STUDIES ON A COMPLEMENT FIXATION TEST FOR HERPES SIMPLEX¹

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Received for publication January 27, 1955

Although the neutralization test is, in general, satisfactory for immunologic studies with a diversity of viruses, it is cumbersome and expensive. The complement fixation test, in contrast, is simple and rapid and has been successfully applied to a number of viral diseases, especially for diagnostic purposes. In the case of herpes simplex virus infections, however, the literature concerning the applicability and usefulness of the fixation method is at considerable variance; on the whole, positive reactions have been elicited with difficulty, and the basic problem lies in the preparation of an adequately potent and specific antigen. Thus, while some early workers (Kraus and Takaki, 1925, 1926; Takaki, Bonis, and Koref, 1926) obtained fixation when testing human or rabbit immune sera against a rabbit brain antigen, others (Greenbaum and Harkins, 1925; Todorovitch, 1925; Bedson and Crawford, 1927; Schultz and Hoyt, 1928; Tang and Castaneda, 1929; Gay and Holden, 1931; Myers and Chapman, 1937), using antigens prepared from rabbit brain or herpetic vesicle epithelium or fluid from guinea pigs and rabbit immune sera of high neutralizing capacity, were unable to confirm these results. The failure of such systems may most probably be attributed to the nonspecific and anticomplementary activity of crude rabbit brain suspensions (Maltaner, 1946) and rabbit serum (Kidd and Friedewald, 1942); whatever the difficulty, specific fixation could be elicited in systems employing suspensions of infected guinea pig pads and guinea pig immune sera (Bedson and Bland, 1929) or sera from patients with herpetic infections (Brain, 1932).

¹ Portion of a thesis submitted to the Graduate Division of the University of California at Berkeley in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Bacteriology.

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The preparation of antigen from guinea pig pads, however, is tedious and expensive. With a recent resurgence of interest in human herpetic infections, attention has naturally focused on the embryonated hens' egg as a source of antigen, and over the past few years several authors have described antigens prepared from the fluids or tissues of infected chick embryos (Hayward, 1949; Fowler, 1950; Dudgeon, 1950; Gajdusek, Robbins, and Robbins, 1952). Because of differences in experiences reported by these investigators, a systematic study of the distribution of virus and of complement-fixing antigen was undertaken, with a view of selecting as an antigen that material possessed of high antigenic potency and sensitivity without nonspecific or anticomplementary activity.

MATERIALS AND METHODS

Virus strains. The MacIntyre and the Armstrong strains were used. The MacIntyre strain was isolated in this laboratory in 1946 from the brain of a fatal case of encephalitis. The original isolation was in adult mice. After 12 intracerebral passages the strain was adapted to the chick embryo by 23 passages on the chorioallantoic (CA) membrane, using a suspension of chorioallantoic membrane as inoculum. Subsequently, the virus was passed 5 times by the yolk sac route, with a yolk sac suspension as inoculum.

The Armstrong strain, which is an egg adapted strain (Francis and Kurtz, 1950), was obtained through the courtesy of Dr. Thomas Francis, Jr., University of Michigan School of Public Health. This strain produces large pocks on the chorioallantoic membrane; it was passaged in this laboratory by the yolk sac route 5 times.

Age of chick embryos and amount of inoculum. For the allantoic route, 10 day old embryos and 0.1 ml of inoculum were used; for the chorioallantoic membrane, 12 day old embryos and 0.2 ml of inoculum; for the amniotic sac, 10 day old

embryos and 0.1 ml of inoculum; and for the yolk sac, 8 day old embryos and 0.25 ml of inoculum. In each case the inoculum consisted of tissue or fluid corresponding to the route of inoculation (homologous material), and from 50 to 100 LD₅₀ of virus, as determined by previous titrations in chick embryos, constituted the dose.

Incubation of embryos. Incubation was done at 37 C. The period of incubation depended on the route of inoculation used. The embryos were removed from the incubator for harvesting when approximately 30 per cent of the number inoculated were dead, which usually occurred on the third or fourth day after inoculation. When the yolk sac route was used, the embryos were incubated for only 2 days because of the high mortality at this time.

Collection of egg materials. Eggs were chilled for 4 hours before harvesting. In each experiment materials were collected from at least 15 embryonated eggs. Twenty ml and 5 ml syringes were used to harvest the allantoic and amniotic fluids, respectively. The membranes were harvested separately with Wilde's ear forceps. Materials of the same kind were pooled in tubes or deep dishes which were kept immersed in ice water (5-6 C). The blood-free allantoic and amniotic fluids were clarified by centrifugation at 2,000 rpm for 20 minutes, and the supernatant fluids were stored at -70 C on dry ice. The chorioallantoic and amniotic membranes were washed 3 times in cold physiological saline solution (pH 7.2) and drained into large petri dishes. The yolk sacs, usually hemorrhagic, were washed 7 times in cold saline, after which they were free of most of the yolk. The membranes were ground in a mortar with alundum, and phosphate buffered saline was added to give a 25 per cent suspension (w/v). This suspension was centrifuged in the cold at 2,000 rpm for 20 minutes to remove heavy particles. Part of the resulting supernatant fluid was taken for virus titrations, and the remainder was stored at -70 C. Just before use, the material was thawed, shaken, and again centrifuged at 2,000 rpm for 20 minutes to remove floccules; the supernatant fluids were then titrated for their complement-fixing activity.

Titration of infectivity. The 25 per cent suspensions of infected extra-embryonic membranes were diluted to 1:2.5 to obtain a dilution of 1×10^{-1} . The extra-embryonic fluids were diluted to 1×10^{-1} (v/v). From these 1×10^{-1} dilutions

of the infected materials, serial 10-fold dilutions were prepared, usually through to 1×10^{-8} . The virus dilutions were kept in an ice water bath during the course of inoculation into mice. Two litters of 2 to 4 day old mice, averaging 6 mice per litter, were used for each dilution, each mouse receiving 0.01 ml intracerebrally. The mice were kept under observation for a period of 14 days. Most of the infected mice died between the third and the sixth day. Deaths which occurred in the first 48 hours after inoculation were not recorded as specific deaths and were not considered in the calculation of the mortality end points (LD₅₀), which were computed by the Reed-Muench formula (Reed and Muench, 1938).

Neutralization test for antibody assay of sera. All sera were stored at 4 C. Acute and convalescent serum specimens of the same patient were examined in the same test. Immediately prior to use in the neutralization tests, the sera were inactivated at 56 C for 20 minutes. The test virus consisted of a 20 per cent suspension (w/v) of infected (MacIntyre strain) mouse brains in 10 per cent normal rabbit serum broth; the supernatant fluid obtained after centrifugation at 2,000 rpm for 20 minutes was distributed into ampules and stored on dry ice. Serial 10-fold dilutions of the contents of several ampules were made in buffered saline, and to 0.3 ml of undiluted test serum was added an equal volume of virus dilution. Rabbit immune and normal sera were used as controls. The serum-virus mixtures were incubated at 4 C for one hour, then inoculated intracerebrally in 0.01 ml amounts into 2 to 4 day old mice; from 12 to 16 mice were used per dilution. The inoculated animals were observed for 14 days, and the mortality end points were calculated by the Reed-Muench formula, no account being taken of mice that died during the first 48 hours after inoculation.

Complement fixation test for antigen titrations and for antibody assay. Kolmer saline was used as diluent. Two units of hemolysin, as determined by titration at 37 C for 30 minutes with 1:30 complement and 2.5 per cent suspension of sheep cells, were used. Complement was titrated in the presence of antigen with incubation at 37 C for 30 minutes; 2 full units were used in the test proper. Sensitized red cells were prepared by mixing equal parts of a 2.5 per cent suspension of washed sheep erythrocytes and an amboceptor dilution containing 2 units of hemolysin per 0.25

ml. All sera were inactivated at 60 C for 30 minutes prior to the test.

For the titration of the antigens, the complement-fixing activity of the antigens in the presence of herpetic antibodies was determined by the optimal antigen dilution method with guinea pig herpes immune serum with a high content of specific antibodies. The optimal antigen titration method (Dean and Webb, 1926; Eagle, 1937) was used since a zone phenomenon has been observed frequently with potent viral antigens, e.g., influenza (Hoyle and Fairbrother, 1937) and mumps antigens (Henle *et al.*, 1947). The serum dilutions (0.2 ml) were added to the tubes first, followed by the antigen dilutions (0.2 ml), and then by two full units of complement contained in 0.2 ml. The following controls were included: (a) immune and normal serum controls for anticomplementary activity of the sera, (b) antigen controls to determine if the antigens had anticomplementary properties, (c) normal tissue control antigens to determine the specificity of the sera, (d) titer control of immune serum (with previously standardized antigen), and (e) complement controls, which consisted of decreasing amounts of complement, i.e., 2 units, $1\frac{1}{2}$ units, 1 unit, and $\frac{1}{2}$ unit (these controls indicate whether proper amounts of complement are being used in the test, and also serve to indicate the appropriate interval at which reading of the test results should be made (*vide infra*)). Incubation was done at 4 C overnight. On the following morning the tubes were warmed to 37 C, and 0.5 ml of sensitized red cells was added to all the tubes. The tubes were shaken and then placed in the water bath at 37 C. When the complement control tube containing one unit of complement showed complete hemolysis (usually about 15 minutes), the tubes were removed from the bath and left undisturbed for one-half hour, at room temperature, so that the cells could sediment. The degree of hemolysis was determined visually from the supernatant fluids. The highest dilution of antigen giving a 3 plus or 4 plus fixation with the highest dilution of serum was considered as the optimal antigen dose and thus constituted "one unit". This is illustrated in table 1, in which the results obtained with a chorioallantoic membrane antigen are shown.

The complement fixation technique employed for the titration of antibodies was basically that used in this laboratory for the diagnosis of viral

TABLE 1

Titration of herpes simplex antigen for complement-fixing activity. Typical results obtained with 25 per cent chorioallantoic membrane suspensions

Antigen Dilution	Immune Serum Dilution						Normal Serum Control	Antigen Control
	1:8	1:16	1:32	1:64	1:128	1:256		
	Degree of fixation							
Undiluted*	4	4	4	4	4	0	0	0
1:2	4	4	4	4	4	0	0	0
1:4	4	4	4	4	4a	0	0	0
1:8	4	4	4	3	0	0	0	0
1:16	2	1	0	0	0	0	0	0
1:32	0	0	0	0	0	0	0	0
Serum control	0	0	0	0	0	0	0	—
Control antigen†	0	0	0	0	0	0	0	0
Previous lot of antigen	4	4	4	4	4	0	0	0

* Undiluted = 25 per cent suspension (w/v) of CA membrane.

† Antigen prepared from normal chorioallantoic membrane.

a = 1 unit of antigen.

and rickettsial diseases (Lennette, Clark, and Dean, 1949). The main deviation from this method was the use of 2 full units of complement instead of 2 exact units. This modification was introduced since with 2 exact units of complement the normal serum controls occasionally did not show complete hemolysis. In each test, at least 3 known positive sera with different antibody titers and 2 known negative sera were used as controls. It was found that 2 serum dilutions for nonspecific controls were necessary because in the sera giving a nonspecific reaction with the normal antigen, the reaction often showed a prozone phenomenon. Nonspecific reactions often appeared in serum specimens that had been stored in the refrigerator for a period longer than 2 years.

Guinea pig immune sera. Immune sera were prepared in guinea pigs by intra-abdominal inoculation of 3 or 5 doses (2 ml each) of a 20 per cent suspension of mouse brains infected with the MacIntyre strain; the injections were spaced 1 week apart. No overt evidence of infection was observed in the animals at any time.

Human sera. One hundred and thirty-one

serum specimens from 72 patients with herpetic and nonherpetic illnesses were examined.

EXPERIMENTAL RESULTS

Yield of complement-fixing antigens from egg materials. It was considered that reproduction of the virus might occur primarily in certain tissues of the chick embryo and that the mechanism of release of virus from the infected cells might affect the concentration of the virus and of the antigen in different parts of the embryonated egg. The route of inoculation was also considered as a factor, as the susceptibility of the tissues with which the virus first comes in contact might determine the time and the pathway required by the virus to spread throughout the egg. Consequently, various routes of inoculation were used, and, in each case, the different tissues and fluids of the embryonated egg were quantitatively examined for infective and complement-fixing properties. A pool of guinea pig immune serum with a high titer of neutralizing antibodies was used in the complement fixation

TABLE 2

Distribution of herpes simplex virus in the embryonated hen's egg following various routes of inoculation

Route of Inoculation	LD ₅₀ Titer (Intracerebral) in Baby Mice of				
	Allantoic fluid	Amniotic fluid	CA membrane	Amniotic membrane	Yolk sac
<i>MacIntyre strain</i>					
Allantoic cavity...	2.5	3.6	5.0	4.9	4.7
Amniotic cavity...	3.0	3.3	5.0	5.2	4.5
CA membrane.....	2.4	2.7	4.5*	4.6	4.7
			3.9†		
Yolk sac.....	5.0	5.8	6.5	5.9	6.3
<i>Armstrong strain</i>					
CA membrane.....	2.0	2.5	4.6*	4.6	3.5
			4.5†		
Yolk sac.....	3.0	3.9	5.6	5.3	5.3

Serial decimal dilutions for titration made from undiluted fluids as starting material. Starting material with membranes was 25 per cent suspension (w/v); this diluted 1:2.5 to give initial 1×10^{-1} dilution for infectivity titrations.

* "Inoculated" area, i.e., portion of membrane showing extensive, usually confluent, lesion.

† "Uninoculated" area, i.e., remainder of membrane after area of lesions excised.

TABLE 3

Complement-fixing capacity of various antigens when tested against standard herpes immune serum (guinea pig)

Route Inoc. of Egg	Complement-Fixing End Point of Serum in Test with				
	Allantoic fluid*	Amniotic fluid*	CA membrane†	Amniotic membrane†	Yolk sac†
<i>MacIntyre strain</i>					
Allantoic cavity...	1:4	1:4	1:16	1:16	1:16
Amniotic cavity...	1:8	1:16	1:32	1:32	1:32
CA membrane.....	1:4	1:4	1:32‡	1:8	1:8
			1:4§		
Yolk sac.....	1:16	1:16	1:128	1:128	1:128
<i>Armstrong strain</i>					
CA membrane.....	1:4	1:4	1:128‡	1:4	1:4
			1:32§		
Yolk sac.....	1:16	1:32	1:32	1:64	1:32

* Titers given are those obtained with undiluted fluids as antigens.

† Titers obtained with membrane antigen diluted 1:4, i.e., 1:4 dilution of 25 per cent suspension of membrane (or, as used in test, 1:16 suspension (w/v) of original membrane tissue).

‡ "Inoculated" area—see footnote to table 2.

§ "Uninoculated" area—see footnote to table 2.

titrations. The first determinations showed that tissue of the embryo itself, although having a high concentration of virus, possessed a high anticomplementary activity. Therefore, only allantoic fluid, amniotic fluid, chorioallantoic membrane, amniotic membrane, and yolk sac were examined in subsequent experiments. A series of replicate experiments using these materials was done with the MacIntyre strain because of its known passage history and relatively low passage level. After sufficient background information had been acquired, comparisons were made with the Armstrong strain using the chorioallantoic and yolk sac routes of inoculation only. The results of infectivity tests are presented in table 2; the complement-fixing activities of the several preparations are given in table 3. It will be observed in table 2 that, regardless of the route of inoculation, the highest concentrations of virus were found, in general, in the membranes. The only exception occurred with the MacIntyre strain; following yolk sac inoculation this strain, unlike the Armstrong, produced a high concen-

tration of virus in the extra-embryonic fluids. It is also of interest, in passing, that multiplication of virus apparently was uniform throughout the chorioallantoic membrane after inoculation onto this membrane—no remarkable differences in titer were noted between that portion of the membrane bearing lesions and that free of pocks or infiltrations. In addition, the titer attained in the chorioallantoic membrane was appreciably higher following yolk sac inoculation with either strain than after direct inoculation of the membrane itself.

The various preparations were examined for their ability to fix complement in the presence of specific immune serum (guinea pig) in order to ascertain the relationship between infectivity and complement-fixing capacity. The results of the fixation tests of one series of experiments are presented in table 3. Each antigen was tested by the optimal dilution method, and since the "unit" (and hence the standard immune serum end point also) differed between antigens, reduction to a common denominator was desirable for tabulation purposes. As egg fluids generally failed to give significant fixation when diluted even 2-fold, the serum end points shown are those obtained with undiluted fluids. The serum end points shown for membrane antigens are those obtained with the 1:4 dilution of the stock material (or 1:16 suspension (w/v) of the original tissue) since the "unit" of many preparations fell in this range; it should be pointed out, however, that some degree of significant fixation, with the more potent antigens, occurred with higher dilutions (*cf* table 1), and that with weaker antigens lower dilutions sometimes gave a 2 to 4-fold (1 or 2 tubes) higher serum end point.

From table 3 it will be seen that the extra-embryonic fluids from eggs inoculated with the MacIntyre strain by the allantoic, amniotic, or yolk sac routes possessed little activity, and that the membranes were appreciably better in this respect, especially those from eggs inoculated via the yolk sac. With the Armstrong strain, no unquestionable difference in activity was encountered between fluids and membranes after yolk sac inoculation, a situation quite different from that observed with the MacIntyre strain. Fluids and membranes from eggs inoculated on the chorioallantoic membrane with either strain possessed low and essentially similar degrees of

Figure 1. Relationship of infectivity to complement-fixing activity of herpes simplex antigens derived from embryonated eggs.

activity, the only exception being the chorioallantoic membrane itself. That portion of the membrane showing lesions yielded an antigen with a high antigenic activity compared to the nonlesion bearing portion, and especially to the fluids (and, with the Armstrong strain, other membranes).

Comparison of tables 2 and 3 indicates that antigenic activity tended, on the whole, to parallel roughly the infectivity. This apparent relationship is better brought out in figure 1, in which the antigenic capacity of a preparation is plotted against its viral content. Since infectivity end points are based on dilutions of the original tissue (w/v) or original extra-embryonic fluids (v/v), it appeared desirable to express the complement-fixing activity in the same terms. Consequently, while 1:1, 1:2, and 1:4 represent original fluids and fluids diluted as indicated, the starting point with membranes was 1:4, i.e., a 25 per cent tissue suspension, so that if it is desired to express dilution in terms of the starting preparation, as is usually done, the reciprocals from 8 to 128 should be divided by 4. The complement-fixing

capacity is expressed in figure 1 as the highest dilution of fluid or membrane which gave significant fixation when tested against a single dilution (1:4) of immune serum (the so-called "straight-line" method of titration). The separation between fluid and membrane antigens is indicated in figure 1, and is readily done since no fluid gave fixation beyond 1:4, whereas membranes fixed complement in at least 1:16 suspension.

As is brought out by figure 1, there is some association between infectivity and antigenic capacity. This is not unusual as it has been observed with most viruses, and, indeed, successful development of a fixation test has depended in many instances on obtaining materials with a high infectivity (adaptation to a host, use of baby mice, etc.). While there is a rather good degree of correlation in figure 1 between infectivity and antigenicity, it does not necessarily follow that fixation of complement is a function solely of the infectious particle itself. In this connection, several authors (Hayward, 1949; Jawetz *et al.*, 1951; Brown, 1953) have reported on the appearance of a noninfectious, soluble antigen in embryonated eggs infected with herpes virus, and the fact that a high viral content is not invariably associated with a correspondingly high antigenic activity (see figure 1 and tables 2 and 3) also suggests that some factor other than the infectious moiety *per se* plays a role as an antigen in the fixation of complement. Thus, as shown in tables 2 and 3, materials derived from eggs inoculated with the MacIntyre strain via the yolk sac possessed very similar concentrations of virus, yet the extra-embryonic fluids exhibited a low antigenic activity, the membranes a high activity. This situation is, in some respects, analogous to that described for mumps virus (Henle, Henle, and Harris, 1947), where the V (viral) antigen appears chiefly in the fluids whereas the S (soluble) antigen is retained in the membranes.

Method adopted for preparation of complement-fixing antigen. After a number of orientative experiments in which the findings described above were utilized, a basic method of preparing antigen was evolved.

Several considerations were taken into account as concerns route of inoculation and source of antigen. Fluids were regarded as unsuitable because of their low fixing capacities, and membranes represented the material of choice. Inocu-

lation onto the chorioallantoic membrane is not only less simple technically than into the yolk sac, but suspensions of membranes with marked infiltration and necrosis proved to be turbid and anticomplementary. Yolk sac inoculation would permit use of both the chorioallantoic and amniotic membranes, and also would utilize embryos at an earlier stage of incubation. It also appeared preferable not to utilize the yolk sac as a source of antigen since it is difficult to free this tissue completely from yolk material, which even in small amounts is apt to give rise to nonspecific fixation (Wertman, 1945).

The following method was adopted. Infected yolk sacs (MacIntyre strain) were ground to a 20 per cent suspension (w/v) in skimmed milk, which has been shown to be a suitable medium for the preservation of this virus (Speck *et al.*, 1951; Allen *et al.*, 1952). After centrifugation in the cold at 2,000 rpm for 20 minutes, 0.5 ml amounts of the supernatant fluid were distributed into ampules which were shell-frozen, flame-sealed, and stored on dry ice. This material was used as seed for the preparation of antigens, and in the course of 2 years its infectivity titer remained practically the same. A 10^{-8} dilution of this stock virus in buffered saline was used; 8 day old embryos were inoculated by the yolk sac route with 0.25 ml (about 50 LD₅₀). From 70 to 150 embryos were inoculated and incubated at 37 C for 2 days. About 5 to 8 per cent of the embryos died in the first 24 hours and were discarded. The time of death of the embryos as a result of infection was quite consistent, the greater number dying between 40 and 50 hours after inoculation. Beginning at 40 hours after inoculation, the eggs were candled every 2 to 3 hours, and those with recently dead or sluggish embryos were placed in the refrigerator. After 50 hours, the remaining live embryos, usually not more than 20 per cent of the original number, were placed in the refrigerator with the others. The eggs were kept in the refrigerator overnight, and then the chorioallantoic and amniotic membranes were harvested and combined in a single pool. The membranes were washed 3 times with cold saline, weighed, and triturated in a mortar with alundum. A 25 per cent suspension (w/v) in buffered saline (pH 7.2) was prepared. After centrifugation in a refrigerated centrifuge at 2,000 rpm for 20 minutes, the supernatant fluid

was removed, ampuled in 15 ml amounts, and stored at -70°C . Prior to use, the contents of the required number of vials were thawed, shaken, and spun in a refrigerated centrifuge at 2,000 rpm for 20 minutes; the supernatant fluid was diluted to the extent indicated by prior titration and used as antigen in the complement fixation test.

The antigen consisted largely of chorioallantoic membrane, as only 10 per cent of the antigen, by weight, represented amniotic membrane. From 0.5 to 0.9 grams of chorioallantoic membrane were harvested from each egg. The pooling of the membranes with the extra-embryonic fluids did not significantly increase the complement-fixing activity of the antigen, and hence extra-embryonic fluids were discarded when the membranes were harvested.

In the course of the present work 17 lots of antigen were prepared; 10 lots had an antigenic titer (1 unit in 0.2 ml) of 1:16 (1 part of membranes plus 15 parts of saline); the remaining 7 lots had a titer of 1:32.

Sensitivity and specificity of the complement fixation test. To determine whether the fixation produced by herpetic antigens was specific, and whether the test was sufficiently sensitive to detect different levels of antibody, guinea pigs were immunized as described above and bled periodically to assay the sera for antibody, and for reactivity to antigens prepared from normal chorioallantoic and amniotic membranes.

A group of 4 guinea pigs was given 3 injections of live virus at weekly intervals and bled at intervals over a 2 month period. The complement-fixing and neutralizing antibody titers were de-

termined on each specimen, and the results are shown in table 4. It will be noted that the pre-inoculation serum contained no demonstrable antibodies and that both neutralizing and complement-fixing antibodies were demonstrable at 2 weeks after the first injection (6 days after the second injection) of virus. From table 4 it appears that by the time of the first postinoculation bleeding, the neutralizing antibody titer had already reached its maximum, and it remained at essentially this level during the 2 month observation period. Complement-fixing antibody, however, seemed to rise up to about the third week, and then to decline (e.g., animals 3 and 4).

In a second group of 3 pigs, which received 5 injections of virus, the results (shown in table 5) were quite similar insofar as complement-fixing antibody is concerned; antibody was detectable in low concentration in blood taken 11 days after the first injection, increased in titer, and by the third month had declined notably although the maximal titers were higher than those elicited by 3 injections. Allowing for differences arising from experimental error inherent in neutralization tests, neutralizing antibody was first detected at the same time as complement-fixing antibody, apparently rose to a higher level during the ensuing month, and persisted at about this level through the duration of the observation period. Although these experiments are limited (additional data concerned with man are presented below), they indicate that the complement fixation test described here is sufficiently sensitive to follow fluctuations in antibody titer, and also

TABLE 4

Complement-fixing (and neutralizing) antibody response of guinea pigs given 3 injections of virus. Evidence for specificity of C-F test

Inoc. Dates (1952)	Bleeding Dates (1952)	Antibody Titers							
		G. P. 1.		G. P. 2		G. P. 3		G. P. 4	
		NI	C-F	NI	C-F	NI	C-F	NI	C-F
May 4	May 3	<0.5	<1:4	<0.5	<1:4	<0.5	<1:4	<0.5	<1:4
May 11	May 17	1.4	1:32	1.7	1:32	1.5	1:64	1.2	1:16
May 18	May 27	1.9	1:64	1.3	1:64	2.2	1:128	1.8	1:32
	June 3	1.7	1:32	1.9	1:64	2.0	1:64	1.9	1:64
	June 24	1.3	1:32	1.6	1:32	1.4	1:32	2.2	1:16
	July 7	1.5	1:16	1.3	1:16	—	—	1.5	1:8

NI = Neutralization index; C-F = complement-fixing titer.

TABLE 5

Complement-fixing (and neutralizing) antibody response of guinea pigs given 5 injections of virus. Evidence for specificity of C-F test

Inoc. Dates (1952)	Bleeding Dates (1952)	Antibody Titers					
		G. P. 5		G. P. 6		G. P. 7	
		NI	C-F	NI	C-F	NI	C-F
April 13	April 19	<0.5	<1:4	<0.5	<1:4	—	—
	April 24	1.1	1:4	1.5	1:8	<0.5	<1:4
April 27, May 4, and May 11	May 17	2.2	1:256	2.6	1:512	—	—
	May 27	2.5	1:256	2.4	1:256	1.9	1:128
May 18	June 3	2.3	1:128	—	—	2.1	1:128
	June 24	2.0	1:128	—	—	2.0	1:64
	July 12	1.7	1:32	2.0	1:32	1.5	1:32

NI = Neutralization index; C-F = complement-fixing titer.

that complement-fixing antibodies, following their appearance, tend to decline rather rapidly.

As to specificity, none of the guinea pig immune sera fixed complement in the presence of normal membrane antigens. Recent experiments (to be published) using human herpetic sera show that such sera react with herpetic antigens but not with heterologous (mumps, Western equine encephalomyelitis) virus egg antigens.

Relationship between neutralizing and complement-fixing antibodies in man. The results of the animal experiments above indicated that the complement-fixing antigens employed were highly specific for the detection of herpes simplex virus antibodies. Since it was intended to apply the method to the study of human infections, however, additional information on specificity as it relates to human sera was desirable. The neutralization test is a basic immunologic tool in virology and has been widely employed for studies on herpes simplex virus; its specificity in detecting antibodies to this virus is generally accepted. It was, therefore, used as the criterion as to whether herpetic infection, past or present, had occurred in any given individual. If, then, the presence of complement-fixing antibodies could be correlated with the presence of neutralizing antibodies, evidence of specificity would be afforded.

For the present purposes, 131 serum specimens were examined by both the neutralization and complement fixation tests. These specimens came from 72 individuals of various ages in whom a herpes simplex virus infection was suspected clinically, or in whom clinically similar diseases

(e.g., herpes zoster, varicella) were suspected. Paired blood specimens (acute and recovery stages of illness) were available on 59 patients; single specimens (acute or convalescent), on 13.

The serologic results are plotted in figure 2, which depicts the observed relationships between neutralization index and complement-fixing titer. In general, there was a tendency toward association between the higher levels of complement-fixing antibody and the higher neutralization indices; although the correlation manifested was by no means absolute, it was better than expected in view of the experimental errors inherent in the techniques used. Whether a better correlation or correspondence might be revealed by use of neutralization tests employing the constant virus-varying serum method, rather than the varying virus-constant serum method used here, would be of interest to determine.

A significant finding was that in none of the sera was complement-fixing antibody encountered in the absence of neutralizing antibody. While the reverse situation was observed, it was confined to only 6 sera (figure 2) all taken during the first 7 days of onset of a primary herpetic infection; when the second specimen on these patients was tested, complement-fixing antibody had appeared, and the neutralization index had risen.

Neither neutralizing nor complement-fixing antibody was detectable in 40 sera. Of these, 4 came from patients in the acute phase of a primary herpetic infection; both types of antibody subsequently appeared, as determined by examination of recovery-phase sera. The remaining

Figure 2. Relationship between neutralizing and complement-fixing antibody in herpetic and nonherpetic illnesses. (Data based on 131 sera from 72 patients.)

36 negative sera represented paired specimens from 18 patients with illnesses due to agents other than herpes simplex virus.

DISCUSSION

The data presented here show that herpes simplex virus spreads throughout the embryonated egg regardless of the route of inoculation, confirming the histopathologic findings of Anderson (1940). Quantitative examination of the extra-embryonic fluids and membranes indicated that the virus is not equally distributed since significantly higher concentrations of infective particles were found in the membranes. In general, however, regardless of where the virus was found, and its concentration, a comparatively high correspondence was found between the concentrations of infective particles and the concentration of complement-fixing antigen. This phenomenon

has been observed frequently with other viruses. This finding does not necessarily mean, however, that the infective particle is responsible for all the antigenic activity. Such a correlation may indicate that the production of the antigen or antigens parallels the number of infectious particles because either (1) the antigen is the virus itself; (2) the antigen is represented by a non-infectious substance whose concentration in the infected tissue is closely related to the number of infectious units; or (3) a combination of both. In this connection, Jawetz *et al.* (1951) have suggested that the herpetic soluble antigen represents a "precursor" of the virus, and Hayward (1949) and Brown (1953) have indicated that most, if not all, complement-fixing activity is due to a soluble antigen. If this is the case, it provides an explanation for the direct relationship between the concentration of live virus and of antigen. Although no inquiries concerned with a soluble antigen or antigens were undertaken in the present work, such studies are desirable because of the immunologic importance of such antigens (*cf.* Henle *et al.*, 1948) in diagnosis and epidemiology.

Of the several routes of inoculation, the yolk sac route appears to be the one of choice, not only from the standpoint of technical simplicity, but also because it yielded membranes with a high antigenic activity. Antigens prepared from membranes (pooled chorioallantoic and amniotic) derived from eggs so inoculated proved to be highly specific and adequately sensitive, as determined in tests with both guinea pig and human sera. Evidence for specificity lies in the finding that in no instance was complement-fixing antibody encountered in a serum in the absence of neutralizing antibody.

While there appeared, in general, to be some indication of a tendency of high complement-fixing titers of human sera to be associated with the higher neutralization indices, no absolute quantitative relationship, i.e., no linear relationship, was observed to exist between the antibody titers obtained by the 2 tests. Gajdusek *et al.* (1952) have also observed this same lack of quantitative agreement.

SUMMARY

A systematic study was made of the distribution of herpes simplex virus and its complement-fixing antigen in the embryonated hen's egg infected by different routes of inoculation. The

virus spreads throughout the embryonated egg regardless of the route of inoculation employed, but of the several routes tried, the yolk sac route yielded the highest concentration of virus and of antigen. Higher concentrations of both virus and antigen were found in the extra-embryonic membranes than in the extra-embryonic fluids. A direct relationship was found between infectivity and complement-fixing activity.

Suspensions of infected chorioallantoic and amniotic membranes were found to possess high concentrations of complement-fixing antigen, were free of nonspecific and anticomplementary reactions with guinea pig and human serums, and were simple to prepare. Preparation of the antigen is described.

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