Lymphocytic Choriomeningitis Infection in Neonatally Thymectomized Mice Bearing Diffusion Chambers Containing Thymus

Abstract. *Normal, nonoperated Swiss mice which had been inoculated intracerebrally with lymphocytic choriomeningitis virus showed a 100-percent mortality within 8 days after virus challenge. Neonatally thymectomized mice, with or without empty intraperitoneal diffusion chambers, were protected from the lethal effects of the virus, with no animals dying within 14 days after inoculation. Cell-tight Millipore diffusion chambers containing newborn thymic tissue, implanted intraperitoneally into neonatally thymectomized mice, restored the susceptibility of 52 percent of these mice to the lethality of the virus infection. The percentage restoration with subcutaneous thymic grafting was similar. A humoral mechanism of action of the tissue in the chamber is proposed.*

The thymus plays a critical role in establishing the integrity of the lymphoid system (*1*) and is an important regulator of lymphocyte production, serving as an active site of lymphopoiesis in the newborn mouse (*2*), and producing a humoral factor or factors capable of stimulating lymphocyte production (*3, 4*). It has been shown also (*5*) that neonatally thymectomized (CBA × T6) F1 mice bearing thymic tissue in Millipore chambers regain the capacity to reject homografts, although these mice still gave evidence of lymphoid depletion.

Recent studies (*3*) from this laboratory have shown that neonatally thymectomized C3Hf/Lw mice which had been implanted with cell-tight Millipore diffusion chambers containing isologous thymic tissue did not show depletion of lymphocytes in the peripheral blood, involution of the lymphoid organs, or any characteristic sign of the wasting syndrome.

In Swiss mice, neonatal thymectomy is an effective means of protecting animals from the lethality of infection with lymphocytic choriomeningitis (LCM) virus (*6*). Neonatal thymectomy did not produce lymphopenia or induce the wasting syndrome in these mice during the period of study, and did not prevent multiplication of virus or formation of complement-fixing antibody. Nonetheless, the intracerebrally inoculated animals failed to develop the characteristic lymphocytic infiltration of the meninges and choroid plexus, which is thought to be elicited by a hypersensitivity reaction and to serve as the stimulus for the lethal convulsions. Therefore, the protective mechanism of thymectomy was attributed to a depression of some type of tissue hyperreactivity involving lymphoid cells.

The current investigation was undertaken to seek further evidence for diffusible factors in the thymus by determining whether thymus-containing diffusion chambers could exert an action in a strain of mice in which neonatal thymectomy did not cause lymphoid atrophy but suppressed immune responses, as evidenced by marked reduction in the mortality from LCM infection. Experiments were therefore performed to assess the mortality of neonatally thymectomized Swiss albino mice which had been implanted with Millipore chambers containing thymic tissue.

Mice of the noninbred Webster Swiss strain were thymectomized within 24 hours after birth. Experimental mice and normal nonoperated controls of the same age were maintained under identical conditions. Mice were weighed, and in several groups total and differential white blood cell counts were obtained from tail blood when the mice were 3 weeks of age, before any further operative procedures, and again at the time of virus challenge and 4 days after inoculation.

At the age of 3 weeks the thymectomized animals were divided into four groups: (i) thymectomized controls, (ii) mice implanted subcutaneously with one whole thymus from a newborn donor of the same strain, (iii) mice implanted intraperitoneally with a Millipore diffusion chamber containing one whole thymus from a newborn donor of the same strain, and (iv) mice implanted intraperitoneally with an empty Millipore diffusion chamber.

The chambers were constructed by a modification of Algire's method (*7*), using a Lucite ring with an outside diameter of 10 mm, an inside diameter of 6.4 mm, and a depth of 1.6 mm, to each surface of which was glued a Millipore disk of 0.45 μ pore size and 150 μ thickness. At the time of autopsy or sacrifice of the mice, there was no gross evidence of disruption of chamber integrity such as perforation of the membranes or separation from the Lucite rings. Using similar chambers, Shelton (*8*) has found no microscopic evidence of cells or parts of cells traversing the membrane wall.

The mouse-brain-passaged CA 1371 strain of LCM virus obtained from Charles Armstrong was used. Mice were inoculated intracerebrally at 4 weeks of age, 7 days after chamber implantation, with 0.03 ml of a 10^{-1} dilution of a 10-percent suspension of infected brain. Titrations were carried out with each experiment and the challenge dose corresponded to 500 to 1000 LD_{50}. The mice were examined twice daily from the 5th to 14th days after inoculation by spinning by the tail. After this period some thymectomized mice began to develop the wasting syndrome and to die of intercurrent disease.

The mediastina of all mice dying during the period of study were examined grossly and by histologic sectioning. Any operated animal found to contain a thymic remnant was discarded from the experiment. At the termination of the experiments, surviving mice were similarly examined for the adequacy of thymectomy.

Three challenge experiments were done and the results, which were comparable, are combined for presentation here.

None of the thymectomized mice developed the wasting syndrome during the period of study. Table 1 shows that there was no significant difference

Table 1. Body weights and peripheral blood counts of Swiss mice.

Item	Neonatal thymectomy with or without empty chamber			Nonoperated controls
	21 days	28 days	32 days	28 days
No. of animals	26	10	12	20
Mean body weight (g)	18	19	19	20
Mean white blood cells	6267	7030	6763	5480
Mean % lymphocytes	69	75	66	71
Mean % neutrophils	31	25	34	29
Mean No. of lymphocytes	4324	5273	4464	3890
Mean No. of neutrophils	1963	1757	2299	1590

Table 2. Mortality within 14 days after intracerebral inoculation with LCM virus.

Group	Ratio: No. mice dead/ No. inoculated	Percent
I. Nonoperated controls	45/45	100
II. Neonatal thymectomy, subcutaneous thymic graft	6/11	54
III. Neonatal thymectomy, diffusion chamber with newborn thymus	16/31	52
IV. Neonatal thymectomy only	0/9	0
V. Neonatal thymectomy, empty diffusion chamber	0/15	0

betwen nonoperated control and thymectomized mice in either body weight or peripheral blood counts at the time of chamber implantation and challenge.

Figure 1 and Table 2 summarize the mortality of mice after intracerebral challenge with LCM virus. Normal nonoperated controls showed the classical mortality curve, with 100 percent of the animals dying within 8 days after inoculation. In contrast, none of nine completely thymectomized mice and none of 15 thymectomized mice with empty diffusion chambers died during the observation period.

The mortality curves for mice bearing subcutaneous thymic grafts or diffusion chambers with thymus were similar, 6 of 11 dying in the former group and 16 of 31 in the latter. In all groups the great majority of mice which died either had convulsed or were found dead in the morning in the typical postictal position.

Mice dying after the 14-day observation period did not show either the typical clinical or pathologic features of LCM infection. After 6 weeks of age, the thymectomized mice began to develop the wasting syndrome and also to die of intercurrent disease.

Of the 15 surviving mice bearing diffusion chambers with thymus, three were tremulous and one convulsed during the 8th to 12th days after challenge.

The brains of the mice dying with diffusion chambers containing thymus showed the lymphocytic inflammatory infiltrate of the meninges, characteristic of LCM infection.

A statistical evaluation of the mortality curves was then done at 14 days. The Fisher exact test (one-tail) was used to calculate the probability values, which are as follows: group II versus groups IV and V, $p = .001$; group III versus groups IV and V, $p = .001$.

Previous studies have shown that subcutaneous implantation of a whole intact thymus in neonatally thymectomized mice at 1 week of age will abolish immunologic unresponsiveness, reverse the changes in hematopoietic tissues, and prevent wasting disease (*9*). In this laboratory, grafting with either isologous or homologous thymus as late as 3 to 4 weeks of age has been found effective in preventing or reversing the changes associated with the wasting syndrome. Subcutaneous thymic grafting is a highly effective method of restoring the lymphoid system and immunologic reactivity of the neonatally thymectomized mouse (*9*). The data presented here show that Millipore diffusion chambers containing thymus and subcutaneous thymic grafting achieved an equal restoration of susceptibility to the lethal effect of LCM infection. The failure of both methods to restore susceptibility in all mice is likely due to the late implantation of thymic tissue at age 3 weeks, rather than in the first week of life.

There are at least two possible explanations for the effectiveness of the chamber containing thymus. The first is that the chamber or its contents may have produced a nonspecific effect on immune or vascular responses which potentiated subliminal changes produced by the virus infection. Fluid-filled chambers have been shown to have an adjuvant effect on the antigenicity of two soluble antigens (*10*), but only when the antigens were administered in direct contact with the diffusion chamber. This possibility is precluded by the fact that mice bearing empty diffusion chambers did not show a mortality greater than that of the thymectomized controls. In our system the antigen was a highly neurotropic strain of virus, administered by the intracerebral route, while the chamber was placed intraperitoneally.

It is conceivable that products of tissue breakdown provided a potentiating effect on LCM convulsions such as observed with endotoxin injection (*11*). This possibility does not seem likely in view of the 7-day interval between chamber implantation and virus inoculation. This should be sufficient time for the mouse to clear the products of tissue autolysis, which occurs immediately after chamber implantation.

A second, more likely hypothesis is that the thymic tissue in the chamber produced a specific diffusible product that enabled the thymectomized animal to produce new lymphocytes in response to the stimulus of LCM infection.

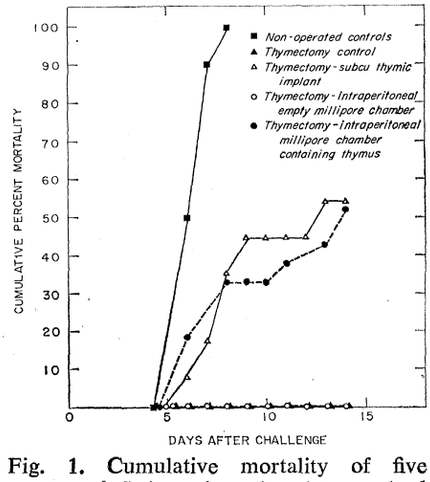

Fig. 1. Cumulative mortality of five groups of Swiss mice after intracerebral inoculation with lymphocytic choriomeningitis virus.

Animals thymectomized at birth failed to show the lymphocytic infiltration of the meninges after challenge with LCM virus (*6*), even though their numerical complement of lymphocytes was normal at the time of virus challenge. Mice which had been implanted intraperitoneally with Millipore diffusion chambers containing thymus and which died after virus inoculation showed the pathognomic lesions of the disease. Therefore, it would appear that the contained thymic tissue produced a noncellular or humoral factor which restored ability to react to infection with a lymphocytic infiltration.

Gowans (*12*) has recently presented evidence that there may be two classes of small lymphocytes: those which are "immunologically committed," in that they already have reacted to antigen, and those which are "uncommitted," in that they are free to establish a new line of dividing antibody-producing cells in response to a new antigenic stimulus.

In accordance with this view, then, the neonatally thymectomized mouse, while not lacking peripheral blood lymphocytes and not showing lymphoid organ atrophy, is unable to produce a "clone" of small lymphocytes to react to the new exposure with LCM virus.

A preliminary report (*3*) showed that a Millipore diffusion chamber containing isologous thymic tissue implanted into neonatally thymectomized mice could prevent the lymphoid atrophy and wasting syndrome which otherwise characterize such animals. This restorative action was attributed to a noncellular or humoral factor that diffused from the thymic tissue in the chamber, the pore size of which did not allow the passage of intact cells.

The data presented here provide further support for a humoral theory of thymic action. The most probable source of this humoral factor is the epithelial-reticular cells of the thymus, which have been shown to survive in the chamber up to 60 days (*3*). Evidence has been reported for activity of the epithelial component of the thymus in lymphocyte production (*13*).

The present data do not permit judgment on whether the diffusible factor is produced by thymus alone or by other tissues as well.

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References and Notes

1. R. A. Good, A. P. Dalmasso, C. Martinez, O. K. Archer, S. C. Pierce, B. W. Papermaster, *J. Exptl. Med.* **116**, 773 (1962); J. F. A. P. Miller, *Proc. Roy. Soc. London, Ser. B*, **156**, 415 (1962).
2. K. Nakamura and D. Metcalf, *Brit. J. Cancer* **15**, 306 (1961).
3. R. H. Levey, N. Trainin, L. W. Law, *J. Natl. Cancer Inst.* **31**, 199 (1963).
4. D. Metcalf, *Brit. J. Cancer* **10**, 442 (1956).
5. D. Osoba and J. F. A. P. Miller, *Nature* **199**, 653 (1963).
6. W. Rowe, P. Black, R. H. Levey, in press.
7. G. H. Algire, J. M. Weaver, R. T. Prehn, *J. Natl. Cancer Inst.* **15**, 493 (1954).
8. E. Shelton and M. E. Rice, *ibid.* **21**, 137 (1958).
9. J. F. A. P. Miller, *Ann. N.Y. Acad. Sci.* **99**, 340 (1962).
10. F. L. Adler and M. Fishman, *Proc. Soc. Exptl. Biol. Med.* **111**, 691 (1962).
11. J. Hotchin, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 479 (1962).
12. J. L. Gowans, D. D. McGregor, D. M. Cowen, C. E. Ford, *Nature* **196**, 651 (1962).
13. R. Auerbach, *Develop. Biol.* **2**, 271 (1960); **3**, 336 (1961).

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Possible Cytoplasmic Change in an Immunologically Competent Tissue of the Chicken

Abstract. *The weight of the bursa of Fabricius of chicks hatched from eggs dipped in a 0.67 percent solution of testosterone propionate in ethyl alcohol was significantly reduced. Also, bursa weights of offspring one and two generations removed from the chickens hatched from the dipped eggs were significantly reduced. The change appears to be cytoplasmic.*

Immunological competence appears to be a function of the mammalian thymus (*1*) and avian bursa of Fabricius (*2*), a lymphoepithelial gland. The weight of the bursa and antibody response of chickens has been eliminated or markedly reduced by dipping eggs in alcohol solutions of testosterone propionate (*3*). Chickens hatched from eggs dipped in testosterone propionate (TP) produced offspring with a reduced bursa size (*4*). The reduced bursa size of the offspring could be attributed to a gene (chromosome) or cytoplasmic change. Data supporting the idea that TP acted by way of the cytoplasm is presented in this paper.

In both of the trials, the parental generation (P_1) refers to chickens hatched from eggs dipped in either ethyl alcohol (EA) or in a 0.67-percent solution of testosterone propionate in ethyl alcohol. In all other generations (F_1 and F_2), the eggs were not dipped. The P_1 generation was produced by removing fertile eggs from the incubator on the 3rd day of incubation and immersing, for 5 seconds, 3.2 cm of the pointed end of the egg in cold solutions (5°C ± 4°C) of TP or EA. The data were analyzed by the analysis of variance. The analysis of covariance was used to analyze bursa weights where body weights differed significantly. Duncan's (*5*) new multiple range test was used to determine significant differences between any two means. Significant difference refers to a statistical difference at the 5 percent level of probability. All means are given with standard deviations.

In the first trial, the P_1 chickens were raised to maturity and mated within their own group to produce the F_1 generation, that is, EA males were crossed with EA females and TP males with TP females. Individuals of the F_1 generation were raised to maturity and mated within their own group to produce the F_2 generation. The bursae were significantly reduced in size in chicks hatched from TP-dipped eggs (P_1) (Table 1). The TP chicks one generation (F_1) and two generations (F_2) removed from the dipping also possessed significantly reduced bursae at hatching. The body weight of the EA and TP females that produced the F_1 and F_2 generations did not differ significantly.

A second trial was conducted to investigate the possibility that the reduction in bursa size in the F_1 and F_2 generations was induced by a change in the cytoplasm. When the P_1 generation was produced, the expected significant reduction in bursa size at hatching was observed in the P_1 chicks (Table 2). An F_1 generation was produced by mating EA males with EA females, EA males with TP females, and TP males

Table 1. The weights of the bursa and body at hatching of P_1, F_1, and F_2 chicks. Significant differences are apparent for the bursa in all three cases.

Ethyl alcohol		Testosterone propionate	
Bursa (mg)	Body (g)	Bursa (mg)	Body (g)
	P_1 (12 birds)		
46±12*	44±3	13±8	44±4
	F_1 (20 birds)		
43.0±7.7*	35.0±3.0	31.9±7.7	35.0±3.0
	F_2 (55 birds)		
49.0±13.0*	42.0±3.0	38.0±9.0	43.0±3.0

* $P \leq .05$