliovirus. The sera were homotypic and contained a very high degree of specificity.

VIII. TITRATION OF SERUM OF AN INDIVIDUAL VACCINATED WITH SALK'S POLIO VACCINE

Each type of poliovirus is capable of causing paralytic poliomyelitis in human beings and of inducing a type specific immunity. The discovery that there are 3 different types of poliovirus is of a fundamental importance, because a vaccine, to be effective, will have to bring about immunity against all three types of the virus.

Flexner and Lewis (41) reported that vaccination of primates was possible. They studied active immunity and passive serum protection. Bodian (42) described neutralization of three immunological types of poliovirus by human gamma globulin. Human gamma globulin did give protection (passive immunity), but this protection was of short duration. It was obvious active immunity against poliomyelitis was a necessity and a possibility, and further, that it was the only answer to this public health problem.

Burnet and Macnamara (43) recognized the immunological differences between strains of poliovirus. Morgan (44), in 1948, was successful in vaccinating monkeys with formalin-inactivated poliovirus. In 1952, Howe (45) published reports on antibody response of chimpanzees and humans to formalin-inactivated trivalent poliomyelitis vaccine. The three

main approaches to the problem of vaccination against poliomyelitis, as suggested by Salk (46) are: (1) a "killed" virus vaccine, (2) vaccination with active virus under cover of antibody, and (3) an avirulent vaccine. Salk and his associates (47) stated that the antigenicity of formalinized tissue culture vaccines, as measured by production of antibody, was comparable to that obtained with similar live virus vaccines grown in tissue culture (monkey kidney cells).

The viruses used in Salk's Vaccine production were grown on an entirely synthetic nutrient medium (No. 199), devised by Morgan, Morton and Parker (48). The growth medium does not contain any animal sera. This permits a cell-free vaccine suitable for use in human beings.

Salk's Vaccine has been very effective in controlling paralytic poliomyelitis. At the beginning, the safety test was inadequate and public acceptance was rather slow. The new and more thorough safety test has done much to increase this acceptance and to rid the public of fear of paralytic accidents due to presence of live virulent virus in a few lots of this vaccine.

The Safety Test includes the following steps:

1. Test for tubercle bacilli in monkey kidney cells.

2. Bacteriological sterility test as described by the National Institute of Health.

a. Cultured in tryptic digest of casein broth, meat mash broth, thioglycollate broth, penicillinase and casein digest broth for 7 days at 37°C. and room temperature.

(1) If contaminated, 2 additional complete test giving entirely negative results are required to clear the pool of suspicion.

3. Antibody-virus neutralization test.

4. For toxicity in guinea pigs (350 grams), observed for 7 days.

5. Virus content via virus titration of pools using Karber's method. CPD_{50} (50 per cent cytopathogenic producing doses) - dilution of virus causing cytopathogenic changes in half of the inoculated cultures. The titers of the pools must be $10^{-6.5}$ or better.

6. Test in rabbits for virus B and lymphocytic choriomeningitis. The rabbits are observed for 2 weeks.

7. The virus pools, after being cleared, are filtered.

a. First using clarifying filters to remove isolated cells, cellular debris and adsorbed or entrapped virus particles.

b. Next it is filtered through a bacteria-retaining filter to remove any occult bacteria or molds slow growing or suppressed by penicillin or streptomycin.

8. Inactivation.

a. Using a 1:4,000 formalin solution subjected to temperatures of 35°-37°C. with ;H 7.0 for one week.

b. Excess formaldehyde is neutralized with sodium bisulfite as it may affect antigen stability.

Finally the vaccine was tested in small animals and monkeys. The vaccine was then tested in children with detectable antibody to any of the three immunologic types of poliovirus. Salk claims that his vaccine is more stable than other biological preparations. Powell and Culbertson (49) reported that if Salk's Vaccine is subjected to a temperature of 55°C. for 10 to 30 minutes, it is deprived of its antigenicity.

The author was vaccinated with Salk's Vaccine. The vaccination schedule was as follows:

1. First injection: 1 ml. of the trivalent formalin-inactivated vaccine, December 1956.

2. Second injection: inoculum 1 ml. given January 1957.

3. Third (last) injection: inoculum 1 ml. given October 1957.

He was bled March 1958. The serum was handled exactly the same as other sera used in this study. The serum was titrated in FL cell culture, using the technics described previously. The purpose of these antibody titrations (3 titrations) was to obtain data concerning present antibody content (average of 3 titers) and to do a comparative study on polio antisera prepared in human beings, rabbits, and monkeys.

The antibody titers in Table 8 represent antibody levels 15 months after receiving the first injection and 2 months after being injected with the third (last) injection. The serum was not toxic in cell culture.

Table 8

Titers of an individual serum that was vaccinated with Salk's polio Vaccine

Serum	VIRUS TYPE		
	MAHONEY	MEF$_1$	SAUKETT
Antibody content of an individual vaccinated with Salk's Vaccine	1,790	550	220

Table 8 shows that there was greater antibody response with type 1 poliovirus than with types 2 and 3, although neutralizing antibodies produced by all three types were titrateable in cell culture.

Each pool of polio antiserum was studied in regard to 50 per cent protective end points for homotypic and heterotypic strains of poliovirus. The tests were made in the FL cell line. The results of the serum-neutralization tests in cell culture, along with the results obtained by Wenner et al. (5), appear in table 9.

Table 9

A summary of antibody titers resulting from 5 different methods of vaccination in 3 different animal species titrated in 3 different cell lines

Method of Preparing Polio Antiserum	SERUM TYPES				
	Type 1	Type 2	Type 3	Animal	Cell line
Schedule A	5,720	5,720	6,400	Rabbit	FL Cells
Schedule B	3,410	5,860	1,290	Rabbit	FL Cells
Schedule C	6,240	11,770	3,850	Rabbit	FL Cells
Polio typing sera prepared in monkeys by Wenner et al.(5)	4,370	1,120	5,590	Monkey	FL Cells
Polio typing sera prepared in monkeys by Wenner et al.(5)	1,600	6,400	6,400	Monkey	Monkey testicular Cells
Polio typing sera prepared in monkeys by Wenner et al.(5)	4,600	25,000	18,000	Monkey	Monkey kidney Cells
Serum of an individual vaccinated with Salk's Polio Vaccine	1,790	550	220	Human	FL Cells

The polio antisera were prepared by three methods of vaccination (Schedules A, B, and C). All titrations and neutralization tests were carried out in the FL Cell line. When compared with the antisera prepared by Wenner et al. (5), the three methods are highly satisfactory.

Wenner et al. (5) titrated the antibody content of the polio antisera in monkey kidney and monkey testicular cells, the resulting titers were as follows: Types 1, 2, and 3 in monkey kidney cells 4,600, 25,000 and 18,000, respectively. Types 1, 2, and 3 in monkey testicular cells were 1,600, 6,400 and 6,400, respectively. The same polio antisera gave these titers in the FL Cell line: 4,370, 1,120 and 5,590 for types 1, 2 and 3, respectively.

The polio antisera prepared using Schedules A, B and C, compare very faborable with the polio antisera prepared by Wenner et al. (5), (reference serum of the committee on Typing of the National Foundation for Infantile Paralysis), when titrated in the FL cell line.

Wenner et al. (5) observed that poliovirus titers in monkey kidney tissue may be 2-4 logs higher than in monkey testicular tissue. It is also true that monkey kidney cells are higher in susceptibility to poliovirus than HeLa or FL cells. Therefore, the author also titrated Wenner's polio antisera in the FL cells, thereby affording a common cell line for the comparison of antibody content of the prepared polio antisera. The results of the author findings appear in table 9.

The 50 per cent serum dilution endpoints (against 100

tissue culture infective doses) were as follows: Type 1, (5,720 Schedule A), (3,410 Schedule B), (6,240 Schedule C) and (4,370 polio antisera prepared by Wenner et al.). Type 2 (5,720 Schedule A), (5,860 Schedule B), (11,770 Schedule C) and (1,120 Wenner's polio antisera). Type 3, (6,400 Schedule A), (1,290 Schedule B), (3,850 Schedule C) and (5,590 Wenner's antisera).

Only type 1 polio antisera prepared using Schedule B have a lower antibody content than the antisera prepared by Wenner et al. (5). All three schedules (type 2 antisera) produced higher antibody response in rabbits than Wenner's type 2 antiserum. Type 3 polio antiserum, prepared by Wenner et al. (5), however, has an antibody content greater than the type 3 antisera prepared by Schedules B and C. Schedule A, type 3 antisera, is slightly higher in antibody content.

Polio antisera prepared by Wenner et al. (5), were found in this laboratory to be devoid of heterotypic activity, although, Wenner et al. (5) reported that types 1 and 3 antisera did show heterotypic activity in mouse neutralization tests made with 100 PD_{50} (100 paralyzing 50 per cent doses) of type 2 poliovirus. Likewise, polio antisera prepared using Schedules A, B, and C only showed cross reaction between types 1 and 3 antisera with type 2 poliovirus.

These findings were consistently observed in 3 separate titrations per serum pool.

IX. DISCUSSION

The data presented show that there was a significant antibody response in rabbits to intramuscular and intravenous injections of live polioviruses (with no adjuvants) grown in cell culture.

The antisera prepared in rabbits by the three vaccination methods (Schedules A, B and C) can be used reliably for typing and identification of polioviruses in cell culture. Type specific neutralizing antibodies were produced with acceptable titers by all of the methods used in this comparative study.

Type 1 antisera prepared by Schedules A and C gave higher titers than the type 1 antiserum prepared by Wenner et al. (5) (reference serum of the Committee on Typing of the National Foundation for Infantile Paralysis), while the type 1 antiserum prepared by Schedule B was slightly lower. Type 2 antiserum, prepared using the vaccination method designated as Schedule C in this study, had a considerably higher antibody level than the others and type 2 antisera of Schedules A and B have antibody titers significantly higher than the reference serum. Type 3 antiserum of Schedule A was slightly higher in antibody

activity than the reference serum, while type 3 antisera of Schedules B and C were appreciably lower.

The antibody response of rabbits to the Schedule A method of vaccination was higher than the antibody response of monkeys used by Wenner et al. (5) in the production of their polio typing sera. Added to the fact that Wenner et al. (5) used adjuvants and an animal appreciably more expensive for their work, Schedule A is obviously a more practical and satisfactory method for the production of polio typing sera by comparison.

While Schedule B method of vaccination gave a slightly lower antibody level for types 1 and 3 sera when compared with the reference serum, type 2 serum was higher in antibody content. This schedule is at least as good as the method of Wenner et al. (5). Again these results were achieved without the use of adjuvants and without a more costly animal. Since the difference in antibody response of these two methods was not significant, Schedule B merits acceptance over Wenner's method of vaccination because of these two facts.

The method of vaccination designated as Schedule C is by far the most practical method of the three used in this study. When compared with the reference serum, the

antibody content is considerably higher, the volume of virus injected is much smaller, and polio typing sera with these titers, 9,320 (type 1), 10,880 (type 2), and 3,500 (type 3) were produced in 46 days using only 10 ml. of virus per rabbit.

Actually by using the rabbit instead of the monkey as the animal in which to prepare the polio typing serum, repeated bleedings with intervals of rest followed by booster doses are not necessary. With the difference of cost of one animal over the other, the rabbit is expendable and can be exsanguinated. Repeated bleedings with the monkey require intervals of rest between bleedings, repeated injections (administering of booster doses), thorough observations of the animal for tuberculosis, pneumonia, temperature changes, paralysis and diarrhea; maintenance of a monkey colony and trained personnels. The mortality rate of monkeys is quite high. Often the monkey expires after bleeding or after being injected with a booster dose and exsanguination is seldom achieved before death ensues. The handling of monkeys is hazardous and required experienced technicians. All of this is very laborious, lengthy and costly.

The advantages of using the rabbit in preference to the monkey for the production of polio typing serum are

very considerable. The rabbit obviously is more practical for this purpose.

All the polio typing sera prepared by Schedules A, B and C were type specific with a very slight degree of heterotypic activity. The only cross reaction observed was: Type 1 serum with type 2 poliovirus and type 3 serum with type 2 poliovirus. However, cross reaction was never observed at dilution of serum greater than 1:10.

Schedules A, B and C are very satisfactory for the production of polio typing sera when compared to the reference sera. Schedules A and C are better than the method of Wenner *et al.* (5), while Schedule B is about as good as the method of vaccination used by Wenner *et al.* (5) for the production of polio typing sera.

The author favors Schedule C because it is much easier to carry out and a very satisfactory typing serum can be prepared in a relatively short time.

The polio typing sera prepared in this study, as compared to the reference sera, are very acceptable for use as polio typing sera.

X. SUMMARY

The comparative study on a simple method for producing antisera with a high degree of neutralizing capacity for polioviruses has been presented. By repeated intramuscular and intravenous injections of rabbits with small doses of poliovirus grown in cell culture (with no adjuvants), antisera with a high degree of homotypic and little or no heterotypic neutralizing capacity have been prepared.

Mahoney (type 1) and MEF_1 (type 2) strains of poliovirus were almost always higher in antigenic potency than was the Saukett (type 3) strain.

All three schedules used in this study were satisfactory when compared with the reference sera. Two of the three methods of vaccination (Schedules A and C) gave higher antibody titers. The third method (Schedule B) was at least as good as the reference sera.

In this study an acceptable polio typing serum was prepared (using Schedule C as the method of vaccination) in 46 days injecting only 10 ml. of live virus (cell culture fluid with no adjuvants) per rabbit.

In this laboratory, Schedule C method of vaccination is being used regularly for the production of typing sera

in rabbits for some of the enteric viruses and other viruses isolated in our laboratory with great success.

The data presented show that an entirely satisfactory polio typing serum can be easily prepared by vaccinating rabbits with live virus antigen (cell culture fluids).

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