A SOLUBLE ANTIGEN OF LYMPHOCYTIC CHORIOMENINGITIS

III. Independence of Anti-Soluble Substance Antibodies and Neutralizing Antibodies, and the Rôle of Soluble Antigen and Inactive Virus in Immunity to Infection

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Lymphocytic choriomeningitis has been shown to belong to the group of virus diseases which is characterized by the occurrence in infected tissues of a soluble antigen separable from the virus (1, 2). The presence of the specific soluble antigen of lymphocytic choriomeningitis was demonstrated in extracts of various organs of acutely ill guinea pigs, mice, and monkeys by means of complement fixation (2). In addition, certain characteristics of the antigen have been determined (3). Moreover, the soluble antigen has been found to be capable of absorbing complement-fixing antibodies from immune serum without appreciably affecting the neutralizing power of the serum (3), an observation which suggested that the anti-soluble substance antibodies are independent of the neutralizing antibodies. The present report deals with evidence of another type which indicates that the two antibodies are separate entities.

In a previous publication (3) the use of the terms anti-soluble substance antibody and complement-fixing antibody as synonymous expressions appeared to be justified by the evidence mentioned and by the fact that practically all of the fixing power of a crude suspension of infected tissue was demonstrable in the non-infectious portion containing the soluble antigen rather than in the preparation of washed virus sedimented from the crude material (2). Although a number of reports have dealt with complement fixation in lymphocytic choriomeningitis, most of them have not distinguished between the rôle of the soluble antigen and that of the infective agent. Since we wish at this time to discuss the antibodies which appear in man and in a number of animal species following infection with the virus of lymphocytic choriomeningitis, a summary of previous findings seems called for.

Armstrong and Lillie (4) in 1934 described a virus which they encountered during passage through monkeys of an agent considered to be the virus of St. Louis encephalitis; this new virus was named lymphocytic choriomeningitis. At that time they reported the occurrence of neutralizing antibodies in the serum of convalescent monkeys. Shortly afterward Armstrong and Wooley (5) detected neutralizing antibodies in the serum of 3 human beings, and Rivers and Scott (6, 7) demonstrated the development of neutralizing substances in the first human beings from whom the virus was isolated directly. The latter workers also reported the occurrence of this type of antibody in convalescent monkeys. The appearance of protective substances in the serum of patients during convalescence was immediately confirmed by Armstrong and Dickens (8) and has been repeatedly shown since that time. Moreover, Lépine, Mollaret, and Kreis (9) demonstrated the development of these antibodies in human beings inoculated with a murine strain of virus for therapeutic purposes. Neutralizing substances appeared both in the patients who developed meningitis and in those who had only an influenza-like disease without later signs of meningeal irritation. Protective substances have also been found in about 12 per cent of the sera from 997 individuals from various parts of the United States without history of involvement of the central nervous system (10) and in 18 per cent of the sera obtained from a widely scattered group of 680 persons in the United States and Hawaii, a number of whom had previously suffered from disease of the central nervous system (11).

Neutralizing antibodies have been detected in several other species of animals following inoculation of the virus of lymphocytic choriomeningitis. Rivers and Scott (6 *a*) and Traub (12) reported the presence of protective substances in the serum of guinea pigs after recovery from the induced disease. Certain species which fail to show clinical evidence of disease after inoculation with the virus do nevertheless develop neutralizing antibodies. For example, rabbits (4, 6, 13, 14), dogs (15–17), and ferrets and pigs (16) are refractory to infection, yet each of these species has been found to possess protective substances subsequent to inoculation (13 *b*, 14–17). Mice appear to be an exception to the rule, since Traub (12) and Traub and Shäfer (18) have observed that these rodents do not possess demonstrable neutralizing antibodies in their serum even when they are immune to reinfection.

Howitt (19) was the first to report complement fixation in the presence of mixtures of hyperimmune serum from guinea pigs or mice and extracts of infected guinea pig tissue. Lépine and Sautter (20) obtained complement fixation with serum from an individual who accidentally contracted the disease in the laboratory. Shortly afterward, Lépine and his associates (21) reported positive reactions with sera from the previously mentioned group of inoculated human beings (9) and from other patients during convalescence as well as with serum from inoculated monkeys and rabbits; however, they did not detect complement-fixing antibodies in the serum of infected guinea pigs. Lépine and his coworkers, like Howitt, used a crude extract of infected tissue as antigen. About this time experiments reported by us (1) showed that anti-soluble substance antibodies develop after infection in man and guinea pigs and are responsible for complement fixation. Subsequently, work elsewhere and in our laboratory confirmed Howitt's original report that serum from hyperimmune mice fixes complement with preparations from infected tissue; crude tissue extracts (18, 22) and soluble antigen (23) were employed.

The present report describes results of investigations on the time of development of anti-soluble substance antibodies and of neutralizing sub-

stances in man and several species of lower animals. In addition, experiments concerning the immunization of guinea pigs with non-infectious material are recorded.

Materials and Methods

Antisera.—Simple immune sera were obtained from animals under light ether anesthesia by intracardiac puncture at various intervals of time after inoculation with a mild strain of the virus of lymphocytic choriomeningitis.

Soluble Antigen.—Solutions of antigen were prepared according to the method described in a previous report (2). A 10 per cent suspension of infected guinea pig spleen was made in physiological saline solution containing 2 per cent inactivated normal guinea pig serum. After the material was freed of coarse particles by means of a horizontal centrifuge, it was cleared of most of the virus by a run in a concentration centrifuge (24) at 30,000 R.P.M. for 20 minutes; it was then filtered through a Seitz pad which reduced the infectivity to a few units per cc. In order to maintain bacteriological sterility, merthiolate was added to a concentration of 1:10,000. Extracts of infected mouse spleen prepared by a similar method were employed in a few instances.

Complement-Fixation Test.—The technique of the complement-fixation test used for the detection of anti-soluble substance antibodies was the same as has been previously described (2). Its salient features are the following: 0.2 cc. each of varying dilutions of inactivated serum were mixed with an equal amount of appropriately diluted solution of antigen; 2 units and in some instances 5 units of antigen which had previously been standardized with hyperimmune guinea pig serum were employed. Following the addition of 2 units of complement, the mixtures were allowed to stand overnight at 3°C. The next morning 0.5 cc. of a 5 per cent suspension of washed sheep erythrocytes and 0.2 cc. of diluted anti-sheep cell amboceptor (2 units of hemolysin) were added to each tube. The degree of hemolysis was finally estimated after the tubes had been left in a water bath at 37°C. for 30 minutes. Suitable anticomplementary and hemolytic controls for each material to be tested were always included. The titer of the serum was regarded as the highest dilution which fixed complement completely in the presence of soluble antigen. A positive guinea pig serum of known titer was run as a control for each group of tests.

Neutralization Test.—The test for the determination of the presence of neutralizing antibodies in serum was performed according to the technique devised by Muench and Scott[1] and used in this laboratory (25) for several years. Briefly it consisted of the subcutaneous inoculation of 4 young normal guinea pigs, weighing 300 to 350 gm. each, with 0.5 cc. of a mixture of equal parts of the serum to be tested and of a 10^{-3} dilution of fresh brain from guinea pigs infected with the W. E. strain of virus. The mixture was incubated in a water bath at 37°C. for 1 hour immediately before injection. Adequate positive and negative controls were included. Daily observations as well as the recording of temperatures were made for 18 days. The test was considered positive if at least 3 guinea pigs survived, negative if 3 succumbed, and equivocal if 2 died; as a rule, all animals in a group responded in a similar manner.

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[1] Unpublished data.

EXPERIMENTAL

Development of Anti-Soluble Substance Antibodies and Neutralizing Antibodies in Man after Infection with the Virus

At the beginning of the present work there existed in our laboratory a number of specimens of serum collected from positive cases of lymphocytic choriomeningitis (7, 25), as well as numerous samples which were considered to be from negative cases inasmuch as they did not neutralize the virus. We were interested in determining how many of these sera were capable of binding complement in the presence of a solution of antigen; accordingly, all available specimens were tested. Although many were found to be anticomplementary after their long period of storage, specimens of serum from 4 of the patients with lymphocytic choriomeningitis were shown to contain anti-soluble substance antibodies. During the past $2\frac{1}{2}$ years serological studies have been performed on material from 12 additional cases of lymphocytic choriomeningitis and from 55 patients who had aseptic meningitis of unknown etiology.[2] None of the latter group developed anti-soluble substance antibodies; moreover, convalescent serum from 26 of these individuals did not contain protective substances.

Results of complement-fixation and neutralization tests on serum from 16 human beings who suffered from lymphocytic choriomeningitis are summarized in Table I. Serum taken 1 to 5 weeks after onset of meningitis from 11 patients gave a positive complement-fixation reaction. Only two (R. R. and J. V.) of these specimens, however, possessed definite neutralizing ability at this time. Serum obtained at a later date from 4 additional patients also fixed complement; thus, 15 of the 16 persons had demonstrable anti-soluble substance antibodies. A second specimen was not available from 1 of the group, but the remaining 15 individuals developed neutralizing antibodies following recovery from the disease. It appears that anti-soluble substance antibodies generally develop several weeks earlier than neutralizing antibodies, that they are still detectable when neutralizing substances have developed, and that they disappear while neutralizing antibodies are still present; the latter, which usually appear 6 to 8 weeks after onset of illness, may persist for at least 5 years after a patient's recovery from the disease.

An exception to the rule is illustrated in Table I by the case of D. S.; although he was proved to have lymphocytic choriomeningitis by neutralization studies, complement-fixing antibodies were not detected in any of the samples of his serum, *i.e.*, those collected at 2, 6, and 12 weeks after onset.

[2] Specimens of serum from these 67 individuals were sent to us by physicians practising in various parts of the United States and Canada.

This individual is the only one we have encountered in whom a discrepancy occurred in the diagnostic data obtained by the complement-fixation and neutralization techniques. Whether D. S. ever possessed complement-fixing antibodies or whether they were present in small amounts for a brief interval of time when blood was not taken is unknown. Complement-fixing antibodies may decline rapidly as illustrated in the case of S. M.;

TABLE I

Development of Anti-Soluble Substance Antibodies and Neutralizing Substances in Serum of Patients with Lymphocytic Choriomeningitis

Patient	Onset of meningitis	Early serum			Convalescent serum			Late serum		
		C-F*	N†	Time after onset	C-F	N	Time after onset	C-F	N	Time after onset
				wks.			*mos.*			*yrs.*
W. E. (7)	Dec., 1934	+	−	3	+	+	4	−	+	5
R. E. S. (7)	" "	+	−	3	+	+	3	−	+	4
B. S. (25)	Aug., 1936	No specimen			+	+	2	−	+	2½
R. R. (25)	Mar., 1937	+	+	3	+	+	2	−	+	1½
M. S.	Oct. "	−	−	½	+	+	2			
M. W.	Dec. "	+	−	5	No specimen			−	+	1
A. S.	Nov., 1938	¶	−	1	+	+	3			
R. Z.	May, 1939	No specimen			+	+	4			
E. P.	Oct. "	+	±	4	+	+	1			
W. D.	Nov. "	+	−	1	+	+	2			
W. P.	Dec. "	+	−	3½	No specimen					
D. S.	" "	−	−	2	−	+	3			
K. P.	" "	+	−	2	−	+	5½			
J. V.	Apr., 1940	+	+	5	+	+	1½			
J. P.	" "	+	−	3	+	+	2			
S. M.	May "	−	−	1	+	+	1½			
		+		3						

* C-F = complement-fixation test.

† N = neutralization test.

¶ Serum anticomplementary.

serum taken 3 weeks after onset of disease titered 1:8, whereas a specimen drawn 4 days later titered 1:4. Results with both sera were reproducible. In our experience, the complement-fixing titer in man has never gone above 1:32 although higher titers do occasionally occur (21).

Development of Antibodies in Species Other than Man after Infection with the Virus

Guinea Pigs.—The development of complement-fixing and neutralizing antibodies was followed in groups of guinea pigs subsequent to infection with 2 strains of virus.

Experiment 1.—10 guinea pigs weighing 300 to 350 gm. each were bled by intracardiac puncture while under ether anesthesia. Their sera when tested individually contained neither anti-soluble substance antibodies nor neutralizing properties. Each animal was inoculated with subcutaneous and intraperitoneal injections of 0.5 cc. and 1.0 cc., respectively, of a 10 per cent suspension of fresh brain from guinea pigs infected with the W. W. S. strain of virus; this strain is of low virulence and seldom kills guinea pigs. 3 to 5 days after inoculation all of the pigs developed a slight amount of fever which persisted for several days, but none of them manifested other signs of disease. Animals were bled at intervals from 2 to 32 weeks after inoculation of virus and their sera were tested for the presence of anti-soluble substance antibodies and for the property of neutralizing

TABLE II

Development of Anti-Soluble Substance Antibodies and Neutralizing Substances in Guinea Pigs after Infection with the W. W. S. Strain of Virus

Guinea pig	Tests before infection		Tests after infection																					
			Interval after infection (weeks)																					
			2		4		5		6		8		10		13		20		27		32			
	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N		
1	−	−	1:32	−	1:64	−	1:64				1:64	+	1:64		D									
2	−	−	1:16	−	1:32	−	1:64	−	1:128	+	D													
3	−	−	1:64	−	1:128	−	1:512	−	1:128	±	1:64	+	1:128		1:128		1:64	+	1:64		D			
4	−	−	1:64	−	1:64	−	1:64	−			1:64	+	1:128		1:32		1:32	+	1:32		1:4	+		
5	−	−	1:16	−	1:32	−	1:64	−	1:128*	−	1:64	+	1:64	+	1:32		D							
6	−	−	1:32	−	1:64	−	1:128	+	1:256	+	1:64		1:128	+	1:128		1:32	+	1:64		1:8*	+		

D indicates death following cardiac puncture.

See text for details of experiment.

* Partial fixation of complement at this dilution.

virus. 4 of the 10 animals died during the first month of the experiment as a result of hemorrhage caused by cardiac puncture. Data obtained on these animals before death were in complete agreement with those on the other 6 animals; consequently these 4 pigs need no further discussion.

From the results of this experiment, shown in Table II, it is apparent that anti-soluble substance antibodies were present in the serum of guinea pigs 2 weeks after inoculation of the virus and that, in general, they reached their highest concentration 5 to 6 weeks after infection; subsequently they gradually declined to a low titer 32 weeks after infection. It is also evident that neutralizing antibodies did not appear until 5 to 8 weeks after inoculation and that these substances were still detectable at 32 weeks. A comparison of the time of appearance of the 2 types of antibody reveals that neutralizing substances began to appear during the period when the titers

of anti-soluble substance antibodies were greatest. The neutralizing power of serum bore no quantitative relationship, however, to the titer of anti-soluble substance antibodies. This is shown by the fact that toward the end of the experiment the serum still neutralized the virus even though complement-fixing antibodies had dropped to a titer as low as or even lower than that observed during the first weeks after infection.

Experiment 2.—A similar experiment was carried out with a group of 5 guinea pigs infected subcutaneously with 0.2 cc. of a 5 per cent suspension of the R. E. S. strain of virus. Although guinea pigs usually survive a subcutaneous inoculation of the R. E. S. strain, the resultant disease is more severe than that encountered in infection with the

TABLE III

Development of Anti-Soluble Substance Antibodies and Neutralizing Substances in Guinea Pigs after Infection with the R. E. S. Strain of Virus

Guinea pig	Tests before infection		Tests after infection															
			Interval after infection (weeks)															
			2		4		6		8		12		20		25		29	
	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N	C-F	N
11	—	—	—		1:64	—	1:128	+	1:16	+	1:128	+	1:64		1:32		1:32	+
12	—	—	1:2*		1:32	—	1:256	—	1:64*	+	1:64	+	1:128		1:128		1:64	+
13	—	—	1:2*		1:64	—	1:128	+	1:64*	+	1:128	+	1:128		1:64		1:32	+
14	—	—	—		1:32	—	1:16	—	1:16	—	1:4	+	D					
15	—	—	—		1:32	—	1:64	±	1:16	+	1:32	+	1:64		1:64		1:64	+

D indicates death following cardiac puncture.

See text for details of experiment.

* Partial fixation of complement at this dilution.

W. W. S. strain of virus. The animals developed fever which in most instances persisted for 2 weeks, during which time they lost weight; however, all of them recovered.

Data on guinea pigs infected with the R. E. S. strain of virus, summarized in Table III, show a slower development of both anti-soluble substance antibodies and neutralizing substances than was observed in Experiment 1. The former were either absent or present in very small amounts 2 weeks after infection, but were demonstrable in moderate amounts at 4 weeks, with a peak occurring at about 6 weeks. 8 weeks after inoculation neutralizing antibodies had appeared in the serum of 4 of the 5 animals and 4 weeks later were demonstrable in the serum of the remaining pig. The slightly retarded formation of antibodies may have been dependent upon the severity of the disease suffered by the animals. In general, the results obtained with serum collected from this group of guinea pigs confirm those of Experiment

1, *i.e.*, complement-fixing antibodies appeared before neutralizing substances and decreased significantly in amount while the latter were still present.

It should be pointed out at this time that not all strains of the virus of lymphocytic choriomeningitis induce an antibody response in guinea pigs similar to that described. Certain strains, *e.g.*, the highly virulent W. E. strain, invariably kill inoculated guinea pigs before demonstrable antibodies appear in their serum. This is true even when high dilutions of infectious material are inoculated; in such instances, either infection occurs followed by death or no disease ensues leaving the animals without antibodies or immunity.

Mice.—It was regularly demonstrated that serum obtained from mice after infection with the virus of lymphocytic choriomeningitis possessed anti-soluble substance antibodies. Such serum, however, did not contain demonstrable neutralizing antibodies.

In one typical experiment, each of 50 young Swiss mice was inoculated intraperitoneally with 0.5 cc. of a 10 per cent suspension of guinea pig brain infected with the W. E. strain of virus. At various intervals of time after infection, a few mice were anesthetized and then exsanguinated by intracardiac puncture. About half of the animals died 6 to 7 days after inoculation as a result of infection. A few of the survivors still appeared ill as late as the 17th day after inoculation, but neither whole blood nor suspensions of spleen taken at this time or 4 weeks later were infectious for normal mice. Extracts of spleens prepared from mice of these 2 groups, *i.e.*, at 17 days and 6 weeks, did not contain demonstrable soluble antigen, although such extracts regularly contain the antigen when prepared from ill mice sacrificed at 6 to 7 days (2).

Anti-soluble substance antibodies in pooled serum taken from groups of mice sacrificed at 8 days titered 1:8; at 10, 13, and 17 days, respectively, 1:64; at 6 weeks 1:32; at 10 weeks 1:32. Pooled normal mouse serum failed to fix complement with the antigen employed, *i.e.*, extracts of infected mouse spleen. Sera collected at 17 days, 6 weeks, and 10 weeks after infection were unable to neutralize the virus.

Monkeys.—During the course of the present work it was observed that monkeys also develop anti-soluble substance antibodies after infection. In 1938, Dr. Lowell T. Coggeshall of the International Health Division of The Rockefeller Foundation encountered an epizootic disease in his colony of monkeys (26), the infectious agent of which was shown by Dr. Thomas Francis, Jr., to be the virus of lymphocytic choriomeningitis (26). Dr. Coggeshall has allowed us to include in this report the results of complement-fixation tests on samples of serum collected from several animals before and after the epizootic. Observations on specimens from one animal are illustrative of the group.

8 samples of serum which were obtained from a monkey over a period of about 14 months were tested. Fortunately, there were 2 specimens from this animal which served

as suitable controls, inasmuch as they were obtained before the epizootic and contained no demonstrable complement-fixing antibodies. A mildly positive reaction (titer, 1:8) was obtained with the third specimen taken shortly after the onset of the disease, while serum taken 4 weeks later titered 1:512. 21, 37, and 42 weeks after the first positive specimen, the titer of the serum was 1:128 in each instance. At 44 weeks the monkey succumbed to tuberculosis; a neutralization test performed by Dr. Coggeshall on serum taken prior to death revealed that it possessed ability to protect against the W. E. strain of virus. Our neutralization test on this specimen confirmed his finding. It has been our experience that convalescent sera stored in a native state quickly lose their neutralizing power but if dried from the frozen state they remain potent for at least 4 years. Since the earlier specimens had been stored in a native state for several months, neutralization tests were not performed with them.

Rabbits.—Anti-soluble substance antibodies were shown to develop in rabbits after injections of virus-infected material, although these animals do not develop clinical signs of disease. Even nursing rabbits, in our experience, do not appear ill after intracerebral injection of active virus; such rabbits, however, are retarded in growth for several weeks when compared with uninoculated animals of the same litter and, in some instances, slight lesions are found in the meninges and choroid plexus.

2 adult rabbits whose sera did not contain complement-fixing antibodies were injected intraperitoneally with 2 cc. each of a 10 per cent suspension of infected guinea pig brain. The animals remained afebrile and in all respects were asymptomatic for the 18 day period of observation. At various intervals of time after receiving the virus, they were bled from the marginal vein in the ear for samples of serum. Before being used each serum was absorbed with packed, washed sheep erythrocytes in order to remove the sheep cell hemolysins which were present. Such absorption did not appreciably alter the complement-fixing titer of the serum.

Anti-soluble substance antibodies, which were present in small amounts 11 days after injection of virus (titer, 1:4 to 1:8), reached a peak about the 4th week (titer, 1:64 to 1:128), and had subsided considerably by the 6th week after injection (titer, 1:8 to 1:16). Sera collected at 4, 6, and 12 weeks after inoculation were tested for neutralizing antibodies; at 4 weeks one sample was negative, while the other was positive; at 6 and 12 weeks both were positive. A specimen of serum taken from a nursing rabbit 2 weeks after intracerebral inoculation of the W. E. strain of virus was found to fix complement in a dilution of 1:32.

Response of Normal Guinea Pigs to Injections of Soluble Antigen and Inactivated Virus

It became of interest to determine whether anti-soluble substance antibodies appeared only after infection with the virus or whether they could be elicited by injections of soluble antigen or by inactivated virus.

Attempts were made to immunize normal guinea pigs with preparations of complement-fixing antigen freed of active virus. For this purpose solu-

tions of antigen prepared according to routine could not be employed because of the presence in them of small amounts of active virus; 0.03 cc. of undiluted antigen inoculated intracerebrally into mice were often non-infectious, but 2 cc. given intraperitoneally to guinea pigs frequently resulted in death. The slight infectivity of the solutions of antigen could be eliminated by heating them at 56°C. for 30 minutes or by treatment with 1 per cent formaldehyde, procedures which did not reduce the complement-fixing power of the solution. Attempts at immunization were entirely unsuccessful when non-infectious preparations of soluble antigen were employed. Only one experiment need be cited.

Each of 6 guinea pigs was injected intraperitoneally with a total of 12 to 15 cc. of a formolized solution of antigen (C-F titer, 1:16) over a period of 4 to 5 weeks. Neither complement-fixing nor neutralizing antibodies were demonstrable 1 to 2 weeks following the last injection. Furthermore, all of the animals developed fever and succumbed when given a standard dose[3] of virus. The results of observations on these 6 animals are included in Table IV.

The immunizing properties of heat-inactivated suspensions of splenic virus which had been washed by differential sedimentation in the ultracentrifuge were next investigated. Suspensions of washed virus were highly virulent for mice and guinea pigs; 0.03 cc. of a 10^{-7} dilution were as a rule lethal for mice when given intracerebrally, and 0.15 cc. of a 10^{-8} dilution injected by the same route killed guinea pigs. 1 cc. doses of washed virus which had been inactivated by heating at 56°C. for 1 hour were injected intraperitoneally into each of 4 guinea pigs at weekly intervals for 6 weeks. None of the animals developed complement-fixing or neutralizing antibodies, and none of them survived a test dose[3] of active virus.

Traub (27) has reported the immunization of guinea pigs by the injection of formolized suspensions of tissues infected with the virus of lymphocytic choriomeningitis. Inasmuch as the method of inactivation of suspensions of pneumococci (28) has been shown to have a bearing on their effectiveness in inducing resistance in animals receiving them, an attempt was undertaken to immunize guinea pigs with suspensions of washed virus inactivated by formalin instead of heat.

Spleens which had been removed from 30 moribund guinea pigs inoculated 6 days previously with the W. E. strain of virus, and then stored in the frozen state at −10°C.

[3] A standard dose of virus consisted of 0.5 cc. of a mixture of equal parts of normal guinea pig serum and of a 10^{-3} dilution of guinea pig brain infected with the W. E. strain of virus. The mixture was inoculated subcutaneously immediately after being heated at 37°C. for 1 hour.

for 2 weeks, were thawed and treated in the routine manner for the preparation of soluble antigen and washed virus (2). The solution of antigen from these organs was formolized and used for the immunization of the normal guinea pigs mentioned earlier. The suspension of washed virus which was infective for mice in a dilution of 10^{-6} had a complement-fixing titer of 1:4. U.S.P. formaldehyde was added to the suspension to a concentration of 1 per cent (0.036 to 0.38 per cent formalin) by volume; after storage at 3°C. for 3 days, the pH of the suspension was adjusted to about 8 by the addition of dilute ammonia water. 2 days later the reaction of the material was found to be slightly acid and it was readjusted to about pH 7.2, where it remained throughout the experiment. This treatment did not alter the complement-fixing activity of the preparation; furthermore, administration of the material to guinea pigs caused no untoward effects.

Each of a number of guinea pigs was injected intraperitoneally with formolized material at weekly intervals; certain groups received 1, 2, and 3 injections of 1 cc. each, while a fourth group was given 4 injections of 1.5 cc. each. During the course of injections these animals and those receiving soluble antigen were housed in a room by themselves where strict isolation precautions were employed. The guinea pigs remained well; daily observations and temperature recordings showed no signs of disease. Serum taken from each animal 1 to 2 weeks after the final injection was titered for anti-soluble substance antibodies. Sera from the groups of guinea pigs which had been injected 3 and 4 times were also tested for the presence of neutralizing substances. Finally, the animals were tested for immunity to a known amount of virus;[3] the groups receiving 1, 2, and 3 immunizing injections were inoculated 1 week after the last dose, while the group receiving 4 injections was inoculated 2 weeks after the last injection. At this time the guinea pigs were moved from the isolation room to one which housed infected animals. A summary of the data obtained in this experiment is presented in Table IV.

From the results shown in Table IV, it is apparent that a single injection of a formolized non-infectious suspension of virus into normal guinea pigs neither stimulated the production of anti-soluble substance antibodies nor induced immunity. 2 injections of formolized virus were followed by the development of complement-fixing antibodies and by some resistance to infection. The administration of 3 doses of material induced about the same amount of anti-soluble substance antibodies as did 2 injections. An increased resistance to infection was noted in the guinea pigs receiving 3 injections of vaccine; all of the animals survived, but 2 of the 3 developed fever following inoculation of active virus. Neutralizing substances were not demonstrable in the serum of these guinea pigs. Finally, animals receiving 4 injections of formalin-treated virus suspension were solidly immune to infection, *i.e.*, they remained afebrile and survived when given a test dose[3] of virus. Moreover, half of the guinea pigs in this group had detectable neutralizing antibodies in their serum at the time when they were tested for immunity. It is interesting to note that the complement-fixing titers of the sera from these animals were lower on the average than those displayed by guinea pigs receiving either 2 or 3 injections. On the

other hand, the injection of each of 9 guinea pigs with 7 cc. of a formolized crude 10 per cent suspension of guinea pig spleen infected with one of several highly virulent strains of virus, including the W. E. strain, has not given results similar to those obtained with formolized washed virus. Partial

TABLE IV

Response of Normal Guinea Pigs to Injections of Preparations of Formolized Washed Virus and of Formolized Soluble Antigen

Material injected	No. of injections	Guinea pig	Results of tests at end of injections			
			C-F titer	Neutralization test	Immunity test	
					Fever	Outcome
Formolized washed virus	1	1	Negative	Not done	+	D
		2	"	" "	+	D
		3	"	" "	+	D
	2	4	1:32	" "	+	D
		5	1:32	" "	+	S
		6	1:8	" "	+	S
	3	7	1:16	Negative	+	S
		8	1:16	"	+	S
		9	1:32	"	−	S
	4	10	1:8	"	−	S
		11	1:16	"	−	S
		12	1:8	"	−	S
		13	1:32	Positive	−	S
		14	1:32	"	−	S
		15	1:4	"	−	S
Formolized soluble antigen	4	16	Negative	Negative	+	D
		17	"	"	+	D
		18	"	"	+	D
	5	19	"	"	+	D
		20	"	"	+	D
		21	"	"	+	D

Serum obtained from each animal before injections was negative in C-F test.
See text for details of experiment.

immunity developed in a few of the guinea pigs but complement-fixing antibodies were not demonstrable.

Response of Hyperimmune Guinea Pigs to a Single Injection of Soluble Antigen

The preceding experiments might suggest that the so called soluble antigen behaves as a hapten rather than as a true antigen since splenic extracts

containing it failed to elicit antibodies. It seemed desirable to test in another way the antigenic properties of the complement-fixing substance by injecting preparations of it into guinea pigs which had recovered from a previous infection. Thus, an anamnestic reaction might increase the antibody response to soluble antigen. This was attempted in the following manner.

10 guinea pigs which had been hyperimmunized with active virus several months previously were found to possess complement-fixing antibodies in low titer, ranging from 1:8 to 1:32. Each of 5 of them was injected intraperitoneally with 3 cc. of a

TABLE V

Effect of Formolized Soluble Antigen and of Formolized Washed Virus on the Complement-Fixing Titer of Serum from Hyperimmune Guinea Pigs

Material injected	Guinea pig	C-F titer	
		2 days before injection	2 wks. after injection
Formolized soluble antigen	1	1:8	1:64
	2	1:8	1:256
	3	1:16	1:256
	4	1:16	1:512
	5	1:32	1:512
Formolized washed virus	6	1:8	1:32
	7	1:8	1:32
	8	1:16	1:64
	9	1:16	1:128
	10	1:32	1:64

None of the animals developed fever after injection of either material.
See text for details of experiment.

formolized solution of antigen. Each of the other 5, chosen to correspond with the first group in regard to the complement-fixing titer of their serum, was given 3 cc. of formolized virus suspension. Both soluble antigen and virus were obtained from the same lot of spleens. Sera collected 2 weeks after the single injection were tested for complement-fixing activity.

The results of the preceding experiment, summarized in Table V, show that in the guinea pigs which received the preparation of formolized soluble antigen, the titer of complement-fixing antibodies increased markedly.[4]

[4] The injection of resting hyperimmune guinea pigs with a preparation of formolized soluble antigen has provided the simplest means so far available for regularly obtaining C-F serum of high titer. In this connection, it might be mentioned that reinjection of hyperimmune guinea pigs with repeated doses of several cc. of a 10 per cent crude suspension of guinea pig brain, rich in active virus, has provided a successful but less reliable means of obtaining potent C-F serum.

Moreover, animals which were given the suspension of formolized washed virus showed a considerable rise in titer of anti-soluble substance antibodies, but the increase was not so great as in the former group.

DISCUSSION

The present observations indicate that in lymphocytic choriomeningitis the anti-soluble substance antibodies and neutralizing antibodies are entirely separate. This was clearly shown by data obtained on the difference in the time of appearance of the 2 types of antibody in human beings and in guinea pigs. The anti-soluble substance antibodies appeared several weeks before neutralizing substances were detectable and diminished in a matter of months, whereas the neutralizing antibodies persisted for a much longer period of time. The fact that mice developed anti-soluble substance antibodies but no demonstrable neutralizing substances adds weight to the concept that the 2 antibodies are distinct. Additional evidence for the identity of each type of antibody is drawn from the results of absorption experiments already reported (3) in which anti-soluble substance antibodies were removed from immune guinea pig serum by specific soluble antigen contained in partially purified extracts of infected spleen. Such absorbed sera were still capable of neutralizing the virus, but did not fix complement or precipitate in the presence of solutions of antigen.

Howitt's data (19) on the time of appearance of complement-fixing and neutralizing antibodies in guinea pigs differ from ours. A possible explanation of her failure to demonstrate complement fixation with certain sera may be due to her use of preparations of infected brain as antigen. Frequent attempts in this laboratory (2) to secure consistently positive results with such preparations have been unsuccessful. On the other hand, we have rarely encountered negative results when a solution of splenic antigen was used in the complement-fixation test with serum from guinea pigs collected at least 3 weeks after infection with the virus. This is in contrast to the inability of Lépine, Mollaret, and Sautter (21) to demonstrate the presence of complement-fixing antibodies in the serum of 12 guinea pigs after infection with the virus. These authors do not state, however, the time at which serum for testing was taken from guinea pigs or the severity of the induced disease; both of these factors are important. Data bearing on these points were given in the protocols of Experiments 1 and 2. Furthermore, the severity of the disease initiated by the W. E. and 2 other strains of virus maintained in our laboratory is such that complement-fixing antibodies have never been demonstrated in guinea pigs infected with them, because death invariably occurs before antibodies become detectable.

When the results obtained with sera from animals, *i.e.*, mice, rabbits, and particularly guinea pigs and monkeys, are compared with data on sera from human beings it is evident that the complement-fixing titers of positive sera from human beings are rather low, since they range from 1:4 to 1:32. No explanation of this difference is at hand, but this finding agrees in a general way with the observation of Lépine, Mollaret, and Sautter (21) who found that undiluted human serum taken some time after inoculation of active virus usually fixed 25 to 30 units of complement. Only rarely did they obtain a high titer, the maximum being fixation of 120 units with serum from a patient who had been reinoculated with the virus. The low titer of complement-fixing antibodies in serum from human beings with lymphocytic choriomeningitis and the fact that complement-fixing antibodies disappear comparatively early, are in direct contrast to results of tests performed on serum from patients with psittacosis by Meyer and Eddie (29). These workers showed that the titer of such serum was as a rule considerably higher than we have found in lymphocytic choriomeningitis, and that complement-fixing antibodies endured in a number of instances for at least several years. The existence of a virus-carrier state has been suggested to account for the persistence of complement-fixing antibodies in patients after recovery from psittacosis (29).

Results of the present experiments indicate that the production of anti-soluble substance antibodies of lymphocytic choriomeningitis can be elicited in guinea pigs by the administration of non-infectious material. Although preparations of heated or formolized soluble antigen did not induce antibodies in normal guinea pigs, the latter material stimulated a marked response in immune animals which had small amounts of complement-fixing substance in their sera. Furthermore, suspensions of washed formolized virus were capable of inducing in normal guinea pigs not only complement-fixing antibodies, but also, under proper conditions, neutralizing substances and immunity to infection. Our observations on neutralizing substances and immunity confirm and extend Traub's (27) experiments in which he obtained resistance to infection following injections of formolized crude tissue extracts made from infected organs. A comparison of his data with ours suggests that formolized washed virus is a more efficient immunizing agent than is formolized crude tissue suspension.

Traub (27) found that the addition of extracts of organs from a heterologous species to formolized vaccine prepared from guinea pig tissue interfered with the response of guinea pigs to inactivated virus of lymphocytic choriomeningitis. A similar finding has been reported by Laidlaw and Dunkin (30) in connection with experiments on distemper vaccines and by

Andrewes and Smith (31) in descriptions of their work on influenza. Nonspecific interference by material consisting of formolized homologous tissue in the present experiments might possibly be responsible for the slight amount of resistance induced by injections of formolized crude tissue suspension when compared with the definite immunity produced by formolized washed virus. In this connection Hartley's (32) observation may be mentioned, namely, that the addition of extracts of guinea pig muscle to flocculated mixtures of diphtheria toxin-antitoxin interfered to some extent with the antigenic response of guinea pigs receiving the material. One might be tempted to extend the idea of interference to account for the failure of normal guinea pigs to respond to the comparatively small amounts of soluble antigen, when contrasted to the amount of tissue protein, in formolized virus-free splenic extracts. The presence of even smaller amounts of the antigen in preparations of inactivated washed virus, as demonstrated by an *in vitro* technique, *viz.*, complement fixation, might nevertheless be sufficient to elicit complement-fixing antibodies in normal guinea pigs because of the presence of relatively small quantities of material antigenically unrelated to the virus, and because of the fact that most substances when adsorbed on the surface of particles develop enhanced antigenic properties. At least some foundation for such an idea may be found in the experiments with formolized crude suspensions of spleen in which no production of complement-fixing antibodies was detectable in pigs receiving such suspensions.

CONCLUSIONS

Anti-soluble substance antibodies and neutralizing substances, which develop following infection with the virus of lymphocytic choriomeningitis, appear to be separate entities. The times of appearance and regression of the two antibodies are different in both man and the guinea pig; the anti-soluble substance antibodies appear earlier and remain a shorter time. Moreover, mice develop them but no demonstrable neutralizing substances.

Injection of formalin-treated, virus-free extracts containing considerable amounts of soluble antigen fails to elicit anti-soluble substance antibodies and to induce immunity in normal guinea pigs; administration of such preparations to immune pigs, however, is followed by a marked increase in the titer of anti-soluble substance antibodies in their serum. On the other hand, suspensions of formolized washed virus are effective in normal guinea pigs in stimulating both anti-soluble substance antibodies and protective substances, and in inducing immunity to infection.

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