STUDIES ON HERPETIC INFECTION IN MICE

I. Passive Protection against Virus Inoculated Intranasally*

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It has been the experience of the present writers that herpes virus given intranasally to 2-week-old mice produces an almost uniformly fatal infection. It has been found, in addition, that mice born of immune mothers are themselves immune to the virus administered by the intranasal route (1). These findings pose several questions. In the present and subsequent reports, we shall describe investigations of the nature of the immunity conferred upon the progeny of immune mothers, as well as investigations of the nature and pathogenesis of herpetic infection of suckling mice. It is the purpose in this first paper to report investigations of the nature and mode of acquisition of the immunity exhibited by mice born of immune mothers.

Andervont (2) showed that inoculation of certain strains of herpes virus into the scarified skin of the mouse was followed by involvement of the central nervous system almost as regularly as inoculation directly into the brain, and demonstrated the protective effect of immune rabbit serum against infection by dermal application. Andervont was also able to show that baby mice born of immune mothers were themselves immune to virus applied to the scarified skin. The immunity of the young proved to be a transient one and for this reason was thought to have been acquired passively from the mother. Gildemeister and Ahlfeld (3) found that a herpes-immune serum prepared in rabbits was able, when given subcutaneously to adult mice, to protect them against subsequent cutaneous inoculation of the virus. McKinley and Holden (4) were able to protect rabbits against subsequent intracerebral inoculation of herpes virus by repeated intravenous injections of immune serum.

Materials and Methods

Mice.—An inbred albino Swiss strain was used. The colony has been free from endemic disease and spontaneous encephalomyelitis has never been encountered.

Virus.—The HF strain of herpes simplex virus was employed throughout. The virus has been carried through 95 consecutive passages in mouse brain. The 66th to 93rd passages were used in these experiments. The term "virus" as used in the present report refers to the mouse-brain antigen.

The virus was prepared for passage by triturating an infected mouse brain with 2.7

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ml. of Locke's solution to make a 10 per cent suspension. No abrasive was used. The suspension thus formed was centrifugalized at low speed and the sediment discarded. The triturate was checked for contamination by culture on a blood-agar plate.

Passage was performed by intracerebral inoculation into mice. The virus proved lethal with uniformity in a concentration of 1×10^{-4}, but was rarely lethal in a concentration of 10^{-5}. After the death of the animals, the brains were removed aseptically and sealed in 50 per cent buffered glycerol. Pieces of the brain and spleen of each animal were cultured aerobically and anaerobically (vaseline seal) in Douglas' broth. If either the brain or the spleen showed bacterial contamination, the brain was discarded. Glycerolated brains were kept in the refrigerator at +4°C. until used They were not preserved longer than 7 to 10 days before use.

Immunity of Suckling Mice Born of Immune Mothers

Immunization and Breeding.—Female mice of about 6 weeks of age were used to form an immune colony. Immunization was accomplished by giving graded doses of unattenuated virus by the subcutaneous, intraperitoneal, and intracerebral routes in the order named. Approximately one-half the mice died of herpetic encephalomyelitis at various stages of the procedure.

After the program of immunization was completed, mice were mated by placing 4 or 5 females in a jar with a male. Each female as she became pregnant was removed to a separate container and the date of birth of the litter noted. Except where otherwise stated, mothers were left with their respective litters until the experiment was terminated. Baby mice born of non-immune mothers (controls) were obtained from stock. The date of birth of each litter was known.

Tests of Immunity of Suckling Mice.—The immunity of baby mice was tested at the age of 2 weeks. It was found by preliminary titrations that when given by the intranasal route only a 10 per cent suspension of virus was uniformly lethal for control mice of this age. (A 1 per cent suspension proved occasionally lethal, a 0.1 per cent suspension but rarely so.) A 10 per cent suspension was used for all intranasal inoculations. The mice were lightly anesthetized with ether—sufficiently to prevent struggling—and about 0.005 ml. was dropped into the nares. A blunt needle was used for this purpose and care was exercised to avoid traumatizing the skin or mucous membrane. For each litter born of an immune mother, a control litter of approximately the same number born of a stock mother was inoculated with the same dose of the same preparation of the virus at the same time. Mice were examined daily, and observation was continued for at least 2 weeks. The mice that succumbed were autopsied by sterile technique and their brains and spleens cultured aerobically and anaerobically in Douglas' broth.

Comparison of the fate of mice born of immune mother with those born of non-immune mothers following intranasal instillation of the virus is shown in Table I. The resistance of those born of immune mothers stands in sharp contrast to the susceptibility of mice of the same age whose mothers were not immune.

Attempts to Recover Herpes Virus from the Fetuses of Infected Female Mice

Whether the resistance of the progeny of immune mothers is the result of infection of the young before birth would depend upon the ability of the virus to pass the placenta. Although, in view of the fact that the blood of actively immunized mice was subsequently shown to contain a high titer of antibody, this now appears unlikely, it seemed pertinent at the time to put the question to test. The result of the experiment is shown in Table II.

TABLE I

Comparison of the Survival Rates of 2-Week-Old Mice Born of Immune Mothers with Those Born of Non-Immune Mothers Following Intranasal Instillation of Herpes Virus

	No. of mice	Survived	Survived
			per cent
Mice born of immune mothers	115	109	94.8
Mice born of non-immune mothers	101	3	2.9

TABLE II

Results of Attempts to Recover Herpes Virus from the Fetuses of Immune and Infected Non-Immune Female Mice

	Maternal brain		Maternal whole blood		Maternal liver and spleen		Whole fetuses		Fetal brains and livers	
Mouse 1	2*				2		4			
Mouse 2	3				3				4	
Mouse 3					3	3,3,4	3			
Mouse 4			3	3,3,3‡	3	3,3,3	3		3	
Mouse 5			3	5,5,6	3	3,3,4	3			

* Number of mice to which tissue was passed.

‡ Day of death of passage mice (blank spaces indicate survival).

Mice 1 and 2 were pregnant, immune females which had received their last immunizing inoculations respectively 31 days and 13 days before they were killed for the experiment. Mice 3, 4, and 5 were pregnant, non-immune females obtained from stock and given a 10 per cent suspension of the virus intracerebrally. Immune mothers were killed with ether. Non-immune mothers were allowed to die of encephalomyelitis. Mouse 3 died 36 hours after infection; mouse 4, 48 hours; mouse 5, 72 hours. The fetuses of all 5 mothers were approximately at the end of the 2nd week of gestation, except those of mouse 2 which were at term. Maternal blood was collected by drawing it into a small syringe through a hypodermic needle from the tip of the exposed ventricle. The needle was filled with a saturated solution of heparin in isotonic saline solution as anticoagulant. The gravid uterus was removed *in toto* and washed in Locke's solution. The amniotic sacs were then ruptured, the fetuses removed and washed again. Removal in this fashion served to minimize the danger

of contaminating fetal with maternal tissues. The material was prepared for passage by trituration with enough Locke's solution to make a 10 per cent suspension. Where the material consisted of whole fetuses or fetal tissues, the entire litter was used, except the litter of mouse 4 which was divided in half. The emulsions formed by trituration were centrifugalized at low speed and the supernatant fluid was removed for passage. Tests for the presence of the virus consisted of the injection of 0.025 ml. amounts intracerebrally into adult stock mice. Test mice were kept under observation for 2 weeks.

In the case of immune mothers, the virus could not be recovered by the method used for either maternal or fetal tissues.[1] In the case of the 3 mothers which were not immune, it is evident that although the virus was present in the maternal blood stream at the time of death it was not obtained from the fetuses or fetal organs.

Demonstration of Antibodies in the Blood of 2-Week-Old Mice Born of Immune Mothers

Fifteen 13-day-old mice, representing 3 litters born of immune mothers, were bled by cardiac puncture under ether anesthesia, the heart having been exposed by dissection. The blood was pooled and the serum removed. The mothers of the 3 litters were bled in similar fashion and their blood pooled. Neutralization tests were done using 0.1 ml. amounts of undiluted serum with equal volumes of varying concentrations of the virus. Blood serum of adult mice obtained from stock was used as control. After thorough agitation by pipetting, the serum-antigen mixtures were placed in the incubator at 37°C. for 90 minutes. At the end of that time, 0.025 ml. of each mixture was given intracerebrally to each of 3 test mice.

The result of the neutralization tests is shown in Table III. Normal mouse serum had no significant neutralizing effect on the virus. The serums of immune females, on the other hand, effectively neutralized the virus when diluted 1:200, as did the serums of their offspring. The scope of the experiment does not permit drawing a contrast of the concentration of immune bodies in the mothers' blood with that of their offspring; but the titer of antibody demonstrable in the blood of the young is at least as high as that of their mothers.

Route of Transmission of Antibodies from Mother to Young—Effect of Foster-Nursing

Four litters of mice born of immune mothers were removed from their mothers within 24 hours after birth and allowed to suckle non-immune mothers. The litters

[1] In subsequent trials, attempts were made to isolate the virus from the brains of actively hyperimmunized mice at varying intervals from 40 to over 150 days after the intracerebral injection of the last immunizing dose. Brains were passed both in the fresh state and after retention in 50 per cent glycerol according to the method of Perdrau (5). The attempts were uniformly unsuccessful.

of the non-immune mothers were transferred to the immune mothers. The mice were left with their foster mothers for 2 weeks, at the end of which time they were tested for immunity by intranasal instillation of virus.

The results are given in Table IV. It is apparent that baby mice tested in this fashion 2 weeks after birth showed no significant immunity acquired by the transplacental route. In the case of mice born of immune mothers but per-

TABLE III

Results of Neutralization Tests Demonstrating the Presence of Antibodies in the Blood of 2-Week-Old Mice Born of Immune Mothers

Final concentration of the virus	Serum of 2-week-old mice born of immune mothers		Serum of immune mothers		Serum of susceptible adult mice (controls)	
	No. of mice	Result	No. of mice	Result	No. of mice	Result
1:20	3	4*	3	4,4,8	3	2,2,2
1:200	3		3		3	3,3,3
1:2,000	3		3		3	3,3,4
1:20,000	3		3		3	4,4,5

* Day of death of passage mice (blank spaces indicate survival).

TABLE IV

Transmission of Antibodies by the Mammary Route from Immune Mothers to Young as the Result of Foster-Nursing

	No. of mice	Survived	Survived
			per cent
Mice born of immune mothers, nursed by susceptible mothers	25	2	8
Mice born of susceptible mothers, nursed by immune mothers	21	21	100

mitted to nurse non-immune mothers, the lapse of time between intranasal inoculation and death was not prolonged beyond that in stock mice of the same age. On the other hand, mice born of non-immune mothers acquired a highly effective resistance if allowed to suckle immune mothers. It is concluded, therefore, that the mammary route plays the effective rôle in the transmission of antibodies from mother to offspring.

Duration of Immunity of Baby Mice

The resistance of mice born of immune mothers to intranasal instillation of the virus was tested in litters of varying age, ranging from 3 to 7 weeks. The mice in each group were removed from their mothers at the end of from 16 to 21 days after birth. As is apparent from Table V, where the results are recorded, the resistance of the young

had regressed considerably by the end of the 4th or 5th week of life, less than 2 to 3 weeks after removal from the mothers.

The experiment was controlled by administering the same dose of the virus intranasally to 48 stock mice between the ages of 6 and 8 weeks. Twenty of these mice succumbed. It appears that to the intranasal inoculation of herpes virus, as to some other viruses, mice may acquire increased resistance with age (6). The results are

TABLE V

Decrease in the Resistance of Young Mice to Herpetic Infection by the Intranasal Route after Cessation of Suckling Their Immune Mothers

Age of young mice	Time after removal from immune mothers	No. of mice	Survived
days	*days*		
23	5	12	12
28	12	16	9
37	19	11	2
47	26	11	4

TABLE VI

The Protection of 2-Week-Old Mice from Intranasal Herpetic Infection by Immune Rabbit Serum

Immune rabbit serum intraperitoneally	No. of mice	Survived	Survived
			per cent
0.5 ml. undiluted	20	17	85
0.5 ml. diluted 1:10	6	6	100
Totals	26	23	88.5
Normal rabbit serum intraperitoneally			
0.5 ml. undiluted	31	4	13
0.5 ml. diluted 1:5	8	0	0
Totals	39	4	10.3

sufficiently decisive, however, to preclude natural immunity as an important issue in their interpretation.

Protection against Intranasal Administration of the Virus Conferred by Immune Rabbit Serum

The results of the previous experiments made it seem likely that baby mice could be protected against intranasal infection with the virus by means of a foreign immune serum.

The immune serum was prepared in rabbits by much the same procedure as that described for the immunization of mice, except that rabbit-brain antigen was used and

the amounts administered were larger. Injections of graded doses were given by the intradermal and subcutaneous routes combined, followed by the intravenous route. Finally, a 10 per cent suspension was rubbed upon the scarified cornea. One month after the development of keratitis, the surviving animals were bled from the heart and the serum pooled. By neutralization tests, the rabbit serum was shown to inhibit the action of mouse-brain antigen diluted 1:200, but not diluted 1:20, when tested by the intracerebral inoculation of mice. Normal rabbit serum exhibited no inhibiting effect.

Baby mice obtained from stock at the age of 2 weeks were given immune rabbit serum intraperitoneally. Control animals of the same age were given normal rabbit serum. Two hours after the administration of the serum, a 10 per cent suspension of the virus (mouse-brain antigen) was instilled intranasally. Deaths were recorded within a 14-day period.

The results are shown in Table VI. It is evident from these protection tests that immune rabbit serum protects suckling mice against infection by herpes virus administered by the intranasal route.

DISCUSSION

The results of all the present experiments blend nicely in one conclusion: the immunity to herpes virus exhibited by the progeny of hyperimmune female mice is the result of the passive transfer of antibodies from mother to young. The demonstration that immunity is acquired principally during extrauterine life by suckling and that it is markedly diminished by the end of the 5th week of life, the possession by the young of a titer of antibody at least equal to that of their mothers, and, finally, the duplication in mice born of non-immune mothers of resistance to intranasally instilled virus by the administration of a foreign (rabbit) immune serum, all point to this conclusion. The failure of herpes virus to pass the placenta of mice is a final argument against the acquisition of an active immunity as the result of intrauterine infection.

There exists to the best of our knowledge no record of any previous attempt to determine whether or not herpes virus is capable of passing the placental barrier of the mouse. Hoff and Shaby (7), however, have presented evidence which they interpret as showing that the virus can at times pass the rabbit placenta. Because of the difficulty with which many viruses pass the placenta, the case of the rabbit should perhaps be reinvestigated.

That, in the mouse, immune bodies are transferred from mother to young by the mammary route has been known for some time. Ehrlich (8), probably the first to investigate the matter by the test of foster-nursing, was impressed by the fact that the immunity of young mice to certain toxic proteins was in greater part acquired by suckling. Ehrlich's experiments do seem to show that young mice born of immune mothers and nursed by non-immune mothers possess a modicum of resistance, but this is manyfold less than that acquired by

nursing immune mothers. This apparent discrepancy between the present experiments and Ehrlich's may well be due to the fact that Ehrlich used a much more sensitive indicator of immunity. Certainly the test of immunity used in the present experiments is much too gross to enable one to detect the presence of a small amount of antibody. Vaillard (9) was able to show that tetanus antitoxin passed from female mice to their offspring in the milk. The antitoxin which he used was prepared in rabbits and administered to lactating mothers after parturition. Culbertson (10) has demonstrated that the transfer of antibodies to *Trypanosoma duttoni* also occurs chiefly after birth.

Our experiment does not gainsay the possibility of passage of some herpes antibody by the transplacental route. It does indicate, however, that the immunity which is effective 2 weeks after birth is acquired by the mammary route. Some antibody may pass from the blood stream of the mother to that of the young before birth, but it must be insufficient in amount or disappear too rapidly to be detected by the method used at the end of the 2nd week of extrauterine life.

The demonstration of a natural passive immunity to a virus of known neurotropic potentiality is not without application. It provides an effective means of testing the rôle of a high titer of circulating antibody in protection of the central nervous system against infection by various routes, and without the introduction of a foreign protein into the animal to be tested. Accurate knowledge of the pathogenesis of intranasally introduced herpetic infection of mice is necessary to assess the value of such a rôle in our experiments. This matter is considered in a subsequent report.

SUMMARY

Passive immunity, naturally acquired from immune mothers or artificially induced through the administration of immune rabbit serum, conferred on suckling mice of the albino Swiss strain a high degree of resistance against herpetic infection following the intranasal instillation of the virus. Antibodies, which could be readily demonstrated in the blood of 2-week-old mice, were received by the offspring of immune mothers primarily by the mammary route. Naturally acquired immunity declined rapidly when suckling was interrupted. Herpes virus was not recovered from the fetuses of either immune or infected, non-immune mothers.

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