INTERFERENCE BETWEEN POLIOMYELITIS VIRUSES IN TISSUE CULTURE*

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Interference of one virus with the multiplication of another has been demonstrated between many pairs of agents, antigenically related or unrelated, infectious or inactive (1). However, with other virus pairs there may be no interference, thus indicating that this is not a universal phenomenon. The feasibility of using tissue culture technics for the demonstration of interference was demonstrated first by Andrewes, using pneumotropic and neurotropic strains of influenza A virus (2), and soon after by Lennette and Koprowski (3) using a number of different viruses. In tissue culture systems all susceptible cells are readily accessible to virus and can be infected at will. The development of tissue culture technics for the cultivation of poliomyelitis virus (4) has made such an *in vitro* host system available for investigating the phenomenon of interference between the poliomyelitis viruses. Observations on the demonstration of reciprocal interference among the three types of poliomyelitis are reported. As control experiments possible interference between poliomyelitis and certain Coxsackie and "orphan" viruses (5) was investigated.

Materials and Methods

Viruses.—Representative strains of the three types of poliomyelitis virus were selected for their pronounced cytopathogenicity *in vitro*. They have been described in earlier reports (6–9). The WS type virus was used in its 12th TC (tissue culture) passage, the Y-SK Type 2 in its 16th, and the Leon Type 3 in its 11th. Their respective titers in testicular cultures were 10^{-4}, 10^{-5}, and 10^{-4} per 0.1 ml. of TC fluid. For the experiments with inactivated virus, the WS strain alone was enployed. It was transferred through 34 passages in monkey testicular roller-tube cultures and one additional passage was made in human embryonic skin-muscle cultures. The virus pool of this 35th passage material gave a TC titer of 10^{-6} per 0.1 ml. in monkey testicular roller-tube cultures.

The Coxsackie viruses used in these studies are also characterized by their ability to grow and produce cytopathogenic changes (fibroblastic degeneration) in monkey testicular cultures. Easton-11 and Easton-30 viruses had been isolated in tissue culture and identified as previously described (8). Harvests of the 9th TC fluid passage of Easton-11, and of the 7th TC fluid passage of Easton-30 viruses were used. Both virus harvests had a TC titer of 10^{-3} per

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0.1 ml. The Cairo-B strain had also been isolated in tissue culture, from rectal swabs collected in Egypt (10). The virus was used in its 7th TC passage, and had a TC titer of 10^{-2}. All three strains had been found to be related antigenically and were classified as members of Type A-9.

In addition to the poliomyelitis and Coxsackie viruses which can be recovered from specimens collected from cases of suspected poliomyelitis, a number of viral agents have also been recovered for which no animal pathogenicity has been established. These agents, tentatively designated as "orphans" to emphasize our ignorance of their natural history, are characterized by their cytopathogenicity for cultures of primate tissues (5). In the interference experiments, 4 antigenically distinct orphan viruses were tested: Grigg, Rende, Cornelis, and Farouk viruses had been isolated in monkey testicular cultures from fecal specimens, and the 5th TC passage of each of the viruses was employed in the present experiments. Cornelis and Farouk viruses each had a TC titer of 10^{-5}, and Grigg and Rende a titer of 10^{-4}.

All viruses were held frozen at −20°C. until used.

Inactivation of Poliomyelitis Virus.—1. Formalin: The WS strain was inactivated by 0.4 per cent formalin at 0°C. according to the method described by Salk (11). Virus was used in these experiments after it had been treated with formalin for 6, 8, 12, and 15 days, and then dialyzed against buffered saline in the cold to remove the formalin. The 6 day treatment yielded active virus in monkey testicular cultures. The 8 and 12 day preparations had only traces of virus: they were negative in testicular cultures and negative upon intracebral inoculation of monkeys; however, monkey pathogenic virus could be recovered by inoculation of monkey kidney cultures. The 15 day treatment yielded non-infective virus by all tests.

2. High intensity electron bombardment (capacitron): The WS virus was irradiated in the frozen and liquid states under atmospheres of helium, air, and oxygen. The conditions of inactivation were similar to those outlined by Dick *et al.* (12). For these experiments viruses were treated with 2.0 to 2.8 × 10^6 R.E.P. under the direction of Dr. W. Huber, Electronized Chemicals Corporation, Brooklyn.[1] After this amount of bombardment, traces of infectious virus still remained in the preparations, the TC_{50} titer being less than 10^0.

3. Ultraviolet radiation experiments: 4 ml. of undiluted TC fluid harvested from the WS-infected cultures were placed in Petri dishes and exposed to ultraviolet light (Sylvania lamp, 30 w. germicidal-B). The dishes were rocked 90 times a minute at a distance of 7 inches from the lamp. Samples were removed at the following seconds: 20, 30, 60, 180, 300, 500, 700, and 900. The irradiated fluids exposed at these various times were inoculated into 12 culture tubes of monkey testicular tissue, and examined for fibroblastic degeneration at the end of 14 days. All the culture tubes inoculated with virus exposed for 30 seconds showed fibroblastic degeneration. 5 of 12 tubes inoculated with virus exposed for 60 seconds exhibited degeneration, while none of the tubes which had received virus irradiated for 180 seconds or longer showed evidence of virus activity.

Antisera.—These were obtained from monkeys immunized against the 3 strains of poliomyelitis virus: Brunhilde, Y-SK, and Leon. A 20 per cent suspension of monkey cord was made, centrifuged at 2000 R.P.M. for 10 minutes, and the supernate mixed with adjuvant (13) in the following proportions: 2 ml. of arlacel, 8 ml. of bayol F, and 10 ml. of virus. Three weekly injections of 2 ml. of the mixture were given intramuscularly. The monkeys were bled 30 days after the last injection. The antisera were frozen at −20°C.

Tissue Cultures.—The technic of preparing roller-tube cultures such as used in these experiments has been described in detail (7). Testes from immature *rhesus* (*Macaca mulatta*) monkeys were the source of tissue. The nutrient medium for initiating outgrowth of cells consisted of 9 parts of Earle-Simms solution (3 parts of Earle's balanced salt solution (BSS), plus 1 part of

[1] Dr. Anthony J. Girardi, United States Public Health Service Fellow, kindly supplied us with this material which he prepared as part of another study.

bovine serum ultrafiltrate) and 1 part of 5 per cent lactalbumin enzymatic hydrolysate as previously described (14). 0.9 ml. of medium was used per culture tube. After 4 to 5 days, immediately before the addition of virus to the cultures, the fluid was replaced by a maintenance medium consising of Morgan, Morton, and Parker 199 (15) diluted with two parts of BSS. The incorporation of Earle's strongly buffered BSS (16) in the medium after the cultures were inoculated, eliminated any necessity for nutrient renewal during the subsequent 2 week period, which was the duration of the experiment. Thus any complicating factors which might have been caused by renewal of nutrient fluid on the interpretation of the outcome of interference were avoided.

Virus Titrations.—0.1 ml. of serial tenfold dilutions of the virus preparation was introduced into a duplicate set of tubes. 12 days after inoculation, the tubes were examined microscopically for evidence of specific degeneration. Either 0 or 4+ degeneration (complete destruction of the normal structure of the fibroblasts, with shrinkage and granulation) was evident at this time. The titer was taken as the final dilution of virus (in the 0.1 ml. inoculum) which produced complete fibroblastic destruction at 12 days. Only very rarely have cultures exhibiting 0 degeneration at this time shown subsequent degeneration.

Plan of the Experiments.—Cultures were inoculated with 0.1 ml. of various doses of one virus, followed at given intervals by 0.1 ml. of various amounts of a second poliomyelitis or other virus, as indicated in the text. Before the addition of the second virus, all cultures were washed 4 times with 5 to 10 ml. of BSS, in order to remove any extracellular or unadsorbed virus of the first inoculum. In this manner, competition of unadsorbed virus with the agent inoculated subsequently could be discounted in the interpretation of the experimental results. Culture fluids and tissues were collected from groups of 2 to 3 culture tubes at varying intervals. Suspensions of tissue were prepared as follows: the fluid phase was removed as completely as possible with a pipette, the tissue was washed 4 times with 5 to 10 ml. of saline, and the tissue fragments were scraped from the bottom of the tube. They were then transferred to a mortar, by means of a capillary pipette, using 0.9 ml. of BSS per tube, and ground with alundum. The suspension was centrifuged at 2000 R.P.M. for 5 minutes. Since each culture tube contained approximately 0.01 gm. of tissue, adding 0.9 ml. of BSS gave an approximate dilution of the tissue of 10^{-2}. The preparations were then titrated for tissue culture infectivity. In order to determine the significance of the difference in titers obtained in this series of experiments, repeat titrations were performed on groups of 2 to 3 culture tubes which had received the same initial infecting dose of poliomyelitis virus. Under these conditions, it was found that the titer produced varied by no more than 1.0 log unit. Hence when the results of titrations of tissue suspensions or fluids are compared, only a difference of 2.0 log units between 2 different samples is considered to be significant.

The interpretation of the interference experiments depended upon a knowledge of the level of virus multiplication in the absence as well as in the presence of the second agent. Therefore, in all experiments, control fluids and tissue suspensions were collected from cultures receiving the first (interfering) virus alone and also from cultures inoculated only with the second (challenge) virus.

In testing harvests from cultures inoculated with two viruses, simple titrations would have given unclear results for one would not have known which agent was being measured. However, if specific antiserum were added at the time the titrations were made, one agent only would be neutralized, and the cytopathic changes observed in the titration series could be attributed to the second virus. Thus in testing for the outcome of interference, the following procedure was adopted: In an experiment involving interference of a Type 1 virus by a Type 2 virus, the growth of the challenge Type 1 virus was followed by titrating fluids and tissue suspensions in the presence of Type 2 antiserum. To serial tenfold dilutions of the sample under test, an equal volume of a 1:5 dilution of a Type 2 serum was added and the mixtures

incubated for 1 hour at room temperature. Each mixture was then inoculated into 2 culture tubes, and the extent of degeneration read at 12 days. The TC neutralization test as employed here has been described in detail (17). A number of controls were also included in each test, and these are described in more detail in the experimental section.

Survival of Virus in Cell-Free Medium at 35°C.—As one of the controls in determining whether interference occurred between two poliomyelitis viruses, the persistence of virus under the conditions of the experiment but in the absence of living cells was investigated. Tubes were made in an identical fashion as the regular cultures with the exception that either no tissue or heat-killed tissue was embedded in the plasma clot. Virus survival was found to be the same in such cultures when fresh medium or "conditioned" medium was present. Conditioned medium refers to fluid removed from cultures in which monkey testicular tissue had grown for a number of days.

EXPERIMENTAL

The Effect of the Time Interval between Addition of the Two Poliomyelitis Viruses on the Outcome of Interference.—10,000 TC doses of Y-SK virus were added to cultures, followed by a second inoculum of 1,000 TC doses of Leon virus, 4 hours, 1 day, or 4 days after the first inoculum. As a control, the extent of multiplication of Y-SK virus alone, and of Leon virus alone, was also obtained under the conditions of the experiment. The outcome of interference was measured by infectivity titrations in roller-tube cultures of fluids and tissues of inoculated cultures removed at different times: 1, 4, 7, and 10 days after inoculation of the second virus.

In testing for the outcome of interference of Y-SK virus with Leon virus, the Leon growth curve was obtained by titrating fluids and tissue suspensions in the presence of Y-SK serum. The following illustrate the types of controls included in this and subsequent experiments:—(*a*) Titrations of cultures which received only Leon virus were also carried out in the presence of Y-SK serum. In preliminary tests, such titrations were always comparable to those carried out in the presence of normal serum, showing that no heterologous cross-neutralization occurs in tissue culture. (*b*) It was necessary to show that the concentration of Y-SK serum used was effective in specifically inhibiting Y-SK virus under the experimental conditions used. Therefore, fluids and tissues collected from cultures inoculated only with the Y-SK virus were tested in the presence of Y-SK serum. Complete neutralization was evident throughout the duration of the experiment. (*c*) That the Y-SK serum had no effect on the Leon virus was shown by subinoculation in the presence of Leon serum of fluids derived from cultures that had received both Y-SK and Leon viruses, and previously had been tested in the presence of Y-SK serum. Subinoculation failed to reveal the presence of either virus. (*d*) Titrations of preparations obtained from cultures inoculated only with Y-SK virus were also made in the presence of normal serum to show that Y-SK virus multiplied at its normal rate. (*d*) In order to evaluate the degree of interference,

the survival of Leon virus in the absence of living, susceptible cells, was determined.

As can be seen from Table I, Part 1, when the challenge virus, Leon, was given 4 hours after Y-SK virus, no interference was demonstrated since the extent of multiplication of Leon virus in the presence of Y-SK virus was

TABLE I

Interference of Y-SK (Type 2) Poliomyelitis Virus with Leon (Type 3) Poliomyelitis Virus

Part	Inoculation of cultures				Serum added at time of titration	Negative log of TC titer of culture fluids and tissue at following days after inoculation of challenge virus								Interference
	First inoculum		Second inoculum 4 hrs. after first			1		4		7		10		
	Virus	TC doses	Virus	TC doses		Fluid	Tissue	Fluid	Tissue	Fluid	Tissue	Fluid	Tissue	
1	Y-SK	10,000	None		Normal	2	3	4	5	3	4			None
					Y-SK	0	0	0	0	0	0			
	Y-SK	10,000	Leon	1000	Y-SK	1	2	3	4	3	4			
	None		Leon	1000	Y-SK	1	2	4	5	4	4			
			1 day after first											
2	Y-SK	10,000	None		Normal	3	4	4	5	3	4	2	3	Partial
	Y-SK	10,000	Leon	1000	Y-SK	2	2	2	<3	1	0*	Und.	2	
	None		Leon	1000	Y-SK	2	5	4	5	3	4	3	2	
			4 days after first											
3	Y-SK	10,000	None		Normal	2	3	3	5	3	4	3	3	Complete
	Y-SK	10,000	Leon	1000	Y-SK	1	0*	1	0*	0	0*	0	0*	
	None		Leon	1000	Y-SK	2	3	4	5	3	4	3	4	
			No tissue (Leon survival)			2		1		0				

* 0 indicates highest concentration that could be reasonably tested. Tissue fragments in each tube w ere suspended in 0.9 ml. to give an approximate concentration of 10^{-1}, the highest concentration tested.

Und. indicates that virus was detected only in the undiluted TC fluid.

comparable to that of Leon alone. However, when given 1 day after Y-SK virus, (Table I, Part 2), the multiplication of Leon was definitely less as evidenced by comparison with its normal growth pattern, and when given 4 days after (Table I, Part 3) no multiplication was demonstrated, indicating complete interference. It will be noted in Part 3, that while some virus was recovered at days 1 and 4 after inoculation, the titers correspond to those obtained when the virus is incubated in the absence of living, susceptible cells.

Furthermore, attempts to demonstrate the presence of Leon virus by subinoculation of negative fluids in the presence of Y-SK serum were not successful. Y-SK virus alone was found in all three experiments to multiply normally (7).

It is evident from these experiments that a quantitative comparison of the two viruses at an early stage and at the completion of an interference experiment, plus a comparison with the survival titers of the challenge virus, are necessary to demonstrate complete, partial, or no interference.

The Effect of Relative Amounts of Two Poliomyelitis Viruses.—In a representative experiment illustrated below, the factor of time was kept constant; *i.e.*, a 1 day interval between inoculation of the two viruses was used. Following the inoculation of 10,000, 1,000, and 100 TC doses of Y-SK virus, 100 and 10,000 TC doses of Leon virus, respectively, were added to each of the above culture series. The outcome of interference was again measured by infectivity titrations in roller-tube cultures of fluids and tissues of inoculated cultures removed at the following times: 1, 4, 7, and 10 days after inoculation of the second virus. Titrations were carried out in the presence of Y-SK serum. Suitable virus and serum controls were included as indicated above.

The results are presented graphically in Fig. 1. Only titrations of the fluids of the experimental cultures are presented, since the tissue titrations revealed essentially similar findings. The titer of fluids from cultures inoculated with the challenge virus alone is indicated by the dotted line, while the titer in the presence of Y-SK virus is indicated by the solid line. It can be seen that using 10,000 TC doses of Y-SK virus, partial interference with 10,000 TC doses of Leon virus was observed, but complete interference with the growth of 100 TC doses of Leon resulted since the small amount of virus recovered here corresponded to that recovered in tubes without living cells. When 1,000 TC doses of Y-SK were used, no interference was obtained with 10,000 doses of Leon, and only partial interference with 100 doses of Leon. A smaller amount of Y-SK, 100 doses, resulted in no interference with either 100 or 10,000 doses of Leon. Not included in the figure are the control titrations of cultures which received Y-SK virus alone, these showing the expected pattern of multiplication illustrated in Table I. This series of experiments show that a gradation of interference effect can also occur when the factor of dosage is varied, as well as when the factor of time is the variable, as was demonstrated in the preceding section.

A more detailed analysis of the factor of dosage in its effect on the outcome of interference is presented in Table II. The data represent the results of an experiment similar to the one illustrated in Fig. 1, except that both 1 and 4 day intervals were employed between inoculation of Y-SK and Leon viruses. Also, the inoculation of 10,000, 1,000, and 100 TC doses of Y-SK

TABLE II

The Effect of Timing and the Relative Amount of the First Virus (Y-SK) and the Second Challenge Virus (Leon) on the Interference of Multiplication of the Second Virus (Leon)

TC doses of Y-SK virus	1 day interval between addition of 10,000 doses of Leon and Y-SK viruses								Extent of interference	4 day interval between addition of 10,000 doses of Leon and Y-SK viruses						Extent of interference
	Fluid titrations* at following days				Tissue titrations* at following days					Fluid titrations at following days			Tissue titrations at following days			
	1	4	7	10	1	4	7	10		1	4	7	1	4	7	
10,000	2	2	0	Und.	2	3	0‡	0‡	Partial	2§	Und.	0	0‡	0‡	0‡	Complete
1,000	3	2	1	2	4	4	3	4	None	1	Und.	<1	0‡	0‡	0‡	Complete
100	2	3	1	3	4	5	2	3	None	3	1	<1	2	0‡	0‡	Partial
None	2	3	1	2	4	4	2	3		2	2	1	4	4	3	
TC doses of Y-SK virus	1 day interval between addition of 1,000 doses of Leon and Y-SK viruses								Interference	4 day interval between addition of 1,000 doses of Leon and Y-SK viruses						Interference
	Fluid titrations at following days				Tissue titrations at following days					Fluid titrations at following days			Tissue titrations at following days			
	1	4	7	10	1	4	7	10		1	4	7	1	4	7	
10,000	1	1	0	0	2	0‡	0‡	0‡	Partial	1§	0	0	0‡	0‡	0‡	Complete
1,000	2	3	1	1	3	3	2	0‡	None	1	0	0	0‡	0‡	0‡	Complete
100	3	3	1	<1	4	4	2	2	None	2	Und.	1	0‡	0‡	0‡	Partial
None	2	3	1	1	4	3	2	2		2	3	1	4	4	2	
TC doses of Y-SK virus	1 day interval between addition of 100 doses of Leon and Y-SK viruses								Interference	4 day interval between addition of 100 doses of Leon and Y-SK viruses						Interference
	Fluid titrations at following days				Tissue titrations at following days					Fluid titrations at following days			Tissue titrations at following days			
	1	4	7	10	1	4	7	10		1	4	7	1	4	7	
10,000	Und.	0	0	0	0‡	0‡	0‡	0‡	Complete	Und.§	0	0	0‡	0‡	0‡	Complete
1,000	Und.	1	Und.	0	2	2	0‡	0‡	Partial	Und.	0	0	0‡	0‡	0‡	Complete
100	1	3	1	<1	3	2	2	0‡	None	Und.	0	0	0‡	0‡	0‡	Complete
None	1	3	1	Und.	3	3	2	0‡		1	2	1	3	4	2	

* Negative logarithm given of TC titer (12th day reading) of culture fluids and tissues collected at days indicated after inoculation of challenge virus. Titers of Leon virus are represented. Control titrations of Y-SK virus, not included in table, showed a normal pattern of multiplications as illustrated in Table I.

‡ 0 indicates highest concentration that could be reasonably tested and found negative. Tissue fragments in each tube were suspended in 0.9 ml. to give an approximate concentration of 10^{-2}.

§ Survival of challenge virus in roller-tubes without tissue.

virus was followed by the inoculation of 100, 1,000, and 10,000 TC doses of Leon virus, respectively, to each of the above series of cultures. The data again indicate that either complete, partial, or no interference may be ob-

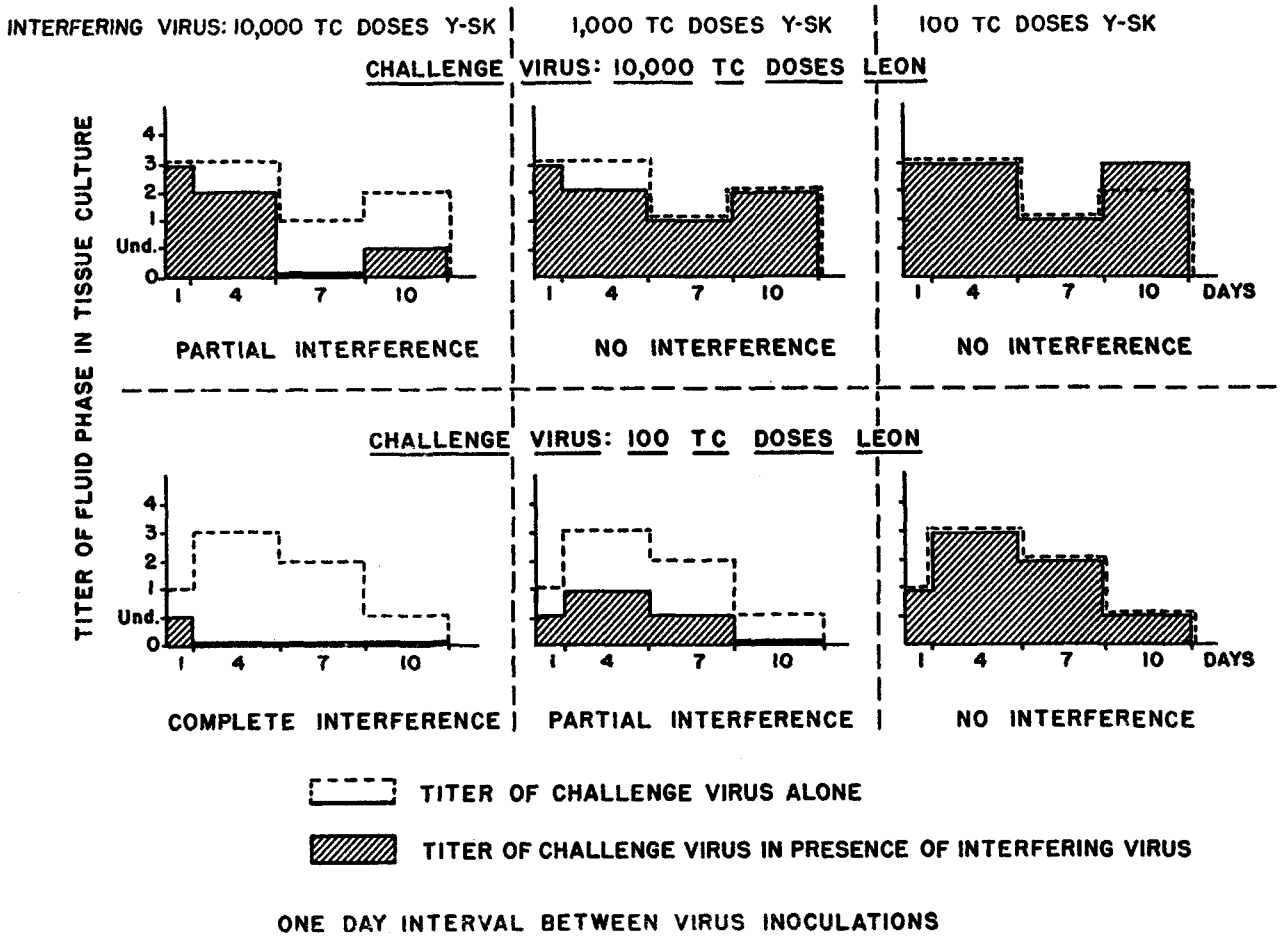

FIG. 1. Effect of dosage on the interference of Type 2 Y-SK virus with Type 3 Leon virus in monkey testicular cultures. The interfering virus was inoculated 1 day before the challenge virus. The titer of the challenge virus in the presence of the interfering virus was obtained by carrying out titrations in the presence of Y-SK antiserum.

tained depending on the factor of dosage of the two viruses, as well as the time used between the inoculation of the viruses. In further experiments, similar findings were also obtained with WS and Y-SK viruses.

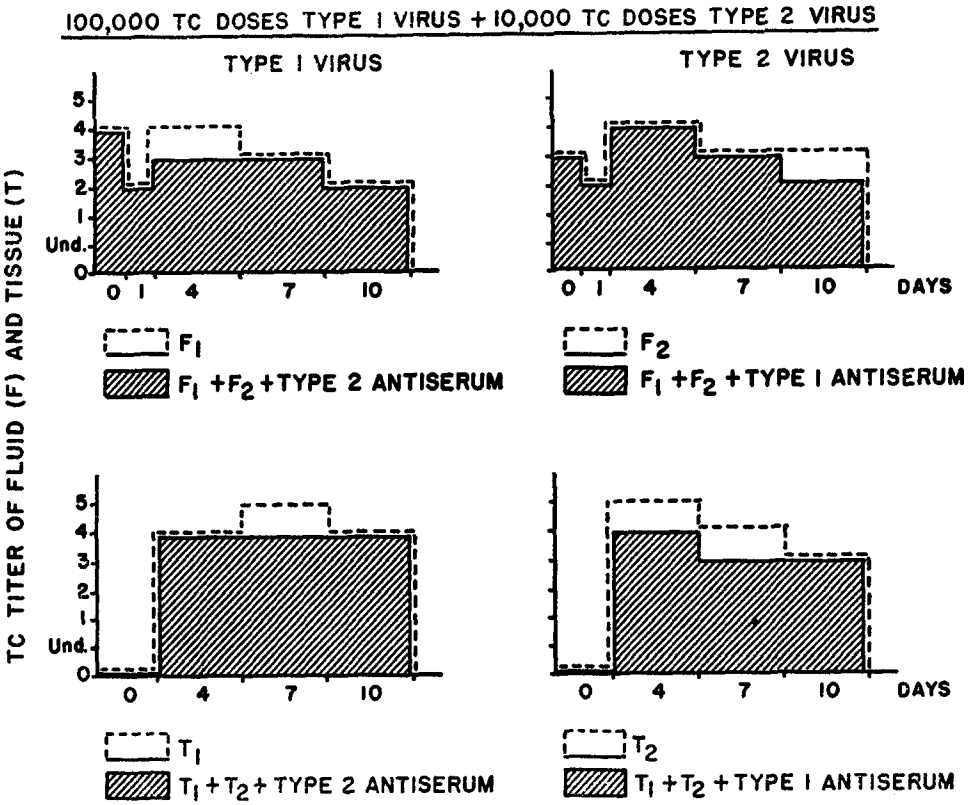

FIG. 2. Growth of Type 1 WS and Type 2 Y-SK viruses inoculated simultaneously in large dosages. F_1 and T_1 indicate fluid phase and tissue of control cultures inoculated with Type 1 virus alone. F_2 and T_2 indicate same for Type 2 cultures. $F_1 + F_2$ + Type 2 antiserum indicates titer of Type 1 virus in fluid phase, for the serum specifically neutralizes only the Type 2 virus present in the mixture; $F_1 + F_2$ + Type 1 antiserum, titer of Type 2 virus in fluid phase; $T_1 + T_2$ + Type 2 antiserum, titer of Type 1 virus in tissue; and $T_1 + T_2$ + Type 1 antiserum, titer of Type 2 virus in tissue.

The Effect of Simultaneous Inoculation of Two Poliomyelitis Viruses.—The problem next investigated was the extent of interference between two poliomyelitis viruses when these were given an equal chance to grow out; *i.e.*, when they were inoculated simultaneously into cultures. In the experiments illustrated in Fig. 2, a large dose of WS virus was inoculated into cultures together with a large amount of Y-SK virus. The growth pattern of each of

the viruses inoculated in the presence of the other virus, as indicated by the solid line, is compared with that obtained when inoculated alone, as indicated by the dotted line. Titrations of fluid (F), shown in the upper part of the figure, and of tissue (T), the lower part, were performed in the presence of specific hyperimmune serum against one or the other of the viruses. The growth pattern of WS virus alone was similar to that obtained in the presence of Y-SK virus. Conversely, the pattern of multiplication of Y-SK virus alone was similar to that obtained in the presence of WS virus.

It was next determined whether the amount of WS virus used above, 100,000 TC doses, would have any effect on the pattern of multiplication of a very small amount of Y-SK virus when the two viruses were inoculated simultaneously. 100,000 TC doses of WS were inoculated together with 1 TC dose of Y-SK virus. The growth patterns of each of the viruses obtained in the presence of the other virus, and when inoculated alone, are presented in Fig. 3. Only partial inhibition of the growth of the small amount of Y-SK virus used appears to have occurred, as revealed by a comparison with its normal growth curve, shown by the dotted line. When both viruses have an equal opportunity to infect susceptible cells in culture, both may grow out. When these and previous experiments are considered, it appears that a period of time is required for resistance to develop in the host tissue.

The Effect of the Excluded Virus on the Multiplication of Interfering Virus.— In his studies on interference between bacterial viruses, Delbrück (18) observed that the excluded virus may reduce the number of particles of the interfering virus liberated upon lysis of the bacterium. This has been called the depressor effect. Experiments were designed to determine whether in the present system excluded virus exerted a similar effect on the interfering virus.

On the basis of the above results, conditions were so arranged that three grades of interference would be produced: none, partial, or complete. To three series of cultures 1,000 TC doses of Y-SK virus were added, followed by a second inoculum of 100 or 1,000 TC doses of Leon virus, either at 1 or 4 day intervals. At 4 and 8 days following the second inoculum, fluids and tissues were collected from infected cultures, and these were titrated in the presence of Y-SK, as well as Leon antiserum. The results are tabulated in Table III.

On the basis of the resulting TC titers of Leon virus determined in the presence of Y-SK serum, varying degrees of interference, from none to complete, with the growth of Leon virus were established. The titers of Y-SK virus produced at 4 and 8 days in cultures which had received Leon virus as the second inoculum, as determined in the presence of Leon serum, were comparable in all cases to those produced in cultures inoculated only with Y-SK virus. Thus partial or complete exclusion of Leon virus had no effect on the resulting titers of the interfering, infecting Y-SK virus, and no "de-

pressor effect" analogous to that shown by Delbrück was obtained under the conditions of these experiments.

Reciprocal Interference among Types 1, 2, and 3 Poliomyelitis Viruses.— As shown in Table II, when 10,000 TC doses of Y-SK virus were used as the interfering virus, and 1 day after, 100 TC doses of the Leon challenge virus were added, complete interference with the growth of Leon virus was

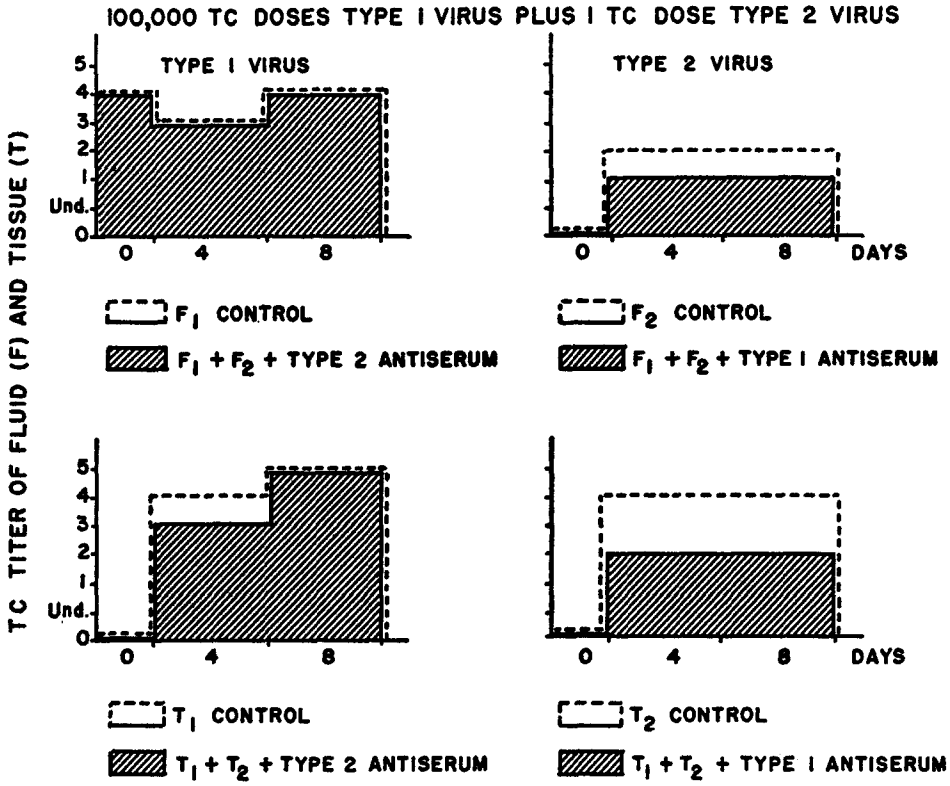

FIG. 3. Growth of Type 1 and Type 2 viruses inoculated simultaneously with a large amount of Type 1 virus and a small amount of Type 2 virus. Legend like that of Fig. 2.

demonstrated. The results of further experiments suggested that under the same conditions Y-SK virus also successfully interfered with WS virus. Therefore, these conditions were utilized in order to test for possible reciprocal interference among the three types of poliomyelitis virus. Fig. 4 shows the data of a representative series of experiments. Titrations of tissue (T) and fluid (F) were performed in the presence of specific antiserum for the interfering virus. From a comparison of (*a*) the growth patterns of the challenge virus inoculated alone, (*b*) the growth patterns of the challenge virus in the

presence of the interfering virus, and (*c*) the survival titers of the challenge virus as indicated by the shaded areas, it can be seen that complete interference may be reciprocally obtained between any two poliomyelitis viruses under the experimental conditions used.

Interference Experiments with Poliomyelitis and Coxsackie Viruses.—Experiments were designed in which three TC adapted strains of Type A-9 Coxsackie virus were tested in interference experiments with poliomyelitis virus, in order to determine the "specificity" of the interference phenomenon

TABLE III

Experiments Designed to Test the Effect of the Excluded Virus on the Multiplication of the Interfering Virus

Part	Inoculation of cultures				Negative log of TC titer of culture fluids and tissues collected at following days after inoculation of challenge virus								Interference with Leon virus	Effect of Leon virus on growth of Y-SK virus
	First inoculum		Second inoculum		4 days				8 days					
			1 day after first		+Y-SK serum*		+Leon serum‡		+Y-SK serum		+Leon serum			
	Virus	TC doses	Virus	TC doses	Fluid	Tissue	Fluid	Tissue	Fluid	Tissue	Fluid	Tissue		
1	Y-SK	1000	None		0	0	3	3	0	0	4	3	None	None
	Y-SK	1000	Leon	1000	2	3	3	4	3	4	3	3		
	None		Leon	1000	3	3	0	0	4	4	0	0		
			1 day after first											
2	Y-SK	1000	None		0	0	3	3	0	0	4	3	Partial	None
	Y-SK	1000	Leon	100	1	2	4	4	1	<2	4	3		
	None		Leon	100	4	3	0	0	3	3	0	0		
			4 days after first											
3	Y-SK	1000	None		0	0	4	3	0	0	4	4	Complete	None
	Y-SK	1000	Leon	100	0	0	2	3	0	0	3	4		
	None		Leon	100	4	4	0	0	3	3	0	0		

* Leon virus is dectected in presence of Y-SK serum.
‡ Y-SK virus is detected in presence of Leon serum.

between two poliomyelitis viruses in tissue culture. Experimental conditions which in previous experiments resulted in complete interference of one poliomyelitis virus with another were utilized. 1 day after infection of four groups of cultures with 10,000 TC doses of WS virus, 100 TC doses of the following viruses were added, respectively: Easton-11, Cairo-B, and Easton-30 Coxsackie viruses, and Y-SK poliomyelitis virus, as a control. Culture fluids were collected at 4, 8, and 12 days after inoculation of the second virus. The justification of collecting only fluids, and not tissues, for the demonstration of the presence or absence of virus, lies in the fact that in no previous experiment had a culture been found which contained virus in the tissue and not in the

fluid. Suitable controls were included. As seen from the data in Table IV, under conditions resulting in complete interference with the growth of Y-SK virus by WS virus, no interference with the growth of the Coxsackie strains was found.

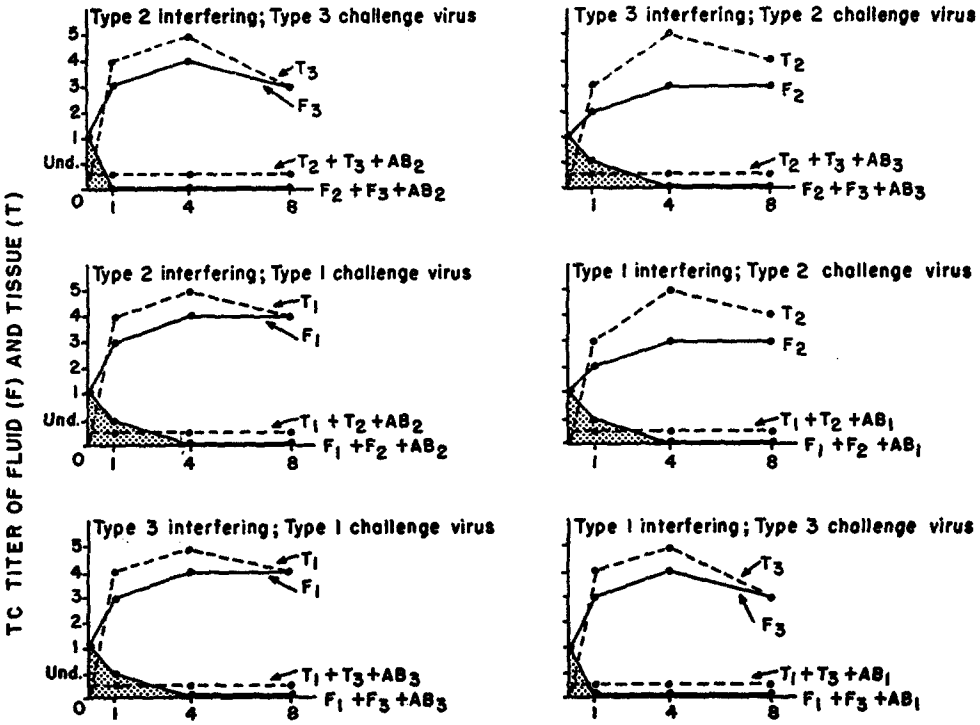

FIG. 4. Reciprocal interference among Types 1, 2, and 3 poliomyelitis viruses. The survival titers of the challenge virus are indicated by the shaded areas. Legend as for Fig. 2. For example, the graph in the upper left indicates the growth curves (F_3, fluid; T_3, tissue) of the challenge Type 3 virus in the absence of the interfering Type 2 virus. $T_2 + T_3 + AB_2$ indicates Type 3 virus in tissue, as Type 2 virus has been neutralized by added homotypic antibody (AB_2), and similarly $F_2 + F_3 + AB_2$ indicates Type 3 virus in fluid phase.

Interference Experiments with Poliomyelitis and "Orphan" Viruses.—As in the experiments with the Coxsackie viruses, experimental conditions which resulted in complete interference of one poliomyelitis virus with another were utilized. 2 days after infection of five groups of cultures with 10,000 TC doses of WS virus, 100 TC doses of the following antigenically distinct viruses were added, respectively: Farouk, Rende, Grigg, and Cornelis orphan viruses, and

TABLE IV

Failure of Poliomyelitis Virus to Interfere with Coxsackie Viruses in Tissue Culture

Part	Inoculation of cultures				Serum	Negative log of TC titer of culture fluids collected at following days after inoculation of challenge virus			Interference
	First inoculum		Second inoculum 1 day after first						
	Poliomyelitis Virus	TC doses	Virus	TC doses		4	8	12	
1	WS	10,000	None		Normal	3	4	3	
					WS	0	0	0	
	WS	10,000	Easton-11	100	WS		3		
2	None		Easton-11	100	WS		3		
	None		Easton-11*	100		Und.	0	0	None
3	WS	10,000	Cairo-B	100	WS		2	2	None
	None		Cairo-B	100	WS		2	2	
4	WS	10,000	Easton-30	100	WS		2	2	None
	None		Easton-30	100	WS		2	3	
5	WS	10,000	Y-SK	100	WS	0	0	0	Complete
	None		Y-SK	100	WS	4	4	3	

* No tissue present. Control for virus survival.

TABLE V

Failure of Poliomyelitis Virus to Interfere with Four Antigenically Distinct Orphan Viruses

Part	Inoculat on of cultures				Serum	Negative log of TC titer of culture fluids collected at following days after inoculation of challenge virus			Interference
	First inoculum		Second inoculum 2 days after first						
	Poliomyelitis Virus	TC doses	Virus*	TC doses		4	8	12	
1	WS	10,000	None		Normal	4	4	3	
					WS	0	0	0	
2	WS	10,000	Farouk	100	WS	3	4	4	None
	None		Farouk	100	WS	3	4	4	
3	WS	10,000	Rende	100	WS	3	4	2	None
	None		Rende	100	WS	3	3	3	
4	WS	10,000	Grigg	100	WS		3	2	None
	None		Grigg	100	WS		2	3	
5	WS	10,000	Cornelis	100	WS		3	3	None
	None		Cornelis	100	WS		3	3	
6	WS	10,000	Y-SK	100	WS	0	0	0	Complete
	None		Y-SK	100	WS	3	4	3	

* The orphan viruses all failed to survive for 4 days in tubes prepared without tissue and incubated at 35° along with the experimental cultures.

Y-SK poliomyelitis virus as a control. Culture fluids were collected at 4, 8, and 12 days after inoculation of the second virus. Suitable controls were included as indicated in Table V.

Under conditions which resulted in complete interference with the multiplication of Y-SK virus by WS virus, no interference was demonstrated with the growth of any of the orphan viruses used. The failure to demonstrate interference under the conditions used does not exclude the possibility that interference may be produced when other conditions of time and virus dosage are employed.

Reciprocal experiments of those described; *i.e.*, interference of WS virus by orphan viruses were also performed. 1,000 to 10,000 TC doses of the above orphan viruses were inoculated into a series of culture tubes. 2 days later, 10 TC doses of WS virus were added. Titrations of the fluids collected 4 and 8 days after the second inoculum revealed that at most a partial inhibition of the growth of WS virus by each of the orphan viruses had occurred.

The Effect of Inactivated Virus upon the Multiplication of Poliomyelitis Viruses.—The effect on the growth of WS poliomyelitis virus by the presence of WS virus rendered non-infective (or almost so) by formalin treatment, by high energy electron bombardment (capacitron), and ultraviolet irradiation was studied. The inactivation treatments are described under Methods.

Undiluted WS virus, grown in culture and subjected to electron bombardment in the capacitron under various conditions, was tested for its interfering property as follows: 0.1 ml. of each preparation was inoculated into each of 8 monkey testicular roller-tube cultures. 1 day later, 0.1 ml. of either 1 or 10 TC doses of active WS virus as indicated was added to 4 tubes of each of the above preparations, leaving 4 culture tubes containing only the electron-irradiated material. Active WS virus alone was also inoculated into 4 culture tubes, as controls. Cultures were examined for fibroblastic degeneration 4, 6, 8, and 12 days after addition of the second inoculum. In no case was interference demonstrated, not even in irradiated preparations which contained very small amounts of residual active virus, as shown in Table VI. Similar results were obtained when the challenge virus was inoculated 4 days after the irradiated virus. Interference experiments with electron-irradiated and active Y-SK virus revealed essentially the same findings.

The WS virus after treatment with formalin and dialyzed to remove the excess free formalin was also tested as an interfering agent. The experimental procedure was similar to that used with the electron-irradiated virus above. Again, as shown in Table VII, no interference was manifest under the conditions used, even though trace amounts of virus were still present in certain of the formalin-treated preparations.

The ultraviolet-irradiated virus was tested after exposures sufficient to inactivate the virus, 180, 300, 500, and 900 seconds. Undiluted, inactivated

TABLE VI

Interference Experiments with High-Energy Electron Irradiated Preparations of Poliomyelitis Virus

Part	Inoculation of cultures			Extent of fibroblastic degeneration observed at following days after second inoculum§				Interference
	First inoculum	Second inoculum 1 day after first						
	Capacitron preparation*	Virus‡	TC doses	4	6	8	12	
1	2.0, liquid, He	None		0	0	1(1/4)	3(2/4)	None
	2.0, liquid, He	WS	10	2	3	4	4	
	None	WS	10	2	3	4	4	
2	2.4, liquid, He	None		0	0	0	2(1/4)	None
	2.4, liquid, He	WS	10	2	3	3–4	4	
	None	WS	10	2	3	4	4	
3	2.8, liquid, He	None		0	0	0	0	None
	2.8, liquid, He‖	WS	1	1	2	2–3	4	
	None	WS	1	1	2	3	4	
4	2.4, frozen, He	None		0	0	2(3/4)	3(3/4)	None
	2.4, frozen, He	WS	10	2	3	4	4	
	2.8, frozen, He	None		0	0	1(1/4)	3(2/4)	None
	2.8, frozen, He	WS	10	2	3	4	4	
	None	WS	10	2	3	4	4	
5	2.0, liquid, O_2‖	None		0	0	0	0	None
	2.0, liquid, O_2	WS	1	1	2	3	4	
	2.4, liquid, air‖	None		0	0	0	0	None
	2.4, liquid, air	WS	1	1	2	3	4	
	None	WS	1	1	2	3	3–4	
6	2.4, frozen, O_2	None		0	0	2(3/4)	3(3/4)	None
	2.4, frozen, O_2	WS	10	2	3	4	4	
	2.8, frozen, air	None		0	0	1(2/4)	2–3(3/4)	None
	2.8, frozen, air	WS	10	2	3	4	4	
	None	WS	10	2	3	4	4	

* Undiluted culture fluid of WS TC-35 which had been subjected to various conditions of capacitron irradiation. 2.0 indicates 2×10^6 R.E.P. Liquid indicates virus held cold but not frozen. Frozen indicates virus held at −70°. Virus irradiated under atmospheres of helium, oxygen, or air as indicated.

‡ Active WS, 35th TC passage.

§ 4 monkey testicular tissue culture tubes were used per inoculum. The fibroblastic degeneration recorded is representative of all of the 4 culture tubes used. If all 4 cultures did not react, a figure in parenthesis after the degeneration number indicates how many cultures of the 4 tubes inoculated did react.

‖ Addition of virus 4 days after first inoculum also resulted in no interference with the active virus.

virus was inoculated in 0.1 ml. amounts into a large number of culture tubes. At intervals of 0, 1, and 3 days after the first inoculum, 10 TC doses of active

TABLE VII

*Interference Experiments with Formalin-Treated Preparations of Poliomyelitis Virus**

Part	Inoculation of cultures			Extent of fibroblastic degeneration observed at following days after second inoculum				Interference
	First inoculum	Second inoculum						
		4 hrs. after first						
	Formalin preparation	Virus	TC doses	4	6	8	12	
1	8 days‡	None		0	0	0	0	None
	8 days	WS	10	2	3	3	4	
	None	WS	10	2	3	3	4	
		1 day after first						
2	8 days	None		0	0	0	0	None
	8 days	WS	10	2	3	3	4	
	None	WS	10	2	3	3	4	
		3 days after first						
3	8 days	None		0	0	0	0	None
	8 days	WS	10	2	3	4	4	
	None	WS	10	2	3	3	4	
		1 day after first						
4	12 days	None		0	0	0	0	None
	12 days	WS	1	1	2	3	4	
	None	WS	1	1	2	3	4	
		3 days after first						
5	12 days	None		0	0	0	0	None
	12 days	WS	1	1	2	3	4	
	None	WS	1	1	2	3	4	

* 6 day treatment with formalin yielded active virus in monkey testicular roller-tube cultures. Active virus was recovered from both of above 8 and 12 day preparations in monkey kidney roller-tube cultures, although not in monkey testicular cultures as indicated. Both preparations were proven to be antigenic in monkeys.

‡ Representative data of experiments using two different formalin preparations given. 4 to 6 monkey testicular tissue culture tubes were used per inoculum. The fibroblastic degeneration recorded is representative of all the cultures used.

WS virus were added to each of 4 tubes of each of the above sets, leaving 4 culture tubes containing only the irradiated virus. Culture tubes receiving only active WS at the above times were also included as controls. The extent of degeneration was followed over a period of 12 days. The time of appearance, as well as the extent, of degeneration in cultures which had received both

the active and inactive viruses were in all cases comparable to those which had received only active WS virus, similar to the data shown in Table VII. Cultures which had received only the irradiated preparations exhibited no degeneration.

DISCUSSION

At least two reports, the first by Paul and Trask in 1933 (19) and the second by Bodian in 1949 (20) already suggested that interference may occur among the poliomyelitis viruses. These studies dealt with the differentiation of immunologic types of poliomyelitis virus by means of reinfection experiments in monkeys. Paralytic infection of monkeys by one strain conferred complete immunity to the same strain, but not always to a second strain, indicating that the latter belonged to a different type. Interference mechanisms might have played a role in the experiments, for when reinoculation by a heterologous poliomyelitis virus type was made shortly after a paralytic infection, some immunity to the second virus was revealed. However, in Paul and Trask's experiments, if a sufficient number of weeks were allowed to elapse between the first and second inoculations, little resistance to the second type was manifest. More recently Casals *et al.* (21) have shown that mice vaccinated with a Type 2 strain exhibit marked resistance to a Type 3 strain; this may also be an example of interference. With the advent of tissue culture technics, the problem of interference has now been investigated more systematically.

Infection of susceptible cells in tissue culture with one type of poliomyelitis virus can render them resistant to the action of a heterotypic virus, as is apparent from the studies reported here. The data presented indicate that under the conditions used, the interfering virus must be given an advantage over the one to be excluded. This may be accomplished by controlling the dosage, *i.e.* the relative amounts of the interfering and challenge viruses, and the time between addition of the interfering and challenge viruses. As was shown, definite quantitative relationships between two viruses were required in order to obtain interference. The fact that reciprocal interference could be demonstrated between Type 1, Type 2, and Type 3 poliomyelitis viruses under the same conditions would suggest that similar mechanisms responsible for the production of interference are involved for all members of the poliomyelitis group.

Only live poliomyelitis viruses were effective as interfering agents. Neutralized mixtures of one poliomyelitis virus type plus its homologous serum did not prevent the growth of a second poliomyelitis virus type, nor did poliomyelitis viruses inactivated by formalin or by ultraviolet or high energy electron irradiation. In fact, the trace amounts of virus present when inactivation did not proceed to complete loss of infectivity also failed to interfere

when larger amounts of active virus were subsequently added. Thus the interfering particle and the infectious particle seem to be identical, or at least to be inactivated together by chemical or physical agents.

Since infected cultures were washed thoroughly at the time of addition of the second virus, interference could not have resulted from the extracellular interaction of one virus with another. Also, the presence in the culture of a multiplying virus *per se* did not interfere with the multiplication of another, as demonstrated in the experiments on simultaneous infection. Therefore, an alteration in the cells, whether only a surface alteration or an intracellular one, must be postulated. This alteration is such that interference between two poliomyelitis viruses may be virtually complete. However in the plasma-clot cultures used, this may not be an absolute effect. For example, when cultures infected with 10,000 TC doses of Y-SK virus were reinoculated after 1 day with 100 TC doses of Leon virus, no subsequent growth of Leon virus occurred. But when cultures infected with the same dose of Y-SK virus were reinoculated with 10,000 TC doses of Leon virus after 1 day, only partial interference with the growth of Leon occurred. This might indicate that the large secondary inoculum partially reverses the state leading to interference. As has been suggested by Ziegler and Horsfall in their studies with influenza viruses (22), there may be a competition for a substance which exists in the susceptible cell in a certain fixed amount, the advantage being shifted in the direction of a large secondary inoculum. However, the effect might be the result of the type of culture used. In plasma-clot cultures, in which both the tissue explant and new cellular outgrowth are present, all susceptible cells are not physically available to the virus precisely at the same time. This question of whether the interference is due to the destructive effects of the virus on the susceptible cells can be better answered by using monolayer cellular cultures in which the cells are grown on glass in the absence of plasma (23–25), a technic which was not available when these interference studies were carried out.

The following evidence indicates that the interference between two poliomyelitis viruses cannot be wholly attributable to a non-specific destructive cellular effect: (*a*) resistance to superinfection was found to be only relative; (*b*) the amount of virus which after 1 day produces complete interference with the growth of a fixed dose of a second poliomyelitis virus type, produces no visible cellular destruction at this time; (*c*) monkey testicular cultures may produce virus for over 30 days, long after all cellular "outgrowth" has been completely destroyed; (*d*) a "specificity" effect of the interference phenomenon between two poliomyelitis viruses in tissue culture is apparent from the lack of interference found between poliomyelitis virus and other viruses which multiply at about the same rate in monkey tissue cultures, namely certain Coxsackie and orphan viruses.

In regard to the factors of dosage and timing studied above, it was found that infection of tissue cultures with a small quantity of virus could result, after a number of hours, in a state which caused interference, but the same state could not be produced by a large amount of virus if the challenge virus were simultaneously introduced. This is undoubtedly due to the rapid rate of virus multiplication under the experimental conditions used, since the results presented indicate that there is a direct relationship between the extent of multiplication of the first virus and the degree of resulting interference. When this is considered together with the facility of obtaining the same extent of interference reciprocally under similar conditions it also suggests that there may not be marked differences in the growth rates of the different poliomyelitis virus types.

SUMMARY

The inhibition of multiplication of one poliomyelitis virus by a poliomyelitis virus of another immunologic type has been established by using tissue cultures of monkey testes. The degree of interference varied from none, to partial, to complete, depending upon the time between inoculation of the interfering and the challenge viruses, and the amount of each virus inoculated. Reciprocal interference was demonstrated between Types 1, 2, and 3 poliomyelitis viruses.

Under conditions which resulted in complete suppression of the growth of one poliomyelitis virus by another, interference by poliomyelitis virus with the multiplication of four antigenically distinct "orphan" viruses and of three antigenically related strains of Coxsackie virus could not be demonstrated.

Poliomyelitis virus rendered non-infective by formalin or by irradiation with high energy electrons or with ultraviolet light, or treated so that only traces of residual active virus remained, failed to interfere with the propagation of active homologous virus.

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