COMPLEMENT FIXATION WITH THE NEUROTROPIC VIRUSES

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A realization of the increasing importance of human infections with the neurotropic viruses has stimulated the interest of investigators toward the development of simple diagnostic procedures for the study of these maladies. The need for such procedures is evident when one appreciates that the central nervous system displays only a limited type of response to infection with the majority of these agents. Indeed, if the pathognomonic histological lesions of rabies and poliomyelitis are excluded, the clinical and pathological data alone are almost never adequate for establishing the type of neurotropic virus disease present in an individual patient and frequently are insufficient to designate the virus responsible for an epidemic (1). Leake (2) has suggested that this group of diseases be diagnosed as "infectious encephalitis" until the responsible virus has been identified in the course of laboratory studies.

Howett (3) demonstrated that the complement-fixation reaction could be employed with experimental materials from Eastern, Western, and St. Louis encephalitis and from lymphocytic choriomeningitis. Diagnostic methods suitable for detecting complement-fixing antibodies in the sera of patients convalescent from choriomeningitis were developed in this laboratory and elsewhere (4, 5). The results obtained by this technique were satisfactorily correlated with those obtained by the classical but laborious methods ordinarily employed in virus research, *i.e.*, isolation of virus and demonstration of neutralizing antibody. More recently Casals and Palacios (6) have found complement-fixing antibodies in the sera of human beings recovered from infection with the viruses of Eastern, Western, and St. Louis encephalitis and of louping ill.

Most of the so called neurotropic viruses possess some viscerotropic tendencies but the latter kind of tropism is generally insufficient in animals to provide highly infectious visceral tissues that can be used for the preparation of complement-fixing antigens. The notable exception is choriomeningitis, for the spleens of guinea pigs moribund with this infection contain relatively large amounts of the specific soluble antigen of the disease which can be used for diagnostic work (5, 7). Furthermore, the great susceptibility of chick embryo tissues to the viruses of Eastern and Western encephalitis has enabled several investigators to obtain from this source antigens which fixed complement in the presence of appropriate viral antibodies (8). It is still essential, however, that infected brain tissue be used as starting material for the preparation of complement-fixing antigens that will react with antibodies against certain of the neurotropic viruses.

Casals and Palacios (6) in their work clarified antigens which were prepared from

infected mouse brain by several methods, *i.e.*, by centrifugation at high speed in the ultracentrifuge, by filtration through a Seitz pad, and by centrifugation at 3500 R.P.M. in an angle-head machine. The combination of the first two methods provided antigens that were devoid of materials which gave non-specific effects. Because of its simplicity the authors recommended centrifugation at 3500 R.P.M. The non-specific effects ordinarily obtained in complement-fixation tests with such antigens were avoided when the antisera were inactivated at temperatures in excess of 56°C.

The present report describes a method for the preparation of complement-fixing antigens from the brains of animals infected with a number of the neurotropic viruses. Antigens prepared in this manner are non-infectious and can be used in the ordinary type of complement-fixation technique, for unlike earlier preparations, they do not react with normal sera inactivated in the usual manner. Additional information on the relationship of certain of the neurotropic viruses is presented at this time.

Materials and Methods

MATERIALS USED IN THE PREPARATION OF COMPLEMENT-FIXING ANTIGENS. —In general, infected brain tissue served as the source of complement-fixing antigen. The need for highly infectious tissue as starting material cannot be overemphasized, since it is a prerequisite for the preparation of satisfactory antigens. Because of the variability of the response of the same groups of susceptible hosts to the different neurotropic viruses, the means of obtaining tissue of maximal infectivity warrants a detailed discussion.

St. Louis Encephalitis Virus: Two strains of the virus of St. Louis encephalitis, *i.e.*, Peterson and Hubbard, were employed in the present work. Both strains[1] were isolated in 1937 (9) and were subsequently maintained by intracerebral passage in mice, and by the storage of infected brain suspensions that had been dried from the frozen state. The Peterson strain which had been through only 17 passages since 1937 was less well adapted to mice than the Hubbard strain which had been through 120 mouse passages; most of the work was done with the latter virus, the former being used only to confirm certain observations.

Suspensions of brain tissue from Swiss mice and from Syrian hamsters (10) infected with both strains of virus served as starting material for the preparation of complement-fixing antigens. The brains of mice inoculated with the Hubbard strain

[1] The Peterson strain of St. Louis encephalitis virus was received in 1937 in its 4th passage (9), while the Hubbard strain of this agent was obtained in its 110th passage in 1941, from Dr. E. H. Lennette, who also supplied us with the Nakayama strain of the Japanese encephalitis virus in the form of 29th mouse passage material and 6th passage hamster brain (11).

The viruses of Eastern and Western equine encephalomyelitis were received from Dr. I. M. Morgan. Dr. K. C. Smithburn gave us a mouse brain infected with the 139th passage of West Nile virus (12).

were generally infectious in dilutions of 10^{-7} or 10^{-8} when injected intracerebrally in mice; the Peterson strain never titered higher than 10^{-6}. As the first step in the preparation of antigen, Swiss mice were inoculated intracerebrally with a 10 per cent suspension of passage material of maximal infectivity. All mice were sacrificed when the first animal in the group developed convulsions, usually about 48 hours after inoculation. Brains were removed aseptically from the chloroformed mice and immediately frozen in a sterile tube which was packed in dry ice. Similar methods were used to obtain infected hamster brain except that these animals were inoculated with mouse passage virus and were sacrificed 48 hours later, before obvious signs of disease appeared. Brain tissue removed from hamsters 48 hours after the inoculation of Hubbard or Peterson virus regularly titered 10^{-6} but that taken on the 4th or 5th day when the animals were sick or moribund was less infectious.[2]

Japanese Encephalitis Virus: Brain tissue for the preparation of antigen was obtained from mice and from hamsters (11) which were sick on the 5th day following an intercerebral inoculation of 10^6 M.L.D. (minimal lethal doses) of virus. As in the case of St. Louis encephalitis virus, hamsters were infected with mouse passage material. Cerebral tissue from sick mice titered 10^{-7} to 10^{-8} while similar material from hamsters usually titered 10^{-6} to 10^{-7}. Most of the antigens used in complement-fixation studies were made from infected mouse brains which were frozen immediately after their removal.

Eastern and Western Encephalitis Virus: These two viruses were received as mouse-adapted strains and were maintained by intracerebral passage in mice. Mice inoculated intracerebrally with a 10 per cent suspension of infected brain tissue became moribund in 24 to 30 hours when given Eastern virus and in about 30 hours when infected with Western virus. As soon as the first animal in the group became moribund or died, all the mice in that lot were chloroformed and their brains removed and frozen for antigen. Such tissue infected with Western virus titered 10^{-8} when injected intracerebrally in mice, while that containing Eastern virus titered between 10^{-8} and 10^{-9}. Infected mouse brains supplied materials which fixed complement with Western and Eastern antisera.

Strains of both viruses were also carried serially for a number of passages in the brains of guinea pigs. Although death occurred in these animals almost as quickly as in the mice, the titer of the guinea pig brains never exceeded 10^{-5}. After several unsuccessful attempts to prepare satisfactory antigens from brain tissue of guinea pigs infected with Eastern and Western encephalitis virus this line of endeavor was abandoned.

During the course of the present studies Syrian hamsters were found to be highly

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[2] Both the Hubbard and Peterson strains of St. Louis encephalitis virus declined in virulence when maintained by continuous intracerebral passage from one moribund hamster to another; the former strain was lost after 10 passages in one series and after 12 passages in another, while the Peterson strain was lost after several transfers. An attempt to adapt the Hubbard strain to growth in the hamster testicle by serial intratesticular passage at 4 to 6 day intervals was only partially successful since an inapparent infection occurred and the agent was lost in the 8th or 9th passage.

susceptible to the viruses of Eastern and Western encephalitis. Hamsters inoculated intracerebrally with 10^7 M.L.D. of Eastern or of Western virus of either hamster or mouse passage strains became moribund in 36 to 48 hours; their brains were infectious in dilutions of 10^{-8}. Antigen for complement-fixation experiments was prepared from brain tissue of hamsters infected with both of the equine viruses.

West Nile Virus: This neurotropic virus, recently isolated by Smithburn and his coworkers, is pathogenic for mice and monkeys (12). The Syrian hamster was found in the present investigation to be as susceptible to the virus as is the mouse.[3] Material for use in the complement-fixation tests was prepared from the brains of mice and of hamsters which had been inoculated intracerebrally with 10^7 or 10^8 M.L.D. of virus, and which were sacrificed when moribund on the 4th or 5th day. Infected cerebral tissue from both species generally titered 10^{-8} when tested by the intracerbral route in mice.

Lymphocytic Choriomeningitis Virus: Soluble antigen of lymphocytic choriomeningitis (14) obtained from the spleens of moriburn guinea pigs infected with the WE strain of virus was employed in most of the present work. In a few instances, suspensions of splenic tissue containing both virus and soluble antigen were used (7). Antigens capable of fixing complement can be prepared from brain tissue infected with this virus (1, 6, 14), nevertheless, it has seemed to us (14), and it still appears, desirable to avoid the use of brain as the source of complement-fixing antigen for diagnostic purposes wherever possible.

Preparation of Complement-Fixing Antigen from Infected Brain Tissue: Centrifugation at relatively high speed was used in order to remove from the suspensions of brain tissue both the anticomplementary material and the substances which gave the non-specific reactions in the complement-fixation tests described by Casals and Palacios (6).

Infectious antigens which were capable of fixing complement specifically in the presence of appropriate antibody were prepared from brain tissue in the following manner. Frozen infected brains were removed from the dry ice storage box and thawed at room temperature. It should be mentioned that freezing and storage at -70°C. were employed only as a means of keeping unchanged the virus and antigen content of the tissues; it made no appreciable difference in the titer of the final antigen or in its anticomplementary action if unfrozen brains were used immediately or if frozen brains were stored for several hours or days at -70°C. Weighed amounts of brain tissue were ground in a mortar without abrasive and a 10 per cent suspension was made by adding a sufficient amount of diluent; this fluid consisted of physiological saline solution with 1 per cent inactivated normal serum from the same species of animal that supplied the brain. Large particles were removed from the suspension by centrifugation in the International machine at 1500 R.P.M. for 10 minutes. The opalescent horizontal supernatant fluid was next placed in capped lusteroid tubes with an internal diameter of 11 to 12 mm. and spun in the new centrifuge of Pickels (15) at 6500 R.P.M. for 20 minutes in the cold room. The clear pink-colored supernatant

[3] More recent investigations have shown that the hamster is almost as susceptible to the peripheral inoculation of Eastern, Western, and West Nile viruses as it is to the intracerebral injection of these agents (13).

fluid was pipetted away from the sedimented and floating materials and placed in fresh lusteroid tubes. These were now centrifuged at 12,400 R.P.M. for 1 hour in the cold room and again the clear fluid was separated from the slight amount of sedimented and floating materials. The final transparent, faintly hemoglobin-tinged solutions were rich in active virus and contained appreciable amounts of complement-fixing antigen. Such preparations will be designated "infectious antigens."

METHODS FOR RENDERING COMPLEMENT-FIXING ANTIGENS NON-INFECTIOUS.—The dangers inherent in the use of infectious material in a technique employing as many steps as does the complement-fixation test need not be emphasized. A number of methods were employed in attempts to render the virus non-infectious without seriously affecting the usefulness of the preparations for *in vitro* studies.

Inactivation of the infectious antigens by irradiation with ultraviolet light in the manner employed recently for another purpose (16) provided a method which was generally applicable to the problem at hand. A quartz-mercury resonance lamp which consisted of seven coiled spirals arranged in the shape of a hollow cylinder with an internal diameter of 7 cm. and an overall height of 15 cm. was used to provide the radiant energy (Fig. 1). The lamp operated at 30 milliamperes and 15,000 volts obtained by a transformer from 110 volt alternating current; it emitted 85 per cent of its radiation in the form of the line 2537 Å. 30 cc. of a clear pink solution of infectious antigen were placed in a quartz test tube having a length of 20 cm. and an internal diameter of 2.1 cm. This tube was then so suspended in the center of the lamp that at least one complete coil encircled the tube above the level of the solution of antigen and another encircled it below.

The rate of inactivation of each of the viruses under discussion was investigated by determining the infectivity of samples of irradiated material which were taken after successive 5 minute periods of exposure. All of the viruses studied, namely, St. Louis, Japanese, Eastern, and Western encephalitis, and choriomeningitis, behaved in essentially the same way. The infective titer dropped rapidly during the first 5 minutes of irradiation from 10^{-7} or 10^{-8} to 10^{-3} or 10^{-4}, and then it declined more slowly during subsequent intervals, until after 20 minutes of exposure the undiluted material was no longer infectious for mice. Following this series of observations all infectious antigens were irradiated for 25 minutes and then tested for active virus by injecting 0.03 cc. of undiluted material intracerebrally into groups of 3 or 6 Swiss mice. The antigens were regularly non-infectious when care was taken to avoid contamination of the upper parts of the tube which were not sufficiently exposed to ultraviolet light. Control antigens prepared from normal brain tissue were also irradiated for 25 minutes.

Inactivation of virus with moderate loss of specific complement-fixing antigen was obtained by heating preparations of Japanese virus at 56°C. for ½ hour. Solutions of infectious antigen became opalescent during heating but were again clarified by centrifugation at 12,400 R.P.M. for 15 minutes; the supernatant fluid contained appreciable amounts of complement-fixing antigen and was not anticomplementary. Heat inactivation of antigens containing St. Louis, Eastern, and Western encephalitis virus left no demonstrable complement-fixing substance in the final clarified solution. The soluble antigen of choriomeningitis is relatively heat-stable (17).

METHODS OF IMMUNIZING ANIMALS FOR THE PRODUCTION OF COMPLEMENT-FIXING ANTIBODIES.—Sera containing antibodies capable of fixing complement in the presence of antigens of the types described above were obtained from one or more species of animal susceptible to each of the viruses, *viz.*, Swiss mice, Syrian hamsters, guinea pigs, or monkeys. All animals except monkeys received infected tissue of the homologous species; monkeys were infected with virus-laden mouse tissue. The basic plan for immunizing animals called for the initial production of a severe clinical disease with ultimate recovery, or for the production of an inapparent infection by the introduction of a large amount of virus by a peripheral route. Some animals were bled at weekly intervals following the original inoculation in order to determine whether complement-fixing antibodies appeared in simple convalescent sera; these animals were subsequently hyperimmunized. It soon became evident that complement-fixing antibodies against certain of the viral antigens could only be induced, in the present experiments, by the repeated injection of active virus. The methods of immunization were sufficiently individualized with each virus to warrant the pre-

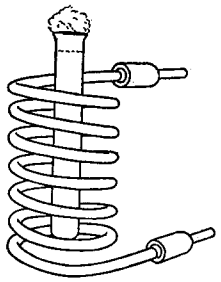

FIG. 1

sentation of a brief illustrative protocol of the procedures that proved successful for each agent. We were interested primarily in the production of complement-fixing antibodies and made no direct attempts to correlate these substances with neutralizing antibodies and with resistance.

St. Louis Encephalitis Virus: Hamsters were inoculated intraperitoneally with 1 cc. of a 10 per cent suspension of infected hamster brain, approximately 10^8 M.L.D. for mice. No obvious illness developed, but resistance was apparently induced since the intracerebral inoculation of 10 M.L.D. on the 10th day failed to produce sickness. Immunization was continued by the intraperitoneal injection of 0.6 to 1.0 cc. of a 10 per cent suspension of infectious hamster brain at intervals of 8 to 10 days. The serum obtained by sample bleedings, taken by cardiac puncture with the animals under ether anesthesia, contained demonstrable complement-fixing antibodies for the first time during the 4th or 5th week after the original inoculation of virus. Immunization with both the Peterson and Hubbard strains of virus gave similar results.

Japanese Encephalitis Virus: Hamsters were inoculated intraperitoneally with 1 cc. of a 10 per cent suspension of hamster brain that was infectious in a dilution of 10^{-7} when titered in mice. No clinical signs of disease appeared during the ensuing

weeks. Complement-fixing antibodies were present in the pooled serum of these hamsters on the 21st day following inoculation but had been absent in sera taken on the 7th and 14th days. Slightly higher antibody titers developed when 4 or 5 intraperitoneal injections of virus were given.

Specific complement-fixing antibodies were also produced in mice. Members of this species were inoculated intraperitoneally with a suspension of infectious mouse brain which was so diluted that it contained approximately 1000 M.L.D. of virus when tested intracerebrally in mice. Four weeks later 6 of the mice were chloroformed and exsanguinated by bleeding from the exposed heart; the pooled serum did not contain complement-fixing antibodies. Intraperitoneal injections of 0.2 to 0.4 cc. of a 10 per cent suspension of infectious mouse brain were given other members of the original group at intervals of about 3 weeks until 4 such inoculations had been made. A number of the mice were then tested for intracerebral resistance to varying amounts of active virus; all animals injected with 1000 M.L.D. of the agent survived. At the time of the immunity test the remaining mice in the original group were exsanguinated. Their pool serum contained complement-fixing antibodies.

Eastern and Western Encephalitis Virus: Antisera against both equine viruses were prepared in guinea pigs, hamsters, and monkeys; however, in none of these animals did complement-fixing antibodies develop until after a number of injections of active virus had been given. The methods of immunization with both agents were essentially identical, hence only the experiments with Western virus need be summarized. Guinea pigs received an initial subcutaneous inoculation of 0.2 cc. of a suspension of infected cavian brain which contained about 10^5 M.L.D. of virus for mice. Subsequent inoculations consisted of the intraperitoneal injection of 1 to 2 cc. of similar viral material given at intervals of 6 to 8 days. Complement-fixing antibodies appeared in 8 to 14 weeks.

Hamsters are highly susceptible to peripheral inoculation of Eastern and Western encephalitis viruses (13), therefore, the original inoculation which was given by the subcutaneous route contained infected hamster tissue diluted to contain only 10 M.L.D. of virus for mice. Subsequently intraperitoneal injections of increasing amounts of virus were administered at intervals of 8 to 10 days, *i.e.*, injections of 10^3, 10^6, and 10^8 M.L.D. of the agent. Complement-fixing antibodies were detected following 3 or 4 inoculations of the more infectious material.

Samples of sera from 2 monkeys, one immunized with Eastern virus and the other with Western virus, were given us by Dr. K. C. Smithburn. These animals were first inoculated by the subcutaneous route with a suspension of infected mouse brain diluted sufficiently to contain only about 1000 lethal doses of virus for mice. Subsequently at intervals of several weeks they received 3 intraperitoneal injections of 1 to 2 cc. of suspensions of mouse brain which titered 10^{-7}. Complement-fixing antibodies appeared following the second injection of Western virus and the third injection of Eastern virus.

West Nile Virus: Although hamsters are highly susceptible to peripheral inoculation of the West Nile virus (13), an occasional animal in titration experiments will survive the injection of an appreciable quantity of this agent, *i.e.*, 10 to 1000 times a lethal dose of the virus calculated on the results of intracerebral titrations in hamsters. Such surviving hamsters had not developed complement-fixing antibodies 2 weeks

following injection but they resisted the intracerebral inoculation of 10 or 100 lethal doses of virus. A group of these resistant animals was given 10^6 M.L.D. of active virus by the intraperitoneal route 2 weeks after the intracerebral test for immunity. Serum obtained the following week from members of the group contained complement-fixing antibodies.

Lymphocytic Choriomeningitis Virus: Antisera rich in antibodies against the soluble antigen of choriomeningitis were obtained from resting hyperimmune guinea pigs which had been stimulated by the intraperitoneal injection of formolized soluble antigen a week or 10 days before bleeding (5).

TECHNIQUE OF THE COMPLEMENT-FIXATION TEST.—Details of the complement-fixation test now employed in our laboratory for the study of virus diseases have been published recently (18). Sera to be tested are inactivated by heating in a 56°C. water bath for 30 minutes. Preliminary tests are run in order to determine the titer of the antibody or of the antigen in unknown materials. In subsequent work 2 or 4 units of antibody are used for the titration of antigen and usually 2 units, but sometimes 4 units, of antigen are employed for the titration of antibody. To appropriate mixtures of antigen and antibody are added 2 units of complement—guinea pig complement need not be titered in the presence of antigen—and the resultant solutions are incubated for 2½ hours in a water bath at 37°C. The hemolytic system, which consists of 0.5 cc. of a 2 per cent suspension of washed sheep erythrocytes and 2 units of hemolysin in 0.2 cc. of saline is then added and the final mixtures are incubated at 37°C. for ½ hour when readings are made. Titers are expressed as the highest dilution of antigen or of serum that gives complete or 3 plus fixation of complement; dilutions are calculated on the basis of the amount of serologically active substance in the 0.2 cc. volume of material employed in the test.

RESULTS

Antigens prepared in the manner described from brain tissue infected with the viruses of St. Louis, Japanese, Eastern, and Western encephalitis and with the West Nile agent, were tested for their capacity to fix complement in the presence of specific antibodies. Illustrative examples of the experimental results obtained with such antigens are presented in Table I.

It is apparent from the tabular data just given that fixation of complement occurred in the presence of appropriate materials from each virus infection. Although higher titers were obtained invariably with infective antigens, the reduction in titer of complement-fixing substances that accompanied inactivation of viruses by irradiation with ultraviolet light was rarely greater than a single dilution, *e.g.*, from 1:16 to 1:8 with the Japanese antigen shown in Table I. Inactivation of virus by heating at 56°C. resulted in a complete loss of complement-fixing antigen in all of the viral preparations except Japanese antigen, and even here the reduction of *in vitro* activity was greater than that which resulted from irradiation (Table I). Important data not included in the above table dealt with control tests. None of the antigens were anticomplementary

even in an undiluted state; moreover, none of them fix complement in the presence of inactivated serum (56°C. for ½ hour) from normal animals of the same species that supplied the antiserum or from several other species. Finally, none of the antisera fixed complement with antigens made from normal brain tissue by the procedure regularly used for the preparation of infective antigens.

TABLE I

Complement Fixation with Brain Antigens

Virus	Immune serum		Type of antigen		Treatment	Complement-fixation tests Dilution of antigen				
	Animal	Dilution	Source	Infective		1:1	1:2	1:4	1:8	1:16
St. Louis (Hubbard)	Hamster	1:4	Hamster	No	Ultraviolet light*	++++	++++	++++	++++	
St. Louis (Peterson)	Hamster	1:4	Hamster	Yes	None	++++	++++	+++	−	−
	"	"	Mouse 1	"	"	++++	++++	++	−	−
	"	"	" "	No	Ultraviolet light	++++	++	−	−	−
Japanese	Hamster	1:10	Mouse 2	Yes	None	++++	++++	++++	++++	+++
	"	"	" "	No	Ultraviolet light	++++	++++	++++	+++	−
	"	"	" "	"	Heated 56°C.	++++	++++	+	−	−
Western	Guinea pig	1:4	Hamster	No	Ultraviolet light	++++	++++	++++	++++	++++
	" "	"	Mouse	"	" "	++++	++++	++++	+++	−
Eastern	Guinea pig	1:4	Hamster	No	Ultraviolet light	++++	++	−	−	−
	" "	"	Mouse	Yes	None	++++	+++	++	−	−
West Nile	Hamster	1:4	Hamster	Yes	None	++++	++++	+++	++	−
	"	"	Mouse 3	"	"	++++	++++	++++	+++	−
	"	"	" "	No	Ultraviolet light	++++	++++	++	+	−

* See text for method of irradiation with ultraviolet light.

Plus signs indicate degrees of fixation.

Specificity of the Complement-Fixation Reaction with the Neurotropic Viruses

The specificity of the complement-fixation reaction obtained with a number of the neurotropic viruses and their antibodies has been studied (3, 5, 6). The subject was reinvestigated at this time for several reasons. In the first place, it was necessary to determine the specificity of the antigens prepared by the new technique. Furthermore, West Nile virus had not been employed previously in serological tests of this type and its relationship to the other neurotropic viruses warranted further study. It will be recalled that Smithburn (19) has recently shown, by means of neutralization tests, that the West Nile,

St. Louis, and Japanese viruses give certain cross reactions. Moreover, Levkovich (20) had previously demonstrated a relationship between the agents of Russian and Japanese encephalitis. We wondered whether the complement-fixation test would offer another means of establishing the presence of common antigenic substances in this group of neurotropic viruses. Experiments designed to determine the immunological cross reactions of our antigens and antisera were investigated in the following manner.

Irradiated antigens were prepared by the method described above from mouse brains infected with Japanese virus and from brain tissue of groups of hamsters in-

TABLE II

Specificity of the Complement-Fixation Reaction in Neurotropic Virus Diseases

Antisera	Non-infectious antigens						Normal antigens	
	LCM (1:2)	Japanese (1:1)	St. Louis (1:1)	W. Nile (1:2)	Western (1:4)	Eastern (1:1)	Hamster (1:1)	Mouse (1:1)
LCM	1:32	0	0	0	0	0	0	0
Japanese	0	1:32	0	0	0	0	0	0
St. Louis	0	0	1:8	0	0	0	0	0
West Nile	0	0	0	1:16	0	0	0	0
Western	0	0	0	0	1:16	1:2	0	0
Eastern	0	0	0	0	0	1:2	0	0
Normal hamster	0	0	0	0	0	0	0	0
Normal guinea pig	0	0	0	0	0	0	0	0

See text for the source and preparations of antigens and for the animal species that supplied antisera.

Numbers in parentheses which follow the abbreviated symbols for antigens represent the dilution of material used in the test.

Numbers in the columns represent the complement-fixing titer of that serum with the antigen designated above. Zero indicates no fixation with a 1:1 dilution of Eastern and of normal sera and a 1:2 dilution of the other sera.

fected with the West Nile, St. Louis (Peterson), Eastern, and Western agents. In addition similar preparations of irradiated material were made from normal brain tissue of mice and hamsters. Finally, a suspension of guinea pig spleens which contained both virus and soluble antigen of choriomeningitis was rendered non-infectious by irradiation. Serial dilutions of the preparations of antigen were titered in complement-fixation tests with their corresponding antisera which were diluted in such a manner that approximately 2 units of the antibody were present in each test tube. Guinea pigs supplied antisera against the Eastern, Western, and choriomeningtis viruses, the remaining antisera were obtained from hamsters. On the basis of the results of these preliminary tests the tissue suspensions were diluted so that two units of antigen were contained in 0.2 cc. volumes. Representative antisera which were known to react with their homologous antigens were then tested with homologous and heterologous antigens in the same experiment. Control tests consisted of titra-

tions of sera from normal hamsters and guinea pigs with the various viral antigens, and the titration of each antiserum with antigens prepared from brain tissue of normal mice and hamsters were also performed in this experiment. The results obtained in such a series of tests are summarized in Table II.

TABLE III

Cross Fixation in Eastern and Western Encephalitis

Experiment No.	Antiserum (guinea pig, dilution 1:4)	Antigen	Complement-fixation tests Dilutions of antigen				
			1:1	1:2	1:4	1:8	1:16
1	Eastern	Eastern (hamster)	++++	++	−	−	−
		" (mouse)	++++	+++	++	−	−
		Western "	++	+	−	−	−
	Western	Eastern (hamster)	++++	++++	++++	+++	+
		" (mouse)	++++	++++	++++	++	+
		Western "	++++	++++	++++	++	−
	Normal	Eastern (hamster)	−	−	−	−	−
		" (mouse)	−	−	−	−	−
		Western "	−	−	−	−	−
	(Monkey)	(Hamster, dilution 1:6)	(Dilutions of serum)				
			1:1	1:2	1:4	1:8	
2	Eastern	Eastern		++++	++++	−	
		Western		++++	++	−	
	Normal	Eastern		−	−	−	
		Western		−	−	−	

Eastern, Western, and normal sera used in the first experiment summarized above were mixed with 1000 M.L.D. of Eastern virus and injected intracerebrally into mice. Eastern antiserum protected all inoculated mice, Western and normal did not.

Eastern antiserum used in the second experiment was found by Dr. K. C. Smithburn to inactivate 10^5 M.L.D. (mouse intracerebral test) of Eastern virus but less than 10 M.L.D. of Western virus.

The results summarized in Table II clearly indicate that cross reactions were absent in complement-fixation tests carried out with materials from infections caused by the West Nile, St. Louis, and Japanese viruses. The reactions were specific for these diseases as well as for choriomeningitis. As might be expected, the Hubbard and Peterson strains of the virus of St. Louis encephalitis were undistinguishable by the complement-fixation technique.

Antigens prepared from the viruses of Eastern and Western encephalitis failed to react with antisera against the other neurotropic agents under inves-

tigation; however, Western antiserum did fix complement to some extent with Eastern antigen (Table II). Additional experiments adequately demonstrated that at least one antigenic structure is common to both equine viruses. Indeed, the results summarized in Table III reveal such extensive cross reactions between these two viruses, which were previously regarded as immunologically distinct (21), that one suspected an accidental contamination had occurred. The Eastern and Western antisera and the strains of virus employed in these experiments were immediately reidentified by means of neutralization tests and results of the classical type were obtained (21), namely, neutralization of the homologous but not of the heterologous virus.

It should be emphasized that the data presented in Table III were derived from experiments that were designed to elicit evidence of cross reactions with the equine viruses. A specific type of complement-fixing reaction was obtained under proper conditions with materials from each disease. Antisera usually reacted to higher titer with homologous antigen; therefore, a dilution of serum was generally found which fixed complement only with the homologous antigen. A phenomenon related to this dilution effect was encountered in sera of hyperimmune animals which were resting after a series of inoculations. In such animals the titer of complement-fixing antibodies decreased progressively and usually dropped to a value of less than 1:2 in a period of 1 to 2 months. At some stage during the decline the serum contained homologous but not heterologous antibodies. Specific reactions were obtained in still another way. It was noted in several instances that the serological substance present in the antigens which reacted with heterologous antiserum disappeared more rapidly during storage at 5°C. than did the material which fixed complement with homologous antiserum. As a result a point was reached at which the antigen behaved as a serologically specific substance.

Observations on Retention of Serological Activity of Materials under Different Conditions of Storage

The diagnosis of human infections caused by known viral agents has become sufficiently standardized so that it presents few difficulties except those of labor and expense to laboratories properly equipped for the work. Nevertheless, the usual diagnostic procedures, *i.e.*, isolation of virus and demonstration of neutralizing antibodies, have not come into wide, general use because workers in comparatively few places are willing to undertake the task. For similar reasons complement-fixation tests of the type described here and elsewhere (6) will be of limited use to the clinician unless they can be performed by general laboratories. At the present time the preparation of antigens and of positive antisera for control tests is too specialized a procedure for the ordinary serological laboratory. The current limitations on the application of these methods could be wiped out, however, if the necessary materials for diagnostic studies

were supplied to regional laboratories. Such a procedure is now feasible for work with choriomeningitis since both the non-infectious specific soluble antigen of this disease and its antibody are relatively stable under ordinary conditions; furthermore, they can be dried from the frozen state, stored, and shipped without deterioration (7). Attempts to apply similar methods in the preservation of antigens of the type described in the present paper were interrupted before the studies were completed.

Irradiated antigens prepared from infected brain tissue usually withstood storage poorly when left at ordinary ice box temperatures. In several weeks the antigens were often useless because of the loss of specific serological activity. In general, Japanese antigen was more stable under these conditions than the equine antigens and they in turn were more stable than preparations of St. Louis virus. The need for reassaying the potency of each stored antigen immeiately before each test run soon became apparent. It has been found, however, that antigens prepared from the viruses of St. Louis, Japanese, Eastern, and Western encephalitis and the West Nile virus can be preserved without change for a number of weeks when stored in the frozen state at $-65°$ to $-70°$C. It was also found desirable to store certain antisera in the CO_2 ice box; both mouse and hamster sera frequently became valueless because of the development of anticomplementary activity when stored in the liquid state at 5°C. for several weeks.

DISCUSSION

The property of brain antigens of fixing complement in the presence of normal serum, even though the antigens are not anticomplementary, has plagued workers in this field for some time (6). Kidd and Friedewald (22) have studied this general phenomenon and concluded that it depends on the presence of a "natural antibody reacting with a sedimentable constituent of normal tissue." The natural antibody of these workers was destroyed by heating at 60–65°C. and the reacting substance in the antigen was sedimented by relatively high speed centrifugation. Casals and Palacios (6) in order to obtain specific reactions with their brain antigens destroyed the substance in serum by inactivation at temperatures of 60–65°C. We have sought for and attained specificity in our tests by eliminating the offending substance from our antigens. The centrifugation procedure which accomplished this separation of unwanted material also provided antigens which were desirable in other respects. For example, the water-clear character of the solutions of antigen proved an especially useful property when irradiation experiments with ultraviolet light were undertaken.

The applicability of the complement-fixation technique for the diagnosis of infections with the neurotropic viruses was obviously restricted as long as highly infectious antigens were employed in the test. During the course of

this work, Casals also undertook the preparation of non-infectious brain antigens and described (23) results which corresponded in general to those reported in this paper.

Conclusions which may be drawn from the results of present studies regarding the immunological relationships between certain of the neurotropic viruses appear at variance with those of other workers. It should be noted, however, that the principal differences arise when the results obtained by the complement-fixation technique are compared with those from neutralization and cross-immunity tests. It is likely that the presence of common antigenic structures in groups of agents can at times be demonstrated more clearly by one technique than by another. Certainly there is no reason to doubt that the Japanese, St. Louis, and West Nile viruses belong in a related group (19) even though this was not shown in the current studies. Furthermore, although the Eastern and Western strains of equine encephalomyelitis are readily differentiated by the older techniques (21), under certain conditions they are indistinguishable by the complement-fixation technique. Howitt (3) also noted some crossing between the equine agents in certain of her complement-fixation studies, while Casals and Palacios did not (6). It is apparent from the present observations that the demonstration of either cross or specific reactions in fixation tests with the two strains depends on the type of experimental materials employed. Mention should be made of the interference type of phenomenon observed in infections with the Eastern and Western strains (24); Schlesinger and his coworkers have interpreted their experiments as indicating the induction of a non-specific resistence. The subject of the interrelationship of the neurotropic viruses obviously requires further study.

The present work was undertaken with the object of developing readily usable materials and methods for the diagnosis of human disease. The results reported at this time are entirely experimental in character; the applicability of the techniques to diagnostic studies is being determined.

SUMMARY

Antigens capable of fixing complement specifically with the appropriate antibodies have been prepared from brain tissue of hamsters and mice infected with the viruses of St. Louis, Japanese, Western, and Eastern encephalitis, and with the West Nile virus. The antigens were freed of the material which reacts with normal serum by means of centrifugation at relatively high speed. In addition, the infectivity of the preparation was destroyed by irradiation with ultraviolet light.

Cross reactions were demonstrated by means of the complement-fixation technique with materials from animals infected with the viruses of Eastern and Western equine encephalitis. No relationship was detectable by this procedure between St. Louis, Japanese, and West Nile viruses. These findings

emphasize the need for further investigation and correlation of the immunological reactions of the groups of neurotropic viruses, since the equine agents are apparently unrelated when studied by neutralization and cross-immunity tests while these methods provide evidence of the presence of common antigenic structures in the St. Louis, Japanese, and West Nile agents.

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