FAMILY STUDIES IN POLIOMYELITIS

by

Richard H. Barger, B.S.

A THESIS

Presented to the Department of Bacteriology
and the Graduate Division of the University of Oregon Medical School
in partial fulfillment
of the requirements for the degree of
Master of Science

June 1955

APPROVED:

[redacted]

(Professor in Charge of Thesis)

[redacted]

(Chairman, Graduate Council)

TABLE OF CONTENTS

INTRODUCTION

The viruses constitute a group of obligate parasites of living cells which, in general, show the following characteristics: 1) They do not grow on artificial media, 2) they are not visible under the ordinary light microscope, 3) they pass filters that retain bacteria, and 4) they produce disease in animals and plants. Cells for the cultivation of viruses may be supplied by intact susceptible animals, may be derived from embryonic tissue of the developing chicken's egg, or from pieces of susceptible tissues. For in vitro propagation, two general classes of tissues are used: one is the minced, surviving, functional type described by Maitland and Maitland (1) and Li and Rivers (2); the other is the rapidly growing cell such as is found in embryonic explants or tumors. Most viruses prefer rapidly growing cells but they may also multiply well in cells which are simply maintained in a functional state (3). For these reasons tissue culture techniques have been extensively utilized by virologists to further the knowledge of these organisms and the diseases produced by them.

Harrison (4) described the first "tissue culture" in 1907 when he visualized cells growing on a glass slide. He watched microscopically the development of nerve fibers from fragments of frog neural tube suspended in frog lymph. The cultivation of viruses in tissue culture was first attempted by Steinhardt (5) in 1913, and later Maitland and Maitland (1) in 1928 who reported varying degrees of success in propagating vaccinia virus. Early preparations consisted

of mixtures of cells not actively metabolizing, such as minced adult tissue in serum and salts. Following these classic experiments the field has progressed rapidly and the present complexities are reflections of the success which has been achieved in the propagation and study of many viral and rickettsial agents.

TYPES OF VIRUSES GROWN

Listed in the following table are most of the medically important disease producing viruses, the tissue they were grown in, the particular type of study involved and the authors who first reported their cultivation.

TABLE I (Modified from Sanders)[54]

VIRUS	TISSUE	STUDY	AUTHORS
Adenoidal-pharyngeal-conjunctival agents	tonsil, adenoid epithelium	P*	Huebner, Rowe, Ward, Parrott, and Bell (6)
Coxsackie	mouse brain, muscle, intestine	P	Slater and Syverton (7)
Dengue	mouse brain, egg embryo	P	Schlesinger (8)
Japanese B encephalitis	embryonic chick liver	P&V*	Warren and Hough (9)
St. Louis encephalitis	embryonic mouse brain	P	Syverton and Berry (10)
Epidemic kerato-conjunctivitis	" "	P	Sanders, X. (11)
Equine encephalomyelitis	egg embryo	P	Syverton, Cox, and Olitsky (12)

Foot and Mouth	embryonic guinea pig skin	P	Hecke (13)
Herpes simplex	rabbit brain and testicle	P	Parker and Nye (14)
Hog cholera	swine bone marrow and lymph nodes	P	Hecke (15)
Influenza	egg embryo	P	Francis and Magill (16)
Louping ill	egg embryo	P	Rivers and Ward (17)
Lymphogranuloma venereum	guinea pig kidney and testicle	P	Meyer and Anders (18)
Measles	minced chick embryo	P	Plotz (19)
Mumps	minced amnion, egg embryo	P V	Weller and Enders (20) Beveridge and Lind (21)
Newcastle	egg embryo	P	Topacio (22)
Poliomyelitis	embryonic human nervous tissue,	P	Sabin and Olitsky (23)
	monkey kidney	V	Salk (24)
Psittacosis	mouse spleen	P	Bedson and Bland (25)
Rabbit myxoma	mononuclear cells	P	Benjamin and Rivers (26)
Rabies	rabbit embryo brain	P V	Kanazawa (27) Koprowski and Black (28)
Serum hepatitis	minced chick embryo	P	Drake, Henle, and Stokes (29)
Yellow fever	chick embryo, rabbit testicle, guinea pig testicle	P	Haagen and Theiler (30)
Vaccinia	rabbit corneal tissue	P	Aldershoff and Broers (31)
Varicella and Herpes zoster	human embryonic skin and muscle	P	Weller (32)

P* refers to propagation, V* to vaccine

Several important viruses have failed to grow in tissue culture. Included in this group are those of infectious mononucleosis, trachoma, common cold, infectious hepatitis, and epidemic kerato-conjunctivitis. It is likely that some requirement is still lacking either in the culture medium or in the techniques thus far used in attempts to propagate these viruses.

OTHER TYPES OF TISSUE CULTURE STUDIES

1. Antigen Production

Immunizing antigens or vaccines have been prepared in tissue culture with varying degrees of success. Repeated passage of mumps virus in chick embryos has resulted in an attenuated strain which has been used for immunization. The 17D strain of yellow fever virus has likewise been attenuated in mouse and chick embryo and employed as an immunizing agent. Complement fixing antigens have been successfully prepared in tissue culture using the HeLa cell for the newly recognized group of viruses called adenoidal-pharyngeal-conjunctival agents (6). Generally speaking, tissue culture offers good opportunity for the preparation of antigens for diagnostic testing in viral diseases.

2. Immunity Studies

Tissue culture virus neutralization is probably the most extensively used test for immunity. Antibody titrations of serum may be performed and neutralization indices calculated by the Reid and Muench method (33). Neutralizing antibodies prevent the virus

from destroying cells of susceptible tissues. Acute and convalescent serums are generally tested simultaneously. Details of this technique will be described.

3. Growth Curves

The fact that viruses depend on the viability of the host cell for their multiplication and their intracellular habitat has made them more difficult to study than bacteria. However, using tissue culture methods, growth curves for mumps (20), influenza (34), and poliomyelitis (35) viruses have been described and have added to our knowledge concerning the mechanism of viral reproduction.

4. Antibiotics and Germicides

Tissue culture can be readily adapted to determine cellular toxicity and at the same time the bactericidal or viricidal activity of a variety of antibiotics and germicides. Herrell and Heilman (36,37) used explants of rabbit lymph node to test cell toxicity and bactericidal action of a sulfonamide drug and two antibiotics. They were also able to determine the antagonistic action of these substances toward one another and to indicate or contraindicate their use in treatment of infectious disease.

POLIOMYELITIS VIRUS IN GENERAL

Prior to the work of Enders and co-workers (38) in 1949, the in vitro propagation of the virus of poliomyelitis was quite irregular. The first notable success in propagating these agents was

achieved by Sabin and Olitsky (23) in 1936, when they maintained the virus for six serial passages, obtained an increase in viral population and produced the disease in monkeys with the final culture material. Nervous tissue from a human embryo minced in Tyrode's solution was used.

1. Etiology

Little was known about the etiological agent of poliomyelitis, until recently, because of the difficulty in cultivating it and the expense of using monkeys for test animals. The disease, on the other hand, was well understood with respect to its pathogenesis, mode of transmission, and production of immunity. Studies on rhesus monkeys were done by Landsteiner and Popper (38) as early as 1909. These men were the first to transmit poliomyelitis to monkeys. They used the intraperitoneal route of injection but were unable to successfully pass the disease to other animals. Flexner and Lewis (40) in 1910 succeeded in making serial passages by intracranial injection of monkeys with infected material.

2. Immunity

Immunity to poliomyelitis was recognized in these same early studies and subsequent ones. Neutralization tests were performed by Flexner and Lewis (41) (42) and Landsteiner and Levaditi (43) in 1910 when it was discovered that serum from monkeys which had recovered from the experimental disease neutralized the virus in vitro. Animals subsequently injected with these mixtures did not acquire the disease. The knowledge gained on the experimental

animal was applied to human cases and similar immunity studies were done. These tests were not well controlled, however, and often only one monkey was used in an experiment. In spite of the discrepancies and variation found in these early studies they formed the ground work for the later more carefully executed investigations from which the bulk of our knowledge was obtained about the virus of poliomyelitis.

3. Vaccines

Vaccines against poliomyelitis have been employed in various forms since 1910. Flexner and Lewis (44) vaccinated one monkey with a live virus suspension subcutaneously and found that the animal resisted an intracerebral challenge with the same virus at a later date. Many investigators since this time have also used live poliomyelitis virus as a vaccine for experimental animals to determine the degree of immunity produced. However, many monkeys developed paralytic poliomyelitis as a result of being vaccinated despite the use of subcutaneous or intradermal routes of injection. The highest antibody titers were produced by intramuscular injection, generally, but also the highest mortality rate (45). Kolmer (46) prepared a vaccine supposedly inactivated by sodium ricinoleate. When used intracutaneously, this vaccine did not produce paralysis in monkeys but in most cases protected them from intracerebral challenge with live virus. (47). When Kolmer's vaccine was tried on humans, 9 subjects out of 10,752 developed paralytic poliomyelitis,

in some cases referable to the injected site. Similar results were observed by Leake (48) using a formalin treated vaccine. In these studies no report is given on the levels of antibody response in the vaccinated group and consequently little was learned about the effectiveness of the vaccine.

Very recently, a formalinized vaccine has been produced, tested, and found to be safe as well as highly antigenic, thanks to in vitro methods of growing large quantities of virus and to strict safety testing.

4. Serologic Types of Poliomyelitis Viruses

Bodian (50) took advantage of the observation that monkeys which survived an attack by one strain of virus could become paralyzed by another strain. He conducted experiments in which groups of animals were vaccinated with two prototype strains, Lansing and Brunhilde. On intracerebral challenge using twelve other strains, he found that one strain (Leon) was unrelated to either Lansing or Brunhilde, three strains were identical to Lansing and not Brunhilde, and eight were related to Brunhilde and not to Lansing. In all cases he found that vaccinated monkeys challenged with heterotypic strains developed paralysis and those challenged with a homotypic strain survived. This was excellent evidence that there exists at least three antigenic types of poliomyelitis virus. Since these experiments in 1949, all strains of poliomyelitis have been found to fall into one of three types. Some well-known prototype viruses are

Brunhilde and Mahoney, (type I), Lansing and MEF-1, (type II), and Leon and Saukett, (type III).

TISSUE CULTURE

Enders and associates have shown that the virus will grow, not only in neural, but in non-neural tissues of quite different types. This series of observations (38,51,52,53) opened the way for mass studies of poliomyelitis not limited by the tremendous number of experimental animals needed for this work. However, in his first successful report on non-neural growth, Enders (38) produced the disease in monkeys to confirm the results of his in vitro experiments. Among the tissues found to propagate the virus of poliomyelitis, nervous and embryonic human tissue were the most susceptible, but testicular tissues of adult humans and monkeys and monkey kidney were also proven useful. Table II (see following page), modified from Sanders' review (54), shows the widely divergent sources of tissues susceptible to the poliomyelitis viruses. In their first successful attempts at growing poliomyelitis viruses, Enders and co-workers (38) utilised three types of embryonic tissue including brain and two non-neural types. Tissue fragments were suspended in a nutrient medium containing three parts balanced salt solution and one part of serum ultrafiltrate. They inoculated these tissues with 0.1 ml. of a mouse brain suspension of Lansing virus. The nutrient fluid was replaced every 4 to 7 days and new sub-cultures were made to fresh tissue every 1 to 3 weeks. An

TABLE II

TISSUE	ORIGINAL VIRUS TYPE GROWN
Human embryonic	
Brain and spinal cord	I, II, and III
Skin muscle	I and II
Intestine	I and II
Adrenal	II
Kidney	II
Thyroid	II
Lung	II
Heart	II
Human non-embryonic	
Testicle	I and II
Kidney	I, II, and III
Uterus	I and II
Tonsil	II
Thyroid	II
Tumors	II
Monkey	
Testicle	I and II
Kidney	II and III
Muscle	II
Brain and spinal cord	II
Lung	II
Intestine	II

increase in the virus was found in all three types of tissue and identification was made by neutralization tests with specific antisera in mice and monkeys.

A second paper by these same investigators (53) described a phenomenon of cellular destruction of infected tissues which was called "cytopathogenic effect". This type of cellular degeneration has since played a prominent role in the isolation and identification of viral agents and in antibody quantitation. Human embryonic tissues were again used and the tissue fragments were suspended in cultures consisting of fowl plasma plus chick embryo extract. Infected cultures showed a decreased respiration as judged by the elevation in pH of the medium. The usual cellular outgrowth from tissue fragments did not occur. Sections were made and varying degrees of degeneration were found in the infected cultures as opposed to the healthy controls. Immune serum from animals and humans was found to inhibit the "CP" effect, probably by neutralizing the infectivity of the virus. Mention is made of the possibility of using this CP effect to indicate the presence of the virus in infected tissues and for isolating the virus from the host, serologic typing of the virus, and screening procedures.

Since these classic experiments, research on the in vitro growth of poliomyelitis virus in non-neural tissues has advanced in three main directions. These may be listed as follows:

1) The use of monkey and human testicular tissue for cultivation and isolation of the viruses and for the detection of specific antibody (55,58).

2) The use of the HeLa cell for the cultivation and isolation of the viruses and for the detection of specific antibody (57).

3) The preparation of a vaccine to be used in the prevention of paralytic poliomyelitis (49).

Monkey Tissues

Smith, Chambers and Evans (55,58) presented evidence for the propagation of Hof and Lansing viruses in testicular tissues of both humans and monkeys. With Lansing strain they found virus still present after a dilution of 1:10 billion. This corresponded to twelve fluid changes of culture medium. Younger, Ward and Salk (56) have given evidence for the propagation of all three types of poliomyelitis virus in monkey testicular tissues. Roller tube cultures were used by these investigators presumably for enhancing the activity of the virus. With this method, separate culture tubes containing tissue fragments suspended in a plasma clot are inserted in a roller drum which rotates constantly, periodically bathing the tissue with medium. Virus material may then be added to these tuber after sufficient outgrowth of new cells has occurred. The optimal temperature range for these techniques is from 35 to 37 degrees Centigrade.

Monkey kidney has also been a very useful substrate for growing, isolating and typing the poliomyelitis viruses. Salk, et al, (49,59) has employed this medium for preparing fluids of very

high virus titer to be used as a vaccine. Youngner (60) described a method by which roller tubes were prepared from trypsin dispersed monkey kidney cells. He found that higher titers were consistently obtained by this method than by simply using minced tissue fragments.

From the significant observations of Robbins and co-workers that pH differences occur in infected versus non-infected cells in tissue culture, a colorimetric test has been devised for use in typings, neutralizations and isolations of the poliomyelitis virus which may be read by changes in pH of the nutrient medium. As mentioned previously, respiration of the cell decreases in infected cultures and with the proper indicator, a rise in pH may be observed. In healthy cells, on the other hand, an increasingly acid pH occurs. In testing by this method a difference of 0.2 pH unit has been stated as being significant when the infected cultures are compared with the controls (53). The method offers the advantages of being faster and easier to read but it has also been found to show considerable variation in duplicate tests.

HeLa Cells

The HeLa cell, isolated and cultivated by Gey (62) has been very widely used as a medium for the growth of viruses, especially poliomyelitis, in vitro. This cell was derived from a human epidermoid carcinoma of the cervix and has been described as being especially noteworthy in the following respects:

1) The cells can be propagated continuously in tissue culture.

2) They were derived from human tissue.

3) They remain viable for prolonged periods in synthetic medium.

4) The cells may easily be shipped long distances without damage.

Syverton and Scherer (57,63) utilized the HeLa cell to grow, isolate, and type poliomyelitis viruses from several epidemics. In addition to poliomyelitis, the HeLa cell has been found to support the growth of the viruses of herpes simplex, vaccinia, pseudorabies, pseudolymphocytic choriomeningitis, Japanese B. encephalitis, Eastern equine encephalomyelitis, and members of the coxsackie group (61).

When the HeLa cell is used to isolate poliomyelitis virus, a suspension of the cells is permitted to settle onto the side of a test tube, under aseptic conditions, and allowed to grow for a few days in fluid medium. The suspected material is then added and if virus is present, the cells rapidly undergo degeneration. Immune serum prevents this cellular degeneration by neutralizing the infectivity of the virus. This fact forms the basis for the neutralization test and also makes possible the identification of the serologic type of the virus present.

A test employing pH change of the medium as an indicator of viral activity has not been made available in the case of the HeLa cell. A personal communication from Dr. Wenner (64) revealed that the cell was quite capricious and that special fluid medium

would have to be employed with regard, probably, to the amount of glucose used. Consequently, this method has enjoyed little application.

Vaccine

The preparation of a suitable vaccine against poliomyelitis has been at least partially realized since the observations of Enders and co-workers that the virus of poliomyelitis could be grown in vitro. Much higher virus titers can be obtained in vitro than in the experimental animal and this fact is a major requirement for a successful poliomyelitis vaccine.

The National Foundation for Infantile Paralysis, (NFIP) in the autumn of 1953, announced their decision to conduct large scale field trials to determine whether or not paralytic poliomyelitis could be prevented by vaccination. A vaccine developed by Dr. Jonas Salk (49) at the University of Pittsburg was used. The vaccine was prepared from fluid of cultures of monkey kidney cells containing the three known types of poliomyelitis viruses which were inactivated with 1:4000 formalin at 36 C. and pH 7. The final product was rendered sterile by filtration. The resulting material was not used if it was found to contain more than 0.35mgm/ml of total nitrogen because of the possibility of extraneous immunizing material being present. Because the virus was grown in monkey tissue, the question arose as to the possibility of Rh antigens being present. Evidence has since been presented that this is

unlikely (59,65). Salk (59) observed that levels of antibody could be attained which simulated those found in natural infections. A booster injection seven months later produced a striking rise in the blood antibody titer.

THE NFIP STUDY PLAN

In the 1954 field trial study of the vaccine, the so-called "placebo control study" was employed in certain participating areas. Under this plan children of the first, second and third grades were combined, one half acting as controls. Vaccine or placebo, as the case may be, was administered to the children under the same lot number but a code number known only to the Evaluation Center would indicate whether any given child was in the control or vaccinated group. Any child in the study group who might subsequently develop poliomyelitis could be immediately identified by the Evaluation Center. Also, family members and other contacts of the study members were taken into consideration in the event of an outbreak of poliomyelitis. All cases occurring within the study group were recorded and reported by the local program director. This information could then be augmented by serologic testing and by virus isolations from stool specimens. Of the study group 2% were bled prior to, two weeks after, and again at the end of the poliomyelitis season. This would provide information relative to: 1) the activity of the vaccine in the vaccinated group and 2) the incidence of natural exposures to poliomyelitis in the control group.

General Techniques Used in Testing

Several methods of testing for serum antibodies to poliomyelitis were recommended by the Evaluation Center for participating laboratories (66). Among these are: 1) The monkey kidney cell metabolic inhibition test of Salk, Younger and Ward. 2) The HeLa suspended cell method of Syverton. 3) The HeLa cell stationary tube method of Syverton. 4) The HeLa suspended cell color change method of Steigman. In the first method a suspension of trypsin dispersed monkey kidney cells is prepared containing 150,000 to 300,000 cells/0.25 ml in a medium containing mixture 199 (69) 95 ml., 2.8% $NaHCO_3$, 3 ml., horse serum, 2 ml., penicillin 100ug, and streptomycin 0.1 mgm./ml. The serum to be tested is mixed with 100 tissue culture doses (TCID 50) of each prototype virus suspension and allowed to stand at room temperature for thirty minutes. After this "neutralisation period" the suspended cells are added to the mixture and the tests are incubated at 35-36°C. Within six or seven days the tests are observed for pH change. Inhibition of virus effect is indicated at pH 7.0 or less and virus activity is shown by a pH above 7.0.

The second method makes use of the HeLa cell described earlier in this paper. Each serum to be tested is diluted in Hanks' solution in the following dilutions: 1:4, 1:8, 1:16, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048, and 1:4096. To each of these serum dilutions 100 TCID-50 of virus is added and the mixture is

incubated at room temperature for one hour. A suspension of HeLa cells in nutrient fluid is then added to each dilution in 0.25 ml. amounts and the tubes are placed in a slanting position in the incubator at 35 to 36 degrees C. Virus controls are set up with each test and are diluted in half log steps in mixture 199. The tests are read microscopically when the virus controls show a TCID-50 of approximately 100. The actual TCID-50 is then calculated according to the Reid and Muench formula mentioned previously.

The third method is much the same as the second except that prepared tubes of HeLa cells which have been growing for several days are innoculated with the serum-virus mixture, and finally fluid medium is added. The fourth method, involving pH changes with HeLa cells, was still under investigation at the time this study was being done and had not been routinely used. The HeLa cell is known to be somewhat capricious in respect to testing by the pH method and the procedure is not perfected to the point of widespread use as yet.

In this laboratory the HeLa cell suspension method has been used with quite favorable results for serum antibody testing and isolations. The prepared tube method using the HeLa cell was also used in isolations and typing of virus from fecal specimens and will be described later.

EXPERIMENTAL STUDIES

STATEMENT OF PROBLEM

To investigate the serum antibody response in families and in cases of naturally acquired poliomyelitis occurring in Oregon and Idaho in 1954, and to correlate these findings with the isolation and typing of the virus. The studies reported here and others to be reported later have been made possible by a grant from the NFIP.

MATERIALS AND METHODS

Strain HeLa cells

The HeLa cells used in these studies were obtained from Dr. Russell Brown, the George Washington Carver Foundation, Tuskegee Institute, Tuskegee, Alabama. The cells are grown on one surface of square, 200 ml. glass, screw-cap bottles and are generally shipped without nutrient medium to avoid excess agitation in transit. Upon receipt of the cell cultures, they are immediately revived by adding 10 ml. of nutrient medium under aseptic conditions. They are then incubated at 35 to 36°C with the cell surface of the bottle down in order that all of the cells are covered by the nutrient fluid. After a day or two under nutrient fluid, the cells may be harvested in the following manner: The medium is decanted and the cells are washed three separate times in 10 ml. aliquots of Hanks' solution to rid them of any antipoliomyelitis

viral substances which may have been present in the medium. They are then treated with 8 to 10 ml. of a 0.5% solution of Difco 1:250 trypsin in mixture 199 for 1/2 to 1 hour. The trypsin releases the cells from the glass surface so that they may be forced through a small bore pipette to break up any cell clumps. This material is then transferred to 50 ml. centrifuge tubes and centrifuged at 1000 to 1200 R.P.M. for about 10 minutes, after which time the supernate is decanted. The cells are then resuspended in Hanks' solution and centrifuged two successive times. At this point the cells are resuspended in the nutrient medium to be used in testing and may quite easily be enumerated in a hemocytometer. The cells are added to the medium in sufficient number so that 1 ml. contains approximately 200,000 cells. 0.25 ml. of the cell suspension containing about 50,000 cells is used in testing serums for poliomyelitis antibodies.

Virus

The prototype viruses used in this laboratory were prepared in the Connaught Laboratories, Toronto, Canada. The strains used were Mahoney(type I), MEF-1 (type II), and Saukett (type III). The viruses were grown in cultures of suspended monkey kidney fragments, were harvested, cultured for bacteria and fungi, and for the presence of virus B by animal inoculation. (Virus B is an infective virus found in the saliva of normal monkeys). Aliquots of each virus type was tested in monkey kidney and HeLa cell tissue

cultures for infectivity endpoints. Ampoules were then sent to each laboratory participating in the poliomyelitis field trials. The virus suspensions are stored at -70°C. in order to retain their potency. In our laboratory, type III virus was found to show decreasing infectivity titers so three further passages of this strain were made against HeLa cells which raised the titre from $10^{-3.5}$ to approximately $10^{-6.5}$. The 50% endpoint (ID-50) of the virus is obtained by doing half log dilutions of the stock material in mixture 199 and placing 0.25 ml. of each dilution in He La cell culture tubes. The cytopathogenic effect produced by the virus on HeLa cells makes possible the calculation of the 50% infective dose by the Reid and Muench method (22). Once the ID-50 has been determined for a given virus, titrations of 0.1, 0.5, 1.0, 5.0, and 10.0 ID-50 are used as virus controls for each test run. The serums of patients to be tested are diluted as mentioned previously in Hanks' solution and 0.25 ml. of this dilution is incubated for one hour at room temperature with 100 ID-50 (0.25 ml.) of virus. After this period a suspension of HeLa cells containing approximately 50,000 cells in 0.25 ml. of nutrient medium is added and the tubes are incubated at 35 to 36°C. When the virus controls show a titer of approximately 100 ID-50, the test is read microscopically, the cytopathogenic effect of the HeLa cell being the indicator of viral activity. Serums with protective antibodies will inhibit this effect and the dilution at which the serum no longer protects the cell from the virus is the endpoint or titer of the serum.

Standard Anti-sera

Antisera were prepared in rhesus monkeys for all three antigenic types of poliomyelitis viruses and were standardized by the determination of neutralization indices and serum protection tests in monkeys, mice and tissue culture (67). These serums were supplied by Dr. Wenner in dry form and were reconstituted by adding 0.5 ml. of sterile distilled water. They are prepared in specified dilutions in Hanks' solution and are included in each test run along with the virus controls and test serums. 0.25 ml. amounts of each dilution are incubated with 100 ID-50 of virus and tested in the same manner as the unknown test serums from cases. The antisera should showapproximately the same protection titers in each test run.

Nutrient Medium

Human adult serum (HAS) is used as nutrient medium for the HeLa cells in both stock cultures of the cells and in testing. We obtained whole blood in 500 ml. amounts from the American Red Cross donor's service and separated the serum aseptically. The serum was given a number, dispensed into aliquots and stored at -40°C. Each Red Cross serum was screened for antibodies to poliomyelitis as it was needed. Those sera found to contain no antibodies were subsequently used as nutrient medium in the tests. The sera with antibodies were used for our own stock cultures of HeLa cells.

In test runs and in stool isolations of the virus, 10% HAS (containing no antibodies) in mixture 199 was employed as

nutrient fluid. In stock cultures 40% NAS in 199 was used. Cultures are made of each Red Cross serum for bacteria and fungi.

Hanks' Solution

Hanks' solution (68), containing glucose and the various salts of sodium, potassium and magnesium, employs phenol red as an indicator and is used in tissue culture techniques for washing tissues and cell cultures free of extraneous substances as well as in diluting serums for testing.

Mixture 199

Synthetic mixture 199 (69) is a complex mixture containing salts, amino acids, vitamins, glucose, nucleic acid components and accessory growth factors. This mixture was found to support the survival of chick embryo tissue for four to five weeks without the addition of any other nutrients. An initial growth period, however, must be initiated by some substance such as serum or exudates. Mixture 199 is used in washing cells to rid them of antiviral substances, in preparing trypsin solution for harvesting HeLa cell cultures, and in medium for use in tissue culture studies.

Test Serums

The bloods which are received for antibody studies are centrifuged and the serum is taken off aseptically, numbered, and stored at -20°C. Before testing, they are dispensed in 0.6 ml. amounts, inactivated at 56°C for 30 minutes, and diluted 1:4 with

Hanks' solution. Twofold dilutions are then made in Hanks' solution until a maximum dilution of 1:4096 is obtained. (Very few serums were found to have protective antibodies beyond this range). The serum dilution is then transferred to three sterile 16mm. screw cap tubes in 0.25 ml. amounts. Each of these tubes is used to test one prototype virus against the particular serum dilution put into it. The proper dose of virus and cell suspension are subsequently added with the aid of an automatic pipette set at 0.25 ml. Strict aseptic technique is observed in the handling of all these materials to prevent both laboratory infections and contamination.

Virus Isolation From Stools

The isolation of poliomyelitis virus from stool specimens of cases and contacts forms an integral part of this study. The stools were sent to us by local health officers from those areas in Oregon and Idaho where poliomyelitis occurred. They were refrigerated when received and processed in the following manner as time permitted (64):

A 20% suspension is prepared from the stool material in distilled water and ground with mortar and pestle. This suspension is centrifuged at about 2000 RPM for 10 to 15 minutes to throw down the crude materials. The supernate is recovered and put into celluloid centrifuge tubes and centrifuged at high speed (7000 to 10,000 RPM) for one hour to separate the remaining bacteria from solution. The supernate is again taken off, this time aseptically,

and put in sterile glass tubes with stoppers. Penicillin and streptomycin are added to each tube in concentrations of 100 ug and 500 ug/ml. respectively. 0.1 to 0.2 ml. of the final stool suspension is added to four tubes containing luxuriant outgrowths of HeLa cells. Nutrient medium is then added containing 10% HAS in 199 and the tubes are incubated at 35 to 36°C. If virus is present in the suspension, degenerative changes leading to complete destruction of the cell occurs within 2 to 3 days. All suspected material is passed in HeLa cells 2 or 3 times to enable the recovery of very small numbers of virus and in turn dilute out any materials in the stool toxic to the HeLa cell.

Immunologic identification of cytopathogenic strains of poliomyelitis virus is accomplished by using the previously mentioned hyperimmune standard monkey antisera, representative of the three known types of poliomyelitis virus. Eight tubes are used in each typing after the method Syverton (63) with minor modifications. Eight culture tubes containing 50-100,000 HeLa cells from 3 to 5 days old are washed three times with Hanks' solution and to them is added: (a) 0.5 ml. of 10% HAS in 199 to all tubes. (b) 0.25 ml. of type I immune serum to tubes 1 and 2. (c) 0.25 ml. of type II immune serum to tubes 3 and 4. (d) 0.25 ml. of type III immune serum to tubes 5 and 6. (e) 0.25 ml. of normal monkey serum to tubes 7 and 8 for control purposes.

The virus material is then added to all tubes in 0.25 ml. amounts and they are incubated at 35-36°C. If virus is present,

the cells in six of the eight tubes will be destroyed in from 3 to 5 days. The healthy cells in two of the tubes indicate the protective effect of the type specific antisera for the cells and the immunologic type of the virus.

RESULTS AND DISCUSSION

During the poliomyelitis season of 1954, studies were done on 28 physically diagnosed cases and their families in an effort to confirm by laboratory testing, the often difficult diagnosis of this disease entity. Thirteen of these cases were considered to be paralytic and fifteen non-paralytic. Acute phase blood and stool specimens were sent by local health officers and a convalescent blood specimen was received approximately one month later. By comparing the poliomyelitis antibody titers of the acute and convalescent bloods and by isolating the virus from stool specimens, it was often possible to determine the etiology of a particular case. Information relative to the prevalence of a certain type virus in a given area and a rough measure of the incidence of sub-clinical infections was obtained. The emphasis here, however, was placed on an attempt to correlate the serologic response with isolation of virus and the interpretation of these findings in making a laboratory diagnosis of poliomyelitis.

Table IV gives an example of a classic serologic response in a family to naturally acquired infection with type II virus. The paralytic case showed a specific antibody rise from 1:128 to 1:512

TABLE IV

SEROLOGIC RESPONSE TO NATURALLY ACQUIRED INFECTION
BY TYPE II POLIOMYELITIS VIRUS

Family Members	Relation	SERUM ANTIBODY TITER I Acute	I Conv.	II Acute	II Conv.	III Acute	III Conv.	Virus Isolated
Harold	case	0	0	128	512	0	0	II
Mary	mother	64	2048	4	2048	64	1024	II
Henry	father	0	0	512	512	0	0	0
Kathy	sister	0	4	4	1024	0	0	II
Alfred	brother	4	0	128	256	0	0	II

and confirms the clinical diagnosis. Two other members of the family acquired the infection and manifested characteristic and homotypic serology. The problem in the mother (Mary) however was considerably more complicated. The same virus (Type II) was demonstrable in her stool specimen and she also showed a homotypic antibody response of 1:4 to 1:2048. She, presumably, was infected with type II virus and constituted a sub-clinical case. In addition, non-specific heterotypic antibody increases for types I and III viruses occurred simultaneously. This poses the question of whether we were dealing with a mixed infection or an anamnestic response to one virus. The latter is considered to be most likely. Nevertheless, a rise in titer of this magnitude would be difficult to evaluate if the actual virus were not available.

In some of the cases it was impossible to demonstrate a specific rise in titer against one virus type. Perhaps the acute specimen was obtained late in the disease and consequently, only a high peak was observed in the initial serum and an equally high or decreasing value in the convalescent sample. In the majority of the families studied, exposure to other types of poliomyelitis viruses had occurred at some time in the past and often titers to heterotypic strains were higher than to the one causing the infection. Because of these variables, it was not always possible to identify the viral type responsible from the serology alone.

In this study, the cases were separated into paralytic and non-paralytic groups. An attempt was made to arrange the results

in such a way as to confirm or rule out the diagnosis of poliomyelitis. It might be noted that the possibility of infection by other viruses or micro-organisms cannot be overlooked. In tables V and VI following, information on the serologic response to poliomyelitis is given for each case and related family members, including close contacts. Roman numerals indicate the virus type isolated or judged to be responsible for any given laboratory finding. For purposes of clarity the titration values have been omitted and an estimate of the diagnostic accuracy of each procedure is listed. It should be emphasized that a final analysis must also include the timing of specimens, the clinical evaluation of the patient, the vaccination status, and whether or not gamma globulin was given. This information was not available to us. The data which were obtained are shown in condensed form in tables V and VI. These tables must be qualified as follows:
1) All cases were considered to be poliomyelitis without reference to clinical symptoms or to evaluation by physiotherapists.
2) Attempts at virus isolation were confined to the poliomyelitis group only, and infections due to such agents as coxsackie, mumps, herpes, lymphocytic choriomeningitis, and the encephalitides, may well have occurred but were not considered. 2) A specific four fold antibody rise toward a single type of poliomyelitis virus was considered indicative of infection with that type.
4) A serum titer of 1:512 or greater was used as evidence of recent infection, the homotypic virus being indicated by a pre-

dominance of that type in each family. Each table is subdivided into three categories as follows: first; results of virus isolations, second; demonstration of specific antibody rises to the homotypic or other types, third; presence of antibody in the serum equal to or greater than a dilution of 1:512. Within each category the type of virus presumably causing the infection, is selected by the results which were obtained. This evaluation is given under "Type" in each category.

Paralytic Cases and Their Families. Poliomyelitis viruses were isolated from 21 of 65 members of the paralytic group and their families, as presented in Table V. Virus was obtained from 7 of 12 (58.3%) of the paralytic cases and 14 of 53 (26.4%) family members. These data show that a considerable number of the family members were infected as subclinical cases. Of the 13 families studied, an etiological agent was isolated from 9, an overall percentage of 69. There were three families with type I infections, one with type II, and five caused by type III virus. In contrast to the ease with which virus could be isolated, a specific antibody rise of four fold or greater was encountered in only three cases and six family members, an overall incidence of 14%. This may well have been due to the timing of the samples, as mentioned previously. Using the antibody response as a single criterion, there were two type I infections suggested, two type II, and three type III. Examination of the table will reveal that the virus isolations could be correlated with the serology in only four

TABLE V

SERUM ANTIBODY RESPONSE AND VIRUS ISOLATIONS
IN PARALYTIC CASES AND THEIR FAMILIES

Family	Type of Virus Isolated			Specific Antibody Rise Four-fold Response				Serum Titer of 1:512 or Higher		
	From Case	From Family	No.	Case	Family	Total	Type	Homo-typic	Hetero-typic	Type
1.	II	II	4/5*	1	2	3	II	4	1	II
2.	III	III	3/7	1	0	1	III	1	6	?
3.	?	?	0/6	0	1	1	I	1	3	?
4.	NS	III	2/4	0	0	0	?	2	1	?
5.	?	?	0/4	0	1	1	II	3	0	II
6.	?	I	4/7	0	0	0	?	5	2	I
7.	III	ND#	1/1	0	ND	0	?	0	0	?
8.	III	III	2/4	0	1	1	III	1	1	?
9.	?	?	0/6	0	1	1	I	4	4	?
10.	I	?	1/6	0	0	0	?	3	3	?
11.	III	III	3/6	1	0	1	III	5	2	III
12.	I	?	1/6	NCS#	0	0	?	4	2	I
13.	?	?	0/3	0	0	0	?	0	0	?
Total			21/65	3/12	6/51	9/64		33/62	25/62	

\# Not Done (family members)
* Type II virus isolated from 4 of 5 members
\# No Convalescent Specimen

TABLE VI

SERUM ANTIBODY RESPONSE AND VIRUS ISOLATIONS IN NON-PARALYTIC CASES AND THEIR FAMILIES

Family	Type of Virus Isolated			Specific Antibody Rise Four-fold Response				Serum Titer of 1:512 or Higher		
	From Case	From Family	No.	Case	Family	Total	Type	Homo-typic	Hetero-typic	Type
1.	III	III	4/7	0	0	0	?	4	5	?
2.	?	III	1/5	0	0	0	?	2	1	?
3.	?	?	0/5	0	0	0	?	0	0	?
4.	?	?	0/3	0	0	0	?	1	0	I
5.	?	?	0/5	0	2#	2	?	1	0	I
6.	?	?	0/4	0	0	0	?	1	0	II
7.	?	?	0/4	0	0	0	?	2	0	II
8.	?	?	0/4	1	0	1	III	1	2	?
9.	NS	?	0/3	0	0	0	?	1	1	?
10.	?	?	0/5	0	0	0	?	3	1	II
11.	?	?	0/6	1	1	1	III	0	2	?
12.	?	?	0/8	0	0	0	?	2	1	?
13.	III	?	1/7	0	0	0	?	1	4	I
14.	I	?	1/5	0	0	0	?	3	0	I
15.	?	?	0/4	0	0	0	?	0	0	?
Total			7/75	2/15	2/60	4/75		22/75	17/75	

Specific rises to types II and III in one member

instances. When the group was examined for serum antibody titers of 1:512 or greater, a total of 33 of 62 (53.2%) showed a homotypic response whereas 25 of 62 (40.3%) showed a heterotypic response. A definitive diagnosis of type could be made in five instances, referable to the serology only. One type III, two type II, and two type I infections were suggested on this evidence alone. The data with respect to high serum titers could be correlated in four instances with the type of virus isolated.

For purposes of comparison, two tables (VII and VIII) have been presented, modified from the Summary Report of the MVIP. Table VII, involving 670 cases from placebo areas, shows virus isolations from 62% of the cases. In the observed areas, including Oregon and Idaho, the figure was 64.4%. This is to be contrasted with the 58.3% value obtained in the experimental studies reported here, with an overall case-family incidence of 32%.

Table VIII indicates cases in whom a four-fold or greater antibody rise wasobtained approximately one month after infection. In the placebo group, for instance, 34.5% of the cases showed a definitive response and in the observed group an increase was found in 41.8% with an overall value of 39% in both areas. In the small series reported here, specific antibody rises were observed in 25% of the cases and in 14% of the family members. Under more favorable conditions this figure might have been higher. As a single diagnostic test, the rise in antibody titer would appear to be a poor second to actual isolation of the virus.

TABLE VII

PERCENT POSITIVE VIRUS ISOLATIONS IN A GROUP OF PARALYTIC AND NON-PARALYTIC CASES*

Diagnostic Category	Total Cases	Total Specimens	Virus Isolation Number	Virus Isolation Percent	Total Specimens	Virus Isolation Number	Virus Isolation Percent
Paralytic	670	277	141	62	340	219	64.4
Non-paralytic	178	79	29	36.7	76	31	40.8

* Modified From NFIP Summary Report (65)

TABLE VIII

SEROLOGICAL RESULTS IN CASES WITH POSITIVE AND NEGATIVE VIRUS ISOLATIONS*

Virus Isolated	Placebo Group No.	%	Observed Group No.	%	Total No.	%
Serology positive	51	34.5	94	41.8	145	39
" probable	54	36.5	74	32.8	128	34.4
" negative	2	1.4	2	0.09	4	1.07
" indefinite	41	27.6	55	24.5	96	25.9
Total	148		225		373	
Virus Not Isolated						
Serology positive	14	1.1	38	21	52	17.1
" probable	49	39.5	52	28.8	101	33.1
" negative	3	2.42	2	1.1	5	1.7
" indefinite	48	46.8	89	49.1	147	48.1
Total	124		181		305	

* Modified From WFIP Summary Report (65)

As shown previously, a high titer of antibody is not indicative of infection by a given type of poliomyelitis virus. The data from the paralytic cases indicate that an anamnestic response occurred almost as frequently as did high titers to the homologous type.

Non-Paralytic Cases and Their Families. Poliomyelitis viruses were isolated from 7 of 75 (9.3%) persons in the non-paralytic case-family group. These included 3 of 14 (21%) of the non-paralytic cases and 4 of 61 (6.6%) of their family members. A total of 15 families were studied and an etiological agent was isolated from 4 of the 15 families, an overall percentage of 26.6%. As for the virus types found, three families were infected with type III and one with type I.

Specific four-fold or greater antibody rises occurred in only 2 of 15 (13%) non-paralytic cases and in 2 of 60 (3.3%) family members. It would appear from these figures that infection with other viruses might very likely have been responsible for some of the cases. If antibody response had been used as the sole criterion of diagnosis, only 2 (families 8 and 11) would have been detected. A correlation with viruses isolated exists in only one instance. In this group 22 of 75 (29.3%) members showed antibody titers of 1:512 or greater to the homologous virus and 17 of 75 (22.7%) to the heterologous type. In seven instances high titers were suggestive of infection as follows: four of type I, and three of type II. However, in only one family did the serology conform

to the type of virus isolated. For comparison, table VII shows that 36.7% of the non-paralytic cases yielded positive virus isolations in the placebo areas and 40.8% in the observed areas. These figures are approximately 17% higher than in the non-paralytic cases reported here (21.4%).

SUMMARY

The antibody response to poliomyelitis, together with virus isolation studies, were carried out in 13 paralytic and 15 so-called non-paralytic cases and their families. The findings obtained were used as the sole criteria for establishing an etiologic diagnosis without considering the clinical status. A total of 140 persons were examined. The laboratory data were categorized as follows: 1) The results of virus isolations, 2) The demonstration of specific antibody rises to the homotypic or heterotypic types of poliomyelitis viruses, and 3) The presence of antibody titers equal to or greater than a 1:512 serum dilution. Viruses were isolated from 58% of paralytic and from 21% of non-paralytic cases. Isolation figures for family members were 26.4% and 6.6% respectively. The lower values in the non-paralytic group are probably due to a higher incidence of infections with agents other than poliomyelitis. The presence of virus in the stools of more than 25% of family contacts of paralytic cases was a correlary observation.

Specific antibody rises of four-fold or greater were observed in 25% of the paralytic cases and in 14% of their family members. In the non-paralytic group these figures were 13.3% and 3.3% respectively. If antibody response alone was used as a criterion for diagnosis, correlation with the type of virus isolated could be found in only 4 members of the paralytic group and in 1 of the

non-paralytic cases and their families. In the paralytic group and their families, serum titers of 1:512 or greater were demonstrated against the homotypic virus in 53.2% of the members as opposed to 40.3% against heterotypic viruses. The non-paralytic case-family group showed similar high titers to the homotypic virus in 29.4% and to the heterotypic in 22.7% of its members. It appears that the serologic response to infection by poliomyelitis, as measured by the blood samples which were obtained, is not as reliable a criterion as virus isolation for a definitive laboratory diagnosis. In fact the serologic and viral types did not always agree in the same persons. The data indicate some of the difficulties in establishing an exact diagnosis of poliomyelitis infection and point up the necessity of correlating both the laboratory and the clinical findings.

BIBLIOGRAPHY

1. Maitland, H.B., and Maitland, M.C. Cultivation of vaccinia virus without tissue culture. The Lancet, vol. 2, pp. 596-597, 1928.

2. Li, C.P., and Rivers, T.M. Cultivation of vaccine virus. J. Exp. Md., vol. 52, pp. 465-470, 1930.

3. Cameron, G. Tissue Culture Technique, ed. 2, Academic Press, Inc., New York, 1950.

4. Harrison, R.G. Observation on the living developing nerve fiber. Proc. Soc. Exp. Biol. Med., vol. 4, pp. 140-143, 1907.

5. Steinhardt, E., Israeli, C., and Lambert, R.A. Studies on the cultivation of the virus of vaccinia. J. Inf. Dis., vol. 13, pp. 294-300, 1913.

6. Huebner, R.J., Rowe, W.P., Ward, T.G., Parrott, R.H., and Bell, J.A. Adenoidal, pharyngeal, conjunctival agents. A newly recognized group of common viruses of the respiratory system. New Eng. J. Med., vol. 251, pp. 1077-1086, 1954.

7. Slater, E.A., and Syverton, J.T. The cultivation of coxsackie virus. Proc. Soc. Exp. Biol. Med., vol. 74, pp. 509-510, 1950.

8. Schlesinger, R.W., Propagation in chick embryos of the Hawaiian strain of dengue virus. Am J. Hyg., vol. 51, pp. 248-254, 1950.

9. Warren, J., and Hough, R.G. A vaccine against Japanese B encephalitis prepared from infected chick embryos. Proc. Soc. Exp. Biol. Med., vol. 61, pp. 109-113, 1946.

10. Syverton, J.T., and Berry, G.P. The cultivation of the virus of St. Louis encephalitis. Science, vol. 82, pp. 596-597, 1935.

11. Sanders, M. Epidemic keratoconjunctivitis. I. Isolation of a virus. Arch. Opth., vol. 28, pp. 581-586, 1942.

12. Syverton, J.T., Cox, H.R., and Olitsky, P.K. Relationship of the viruses of vesicular stomatitis and of equine encephalomyelitis. Science, vol. 78, pp. 216-217, 1933.

13. Hecke, F. Zuchtung des maul-und klauenseuchcerregers. Zentrabl. Bakt. Abt. 1., vol. 102, pp. 283, 1931.

14. Parker, F., and Nye, R.N. Studies on filterable viruses. II. Cultivation of herpes virus. Am. J. Path., vol. 1, pp. 337-340, 1925.

15. Hecke, F. Die kunstliche vermehrung des schweinepestvirus mittels gewebekulturen. Zentrabl. Bakt. Abt. 1., vol. 126, pp. 517-526, 1932.

16. Francis, T., and Magill, T. Cultivation of human influenza virus in an artificial medium. Science, vol. 82, pp. 353-354, 1935.

17. Rivers, T.M., and Ward, S.M. Cultivation of louping ill virus. Proc. Soc. Exp. Biol. Med., vol. 30, pp. 1300-1301, 1933.

18. Meyer, K., and Anders, H.E. Versuche zur zuchtung des lymphogranuloma inguinale-virus. Klin. Wehnschr., vol. 11, pp. 318, 1932.

19. Plotz, H. Cited from reference #33.

20. Weller, T.H., and Enders, J.F. Production of hemagglutinin by mumps and influenza A viruses in suspended cell tissue cultures. Proc. Soc. Exp. Biol. Med., vol. 69, pp. 124-128, 1948.

21. Beveridge, W.I.B., and Lind, P.E. Mumps 2. Virus haemagglutination and serological reactions. Aus. J. Exp. Biol. Med. Sci., vol. 24, pp. 127-132, 1946.

22. Topacio, T. Cited from reference #33.

23. Sabin, A.B., and Olitsky, P.K. Cultivation of poliomyelitis virus in vitro in human embryonic nervous tissue. Proc. Soc. Exp. Biol. Med., vol. 34, pp. 357-359, 1936.

24. Salk, J.E. Studies in human subjects on active immunization against poliomyelitis. I. Preliminary report of experiments in progress. J. Am. Med. Ass., vol. 151, pp. 1081-1098, 1953.

25. Bedson, S.P., and Bland, J.O. A morphological study of psittacosis virus with the description of a developmental cycle. Br. J. Exp. Path., vol. 13, pp. 461-466, 1932.

26. Benjamin, B., and Rivers, T.M. Regeneration of virus myxomatosum (Sanarelli) in the presence of cells of exudates surviving in vitro. Proc. Soc. Exp. Biol. & Med., vol. 28, pp. 791-792, 1930-31.

27. Kanazawa, K. Cited from reference #33.

28. Koprowski, H., and Black, J. Studies on chick adapted rabies virus. III. Duration of immunity in vaccinated dogs. Proc. Soc. Exp. Biol. Med., vol. 80, pp. 410-415, 1952.

29. Drake, M.E., Henle, W., Henle, G., and Stokes, J. Evidence for propagation of virus of serum hepatitis in chick embryo. Fed. Proc., vol. 11, pp. 467, 1952.

30. Haagen, E., and Theiler, M. Studies of yellow fever virus in tissue culture. Proc. Soc. Exp. Biol. Med., vol. 29, pp. 435-436, 1932.

31. Aldershoff, P.H., and Broers, C.M. Contribution al 'etude des corps intra epitheliaux De Guarnieri. Ann. Inst. Pasteur, vol. 20, pp. 779-784, 1906.

32. Weller, T.H. Serial propagation in vitro of agents producing inclusion bodies derived from varicella and herpes zoster. Proc. Soc. Exp. Biol. Med., vol. 83, pp. 340-346, 1953.

33. Reid, L.J., and Muench, H. A simple method of estimating fifty percent end points. Am. J. Hyg., vol. 27, pp. 493-497, 1938.

34. Henle, W., and Rosenberg, E.B. One step growth curves of various strains of influenza A and B viruses and their inhibition by inactivated viruses of the homologous type. J. Exp. Med., vol. 89, pp. 279-285, 1949.

35. Syverton, J.T., and Scherer, W.F. Studies on the propagation in vitro of poliomyelitis viruses. II. Growth curve and its relation to cytopathogenicity. J. Exp. Med., vol. 96, pp. 369-388, 1952.

36. Herrell, W.E., and Heilman, D. Cytotoxic and antibacterial activity of gramicidin and penicillin; comparison with other germicides. Am. J. Med. Sci., vol. 206, pp. 221-226, 1943.

37. Herrell, W.E., and Heilman, D. Tissue culture studies on cytotoxicity of bactericidal agents. Am. J. Med. Sci., vol. 205, pp. 157-162, 1943.

38. Enders, J.F., Weller, T.H., and Robbins, F.C. Cultivation of Lansing strain poliomyelitis virus in cultures of various human embryonic tissues. Science, vol. 109, pp. 85-87, 1949.

39. Cited from Schaeffer, M., and Muckenfuss, R.S., Experimental Poliomyelitis, ed. 1. The Science Press Printing Co., Lancaster, Pa., 1940.

40. Flexner, S., and Lewis, P.A. The transmission of acute poliomyelitis to monkeys. J. Am. Med. Ass., vol. 53, p. 1739, 1909.

41. Flexner, S. The contribution of experimental to human poliomyelitis. J. Am. Med. Ass., vol. 55, pp. 1105-1113, 1910.

42. Flexner, S., and Lewis, P.A. Experimental poliomyelitis in monkeys. J. Am. Med. Ass., vol. 54, pp. 1780-1782, 1910.

43. Cited from Schaeffer, M., and Muckenfuss, R.S. Experimental Poliomyelitis, ed. 1, The Science Press Printing Co., Lancaster, Pa., 1940. (P13).

44. Flexner, S., and Lewis, P.A. Experimental poliomyelitis in monkeys. 7th note, Active immunization and passive serum protection. J. Am. Med. Ass. vol. 54, pp. 1780-1782, 1910.

45. Boyd, T.E. Immunization against poliomyelitis. Bact. Rev., vol. 17, pp. 349-369, 1953.

46. Kolmer, J.A., Klugh, G.F., and Rule, A.M. A successful method of vaccination against acute anterior poliomyelitis. Further report. J. Am. Med. Ass., vol. 104, pp. 456-460, 1935.

47. Kolmer, J.A., and Rule, A.M. A successful method of vaccination against acute anterior poliomyelitis. Preliminary report. Am. J. Med. Sci., vol. 188, pp. 510-514, 1934.

48. Leake, J.P. Poliomyelitis following vaccination against this disease. J. Am. Med. Ass., vol. 105, p. 2152, 1935.

49. Salk, J.E. Formaldehyde treatment and safety testing of experimental poliomyelitis vaccines. Am. J. Pub. Health, vol. 44, pp. 563-570, 1954.

50. Bodian, D. Differentiation of types of poliomyelitis viruses. III. The grouping of fourteen strains into three basic immunologic types. Am. J. Hyg., vol. 49, pp. 234-245, 1949.

51. Weller, T.H., Enders, J.F., Robbins, F.C., and Stoddard, M.B. Studies on the cultivation of poliomyelitis viruses in tissue culture. I. The propagation of poliomyelitis viruses in suspended cell cultures of various human tissues. J. Immunol., vol. 69, pp. 645-671, 1952.

52. Robbins, F.C., Weller, T.H., and Enders, J.F. Studies on the cultivation of poliomyelitis viruses in tissue culture. II. The propagation of poliomyelitis viruses in roller-tube cultures of various human tissues. J. Immunol., vol. 69, pp. 673-694, 1952.

53. Robbins, F.C., Enders, J.F., and Weller, T.H. Cytopathogenic effect of poliomyelitis viruses in vitro on various human embryonic tissues. Proc. Soc. Exp. Biol. Med., vol. 75, pp. 370-374, 1950.

54. Sanders, M., Kiem, I., and Lagunoff, D. Cultivation of viruses, a critical review. Am. Med. Ass. Arch. Path., vol. 56, pp. 128-225, 1953.

55. Smith, W.M., Chambers, V.C., and Evans, C.A. Growth of neurotropic viruses in extraneural tissues. Northwest Med., vol. 49, p. 368, 1950.

56. Younger, J.S., Ward, E.N., and Salk, J.E. I. Studies on poliomyelitis viruses in cultures of monkey testicular tissue. Am. J. Hyg., vol. 55, pp. 291-300, 1952.

57. Syverton, J.T., and Scherer, W.F. Utilization of a stable strain of human epithelial cells (HeLa of Gey) for diagnosis of poliomyelitis. Fed. Proc., vol. 12, p. 462, 1953.

58. Evans, C.A., Smith, W.M., Chambers, V.C. Multiplication of poliomyelitis virus in monkey testicular tissue. Bact. Proc., p. 93, 1951.

59. Salk, J.E., Ward, E.N., and Younger, J.S. II. Studies in human subjects on active immunization against poliomyelitis. Am. J. Pub. Health, Vol. 44, pp. 994-1009, 1954.

60. Youngner, J.S. Monolayer tissue cultures. I. Preparation and standardization of suspension of trypsin-dispersed monkey kidney cells. Proc. Soc. Exp. Biol. Med., vol. 85, pp. 527-530, 1954.

61. Syverton, J.T., and Scherer, W.F. The multiplication of viruses other than poliomyelitis in a stable strain of human epithelial cell, strain HeLa. Bact. Proc., vol. 53, pp. 42-43, 1953.

62. Gey, G.O., Coffman, W.D., and Kubicek, M.T. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res., vol. 12, p. 264, 1952.

63. Syverton, J.F., Scherer, W.F., and Elwood, P.F. Studies on the propagation in vitro of poliomyelitis viruses. V. The application of strain HeLa human epithelial cells for isolation and typing. J. Lab. Clin. Med., vol. 43, pp. 286-302, 1954.

64. Information received by personal communication from Dr. Herbert Wenner.

65. The National Foundation for Infantile Paralysis. An evaluation of the 1954 poliomyelitis vaccine trials. (Summary report) Edwards Bros. Inc., Ann Arbor, Mich.

66. The National Foundation for Infantile Paralysis. General Outline of materials and procedures recommended for use in the participating laboratories. (Poliomyelitis vaccine evaluation studies, 1954).

67. Wenner, H.A. Preparation and standardization of antiserums with the three known types of poliomyelitis viruses. Am. J. Hyg., vol. 59, p. 221, 1954.

68. Hanks, J.H., and Wallace, R.E. Relation of oxygen and temperature in the preservation of tissue by refrigeration. Proc. Soc. Exp. Biol. Med., vol. 71, pp. 196-200, 1949.

69. Morgan, J.F., Morton, H.J., and Parker, R.C. Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic medium. Proc. Soc. Exp. Biol. Med., vol. 73, pp. 1-8, 1950.