Complement Fixation with Brunhilde and Lansing Poliomyelitis Viruses Propagated in Tissue Culture.* (19357)

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It has recently been demonstrated that the complement fixation reaction can be used for the detection of antibodies against the Lansing type of poliomyelitis virus. Suspensions of infected tissues of the central nervous system, from adult(1) and infant(2) mice and cotton rats(3-5) were used as sources of antigen. Such starting materials inherently have several disadvantages, however, since they require, because of their high content of tissue components, cumbersome or time-consuming methods of purification, such as lipoid extraction, ultracentrifugation or methanol precipitation before they may be employed as antigens. Moreover, only the Lansing-like strains that can be propagated in these rodents have so far proved capable of fixing complement.

Tissue culture technics have provided a means of obtaining large quantities of virus-containing fluid that are relatively free of contaminating proteins and other tissue derivatives(6). Accordingly, an investigation was undertaken to determine whether these materials might provide antigens for use in a practical complement fixation test. The results so far obtained are presented.

Preparation of antigens. Supernatant fluids from tissue cultures infected with either the Lansing or the Brunhilde strain were used as starting materials for the concentration procedure. The virus was grown in suspended cell cultures(6) or roller cultures (tubes or 250 ml centrifuge bottles(7)). A control antigen was prepared in the same way from pooled non-infected control cultures included in the same experiments. Human tissue was employed. It consisted of either embryonic skin and muscle, embryonic intestine, adult uterus, or kidney. The latter was obtained from operations for hydrocephalus(8) in small children, or, in some instances, removed at the autopsy of children a few hours after death. Usually the supernatant fluids were pooled from several experiments involving different tissues from several embryos and children. It is logical to assume that the starting material for the preparation of antigen should contain a high concentration of virus and should be as free as possible of other substances. The supernatants from suspended cell cultures in which an ultrafiltrable medium is used(9), fulfill the second requirement, but in our experience usually contain less virus (Lansing virus; mouse titers ranging from 10^{-3} to 10^{-4} calculated per ml fluid) than roller cultures (mouse titers ranging from 10^{-4} to $10^{-5.5}$). The roller medium, however, usually contains the complex entities, embryo extract and horse serum. Recently the advantages afforded by both methods have been retained by the use of roller cultures in which the complex medium used as routine in such cultures is replaced by the simple medium after a satisfactory outgrowth of cells has occurred and after a large quantity of virus has been introduced as inoculum.

The ultrafiltration method described by Seibert(10) was applied to the concentration of the antigen. Alundum candles, impregnated with a 7% collodion solution in glacial acetic acid and washed, provided the filters. The filter candle was placed in a pharmaceutical graduate with a narrow bottom containing the tissue culture fluid. The filterable material was then removed through the filter by suction. In this way the fluid was almost completely removed. The non-filterable residue on the surface of the filter and at the bottom of the graduate was washed twice with 200 ml of the veronal buffer employed in the complement fixation test using the same procedure. After the last washing the vacuum was broken and the filter-outlet connected to a flask containing buffer and

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placed about 50 cm higher than the filter. The flow of fluid was thus reversed, washing the nonfilterable material off the surface of the filter. When a volume of 10-20 ml was attained it was again removed by suction. Finally, the nonfilterable material was taken up in about 2 ml of fluid passed in the reverse direction through the filter. This concentrate was clarified by centrifugation at 13,000 rpm for 15 minutes in a Servall SS1 centrifuge. All these procedures were carried out at about 4°C. The preparations have so far been concentrated from 60 to 600 times. Should drying of the virus occur during the ultrafiltration procedure no harmful effect may be anticipated since it has been determined that drying in air even at room temperature does not impair the antigenic activity of the concentrate. The resulting product, when derived from cultures containing the simpler medium, was clear or slightly opalescent, colorless or brownish, and slightly viscous when concentrated 600 times. On the other hand a similar preparation concentrated 60 times from fluids containing 5% horse serum and 10% beef embryo extract was very viscous. However, it could be used in the complement fixation tests. The concentrated antigens were frozen and stored at —20°C.

Antisera. Sera from monkeys and from human beings were tested. All were stored at —15 to —20°C. Monkey antisera against Lansing, Brunhilde and Leon virus, respectively, were kindly supplied by Dr. Jonas Salk. They were prepared by pooling sera from monkeys immunized with infected monkey cord together with adjuvant(11). Certain experiments, however, were carried out with the paired sera of a rhesus monkey before and after hyperimmunization in this laboratory with Lansing monkey cord without adjuvant. The Brunhilde serum had a 50% neutralizing endpoint of 1:640 against 100 50% paralyzing doses of Brunhilde virus(12). These sera, as well as many other monkey sera examined were found to be more or less anticomplementary in low dilutions. The anticomplementary effect was eliminated or markedly reduced in the following manner. The sera were diluted 1/2.5 in veronal buffer, heated at 60°C for 20 minutes, cooled in ice water and an equal volume of 10% crystalline bovine albumin added. The mixture was heated again at 60°C for 20 minutes. No significant difference was observed in the antibody titers (1/128) of a specimen of Brunhilde serum heated in this manner and another diluted ½ in veronal buffer and heated once at 56°C for 30 minutes. Paired sera were tested from poliomyelitis patients from whom virus had been isolated in tissue culture(13). Sera from 6 cases in the Brunhilde epidemic which occurred in Boston in 1949 were used together with the sera of 6 patients from the predominantly Leon epidemic of 1950. These sera diluted ½ in buffer were inactivated at 56°C for 30 minutes. Thereafter none of them proved to be anticomplementary to a significant degree.

Technic of the complement fixation test. The drop method described by Fulton and Dumbell(14) was adopted. It provides a simple and reliable technic and is especially valuable because of the small amounts of reagent needed. The following modifications that we have introduced should be mentioned: The preparation of glass dropping pipettes is laborious and they are easily broken. Accordingly we employed a No. 19 needle from which the beveled tip had been removed. It was fitted to a 1 ml Yale syringe from which the upper flange had been cut off to accommodate more readily a rubber bulb. Using distilled water this device delivers 20 mm^3 per drop.

TABLE I. Summary of Attempts to Remove Anticomplementary Activity of the Antigen.

Complement incubated in presence of:*	Complement titer
Veronal buffer	300
Untreated antigen†	14
Ether extracted antigen‡	20
Suspension of ether extract‡	300
5% bovine albumin	400
Antigen + 5% albumin	14
Antigen, 60°C 30 min	400

* Serial dilutions of complement were incubated overnight at 0°C.

† Antigen made from Lansing-infected roller bottles with embryonic skin-muscle; in this experiment with a concentration factor of 28 times.

‡ .3 ml of antigen extracted three times with 2 ml ether at 0°C. .3 ml of veronal buffer added to ether phase. Ether removed by evaporation *in vacuo*.

TABLE II. Effect of Heat on Anticomplementary and Antigenic Activity.

Lansing antigen* heated at	No serum	Monkey sera, diluted 1:5				
		Normal	Lansing 1	Lansing 2	Brunhilde	Leon
60°C 30 min	†	†	±	—	—	—
56°C	†	†	±	+	†	†
54°C	‡	—	±	+	‡	‡
52°C	+	—	+	—	—	—
50°C	+	—	+	—	—	—
No antigen		†	†	†	†	†

* Antigen concentrated 600 times from a pool of Lansing-infected suspended cell culture fluids.

† Complete hemolysis.

‡ Trace of unhemolyzed cells.

— = Not done. Serum Lansing 1 from same monkey as normal serum but after hyperimmunization (monkey cord without adjuvant). 2.5 units of complement added.

TABLE III. Complement Fixation Tests with Lansing and Brunhilde Antigens and Three Types of Monkey Antisera.

Antigen (55°C 30 min)	Titers of monkey sera		
	Lansing	Brunhilde	Leon
Lansing*	20	<5	<5
Brunhilde†	<5	≥40	<5
Normal‡	<5	<5	<5

* Same as in Table II.

† Concentrated 270 times from a pool of Brunhilde-infected suspended cell culture fluids.

‡ Concentrated 600 times from the control cultures carried in parallel with cultures infected with Lansing and Brunhilde viruses.

3 units of complement added.

The relatively small number of tests included in the present experiments made it unnecessary to employ the elaborate rack and boxes described by Fulton and Dumbell. Instead, enameled metal boxes were used. Two boxes were employed: one was kept at 37°C and the other in the ice box. The bottoms were covered with a layer of moist absorbent cotton. During the test ice cubes were placed in the bottom of the cold box. One to three sheets of lucite (9″ x 11″ x 1/8″) were supported by rubber stoppers in these boxes which were covered tightly with a sheet of "Parafilm."‡ The concentration of sheep's red cells (0.4%) was twice that used by Fulton and Dumbell. This modification gave clearer readings, but probably somewhat reduced the sensitivity. The same batch of pooled guinea pig serum was used throughout. This was stored in sealed ampules in the CO_2 box. Most of the titrations of antibody were carried out in the conventional manner, employing constant quantities of antigen and complement and increasing dilutions of serum. The complement titer which was assumed to remain essentially unchanged was, however, verified by titration performed contemporaneously with each test. The amounts of complement used in the test were thus found to vary between 2 and 3 units as indicated in the tables. The unit of complement was taken as the least amount of guinea pig serum that caused complete lysis of the cells. All necessary controls were included. The antibody titer is expressed as the highest dilution of serum giving about 50% or more inhibition.

Elimination of anticomplementary effect of antigens. The antigens, as well as the original tissue culture fluids, proved to be anticomplementary. Although an indication of specific complement fixation was observed with the Lansing antigens that were the least anticomplementary, it was obvious that this effect had to be minimized or abolished if conclusive results were to be expected. In attempts to accomplish this, the procedures mentioned in Table I were followed. It is evident that extraction with ether or addition of albumin were without effect, whereas heat treatment was completely effective. The results presented in Table II indicate that a temperature of 54-56°C for 30 minutes was necessary to abolish the anticomplementary activity. Within the temperature range 54-60°C, however, the capacity of the antigen to fix complement in the presence of homologous antibody was not abolished. Heat treatment (55-56°C for 30 minutes) has so far been

‡ Manufactured by Marathon Corp., Menasha, Wisc.

TABLE IV. "Box Titration" of Brunhilde Antigen with Homologous Monkey Antiserum.

Dilution of antigen	Dilution of antiserum								
	4	8	16	32	64	128	256	512	No serum
2	—	+	+	+	+	+ to ±	†	†	†
4	+	+	+	+	+	+	†	†	†
8	+	+	+	+	+	+	±	†	†
16	+	+	+	+	+	+	+ to ±	†	—
32	+	+	+	+	+	+ to ±	±	†	—
64	+	+	+	±	±	†	†	†	—
128	+	±	‡	‡	†	†	†	†	—
No antigen	+ to ±	†	†	†	—	—	—	—	—

† Complete hemolysis. ‡ Trace of unhemolyzed cells. — = Not done.
Same antigen and antiserum as mentioned in Table III. 2 units of complement added.

TABLE V. Complement Fixation Tests on Sera from Cases of Poliomyelitis.

Name	Age, yr	Type of case	Type of virus isolated	Time after onset, days	Serum titer: Brunhilde antigen*	Serum titer: Cont. antigen†
F.	6 6/12	Paralytic	Brunhilde‡	8	16	—
				54	16	<2
D.	5 2/12	"	" ‡	8	<2	—
				16	8	<4
				440	<2	2
Du.	10 11/12	"	"	5	2	—
				19	16	<2
J.	2 10/12	"	"	4	16	—
				22	32	<2
Fa.	6 5/12	Nonparalytic	"	5	2	—
				26	<2	<2
I.	9 9/12	Paralytic	"	7?	<2	—
				29	16	<2
L.	11 6/12	"	"	4?	<2	—
				28	4	<2
S.	5 5/12	"	Leon	2	<4	—
				19	<2	2
M.	3	"	"	2	<2	—
				29	<2	<2
K.	1 2/12	"	"	5	<2	—
				30	<2	<2
Ma.	10 11/12	Nonparalytic	"	2	≥8	—
				22	16	2
R.	4 11/12	Paralytic	"	5	8	—
				25	4	<2

* Same as in Table III; diluted 1/10.
† " " " " " 1/4.
‡ Brunhilde-like virus isolated from a pool of stools from two siblings F. and D. 2-2.5 units of complement added.

effective in the case of 4 different batches of antigen (Lansing, Brunhilde and normal control antigens) concentrated from 110 to 600 times. This procedure also eliminated the anticomplementary effect of crude tissue culture fluids. The heated antigens could be stored at —20°C and repeatedly thawed and frozen without changing their qualities.

Specificity of the reaction. A. Monkey sera. The specificity of Lansing and Brunhilde antigens is clearly illustrated by the results recorded in Table III. A "box titration" (Table IV) of the Brunhilde antigen against the homologous monkey hyperimmune serum revealed an optimal antigenic activity at the dilution 1/16. This corresponds to about a 20-fold concentration of the antigen present in the crude tissue culture fluid.

B. Human sera. Acute and convalescent serum specimens from 12 cases of poliomyelitis were tested for antibodies against the Brunhilde antigen. The convalescent specimens were in addition tested against the control antigen. The results are shown in Table V. Of the 7 patients yielding Brunhilde-like viruses, 6 had complement fixing antibodies and 4 developed a significant rise (at least 4-fold) in titer during convalescence. In the sera of 3 of these patients an increase in antibody was not demonstrated. In 2 of them, however, relatively high titers were characteristic of both serum specimens—a finding which suggests that the maximum response had occurred by the time the first specimen was taken. In view of our limited experience no entirely satisfactory explanation can now be given for the failure of the third patient (Fa.) to develop antibody. It might be mentioned, however, that this patient, as well as J. and L., had been treated with ACTH during the acute phase. The results afford some evidence that antibody tends to develop early in the disease. How long it may persist in detectible quantities must await further study, but the findings with the sera of patient D. suggest that in some individuals this period may not be long.§ None of the sera mentioned in Table V reacted significantly with the control antigen. Moreover, the few insignificant reactions noted usually did not correlate with the reactions with the virus antigen.

The sera of 2 of the 5 patients with Leon-like virus fixed complement with the Brunhilde antigen. No evidence of increase in titer, however, was observed. The significance of these antibodies is as yet obscure. The following possibilities to account for their presence come to mind: a) they were the result of a previous infection with Brunhilde-like virus; b) infection with a Leon-like virus in human beings may elicit antibodies which cross react with Brunhilde virus; c) these patients may have undergone a mixed infection with both viruses, although only Leon-like viruses were isolated. In one of the patients (Ma.) from whom a Leon-like virus was isolated but in whom no increase in antibody was demonstrated, the acute phase specimen was obtained within 2 days after the onset. This circumstance renders hypothesis b) and c) less probable.

Summary. Antigens which gave specific complement fixation were concentrated from the supernatant fluids of tissue cultures infected with poliomyelitis viruses (Lansing and Brunhilde strains) by a method of ultrafiltration. Their anticomplementary activity was abolished by heating. The drop method of Fulton and Dumbell was used and offered a practical way of performing the tests with minimal amounts of antigen. Specific antibodies were demonstrated in the sera of hyperimmunized monkeys as well as in sera of poliomyelitis patients.

§ Since this paper was submitted for publication additional specimens of sera taken 1.5-2.5 years after the illness have been obtained from all patients with antibodies against Brunhilde except patient I. When tested at the same time as the earlier specimens they all exhibited a marked drop in complement fixing antibody.

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