Immunologic Response and Pathogenesis of Japanese B Infection In Peripherally Inoculated Normal and Cortisone Treated Hamsters.* (23103)

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Most Syrian hamsters are not susceptible to clinical disease when inoculated subcutaneously with Japanese B encephalitis virus (JBE)(1). In our hands the mortality rate following a subcutaneous injection of a 10^{-1} dilution of mouse brain suspension is usually 10% and rarely do any animals developing clinically detectable signs ever survive. Since the immune mechanism is well established as a principal factor in the defense against certain invading viruses(2,3), it seemed logical to consider the relation it might bear to the low susceptibility to JBE virus exhibited by hamsters when inoculated by the subcutaneous route. It was conceivable that early formation of antibody might be responsible for arresting infection prior to development of observable signs of disease. It was found that cortisone markedly increased susceptibility of hamsters to subcutaneous inoculation of JBE virus(4). As the mode of action of cortisone is most probably through inhibition of antibody production, this lent support to the hypothesis. Investigations were undertaken to determine rate of antibody formation and virus multiplication in the brain and blood in normal and in cortisone treated hamsters inoculated with the virus subcutaneously. It was expected that these experiments might shed some light on the pathogenesis of the disease as well as on the protective mechanism.

Materials. A frozen suspension of the JBE Nakayama strain of virus in mouse passage 45 was used throughout. This had an LD_{50} of 10^{-7} when titrated intracerebrally in mice. Syrian hamsters obtained from the Lakeview Hamster Farm and about 8 to 10 weeks old, and randomly selected from either sex were used. White mice of the CFW strain, 3 to 4 weeks old, were employed for all virus titrations and neutralization tests. *Observations on JBE virus infection following subcutaneous inoculation of virus in normal hamsters. Exp. 1.* As an orienting preliminary experiment, 24 hamsters were inoculated subcutaneously with 0.1 cc of a 10^{-1} dilution of virus. On the 1st, 2nd, 4th, 6th, 8th, 11th and 21st days after inoculation 3 hamsters were picked at random among those not appearing ill. They were exsanguinated under anaesthesia and the sera from each day's group were pooled. Brains were also removed and pooled. On each of these days, a portion of the pooled sera and of the pooled brain suspensions was tested quantitatively for virus. Other portions of the serum pools of all hamsters from the 4th day on were tested at one time for presence of neutralizing antibodies by the intracerebral mouse test, using the serial virus dilution method(5). In this experiment 3 hamsters were found sick on the 7th day after inoculation. They were sacrificed at that time, their brains were pooled and tested quantitatively for virus just as in the case of those which were apparently normal. No others were observed to be ill at any time.

Results. JBE virus was present in the sera of hamsters bled on 1st and 2nd day after inoculation and disappeared from the sera by the 4th day (Table I). Beginning on 4th day after inoculation virus was observed in brains of those animals with no signs of illness, reaching its maximum titer of 10^{-4} on 6th to 8th day and decreasing by the 11th day. No virus was found on 21st day. By contrast, brains of those observed to be sick and sacrificed on 7th day showed a titer of $10^{-5.7}$. Results of neutralization tests to JBE virus on the hamster sera are also shown in Table

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TABLE I. Normal Hamsters: Log Titer of Neutralizing Antibodies* and of JBE Virus in Pools of Sera, and of Virus in Pools of Brains at Intervals after Subcutaneous Inoculation of Virus.

Material	Tested for	Interval in days 1	2	4	6	7	8	11	21
Serum	Virus	3.0	1.8	neg†	neg	———	———	§———	———
Brain	"	neg	neg	2.5	4.1	5.7‡	4.1	1.5	neg
Serum	N.A.*	———§———		.6	.9	§	1.0	1.0	1.7

* Tests for N.A. (neutralizing antibodies) made by intracer. method, expressed as log neutralization index. † neg = no virus detected—brains in 20% suspension and blood sera undiluted. ‡ Sick hamsters. § Not tested.

I. Neutralizing antibodies to JBE of a titer usually accepted as significant in clinical diagnostic work with this type of test (neutralization index 50 or 1.7 logs)(5) did not appear until possibly on the 21st day. This is partly explained by the fact that the virus from the ampoule used in this neutralization test was found to have a titer which had fallen since previous titrations of other ampoules. Its titer in the control normal rabbit serum was only 10^{-6}. Since this was but an orienting experiment, the neutralization tests were not repeated.

Exp. 2. Another experiment was performed using a larger number of hamsters. Sixty animals were inoculated subcutaneously each with 0.1 cc of a 10^{-1} dilution of JBE virus. Three were sacrificed at each of several different intervals after time of injection, starting at 6 hours, then 12 hours and 24 hours, then daily up to the 13th day, and finally weekly up to the 42nd day. The hamsters sacrificed were dealt with as in the 1st experiment except that tests for virus on serum and brains were made separately rather than by pools. The sera which did not contain detectable virus were tested for presence and titer of neutralizing antibodies to JBE virus, for the most part in pools by interval day, but all tests were made at one time. The sera from the 4th through the 8th days were also titrated using the method of intraperitoneal inoculation of suckling mice which is believed by many to be more sensitive for detecting small amounts of antibody(6).

Results. Viremia was already present 6 hours after subcutaneous inoculation of virus, reached its maximum at about 24 hours and had disappeared by the 3rd or 4th day (Table II). Virus appeared in the brain on the 4th day, reached its maximum ($10^{-2.7}$ to $10^{-3.5}$) around the 7th or 8th day and disappeared after the 10th or 12th day. In this experiment 2 hamsters were found sick and paralyzed on the 6th day after inoculation. The titer of the virus in their brains (not shown in table) was $10^{-5.6}$ for one and $10^{-5.4}$ for the other, higher than the levels found in any of those observed not to be ill. Neutralizing antibodies as detected by the intracerebral test were at a "significant," detectable level by the 5th day after inoculation, fluctuated during the next week, and then rose rather steadily to reach a maximum titer around the 27th or 34th day after inoculation (Table II). Intraperitoneal type neutralization tests performed on the sera of the 4th through the 8th day, also shown in the table, confirmed the very early appearance of serum antibodies; a neutralization index of 1,000 in the sera of the 4th and 5th days, and greater than 10,000 in sera of the 6th, 7th and 8th days.

Observations on JBE virus infection following subcutaneous inoculation in cortisone treated hamsters. An experiment was performed on the same lines but using hamsters treated with cortisone in a dose of 0.2 mg/g body weight given over a period of 4 days(4). Each of the 40 hamsters used in this experiment was inoculated with 0.1 cc of a 10^{-1} dilution of JBE virus subcutaneously 1 day after the 1st cortisone injection. Three animals were sacrificed daily regardless of their clinical status. During the last few days all hamsters were ill.

Results. Virus was observed in the sera of hamsters beginning on the 1st day after inoculation, reached its maximum about the 3rd day and disappeared from the sera after the 4th

TABLE II. Normal Hamsters: Log Titer of Neutralizing Antibodies* and of JBE Virus in Sera, and of Virus in Brains at Intervals after Subcut. Inoculation of Virus.

Material	Test for	Hamster No.	Interval in hr: 6	12	24	Interval in days: 2	3	4	5	6	7	8	9	10	11	12	13	20	27	34	41
Serum	Virus	1		1.6	2.0	1.2	neg†	neg	neg	——	——	——	——	——	——	‡					
		2	.5§	1.2	2.5	1.2	.3	"	"							"					
		3		‡	2.9	1.0	neg	"	"							"					
Brain	"	1	neg	neg	neg	neg	neg	2.4	2.4	1.5	2.5	2.7	1.8	2.6	neg	neg	——	——	‡	——	——
		2	"	"	"	"	"	1.5	1.6	1.4	3.1	.7	1.7	1.4	"	.7			"		
		3	"	"	"	"	"	1.1	2.5	1.4	2.5	3.5	1.6	2.8	"	neg			"		
Serum	N.A.* i.c.‖ 1,(2,3)¶		——	——	‡	——	.6	1.0	1.8	2.2	2.5	2.2	1.8	2.3	1.0	1.2	2.0	2.3	1.6	3.0	2.6
	i.c. 2 ¶		——	——	——	——	——	——	——	——	——	——	——	——	——	——	1.6	2.5	2.7	——	——
	i.c. 3 ¶		——	——	——	——	——	——	"	——	——	——	——	——	——	——	1.9	1.7	3.4	"	
	i.p.** 1,2,3§		——	——	——	——	——	3.0	3.0	>4.0	>4.0	>4.0	——	——	——	——	——	——	——	——	——

* Tests for N.A. (neutralizing antibodies) made by intracer. method and expressed as log neutralization index.
† neg = no virus detected—brains in 20% suspension and blood sera undiluted.
‡ Not done.
§ Pool of sera from hamsters 1, 2 & 3.
‖ i.c. = intracerebral mouse neutralization test.
¶ Pool of 3 sera, except sera on 13th, 20th and 27th days, which were individual sera.
** i.p. = intraper. infant mouse neutralization test.

day (Table III). Virus was detected in the brains of the hamsters beginning on the 3rd to the 5th day after inoculation, reached its maximum ($10^{-6.8}$ to $10^{-7.2}$) on the 7th or 8th day, and remained at a high level (in most instances above 10^{-5}) to the end of the experiment when all animals were moribund or dead. Also found in Table III are the results of neutralization tests on the sera not found to contain virus. Neutralizing antibodies attained a "significant" level (neutralization index of 50 or 1.7 logs) in only 2 animals, one on the 7th and one on the 11th day.

Discussion. Comparison of the results obtained with cortisone and of those obtained from the experiment with normal hamsters showed the following:

(a) In cortisone treated hamsters viremia persisted for a longer time, for instead of disappearing after the 2nd or 3rd day after inoculation, as in normal animals, it did not disappear until after the 4th day in those treated with cortisone. Furthermore, the titer of virus detected in the blood continued to rise after it had essentially disappeared in normal hamsters and attained at that time a higher titer than that of the earlier peak in normal animals.

(b) Virus was observed in the brains of cortisone treated hamsters slightly earlier (on the 3rd day instead of the 4th) reached its maximum on about the 7th day, behaving essentially like that of the few normal hamsters which became ill, and continued to be on a high level of about 10^{-5} or 10^{-6} to the end of the experiment. These levels were much higher than those attained in most of the hamsters not given cortisone. Virus levels in the brains of normal hamsters were somewhat higher, however, on the 4th and 5th days than in those of the cortisone treated animals. No explanation for this is obvious but it is quite possibly due to individual variation and of no significance.

(c) Neutralizing antibodies appeared considerably later and generally did not attain "significant" levels in cortisone treated hamsters. The antibody response of those not given cortisone was of a higher order, as had been anticipated.

TABLE III. Cortisone Treated Hamsters: Log Titer of Neutralizing Antibodies and of JBE Virus in Sera, and of Virus in Brains at Intervals after Subcutaneous Inoculation of Virus.

Material	Test for	Hamster No.	Interval in days 1	2	3	4	5	6	7	8	9	10	11
Serum	Virus	1	1.6	1.3	3.0	1.0	neg	neg					
		2	1.2	1.0	1.5	1.5	"	"					
		3	1.3	1.0	4.9	3.0	"	"					
		Mean*	1.4	1.1	3.1	1.7	"	"					
Brain	"	1	neg	neg	neg	neg	.3	2.0	7.2	5.5	5.0	6.1	6.0
		2	"	"	.3	"	.5	.7	6.5	6.8	6.1	5.2	5.5
		3	"	"	1.3	1.9	.5	1.3	7.0	5.6	6.3	5.1	4.8
		Mean	"	"	.5	.6	.4	1.3	6.9	6.0	5.8	5.4	5.4
Serum	N.A.	1					.7	.0	.4	1.4		.5	1.7
		2					.7	.5	1.5	1.0	1.0	1.0	1.3
		3					.3	.7	2.0		1.3	1.3	1.2
		Mean					.6	.4	1.3	1.2	1.2	.9	1.4

* Geometric mean.
See Tables I and II for interpretation of other symbols and abbreviations.

Hodes and Webster(7) observed that mice inoculated subcutaneously with the virus of SLE failed to develop clinical evidence of encephalitis, but quickly developed immunity against subsequent intracerebral inoculation. They claimed that this immunity, however, was not accompanied by development of virus neutralizing antibodies in the peripheral blood. Such antibodies were noted some 8 weeks after subcutaneous inoculation, at which time, however, immunity to intracerebral inoculation was lost.

Brown, *et al.*(2) showed that humoral antibodies against the virus of SLE can be demonstrated in the sera of hamsters inoculated by the subcutaneous, intracerebral, intravenous and oral routes provided the animal survived the infection 7 days or more. They showed that after subcutaneous inoculation of SLE virus in hamsters, antibodies might appear in the serum within 48 hours after inoculation. They persisted at a high titer for many weeks. Our work tends to confirm this rather than that reported in mice by Hodes and Webster, although viremia was still present in our animals at 48 hours so no test was made for neutralizing antibodies.

From the results of our experiments on normal hamsters inoculated subcutaneously with JBE virus, it appears most probable that the early appearance of antibodies is the main factor that renders hamsters insusceptible to disease from subcutaneous inoculation. This early production of antibodies probably prevented free virus multiplication in the hamster brains and kept the virus at a low level (below 10^{-4}), a level which does not lead to apparent disease. Only in a very low percentage of hamsters, which possibly failed to develop early antibodies, did the virus multiply freely in their brains, reach a titer of about 10^{-5} and lead to obvious illness and death.

Through the action of cortisone which most probably is by inhibition of antibody production, susceptibility of hamsters to subcutaneous inoculation of JBE virus was increased. It appears that cortisone delayed and inhibited antibody production so that the virus multiplied freely and remained for a longer time in the blood, and appeared earlier and reached a higher titer in the brain.

It is interesting to note that this virus infection of hamsters which has been studied following subcutaneous injection and appears to cause clinical illness and death by damage to the central nervous system, is associated with viremia and that the eventual course—clinical illness and death—does not appear to depend upon the breakdown of any supposed "blood-brain" barrier. As in the human host, by natural infection, most normal hamsters are infected and respond immunologically but without disease, while a few become ill. In the hamster virus appears very early in the blood, possibly multiplying there or elsewhere

and released into the blood. Then with apparent absolute regularity it is found in the brain, probably multiplying there. However, this involvement of the brain generally remains at a subclinical level and after what may very likely be a lively combat between virus multiplication and antibodies, with serum antibody levels and brain virus levels fluctuating considerably as a result, the infection with brain involvement is usually overcome. Obviously antibody does not prevent virus from penetrating a "blood-brain barrier" in the normal animal for the virus regularly appears in the brain. This is not a reflection of what is in the blood, for by the time it appears in the brain it is no longer detectable in the blood. Nevertheless, antibody may play a very important role in controlling the extent of virus invasion in the central nervous system provided it is available sufficiently soon. Cortisone, as used, probably effects an adequate delay in this response to change the outcome so that essentially 100% of the animals become ill and die.

Summary. Most hamsters are not susceptible to disease following subcutaneous inoculation of JBE virus, probably due to early production of antibodies. Normal hamsters when inoculated with JBE virus subcutaneously, developed viremia 6 hours after inoculation. This viremia reached its maximum after 24 hours and disappeared by the 4th day. Virus appeared in the brain on the 4th day and reached its maximum around the 7th or 8th day and disappeared after the 10th or 12th day. Neutralizing antibodies to JBE virus were detected in their sera beginning on the 4th or 5th day and reached their maximum titer around the 27th or 34th day after inoculation. In cortisone treated hamsters viremia reached its maximum by the 3rd day and disappeared after the 4th day. Virus was detected in the brains of these hamsters from the 3rd day after inoculation, reached its maximum on the 7th or 8th day and remained at a high level to the end of the experiment. Neutralizing antibodies to JBE virus attained a significant level (1.7 logs) in only 2 animals and that on the 7th and the 11th days. It seems probable that cortisone, through partial inhibition of antibody production, allowed the virus to multiply freely in the brain and so rendered hamsters highly susceptible to subcutaneous inoculation of the JBE virus.

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