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The cultivation of the poliomyelitis viruses in tissue culture

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In this lecture it is our purpose to recall our principal observations on the behavior of poliomyelitis viruses in tissue culture and the manner in which they were made. To do this seems appropriate, since it is for them we have jointly been awarded the highest of honors in our field - the Nobel Prize in Physiology or Medicine. But before we begin we earnestly desire to point out that the results of our research are not alone the product of our triple thought and effort. As nearly always in the undertaking of science many others who have worked with us have contributed of their minds and labor. And so we would think of them as here with us now, sharing in these great happenings.

Antecedents to the Investigation of Poliomyelitis Virus

As observed long ago by Claude Bernard the history of many discoveries in the more exact sciences may be clearly and tersely told because such discoveries depend upon ideas logically derived from well-established general principles or theories. Such ideas give rise to a series of deductions the validity of which must, of course, be then experimentally corroborated. Biology including medicine has long sought to range itself with physics and chemistry by defining general principles that govern the reactions of living organisms. Within certain areas such as genetics and biochemistry much progress has been made toward these ultimate goals. In other biological disciplines, however, as in our own of viruses and viral diseases the situation in certain respects does not differ too greatly from that of the state of medicine in the time of Bernard. Medicine he described as a science in its infancy where complex and obscure questions were still to be studied and where experimental ideas did not always emerge from the rather vague conceptions then current. Under these circumstances, he wrote, "physiologists should not be afraid to

act somewhat at random, so as to try - permit me the common expression - fishing in troubled waters. This amounts to saying that, in the midst of the functional disturbances which they produce, they may hope to see some unexpected phenomena emerge which may give direction to their research".

In our own studies we did not depend entirely upon fishing in troubled waters, but that we profitably indulged at one point in this sport cannot be denied. Indeed, as so often in the realm of experimental medicine, many of the antecedent conditions which finally focused our investigations upon the specific problem of the growth *in vitro* of poliomyelitis virus in extraneural cells would have been quite indefinable *a priori.* It may, therefore, be pertinent to recall at this time some of the factors which led to these observations.

In 1937 Dr. Enders working in the Department of Bacteriology and Immunology at the Harvard Medical School turned from the study of bacterial immunity to an investigation of the growth of herpes simplex virus. Experience with the herpes virus served to establish an enduring preoccupation with pathogens of this class and was followed by attempts to isolate the agent of measles. In these experiments the tissue-culture method was employed with uncertain results. But the conviction was gained that it represented a basic tool for the study of viruses of which the possible applications were almost unlimited. For this as well as other reasons the roller-tube method used about this time by Gey and Bang for the cultivation of the virus of lymphogranuloma venereum was selected as an ideal means of investigating over a prolonged period the relationships between a virus and its host cells. One of the participants in this research in which the agent of vaccinia was employed was Thomas Weller[1]. Dr. Weller, then a fourth-year medical student at Harvard, joined the group primarily because of his concern with the larger parasites of man. He wished to determine whether one of these, *Trichinella spiralis,* could be made to pass through its developmental cycle in the presence of living tissue cells. While carrying out these studies he also acquired an interest in the viruses.

These preliminary explorations of the value of the tissue-culture method were interrupted by the War, although by 1941 further observations stimulating to us had been made at the Harvard Medical School on the behavior in roller-tube cultures of the viruses of equine encephalomyelitis, herpes simplex, and influenza virus, and on that of varicella independently by Dr. Weller and Dr. L. C. Kingsland, Jr., at the Children's Hospital in Boston.

After the war Dr. Enders was asked to establish a laboratory for research in infectious diseases at the Children's Hospital. Dr. Weller participated in the

establishment of the laboratory and we were soon joined by Dr. Robbins who had recently returned from duty in Italy where under his direction an epidemiologic study of a disease resembling Q fever had resulted in the identification of the responsible rickettsia for the first time in that country. Under these circumstances congenial to the free implementation of our interests the first experiments in the new laboratory were begun in April 1947.

Investigation of the potentialities of tissue culture for the propagation of viruses was naturally resumed and for this purpose the agent of mumps was first selected, since recent observations had provided the essential techniques whereby it could be easily studied in the laboratory. It was soon demonstrated that growth readily occurred in a simple suspended cell culture consisting of fragments of chick amniotic membrane nourished with a balanced salt solution and ox serum ultrafiltrate[2]. Viral increase was easily followed by measuring the amount of hemagglutinin that emerged in the culture fluid after inoculation of small quantities of the agent. A modification of the usual technique for handling such cultures was introduced that permitted the demonstration of the slow-growing mumps virus. Instead of transferring material from one culture to another after an interval of three or four days as had been the procedure of most previous investigators, the tissues were preserved while the nutritive medium was removed and replenished at frequent intervals. In this way viability of the cells could be maintained for 30 days or longer and full opportunity allowed for viral growth to occur while a substantial dilution of the original viral inoculum was effected.

In 1948 when this work was completed we had no immediate intention of carrying out experiments with poliomyelitis viruses. Nevertheless from time to time we had considered the mounting evidence which had been well summarized by Green and Evans in favor of the possibility that these agents might not be strict neurotropes. Thus, for us, along with others it was becoming increasingly difficult to visualize the nervous system - as site of manufacture of the enormous quantities of virus that was found in the feces of many patients. Such ideas were in our minds when the decision was taken to use a mixture of human embryonic skin and muscle tissue in suspended cell cultures in the hope that the virus of varicella might multiply in the cells of its natural host. In this way such cultures were made available while close at hand in the storage cabinet was the Lansing strain of poliomyelitis virus. Thereupon it suddenly occurred to us that everything had been prepared almost without conscious effort on our part for a new attempt to cultivate the agent in extraneural tissue.

Demonstration of Multiplication of Poliomyelitis Viruses in Cells of Extraneural Tissues

Accordingly the virus in the form of an infected suspension of mouse brain was introduced into several of these cultures of human tissue which were then handled exactly as in the experiments with the mumps agent. By inoculation of mice with the fluids removed from the original cultures as well as with those taken from subsequent serial passages *in vitro* it soon became apparent that multiplication of the virus regularly occurred. Introduction of fluid from the third passage into the brains of monkeys was followed by the appearance of typical flaccid paralysis of the legs[3]. These findings, surprising as they were in the light of the failure of our predecessors to propagate the virus in extraneural tissue, left no doubt in our minds that this could be accomplished.

The increase in the Lansing virus content of tissue cultures of this sort is illustrated in Fig. 1. These results were obtained by titrating at intervals of four days the pooled fluids removed from four cultures all of which were inoculated with the same quantity of infected mouse brain. The upper curve represents the negative logarithm of the mouse titers, the lower the calculated amounts of virus, expressed as LD_{50}/ml for mice, that emerged in the fluid phase during each interval of four days. It is evident that under these conditions significant quantities of virus appear only after a period of eight days and that the maximal yield is obtained between the 12th and 16th days. These findings were soon confirmed and extended: *(a)* by continued serial passage in human embryonic skin and muscle tissue of this as well as another strain of Lansing virus; *(b)* by the successful propagation of representatives of Types I and III viruses[4,5]; and *(c)* by identification of the agents grown in tissue culture in virus neutralization tests.

Since the tissue employed in these experiments was removed from the extremities it was considered to be free of intact nerve cells. It was concluded, therefore, that growth of poliomyelitis virus in cultures of this material occurred in cells of extraneural origin. This conclusion has been amply supported by direct observations of the effect of the viruses on various types of cells which we will mention hereafter as well as the later demonstration by Scherer and Syverton of multiplication in presumably pure lines of fibroblasts and cells of epithelial origin. It has been made evident, then, that *in vitro* at least poliomyelitis viruses are not strict neurotropes.

During this earlier period of research, growth of representative strains of

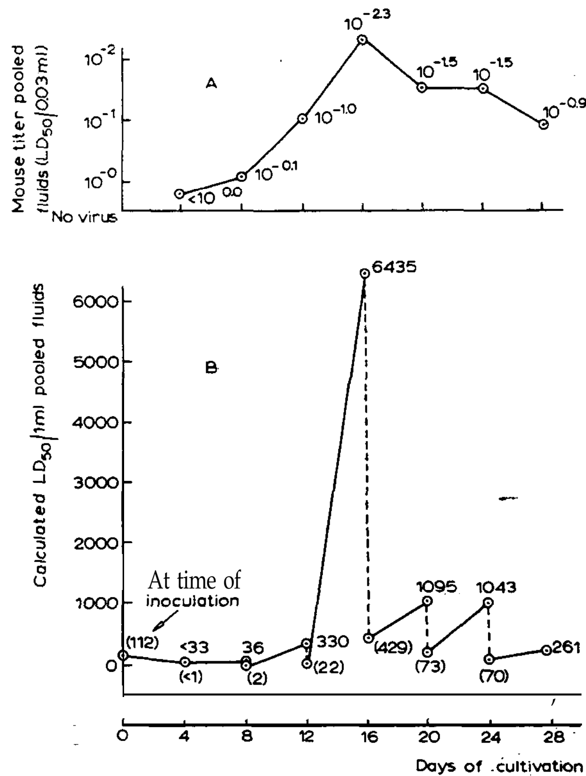

Fig. 1. Mouse infectivity of pools of fluids removed at four-day intervals from suspended cell cultures of human embryonic skin-muscle tissue inoculated with Lansing mouse-brain virus. (From *J. Immunol.*, 69 (1952) 652.)

virus was demonstrated in a variety of other human embryonic tissues in suspended cell cultures[6]. These included intestine, liver, kidney, adrenal, brain, heart, spleen, and lung. Multiplication also occurred in postnatal renal and preputial tissue. In confirmation of the results of Sabin and Olitsky[7] reported in 1936 growth of virus was also obtained in cultures of human embryonic brain. Later when we applied the roller-tube technique[8] to the cultivation of these agents, for reasons that will be indicated shortly, it was found that viral increase occurred more rapidly and tended to reach somewhat higher maxima when compared with those in Fig. 1. The curves in Fig. 2 illustrate these differences. The data included in Fig. 2 were obtained by titration in mice, of fluids removed successively from roller-tube cultures of human embryonic skin and muscle tissue inoculated with Lansing virus. It is worthy of remark that the highest concentrations of virus attained in the

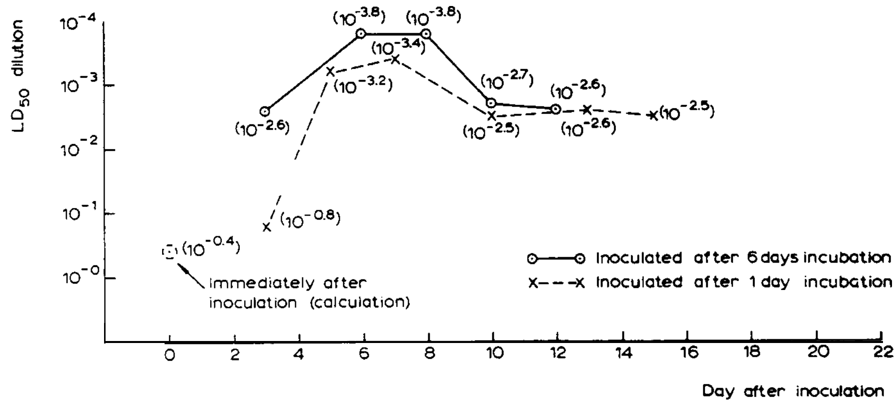

Fig. 2. Multiplication of Lansing virus in roller-tube cultures of human embryonic skin-muscle tissue inoculated at two intervals after preparation. Mouse infectivity titers of pools of fluids removed at various intervals after inoculation.
(From *J. Immunnol.*, 69 (1952) 689.)

tissue-culture fluid are equivalent to or exceed those encountered in the nervous system of the infected mouse.

The Cytopathogenic Effect of Poliomyelitis Viruses

The results we have just described appeared to be of basic significance since they left no doubt that the three known antigenic types of poliomyelitis viruses could be grown without difficulty apparently in a wide variety of human cells. Their ultimate value, however, in the future study of these agents both from the biologic and practical points of view would have been greatly limited had phenomena not been discovered which clearly and accurately indicate the occurrence of viral multiplication within the tissue-culture system itself. Thus it is probable that if it had remained necessary to inoculate experimental animals in order to demonstrate virus in the culture, the method would have been largely utilized as a convenient means for the preparation of virus suspensions.

Fortunately, signs of viral activity in the cultures themselves were observed soon after it was established that multiplication took place. Examination of stained sections of fragments of human embryonic intestine and skin and muscle removed from the early suspended cell subcultures of the Lansing strain revealed widespread cellular degeneration. In contrast, uninoculated

tissues maintained under the same conditions were found to contain many cells in apparently excellent condition. Subsequent study of the effect of Type I and Type II viruses in this tissue as well as in cultures of embryonic intestine and brain convinced us that after a prolonged period (16 to 32 days), growth of virus resulted in cell death and disintegration. Fibroblastic and epithelial cells at least were affected by the virus.

At about the same time these cytopathogenic effects of the virus, as we later called them, were observed, we perceived an indirect manifestation of cell injury. It consisted of an accelareted decline in the metabolic rate of tissues infected with virus as expressed by a progressive reduction of acid formations. This phenomenon was demonstrated in a simple manner by comparing at intervals the pH of the medium in inoculated cultures with

*Dilution of virus inoculated**	*Days after inoculation of culture*						
	3	*7*	*11*	*15*	*20*	*24*	*28*
10^{-1}	6.8**	6.8	6.9	7.1	7.2	7.3	7.3
10^{-2}	6.8	6.8	6.9	7.1	7.2	7.3	7.3
10^{-3}	6.8	6.8	6.8	7.0	7.1	7.3	7.3
10^{-4}	6.8	6.8	6.8	6.9	6.9	7.2	7.3
10^{-5}	6.8	6.8	6.8	6.8	6.8	6.8	6.9
No virus	6.8	6.8	6.8	6.8	6.8	6.8	6.9

* The virus consisted of a suspension of infected mouse brain. Three cultures were each inoculated with 0.1 cc of each dilution.

** Average of individual pH readings of the medium in three flasks. Supernatant fluids were removed and fresh medium added on the days indicated. Readings were made just before this was done. The pH of the fresh medium varies from about 7.7-7.5.

Fig. 3. Determinations of the pH of the fluid phase of flask cultures of human embryonic brain inoculated with Lansing virus. (From *Proc. Soc. Exptl. Biol. Med.,* 75 (1950) 373.)

that in control cultures containing no virus. Experiment showed that a satisfactory correlation usually existed between a high pH and the presence of virus in the culture fluid. With the establishment of this correlation it became possible to apply the phenomenon to the titration of the infectivity for the tissue culture of a given suspension of virus just as one employs a series of susceptible animals for the same purpose. In Fig. 3 the results obtained in the titration of Lansing virus grown in tissue culture are presented as an example of how this indirect manifestation of viral cytopathogenicity can be

invoked to determine the smallest quantity of the agent capable of multiplying in suspended cell cultures of human embryonic brain fragments. It is evident that in this instance the material could be diluted 10^4 times and still inhibit cell metabolism. Subsequently Rhodes and his associates showed that reduced acid production was correlated with a progressive decrease in glucose utilization.

These indices of viral multiplication in suspended cell cultures, useful as they were in our earlier investigations, proved inconvenient in two respects. The direct observation of cell injury required the fixation, sectioning and staining of the fragments - a laborious process, while the difference in pH required the lapse of about two weeks or longer before it became evident. Accordingly, other means were sought by which viral cytopathogenicity might be conveniently and rapidly demonstrated. At first we explored the method of explantation which had been used in the past by other workers, in particular Huang, to test the effect of viruses on cell viability. This consists in placing the fragment taken from a suspended cell culture in a drop of clotted chicken plasma and observing whether or not cell outgrowth occurs upon subsequent incubation. Results of experiments with poliomyelitis virus carried out in this manner were unequivocal. When infected fragments were removed eight days or longer after infection and explanted, cell growth failed to occur, or if it did begin, soon ceased to progress and was followed by rapid degeneration. In the control preparations the growth of cells proceeded in a normal manner. It was also determined by this means that the addition of type-specific antiserum to the suspended cell culture before the virus was introduced protected the cells from the destructive effect of the latter.

These observations appeared to be important from several points of view. In the first place it was made evident that the cytopathogenic effect of the virus could be directly and easily observed in the untreated culture. Thus another and more direct criterion of viral multiplication was made available and an easier method of assaying infectivity was foreshadowed. The demonstration of the inhibitory effect of homologous antiserum not only afforded convincing evidence that the cytopathic changes observed were caused by the virus but also indicated that it would be possible to determine *in vitro* the antigenic type of poliomyelitis viruses, as well as the presence of type-specific antibody in human and animal sera.

The explant procedure, however, was still rather cumbersome and a considerable lapse of time was required before results could be obtained. Could

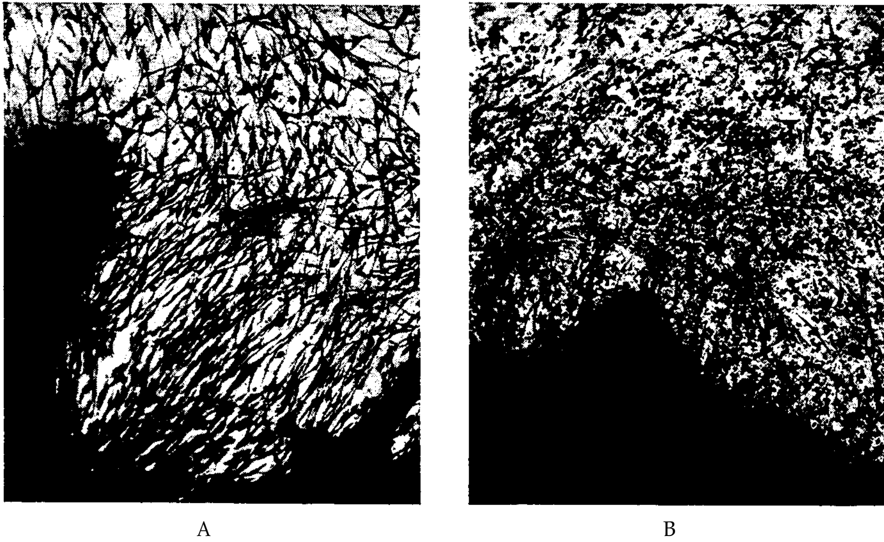

Fig. 4. Preparations from roller-tube cultures of human embryonic skin-muscle tissue. (A) Control uninoculated culture. (B) Culture three days after inoculation with Type I virus (Brunhilde strain). (Hematoxylin and eosin stain.)

not the same phenomena, we inquired, be elicited more simply and more rapidly in roller-tube cultures which we knew provided large sheets of proliferating cells? Accordingly viruses representing the three types were inoculated in roller-tube cultures of human embryonic skin and muscle. Within three to five days, degenerative changes were evident which thereafter involved the entire cell population. The inclusion of type specific antiserum in the system exhibited the same inhibitory effect as in the explant experiments. The cytopathogenic properties of Type I virus (Brunhilde strain) in roller-tube cultures of human embryonic fibroblastic-like cells are shown in Fig.4 (A and B). Because of the speed with which these changes develop and the readiness with which they may be observed the roller-tube technique was used in most of our subsequent investigations.

Early Applications of the Basic Phenomena

We next investigated whether these basic phenomena, i.e., growth of virus and its cytopathogenic activity *in vitro,* might be employed in various procedures which hitherto could only be carried out through the use of large

numbers of experimental animals. This objective seemed desirable since the only hosts then known to be susceptible to infection with Types I and III viruses were certain of the primates.

We have already indicated how the cytopathic changes induced by the poliomyelitis virus can be applied to the quantitative determination of viral infectivity. Additional experiments quickly made it clear that by inoculating roller-tube cultures with increasing dilutions of a suspension of virus, it was possible to define a minimal infecting dose. This dose is now usually expressed as that quantity of a viral suspension which will induce cytopathic changes in one-half the cultures to which it is added. 'With the definition of an infective dose of virus, it became feasible to measure the neutralizing capacity of poliomyelitis antisera by determining the least amount of a serum that will prevent the cytopathogenic activity of a known quality of virus. Although we had carried out such experiments the first published results of the titration of neutralizing antibodies were presented by Melnick and his co-worker[10] and Salk and his associates[11].

The isolation and typing of poliomyelitis viruses from man has been in the past a laborious and time-consuming procedure since it depended upon the intracerebral inoculation of monkeys. Therefore, we sought to determine whether these agents might not be recovered from feces or suspensions of the spinal cord. It was soon learned that through the addition of the antibiotics streptomycin and penicillin followed by centrifugation contaminating bacteria and other micro-organisms could be suppressed. Materials treated in this manner were at first added to suspended cell cultures of human embryonic skin and muscle or postnatal human kidney[12]. The presence of a cytopathogenie agent was revealed by differences in pH that occurred in these cultures as compared with controls. Subsequently the antigenic type of the agent thus isolated was determined by the explant method or in roller-tube cultures using mixtures of the virus and antisera for the three known types. After these original isolations were made, however, roller-tube cultures were adopted as routine for isolation as well as typing of the virus since the procedure is less complicated and results are obtained more rapidly. Indeed when large amounts of virus are present it has been possible by simultaneously carrying out both steps in this system to demonstrate poliomyelitis virus in the feces within 48 hours.

At this point it seems appropriate to remark that the discovery of the antibiotics has, as in so many other areas, worked a revolution in the field of tissue culture. Through the use of these substances it is now not only possible

to apply tissue cultures to the routine isolation of viruses from materials heavily contaminated with micro-organisms, but it has become feasible to use them under conditions and in numbers which in the past would have been quite unthinkable. Here then we have another example of how one discovery leads to many others often of quite a different nature.

The need of a serological test for poliomyelitis which could be more easily carried out than the test for virus-neutralizing antibody has long been felt. Various workers, therefore, had attempted to prepare satisfactory antigens from infected nervous tissue suitable for use in a complement fixation test. This objective was not attained, however, until Casals and Olitsky in 1951 adapted Type II virus to suckling mice and showed that the brains of these animals contained antigen in sufficient concentration to fix complement with poliomyelitis antisera. Since the tissue culture had just been shown to provide a convenient means of producing large quantities of virus of all three antigenic types in a menstruum relatively free of tissue contaminants, we decided to see whether the virus-containing fluids might not be used as complement fixing antigens. The exploration of this problem was chiefly carried out by Dr. Arne Svedmyr of Stockholm who had come to our laboratory in 1951. Fluids were concentrated by a simple method of ultrafiltration and employed as antigens in complement fixation tests, first with type-specific antisera prepared in monkeys and then with sera from patients with poliomyelitis. In both instances the capacity of the concentrated virus suspensions to fix complement in the presence of poliomyelitis antibodies was demonstrated[13,14]. With the monkey sera the reactions were clearly type specific in character. In contrast human sera often reacted with all three types of antigen although they were known to contain neutralizing antibody specific for only a single type. This tendency of certain human sera to react in this broad manner at present limits the usefulness of the complement fixation test as a diagnostic measure.

Investigations by numerous workers in the past had shown that poliomyelitis virus derived from infected nervous tissues when inoculated by a peripheral route might induce resistance to subsequent infection via the nervous system. The virus suspensions used in this way contained of necessity large amounts of nervous tissue derivatives. Because of the absence of such materials in the infected fluids of tissue cultures it early became of interest to determine whether they might act as immunizing agents. Accordingly, fluids from suspended cell cultures of human embryonic skin and muscle infected with the Lansing strain were inoculated intraperitoneally into mice.

Three weeks later the mice were injected intracerebrally with from 10 to 50 LD_{50} doses of the homologous virus. In repeated experiments about one-half of the animals remained well whereas the mortality rate in the controls varied from 90 to 100 per cent. We concluded on the basis of these findings that virus propagated in tissue culture was capable of inducing immunity. A similar prophylactic effect resulting from peripheral inoculation of poliomyelitis tissue-culture virus was described a little before this time by Milzer and his co-workers[15].

The possibility that tissue-culture virus in some form might eventually prove of value as a prophylactic agent naturally occurred to us when we first became convinced that the agent could be grown in extraneural tissues. We, therefore, were prepared to observe any indications of changes in pathogenicity for animals that might follow during serial passages. It was indeed not long before we observed that the mouse virulence of the Lansing strain which had been passed three times in suspended cell cultures of skin and muscle had markedly decreased. Although this loss might have been occasioned by a reduced capacity to multiply *in vitro,* it was soon shown that this was not the case, since the titer of viral infectivity as determined by the tissue-culture method remained unchanged. Similar experiments with a Type I virus (Brunhilde strain) indicated that between the 2nd and 10th passages its pathogenicity for rhesus monkeys had diminished significantly. Subsequent passages in roller-tube cultures rapidly led to a further decrease in the capacity of the agent to produce paralysis in monkeys following intracerebral inoculation. The data recorded in those experiments are summarized in Fig. 5. The total decline in virulence was calculated to be of the order of

Material titrated	*Monkey infectivity (PD_{50}/1 ml)*	*Tissue-culture infectivity (ID_{50}/1 ml)*	*Ratio: TC infectivity/ monkey infectivity*
Monkey cord	10^5*	10^5	(1)
Susp. cell cult. pass. 2	10^3	10^5	100
Susp. cell cult. pass. 10	10^1	10^5	1,000
Roller cult. pass. 1	<1	10^4	$>$ 10,000
Roller cult. pass. 2	<1	10^5	$>$100,000

* Usual titer of infected monkey cord as obtained by various workers.

Fig. 5. Results of titrations in monkeys and in tissue cultures of materials from a tissue culture line of Type I virus (Brunhilde strain).

100,000 to 1,000,000 fold, since again no change had occurred during these passages in the titer of the virus as determined in tissue culture. It is worthy of note that in these experiments in which the final drop in monkey pathogenicity occurred, the roller-tube cultures were inoculated with the smallest amount of virus capable of inducing cytopathic changes.

From these observations we concluded that, as with other viral agents, the virulence of poliomyelitis virus is not a fixed attribute but on the contrary may readily be altered under appropriate conditions.

Subsequent Development of Techniques and Applications

The appearance in 1950 of reports by Milzer and co-workers and by Smith and associates[16] confirmed our observations on the capacity of the poliomyelitis viruses to multiply *in vitro* in human tissues of non-nervous origin. These papers proved to be the forerunners of a number of significant contributions from other laboratories in the United States that rapidly introduced procedural modifications extending the general usefulness of the method. Here specific reference may be made to only a few of these reports; however, particular mention should be made of the contributions of Evans and his associates in Seattle, of the group working under Paul and Melnick at New Haven, of Li and Schaeffer at Montgomery, of Sabin in Cincinnati, of Salk and Youngner in Pittsburgh, and of Syverton and Scherer in Minneapolis. It is to be stressed, however, that at present tissue-culture methodology as applied to research on poliomyelitis continues to undergo rapid changes.

Prior to 1950, poliomyelitis research was of necessity concentrated in relatively few centers that possessed facilities for handling large numbers of monkeys. Following the demonstration that multiplication of poliomyelitis viruses could be accomplished *in vitro* in monkey-testicular[17,18] and in monkey-kidney tissues[19], it was a natural development for those groups working in these centers to concern themselves with the practical application of methods utilizing monkey tissues. Meanwhile other groups devoted much effort toward establishing strains of standardized cells of human origin and to the investigation of their potentialities in the study of the poliomyelitis viruses. Scherer, Syverton and Gey[20] introduced the use of the HeLa strain of human cells originally isolated from an epidermoid carcinoma of the cervix. Swim and Parker, among others, maintained lines of normal cells in continuous culture, and demonstrated the susceptibility of cells so cultivated to the cyto-

pathic action of the poliomyelitis viruses. Concurrently, for certain objectives, simplified methods of preparing cultures were introduced, such as the planting of tissues on the wall of small tubes, that were subsequently slanted during incubation. Considerable effort has been expended on the development of synthetic media with the objectives of achieving standardization, of eliminating viral inhibitory substances, and in particular, for the purposes of preparing materials for immunization, of eliminating extraneous antigenic substances. Most widely used has been the synthetic-tissue culture medium, known as 199, developed by Morgan, Morton and Parker. We will now describe certain of the more interesting and important applications of the method.

Assay of infectivity and selection of strains of altered pathogenicity

Dulbecco and Vogt[21] in a series of ingenious experiments have devised methods to obtain *in vitro* isolated plaques of cellular degeneration analogous to those produced by bacteriophage and have presented evidence indicating that each plaque represents the effect of a single infective unit of virus. According to their procedure cell suspensions are prepared by exposing tissues to trypsin. Kidney cells obtained in this manner when planted in flat dishes, yield homogeneous sheets of growth. After inoculation, the cells are covered with a thin layer of agar, thus limiting the spread of the cytopathogenic virus. This technique provides a new method for the more accurate assay of viral activity. Of greater importance is the means it offers of establishing clones of virus in connection with the search for variants possessing altered pathogenic properties.

On theoretical grounds, the desirable immunizing agent of the future for poliomyelitis may well contain live stable avirulent variants of each of the three main antigenic types of virus. It has already been noted that variations in virulence may accompany prolonged propagation *in vitro*, and that the selection of populations with altered characteristics may be expedited by the utilization of the limiting dilution inoculation technique. Investigation along these lines is being carried out by Sabin[22]. Employing massive inocula and passage at 24 hour intervals, together with final passage at the dilution endpoint, strains have been developed from all three types that exhibit almost complete loss of virulence on inoculation into primates. It would thus appear that the development of immunizing materials against poliomyelitis, comparable to the live avirulent strain of yellow-fever virus, through the use of *in vitro* techniques is already more than a theoretical possibility.

Development of immunizing materials employing inactivated poliomyelitis viruses prepared by in vitro methods

In 1953, Salk and his co-workers reported experiments on active immunization in human subjects employing a polyvalent vaccine containing virus treated with formalin. A striking antibody response followed the subcutaneous inoculation of three doses of vaccine[19,23]. A large-scale field evaluation trial of the vaccine was sponsored by the National Foundation for Infantile Paralysis and is now in progress. Under the direction of Dr. Thomas Francis, Jr., the Evaluation Center is maintaining records on 1,800,000 children; of these 440,000 have received three injections of the Salk vaccine and 210,000 injections with a placebo. The study also includes a group of 20,000 children in Finland. Analysis will be made of the antibody responses in a significant number of those in the study group, as well as intensive laboratory investigation of all cases of poliomyelitis or suspected poliomyelitis in the population under study. To accomplish the large volume of work involved additional procedural modifications have been developed. Salk, Youngner and Ward[24] have recently described a test based upon differences in pH for titration of virus as well as for antibody assay, wherein a cellular substrate of trypsinized monkey kidney cells is employed.

A somewhat different approach to the problem of immunization has been carried out concurrently by Milzer, Levinson and associates[25] who have developed a trivalent tissue culture vaccine inactivated by ultraviolet irradiation. Injection of this material into human volunteers has been followed in most instances by a significant increase in antibody.

Diagnostic procedures based upon the use of tissue cultures

As we have indicated the tissue culture offers two techniques for the diagnosis of suspected cases of poliomyelitis; isolation of virus may be accomplished, or increase in specific antibody may be demonstrated. In the brief period since these procedures were first described, many reports have accumulated concerning the isolation of viruses by this technique. For example, in the United States Syverton, Scherer and Elwood recorded the isolation of 300 strains from patients with a clinical diagnosis of poliomyelitis. Here in Stockholm a series of isolations have been accomplished by Gard and Johnson, Svedmyr and Kjellen, and by Wesslen. Elsewhere throughout the world these techniques are being applied, as revealed by the reports of Goffe and

Le Bouvier, Lepine and Barski, Monaci von Magnus, Gear and Malherbe, Lahelle, Bazeley, and Kitaoka. Information is, therefore, becoming available for the first time concerning the distribution of the three types of virus throughout the world. Serological tests based on the tissue-culture method are now also beginning to be used as diagnostic aids.

Epidemiological studies utilizing in vitro procedures

Since the introduction of these simple techniques it has become feasible for the epidemiologist to study, with the assistance of the laboratory, the spread of poliomyelitis in populations. Two recent developments in this field deserve particular comment. Melnick and Ledinko[26] have for the first time been able to carry out serological studies on a large population group and to establish definitely the ratio of paralytic to nonparalytic cases. The second development which resulted from the combined observations of several workers consists in the further documentation of the fact that poliomyelitis is hyperendemic throughout the poorly sanitated tropical areas of the world.

Discovery of new cytopathic viruses resident in the human intestinal tract

As a direct consequence of the wide application of *in vitro* procedures to the problem of poliomyelitis virus isolation, a variety of cytopathogenic viruses have been isolated from fecal materials of human origin. Following the original recovery of representatives of this group in our own laboratory, many workers have isolated agents whose relationships to each other as well as their etiological and epidemiological significance still remain to be elucidated. Certain of these have been shown to be Coxsackie viruses; others do not fall in that category. Regardless of the significance of these viruses as eventually defined, it is apparent that through the use of tissue culture a new approach has been provided for the study of the viral inhabitants of the human inrestinal tract.

Application of in vitro Techniques to the Investigation of other Virus Diseases of Man

While methods of tissue culture have been applied extensively to the study of viruses since 1928, when the Maitlands described the suspended cell tech-

nique, it is apparent that there has been a stimulation of interest in this approach within the past few years. The study of known viruses pathogenic for man in cultures of human tissue have been profitably applied by various workers as illustrated, for example, in the report of Kjellén and Svedmyr[27] who conclude that this system is most suitable for the primary isolation of the virus of herpes simplex.

Yet, it is in the investigation of several viruses that manifest a high degree of host specificity for man that some of the most interesting developments have occurred. Thus, it has now been amply demonstrated that for certain purposes, lacking a susceptible experimental animal, the culture composed of human tissues may be substituted for the human subject. Here it is pertinent only to outline briefly certain of these developments.

Varicella and herpes zoster

Concurrently with the poliomyelitis studies, efforts directed at the isolation *in vitro* of the etiological agent of varicella were continued in our laboratory. In 1952, Weller and Stoddard reported that the inoculation with varicella vesicle fluid of suspended cell cultures of human tissues was followed by the development of intranuclear inclusions although attempts at serial propagation of the agent proved unsuccessful. Then, profiting by the accumulated experience with the roller-tube technique, it was demonstrated that specific cytopathic changes followed inoculation of such cultures of human tissues with vesicle fluid materials from cases of varicella or of herpes zoster. With this culture system, serial propagation *in vitro* of the agents could be accomplished as demonstrated by the regular appearance in subcultures of cytopathic changes that were focal in nature and associated with the presence of intranuclear inclusions[28]. Employing the fluorescent antibody procedure of Coons[29], and more recently utilizing culture materials as a complement fixing antigen, immunologic evidence has been obtained to support the thesis that the etiologic agents of varicella and herpes zoster have been isolated and propagated *in vitro.* It is of special interest that to date no morphologic or antigenic dissimilarities have been noted that would permit differentiation of the agents recovered from these two diseases.

Epidemic exanthema of Neva and Enders

In 1951, an epidemic exanthem occurred in Massachusetts that was charac-

terized by fever of short duration, a macular to maculo-papular eruption and the absence of significant adenopathy or Koplik's spots. Cytopathogenic agents were isolated in tissue cultures from the feces of seven patients and the development of specific neutralizing antibodies in paired serum specimens was demonstrated[30]. The evidence now indicates that these studies have resulted in the identification of a new clinical entity.

Measles agents

Recently Enders and Peebles[31] reported that the inoculation of roller-tube cultures of human renal cells with blood or throat-washings collected from patients with measles during the 24-hour period following appearance of the rash, resulted in unusual cytopathic changes. Characteristically, large syncytial cell masses developed that on staining were found to contain numerous nuclei with intranuclear inclusion bodies. The appearance in measles of a virus-neutralizing antibody reacting specifically with the agents thus isolated has been demonstrated. Also, fluids from infected cultures contain an antigen that fixes complement in the presence of serum from persons convalescent from the disease. It has also been possible to assay the concentration of neutralizing and complement fixing antibodies reacting with these agents in various lots of human gamma globulin.

New respiratory agents from man

The application of tissue-culture procedures has revealed the existence of a variety of new viruses that inhabit the respiratory tract of man. Rowe and his associates[32] noted spontaneously occurring cytopathic changes in cultures of human adenoid tissue. Further investigations by Huebner and co-workers have resulted in the isolation of a variety of cytopathogenic agents that may be divided into at least six groups by immunologic means. Similarly, studies by Hilleman and his associates[33] of cases of acute respiratory infection have led to the recognition of a new group of viruses, the pathogenic attributes of which are now being elucidated.

We shall now bring to an end this fragmentary account of our results and the investigations of others that have widely extended these results and given them greatly added significance and value. We regret that the exigencies of time and space have prevented in many instances the inclusion of specific references as well as more adequate descriptions of their contributions. It is a

satisfaction to us, however, that the volume of these contributions is so great since it testifies to the wide recognition of the value of the tissue-culture method in the exploration of the fascinating phenomena offered the investigator by the virus diseases.

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