IMMUNITY TO YELLOW FEVER ENCEPHALITIS OF MONKEYS AND MICE IMMUNIZED BY NEURAL AND EXTRANEURAL ROUTES*

By JOHN P. FOX, M.D.

(From the Laboratory of the Yellow Fever Research Service, Rio de Janeiro, Brazil)

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The production of an effective active immunity to viruses which attack the central nervous system constitutes a problem which differs in important respects from that presented by other virus diseases. A developing systemic immunity, evidenced by the presence of considerable quantities of specific antibodies in the serum, may not prevent an established neural infection from continuing to a fatal evolution, while a pre-existing systemic immunity not only fails to confer uniform protection to more than small doses of neurally inoculated virus but also may fail at times to protect against virus inoculated by extraneural routes. Complete immunity, manifested by uniform resistance to neurotropic virus directly introduced into the nervous system, usually has been achieved only in those animals which have survived a previous neural infection. Indeed, in some cases such complete immunity may be demonstrated only when the challenge dose is placed in that part of the nervous system which actually was invaded during the previous infection. While a complete review of this problem is not contemplated, a number of observations in support of these generalities are cited in the following.

In the field of rabies, the development of absolute immunity to neural infection has not been reported. Immunization of mice by the intraperitoneal route with virus adapted to tissue culture does not enable the animals to resist more than 100 M.L.D. of virulent virus inoculated intracerebrally (1). Indeed, the measurement of the *relative* resistance of intraperitoneally immunized mice to intracerebral challenge has been made the basis for a recently devised technique for testing the potency of individual vaccine preparations (2). In dogs, studies of the effectiveness of various antirabic vaccines (3–5) have been made using an extraneural site, the masseter muscle, for challenge inocula, since direct neural inoculation was considered to constitute too severe a test. Even so, an appreciable number of animals in each vaccination group developed fatal infections.

With the viruses of equine encephalomyelitis similar observations have been made. Monkeys surviving an extraneural infection and shown to possess specific antibodies

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in their sera did not uniformly resist later cerebral infection (6), while other monkeys whose serological immunity had been stimulated by formalized vaccine were found in some instances to be still susceptible to virus administered by the nasal route (7). Also, neural infections in monkeys have been observed to progress to their usual fatal outcome in spite of the development of significant levels of serum antibody (6, 7). Studies in mice and rabbits (8, 9) have demonstrated particularly well the relative nature of the immunity produced by extraneural immunization, since a direct correlation was established between the degree of intracerebral resistance and the titer of the circulating antibodies. On the other hand, guinea pigs which had experienced a non-fatal neural infection following the inoculation of freshly isolated strains of Western equine encephalomyelitis virus were found completely immune to reinoculation (10).

Some of the most interesting observations have been made with poliomyelitis virus. Monkeys with relatively high levels of circulating antibody, acquired either by active intravenous immunization or by passive means, have been found to be susceptible nonetheless to intracerebrally injected virus (11). Even monkeys recovered from previous attacks are not wholly resistant to subsequent infection by the intracerebral or intranasal routes, although the greatest number of second infections have been produced with virus of heterologous strains (12, 13). An explanation for these experimentally induced reinfections in monkeys, and also for a number of well authenticated instances of second attacks in human beings has been indicated by recent studies on recovered monkeys (14). These studies have shown that resistance to reinfection is limited to those regions of the nervous system which actually were invaded by virus during the initial infection; parts of the nervous system not previously invaded remain susceptible to subsequent infection resulting either from direct neural inoculation of virus, or, when that portal had not been employed in initiating the prior attack, inoculation by the nasal route. Finally, observations made with the virus of mouse encephalomyelitis, which closely resembles that of poliomyelitis, have shown that cerebral infection with an avirulent strain produces a much more substantial immunity to virulent virus subsequently inoculated intracerebrally than does immunization by extraneural routes (15).

Although the virus of yellow fever typically does not produce a disease of the central nervous system in man, it has a well recognized neurotropic character which can be either augmented (16–18) or greatly reduced (19, 20) under proper conditions. Because of this neurotropism, it is possible that certain observations made with yellow fever virus may be of significance to workers engaged in studying the problem of immunization against other viruses which primarily attack the central nervous system.

The number of pertinent observations with yellow fever virus so far reported is small. The development of serum antibodies has been observed in monkeys which subsequently died of encephalitis following extraneural inoculation of virus of the highly neurotropic (but no longer viscerotropic) mouse-fixed variant of the French strain (21). Immune serum, inoculated intraperitoneally in quantities sufficient to

prevent the development of visceral yellow fever, did not prevent the evolution of a fatal encephalitis in monkeys infected by the cerebral route with virus of a pantropic strain (18). Even when potent immune serum was injected into the cisterna magna some hours prior to the intracerebral inoculation of French neurotropic virus, only a small number of monkeys were protected (22). Although active immunization with subcutaneously inoculated virus of the relatively avirulent 17D strain has been reported to render a high proportion of monkeys resistant to intracerebrally inoculated virus of the French neurotropic strain (20, 23), just the contrary is reported in the present paper. Finally, recent observations with mice have indicated that animals which survive an intracerebrally induced infection with yellow fever virus are very resistant to later inoculation by the same route of large doses of virus of a highly neurotropic strain (24).

In the present paper are reported further observations on the resistance of monkeys and mice, immunized by neural and by extraneural routes, to intracerebrally inoculated virus of the neurotropic variant of the French strain.

Materials and Methods

Yellow Fever Virus.—The strains of yellow fever virus employed were the following:

17D.—Now commonly used for human vaccination, this avirulent strain was evolved from the pantropic Asibi strain during a long series of passages in tissue culture (19, 20). Although producing a usually fatal encephalitis when inoculated intracerebrally into mice, it has lost almost completely both its neurotropic and viscerotropic virulence for monkeys. Even when inoculated intracerebrally in these animals, it produces an encephalitic process usually so benign that the only clinical manifestation is a febrile reaction (20, 23, 25). Although a number of substrains of 17D virus with relatively well defined individual characteristics are now recognized (24–27) and have been employed in the present work, only one, substrain 17DD low, is possessed of special attributes pertinent to the present study. This substrain is noteworthy for the frequency with which it produces non-fatal infections in mice (24). In general, the virus source consisted of vaccines prepared from infected chick embryos in the routine manner (28), although in one instance virus was derived from freshly suspended brains of infected mice.

Asibi.—This pantropic strain, the history of which has been well traced elsewhere (19), is highly virulent for monkeys, producing in these animals a usually fatal, visceral disease. Fresh or desiccated serum from infected monkeys, or *Stegomyia* mosquitoes infected by feeding on monkeys constituted the virus source.

Jungle Virus.—Several strains of unmodified virus, isolated from human cases of jungle yellow fever, have been used. Although less virulent for *rhesus* monkeys than the Asibi strain, the jungle strains often produce a fatal disease of the visceral type. The virus sources employed have been serum from the original human cases or from subsequently infected monkeys.

French Neurotropic.—This is the highly neurotropic, mouse-fixed variant of the French strain (16), here employed in from the 500th to the 600th serial passage in mice. Even when inoculated in minimal doses by the intracerebral route, this virus

produces a regularly fatal encephalitis in both mice and monkeys. Freshly taken brains from infected mice, triturated and suspended in physiological saline, have served as the virus source.

Eastern Equine Encephalomyelitis Virus.—One strain of this virus has been employed in verifying the specific nature of the resistance of animals to intracerebrally inoculated yellow fever virus. This strain was obtained originally by Dr. E. H. Lennette from the laboratory of Dr. P. K. Olitsky of The Rockefeller Institute and has been maintained since in serial passage in mice.

Monkeys.—The monkeys were all of the common *rhesus* variety (*Macaca mulatta*) and usually weighed from 2 to 3 kilos.

Those immunized by the intracerebral route had received inoculations into the left frontal lobe of 0.5 ml. of a 1:2 or 1:10 dilution of routinely prepared vaccine containing 17D virus.

Extraneurally immunized monkeys fell into three groups. The largest of these consisted of animals utilized in various experiments, which had received 17D virus in subcutaneous doses of varying size. Another group included monkeys which had survived subcutaneous inoculation with virus of one or another of several jungle strains. The third group was composed of monkeys which had survived infection with the usually lethal Asibi strain, induced either by the bite of infected mosquitoes or by the subcutaneous inoculation of infected monkey serum.

Mice.—All mice used were adults (over 35 days of age) of the Swiss strain, bred and raised in this laboratory. Those immunized intracerebrally represented animals which had survived infection with 17D virus, usually of substrain 17DD low. The intraperitoneally immunized mice were prepared as described in the text.

Virus Titrations.—Virus titers were calculated by the 50 per cent mortality or infectivity end-point method (29) and were based upon the intracerebral inoculation of 6 or 12 mice per serial fourfold or tenfold dilution of the preparation being titrated. As the diluent, 10 per cent normal human or monkey serum in saline was employed.

Determination of Protective Antibodies.—The presence of antibodies against yellow fever was determined either, as in the case of a number of monkey sera, by the intraperitoneal technique employing adult mice (30); or, as in the case of the remaining monkey sera, the mouse sera, and saline suspensions of mouse brains, by a more sensitive intraperitoneal technique employing young mice (26, 31). Titrations, in either case, were performed by testing serial fourfold dilutions in groups of 6 or 12 mice.

Observations in Monkeys

The following experiments were carried out with the purpose of comparing the resistance to intracerebrally administered French neurotropic virus of monkeys immunized by the intracerebral inoculation of 17D virus with that of monkeys immunized by the extraneural inoculation of 17D virus or by nonfatal systemic infections with the pantropic Asibi and jungle strains.

The Resistance of Monkeys to Intracerebral Doses of French Neurotropic Virus

Experiment 1.—This preliminary experiment comprised a total of 12 immune monkeys which were inoculated intracerebrally with graded doses (from 1.4×10^5

to 1.4×10^2 M.L.D. for mice) of French neurotropic virus. The animals, all of which had received their immunizing infection approximately 2 months previously, were divided into three equal groups representing monkeys immunized by the intracerebral inoculation of 17D virus (various substrains), by the subcutaneous inoculation of 17D virus, and by the subcutaneous inoculation of virus of jungle origin. Although not included in this experiment, normal monkeys are known to develop invariably fatal encephalitis following intracerebral challenge doses much smaller than those here employed. Immediately before inoculation of the challenge dose, each animal was bled to obtain serum for examination in the protection test. These sera were titrated in one or the other of two apparently comparable runs of the adult mouse test, using 6 mice per each fourfold dilution of serum.

The results are shown in Table I. Of the 4 intracerebrally immunized monkeys, 1 (No. 2) showed a possibly significant febrile reaction, and none developed any other signs of encephalitis. Of the animals immunized subcutaneously with 17D virus, *rhesus* 5, which had received the largest challenge dose, died on the 3rd day with tuberculosis; monkeys 6 and 7, which had received the next smaller doses, developed typical, fatal encephalitis; and only No. 8, which had received the smallest dose, completely resisted the challenge. In the final group, which had survived infection with unmodified jungle yellow fever virus, again only the monkey receiving the smallest challenge dose (No. 12) was completely resistant; monkeys 9 and 10 developed typical, fatal encephalitis, while No. 11 developed a permanent quadriplegia and was sacrificed after 30 days.

Table I also indicates the serum-antibody titers of these monkeys just prior to the challenge inoculation. Study of these titers makes it clear that the superior resistance of the intracerebrally immunized animals was not based upon a corresponding superiority in the pre-existing level of serological immunity. Monkeys 1 and 2, for example, whose serum-antibody titers were 14.5 and 7.5, resisted inocula of 1.4×10^5 and 1.4×10^4 M.L.D. respectively, whereas monkeys 9 and 10, whose serum titers (14.5 and 9) were nearly identical with those of Nos. 1 and 2, succumbed to exactly corresponding challenges.

On the other hand the results suggest that the level of serological immunity may have some relation to the resistance to encephalitis of animals immunized as the result of extraneural infection. Comparing only those animals which received equivalent challenge inocula, *i.e.*, No. 6 with No. 10 and No. 7 with No. 11, it is seen that the encephalitis in those with the higher antibody titers was of later onset and much longer duration.

Experiment 2.—In a second, larger experiment 14 intracerebrally immunized monkeys were challenged with graded intracerebral doses of French neurotropic virus ranging from 1.2×10^8 to 1.2×10^4 M.L.D. for mice. These animals had received immunizing inocula of 17D virus (substrain 17D-NY 104) 5 weeks (11 animals), 9 months (monkey 18), and 17 months (monkeys 21 and 24) previously. In addition, 4 monkeys, immunized 3 months previously as the result of non-fatal systemic infec-

TABLE I

Resistance of Subcutaneously and Intracerebrally Immunized Monkeys to Intracerebrally Inoculated French Neurotropic Virus Protocol of Experiment 1

Immunization		Monkey No.	Serum-antibody titer	Challenge inoculum (M.L.D. for mice)	Clinical observations* on day post-inoculation														Comment
Route	Virus				2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Intracerebral	17D	1	14.5	1.4×10^5															Normal at 30 days
		2	7.5	" $\times 10^4$					F	F	F	F							" " " "
		3	‡	" $\times 10^3$					F										" " " "
		4	8	" $\times 10^2$															" " " "
Subcutaneous	17D	5	4	1.4×10^5		D													Died of tuberculosis
		6	4	" $\times 10^4$		F	F	F	F, E	F, E	D								" " encephalitis
		7	8	" $\times 10^3$				F	F	F, E	F, E	D							" " "
		8	14	" $\times 10^2$															Normal at 30 days
	Jungle virus	9	14.5	1.4×10^5		F	F	F	F	F	F, E	D							Died of encephalitis
		10	9	" $\times 10^4$				F	F	E	E	E	E	E	D				" " "
		11	128	" $\times 10^3$						F	F	F, E	F, E	E	E	E	E	E	Quadriplegia, sacrificed at 30 days
		12	36	" $\times 10^2$															Normal at 30 days

* F = fever, D = dead, and E = signs of encephalitis.

‡ End-point not reached in lowest dilution tested.

tions with Asibi virus, were given challenge doses of from 1.2×10^6 to 1.2×10^3 M.L.D.; and 10 monkeys, inoculated subcutaneously with virus of substrain 17D-NY 104 5 weeks previously, were challenged with inocula containing from 1.2×10^6 to 1.2×10^2 M.L.D.

As in Experiment 1, pre-challenge sera from all of the animals were titrated for protective antibody. In this case, however, the protection test technique employing young mice was used, 12 being inoculated for each fourfold dilution of serum. It must be noted that, because the different technique resulted in a test of greater sensitivity, the titers presented for Experiment 2 are not to be compared with those presented for Experiment 1.

The results are presented in Table II. As in the previous experiment, all of the animals previously inoculated with 17D virus by the intracerebral route resisted the test inocula, while most of those which had been inoculated with the same virus by the subcutaneous route succumbed. On the other hand, the 4 animals immunized with the pantropic Asibi virus all resisted the challenge doses, in contrast to the results obtained in the preceding experiment with the animals immunized with pantropic virus of jungle origin.

Although, as has been reported elsewhere (25), the monkeys immunized by the inoculation of 17D virus intracerebrally manifested a higher average level of antibody in the preinoculation sera than did those immunized with the same virus by the subcutaneous route, it is again evident that the uniform resistance of the former group cannot be explained on a serological basis. At least 6 animals in the intracerebrally immunized group yielded serum-antibody titers no higher than those observed in some of the subcutaneously immunized animals which succumbed to the challenge dose. It is possible, however, that the relatively high levels of serum antibody in monkeys 37 and 38, immunized by the subcutaneous inoculation of 17D virus, and in monkeys 27 to 30, the Asibi immunes, may have played some rôle in their resistance.

Although it appears from the two preceding experiments that complete resistance to cerebral infection with neurotropic yellow fever virus results from a prior cerebral infection with 17D virus, it should be noted that the challenge inocula in both experiments were placed in the same site, the left frontal area, as the immunizing inocula. This is of significance since it has been shown that absolute resistance to second infections with the virus of poliomyelitis is observed only when the challenge inoculum is placed in that part of the central nervous system actually affected during the initial infection (14). A third experiment, therefore, was undertaken to study the resistance of intracerebrally immunized monkeys to neurotropic virus placed in a different part of the nervous system.

Experiment 3.—8 monkeys, which had been inoculated with 17D virus by the intracerebral route 9 months before, were given challenge inocula containing 3.3×10^8 M.L.D. of French neurotropic virus. In 4 animals the challenge dose, like the pre-

TABLE II

Resistance of Subcutaneously and Intracerebrally Immunized Monkeys to Intracerebrally Inoculated French Neurotropic Virus
Protocol of Experiment 2

Immunization		Monkey No.	Serum antibody titer	Challenge inoculum (M.L.D. for mice)	Clinical observations* on† day post-inoculation														Comment‡
Route	Virus				2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Intracerebral	17D	13	330	1.2×10^8															Normal at 30 days
		14	286	"															Quadriplegia when test dose given—unchanged
		15	98	1.2×10^7															Normal at 30 days
		16	177	"			F	F	F	F	F	F	F	F	F	F	F	F	Developed diarrhea, otherwise normal
		17	373	"															Normal at 30 days
		18	200	1.2×10^6															Normal at 30 days
		19	104	"															" " " "
		20	108	"															" " " "
		21	16	1.2×10^5															Normal at 30 days
		22	153	"															" " " "
		23	45	"															" " " "
		24	36	1.2×10^4															Normal at 30 days
		25	708	"															" " " "
		26	195	"				F						F			F		" " " "

	Asibi	27	165	1.2×10^6																Normal at 30 days
		28	>1024	1.2×10^5				F		F	F		F	F	F	F	F			" " " "
		29	400	1.2×10^4																" " " "
		30	474	1.2×10^3	F	F	F	F	F	F	F		F	F	F	F	F			Developed diarrhea, otherwise normal
Subcutaneous	17D	31	25	1.2×10^6		F	F	F	E	D										Died of encephalitis. Brain in mice showed 5/6 virus
		32	138	"			F	F	F	F, E	E	E	E	D						Died of encephalitis. Brain in mice showed 0/6 virus
		33	123	1.2×10^5			F	F	F	F, E	E	E	E	D						Died of encephalitis. Brain in mice showed 0/6 virus
		34	40	"		F	F	F	F	E	E	E	E	E	E	E	E	E	E	Died of encephalitis on 18th day. Brain in mice showed 0/6 virus
		35	36	1.2×10^4	F	F	F	F	F	E	D									Died of encephalitis. Brain in mice showed 6/6 virus
		36	16	"	F	F	F	F	E	D										Died of encephalitis. Brain in mice showed 6/6 virus
		37	128	1.2×10^3																Normal at 30 days
		38	165	"			F	F	F	F				F	F	F	F	F		" " " "
		39	118	1.2×10^2					F	F	F, E	E	E	D						Died of encephalitis. Brain in mice showed 5/5 virus
		40	25	"			F	F	F	F, E	E	E	E	D						Died of encephalitis. Brain in mice showed 5/5 virus

> = end-point not reached in highest dilution tested.

* F = fever, D = dead, and E = signs of encephalitis.

‡ Brains of monkeys dying were examined histologically and in every case were found to contain lesions of encephalitis. 10 per cent emulsions of brain were also inoculated into mice, intracerebrally. The mortality ratio (mice dying to mice tested) is shown in each case.

ceding immunizing inoculum, was placed in the left frontal lobe, while in the other 4 a site in the right frontal lobe was employed. All of the animals were carefully observed for a period of 30 days.

One monkey in each group developed fever (a rectal temperature over 40°C.) on the 4th day but in both cases this promptly subsided. No other indication of an encephalitic reaction was observed.

From this experiment it can be concluded that, in monkeys inoculated with 17D virus intracerebrally, the subsequent resistance of the central nervous system to infection with neurotropic virus is not limited to the area receiving the immunizing inoculum.

Resistance of Monkeys to the Virus of Eastern Equine Encephalomyelitis

That resistance to cerebral infection with a highly neurotropic virus may have a non-specific basis has been recently pointed out (9). To test this possibility in the present case the virus of Eastern equine encephalomyelitis was employed. Intracerebral challenge inocula of this virus containing from 1.7×10^6 to 1.7×10^4 M.L.D. for mice were administered to 3 of the monkeys whose survival of a maximum challenge dose of neurotropic yellow fever virus had been demonstrated 6 months previously in Experiment 3.

All three monkeys died within 3 to 4 days with typical signs of encephalitis. This result indicated the probably specific nature of their previously demonstrated resistance to French neurotropic virus.

Observations in Mice

The observations just reported have led to the conclusion that, in marked contrast to the behavior of monkeys immune to yellow fever as the result of systemic infections, monkeys whose immunity has resulted from actual cerebral infection manifest an absolute resistance to direct challenge of the nervous system with virus of a highly neurotropic strain. Furthermore, this resistance is probably to be explained on the basis of a mechanism of localized nature since it apparently is independent of the degree of pre-existing systemic immunity as measured in terms of serum-antibody titers.

It has recently been shown that mice which survive cerebral infection with yellow fever virus of several strains also are highly resistant to maximal intracerebral doses of virus of the virulent French neurotropic strain (24). It remained to compare the resistance of such mice with that of mice immunized by extraneural routes; and also to extend the studies in this inexpensive animal in an effort to demonstrate the mechanism of the localized resistance which intracerebrally immunized animals apparently possess.

Resistance to Neurotropic Yellow Fever Virus

A study was first made of the comparative resistance to French neurotropic virus of mice surviving a previous cerebral infection, and of mice immunized

by extraneural inoculation, for which the intraperitoneal route was chosen for its obvious convenience.

Experimental.—Since it was proposed to collect the intracerebrally immunized mice from survivors of infections with 17D virus, this strain (substrain 17DD low) was also employed for purposes of intraperitoneal immunization. Preliminary experiments revealed that intraperitoneal doses containing at least 4×10^4 M.L.D. of virus were necessary to provoke regularly a serologically detectable immune response; and that reinoculation with a large virus dose at about the peak of the primary immune response (21 days later) resulted in a greatly augmented serum-antibody level.

Following this basic technique, two large lots of intraperitoneally hyperimmunized mice were prepared, the first and second inocula in both cases containing between 10^5 and 10^6 M.L.D. of virus. These immunizing inocula consisted of suspensions of

TABLE III

The Resistance of Intraperitoneally and of Intracerebrally Immunized Mice to Intracerebral Challenge Doses of French Neurotropic Virus

Method of immunization	Mortality ratios of mice challenged with doses of (M.L.D.)						
	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	5×10^1	All doses
Intraperitoneal:							
One inoculation	10/20	9/11	4/11	4/10	5/11	13/22	45/85
Two inoculations	10/57	9/24	7/24	6/33	—	0/17	32/155
Cerebral infection	1/82	—	—	—	—	—	1/82
Normal controls	42/42	42/42	41/41	41/41	42/42	41/41	249/249

infected mouse brain rather than the usual chick embryo preparations to avoid the possible occurrence of anaphylactic reactions. At intervals during the course of the immunization, mice from these lots together with animals which had survived cerebral infection were subjected to intracerebral challenge with French neurotropic virus, using as controls normal mice of the same age as the intraperitoneal immune mice. The results have been expressed as the ratio of the number of mice dying as the result of the challenge inocula to the number tested (mortality ratio).

Results.—The observations have been summarized in Table III. It is evident that some degree of resistance to cerebral infection was conferred by intraperitoneal immunization, since many mice so immunized survived the challenge inocula whereas all of the normal controls succumbed. Furthermore, this resistance was clearly augmented by the hyperimmunizing inoculation. Of the 85 mice tested after a single immunizing infection, 45 (or 53 per cent) developed a fatal encephalitis whereas only 32 (or 21 per cent) of the 155 hyperimmunized mice succumbed. This resistance, however, was complete only in the groups of hyperimmunized mice which received the smallest challenge dose (5×10^1 M.L.D.). Otherwise, little relation is evident between the size

of the challenge inoculum and the proportion of mice succumbing. In sharp contrast to this picture of irregular and incomplete resistance are the results observed for the mice whose immunity had resulted from previous non-fatal cerebral infection. Of 82 such mice tested, only 1 failed to survive.

Resistance to Eastern Equine Encephalomyelitis Virus

To parallel the similar study in monkeys, an attempt was made to verify the specific nature of the resistance of yellow fever-immune mice to cerebral infection with a highly neurotropic yellow fever virus.

Experimental.—In graded doses containing from approximately 5 to 5 $\times$ 10^3 M.L.D. as determined by titration in normal mice (using 12 mice per decimal dilution), Eastern equine encephalomyelitis virus was administered intracerebrally to 81 mice which had survived a challenge inoculum of French neurotropic virus given from 12 to 21 days previously. These mice represented survivors from a miscellaneous group of experiments; they included not only animals which had survived previous cerebral infection with yellow fever virus of several strains but also nearly 50 mice which had received, while from 3 to 7 days of age, subcutaneous or intraperitoneal inoculations of Asibi virus.

Results.—Of 45 mice receiving 5 $\times$ 10^2 or 10^3 M.L.D. of equine virus, all died; of 24 mice inoculated with 5 $\times$ 10^1 M.L.D., 16 died; and of 12 mice given 5 M.L.D., 2 died. Thus, it would appear that these yellow fever-immune mice were capable of resisting about 10 M.L.D. of equine virus. It was noted also that the average time of death of these mice was significantly longer than that of normal control mice receiving equivalent virus doses. Deaths among the test mice occurred on from the 2nd to the 6th day, with an average of 3.3 days, whereas deaths among the control mice occurred on from the 2nd to the 4th days with an average of 2.7 days.

These observations indicate that mice known to resist large intracerebral doses of neurotropic yellow fever virus may be capable of a slight though definite resistance to a heterologous neurotropic virus of considerable virulence. Perhaps this non-specific resistance is to be explained by a residual process of inflammation resulting from the rather recent challenge inocula to which the mice had been subjected. Whatever its explanation, however, the degree of non-specific resistance demonstrated is very small as compared to that of the previously demonstrated resistance to neurotropic yellow fever virus.

Protective Antibodies in the Sera and Brains of Intracerebrally and Intraperitoneally Immunized Mice

In an attempt to explain the basis for the superior and largely specific resistance of the nervous system of animals surviving a previous neural infection,

studies were made of the comparative protective capacity against yellow fever virus of serum pools and brain suspensions from neurally and extraneurally immunized mice.

Experimental.—On the same occasions that the intracerebrally and intraperitoneally immunized mice were tested for their resistance to intracerebrally inoculated French neurotropic virus, identically immunized mice, in groups of from 4 to 8, were exsanguinated from the heart to obtain serum for pools. The brains of these mice were also collected, pooled to correspond with the respective sera, and then triturated and suspended in physiological saline in a 10 per cent concentration. These serum and brain suspension pools were titrated for their protective action; 12 young mice were used per fourfold dilution of each pool. In all cases, corresponding serum and brain suspension pools were examined in the same protection test runs.

Results.—The results of these examinations are recorded in Table IV.

Turning first to the sera, the important fact is that in general the titers of the pools from the intraperitoneally hyperimmunized mice, averaging 595, greatly exceeded those obtained for the pools from mice immunized intracerebrally, which averaged only 114. Thus, while the high levels of serum antibody observed in the hyperimmunized mice may have been responsible for their being relatively more resistant than mice immunized with but a single intraperitoneal inoculation, no similar basis can be advanced for the nearly complete resistance of the intracerebrally immunized animals.

Examination of the results obtained for the brain suspension pools reveals that, although the serum-antibody levels of the hyperimmune mice were much higher than those of the mice surviving cerebral infection, a significant though low degree of protective activity was demonstrated only in the brain suspensions from the latter group. On the basis of the average figures presented (titers recorded as less than 2 were assigned the arbitrary value of 1), the titers of brain suspensions from the cerebral immunes (6.4) were 5.3 times greater than those of the hyperimmune mice (1.2). This observation strongly suggests that the local mechanism responsible for the uniform resistance of the neurally immunized mice (and presumably monkeys as well) is based at least in part upon a concentration of specific protective antibodies in the neural tissue fluid.

The Development of Immunity in Intracerebrally Inoculated Mice

Since mice which survive an intracerebral infection are the exception rather than the rule, it is obviously impossible to study the development of the immune process contributing to their survival. It is possible and of some interest, however, to study the development of immunity in mice inoculated intracerebrally with 17D virus of a substrain (17DD low) which is known to produce relatively frequent non-fatal infections.

Experimental.—Two groups of 60 normal adult mice each were inoculated with virus doses (determined in a coincidental titration) of 512 and 32 M.L.D. respectively.

TABLE IV

Protective Antibody Content of Pooled Sera and Brain Suspensions from Intracerebrally and Intraperitoneally Immunized Mice

Immunization (17D virus) by:	No. of mice	Antibody titer of pools	
		Sera	Brain suspensions
One inoculation intraperitoneally	6	22	—
	6	64	—
	12	43*	—
Two inoculations intraperitoneally	3	250	<2
	3		<2
	3	930	<2
	3		<2
	6	950	1.3
	6	314	3.0
	6	325	1.2
	6	800	0
	36	595*	1.2*
Prior cerebral infection	5	256	5.1
	4	158	6.1
	3	74	10.0
	3		8.0
	6	39	5.3
	7	47	3.5
	6	182	5.0
	6	132	5.7
	6	81	6.2
	8	136	15.2
	8	98	4.0
	6	38	4.0
	6	126	5.7
	74	114*	6.4*

< = less than the lowest dilution tested.

* Average titers.

At daily intervals thereafter, sera and brains, formed into corresponding pools, were obtained from representative mice sacrificed from each group by exsanguination. The brains were immediately triturated, suspended in physiological saline in a 20 per cent concentration, and titrated for their content of active virus; 6 normal mice

were inoculated per decimal dilution. Subsequently, when their infectivity had been lost, residual portions of the brain suspension pools and the corresponding serum pools were examined for their protective activity in the young mouse protection test, the undiluted specimens being tested in 6 mice each. The results of these examinations have been expressed as the ratio of the number of mice protected to the number tested (protection ratio or PR).

TABLE V

The Development of Infection and Immune Response in Mice Inoculated Intracerebrally with 17D Virus

Day post-inoculation	512 M.L.D. inoculated				32 M.L.D. inoculated			
	No. of mice sacrificed*	PR of serum pools	Brain suspension pools		No. of mice sacrificed*	PR of serum pools	Brain suspension pools	
			PR	Virus titer‡			PR	Virus titer‡
1	3w	0/6	0/6	>1	3w	0/6	0/6	0
2	3w	0/6	2/6	>1	3w	1/6	2/6	>1
3	3w	0/6	2/6	>100	3w	0/6	0/6	>100
4	3w	1/6	1/6	10,000	3w	0/6	1/6	3,200
5	3w	0/6	1/6	100,000	3w	0/6	3/6	10,000
6	5w	0/6	2/6	320,000	3w	5/6	2/6	40,000
7	4w	6/6	1/6	70,000				
	5s	6/6	1/6	200,000	3w	3/6	4/6	80,000
					5w	5/6	2/6	70,000
8	5s	6/6	1/6	200,000	3s	5/6	0/6	400,000
9	3s	6/6	1/6	25,000	3s	6/6	1/6	600,000
10	2s	x	1/6	250,000	7s	5/6	0/6	25,000
11	All dead	—	—	—	3s	6/6	1/6	7,000
12	—	—	—	—	5s	5/6	0/6	400,000

> = end-point not reached.

x = serum specimen not sufficient for testing.

* w = mice sacrificed appeared well, s = mice already sick.

‡ Titers given are of 20 per cent suspensions. For full brain titers, multiply by five.

On the 7th and 8th days separate pools of brains and sera were made for mice still apparently normal and for mice already sick. The observations were terminated on the 10th and 12th days by the death from encephalitis of the remaining mice.

Results.—Table V contains the results. Definite protective capacity was demonstrated in the sera collected on and after the 6th or 7th days. In spite of this the active virus content of the brains continued high or even increased, and the disease progressed to its usual fatal termination in all the mice not sacrificed for study.

The observations as to the protective capacity of the brain suspensions are inconclusive since PR's of 2/6 were obtained for specimens collected as early as the 2nd day after inoculation yet were not followed by a series of more certainly positive results. These inconclusive results, for the most part, probably represent a reduction in the effectiveness of the standard test dose of virus due to its partial inactivation when diluted in the saline-brain suspension. It is possible, however, that the PR's of from 2/6 to 4/6 which were obtained for the 5, 6, 7, and 8 day specimens from mice inoculated with 32 M.L.D. indicate specific protective effect. If this be true, the negative results obtained for specimens collected subsequently, and the failure to detect a similar indication in the 512 M.L.D. series may have been due to a masking of antibody in the cerebral tissue fluid by its full combination with the 17D virus present originally in the brains in relatively high concentration.

Taken in their entirety, the results of this experiment make little contribution to the elucidation of the resistance of intracerebrally immunized mice. They do, however, provide one more example of the failure of a developing systemic immunity to modify the course of an already established neural infection.

DISCUSSION

The evidence presented suggests that the problem of immunizing the nervous system against a neurotropic strain of yellow fever virus—a somewhat academic problem, to be sure—is not dissimilar to that with respect to primarily neurotropic viruses. Neural infections proceed in their fatal evolution in the face of a developing systemic immunity; pre-existing systemic immunity confers only irregular protection against direct neural infection with virulent virus; and only following prior neural infection does complete immunity of the central nervous system become established.

The usual failure, observed in the present experiments, of monkeys immunized by the extraneural inoculation of 17D virus to resist neural infection with French neurotropic virus is at some variance with the experience of other workers (20, 23) who reported the occurrence of but 1 fatal and 2 non-fatal cases of encephalitis among eleven animals subjected to intracerebral challenge. The amount of virus contained in the challenge inocula, however, was not indicated in these reports and may have been relatively small.

It has been demonstrated recently that in animals immunized by extraneural routes resistance to neural infection with the viruses of equine encephalomyelitis is directly related to the presence of specific antibodies in the nervous system in demonstrable concentrations, and that this in turn is contingent upon a titer of serum antibody of at least 300 (9). In the present case, some parallelism was evident between the resistance of extraneurally immunized animals and their level of serum antibody. However, in experiments with mice, antibody could not be demonstrated regularly in the brain even in cases in which the serum-antibody titer greatly exceeded 300.

In contrast to the relative nature of the resistance of extraneurally immunized animals, that manifested by animals which have undergone a prior neural infection is so absolute as to suggest that they are completely refractory. The facts that the resistance of cerebrally immunized animals is long lasting (at least 17 months in the case of monkeys and 114 days in the case of mice) and is not equally valid against the virus of Eastern equine encephalomyelitis make it unlikely that this resistance is based upon a non-specific mechanism analogous to the refractory state of damaged anterior horn cells to infection with poliomyelitis virus (32), or to the resistance of the regenerating nasal mucosa of ferrets to influenza virus (33). Finally, the resistance observed has no evident relation to the degree of the coincidentally existing systemic immunity.

The best explanation, therefore, for the superior resistance to cerebral infection with yellow fever virus manifested by intracerebrally immunized animals would seem to be that it is based upon a specific local mechanism. The persistance of yellow fever virus has recently been demonstrated in the brains of monkeys which remained apparently normal following intracerebral inoculation with 17D virus and which died with tuberculosis 2, 3, or 5 months after inoculation (34). Although no virus could be found in the brains of equivalently inoculated but non-tuberculous monkeys which were deliberately sacrificed, and although numerous attempts to demonstrate virus in the brains of mice surviving 17D virus infection were also unsuccessful (24), the above observation suggests that the resistance might be based on a blocking effect, similar to that observed by Hoskins (35), produced by small amounts of living 17D virus persisting indefinitely in the brain. On the other hand, the data contained in the present paper suggest that the local mechanism may have a truly immunological basis, since brains from cerebrally immunized mice were found to contain much more protective antibody than brains from intraperitoneally hyperimmunized mice, even though the latter manifested much higher titers of antibody in their sera.

The concept of the production of a local tissue immunity is an old one and has been adequately discussed elsewhere (36–42). In the past, however, the concept has been limited chiefly to immunity against bacterial infections and to tissues other than those of the nervous system, although workers with poliomyelitis virus, at least, have differentiated tissue resistance from systemic humoral immunity (11, 13, 14, 43, 44). Considerable discussion as to the mechanism of local tissue immunity has been entered into, particularly as to the relative importance of cellular *versus* humoral factors. In at least two instances, however, the production of antibodies in the local site of immunization has been demonstrated (41, 42).

In the present instance the data do not permit any conclusions as to the source of the antibodies demonstrated in the brain. Under normal conditions, apparently because of the effective barrier action of cerebral capillaries to the globulin molecules in the serum, the tissue fluid of brain and the cerebrospinal

fluid contain only very small amounts of antibody in comparison with the content of the circulating blood and of tissue fluid from other tissues (45). However, the encephalitic process from which all of these neurally immunized animals had recovered is in essence a type of inflammatory reaction which might be expected to have altered greatly the normal vascular permeability. Since antibodies are known to accumulate in areas of active inflammation (46), it is possible that the antibodies found in the mouse brains may have accumulated while the encephalitic process was still active. The re-establishment of the normal barriers upon recovery might then have served to prevent the dispersion of this unusual accumulation of antibodies at a rate faster than the slow rate of antibody accumulation in the brain under normal conditions.

Finally, the attainment in monkeys of complete resistance to neural infection with virulent yellow fever virus by the prior neural inoculation of a virus strain relatively avirulent for monkeys suggests a line along which investigations with primarily neurotropic viruses might be directed. Although direct immunization of the nervous system would appear to be a drastic procedure, the development of relatively non-neurotropic strains of such viruses as those of rabies and poliomyelitis in particular might justify its application under certain conditions. Such direct immunization might, for instance, be reasonably applicable and yield more certain results than present methods in the face of known exposure to rabies.

SUMMARY AND CONCLUSIONS

Monkeys and mice surviving cerebral infection with yellow fever virus of relatively avirulent strains have been found to resist maximal intracerebral doses of yellow fever virus of a highly neurotropic strain. Such animals, however, do not resist more than very small doses of intracerebrally inoculated virus of Eastern equine encephalomyelitis.

Animals immunized by extraneural routes, on the other hand, are not uniformly resistant to neural infection with neurotropic yellow fever virus. Monkeys which have undergone systemic infection with virus of the avirulent 17D strain or of several jungle strains resist only small intracerebral doses of neurotropic virus; while mice, even when possessed of very high serum-antibody levels as the result of intraperitoneal hyperimmunization, manifest only an irregular resistance to intracerebral challenge inocula.

The difference in the resistance of neurally and extraneurally immunized animals is not related to similar differences in the levels of protective antibody in the sera. Indeed, the average of the serum-antibody titers of the hyperimmune mice is several times that of the intracerebral immunes.

A possibly significant relation does exist, however, between the resistance of mice to neural infection and the content of protective antibody in the brain. The protective activity of suspensions of brains from mice surviving cerebral

infection was found to be several times that of brain suspensions from the hyperimmunized animals.

It is concluded that the superior resistance to neural infection of animals whose immunity results from a previous non-fatal infection of the nervous system is effected by a specific local mechanism which is based at least in part upon an increased concentration of antibody in the cerebral tissue.

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