Induction of Autoimmune Hemolytic Disease by Diffusible Substances from the Thymus of NZB/B1 Mice

By Jason M. Masters and Carroll L. Spurling

The development by Bielschowsky1 of the NZB/B1 strain of mice has provided a unique animal model for the study of autoimmune disease.2-10 The regular spontaneous appearance of Coombs-positive hemolytic anemia and other serologic markers of autoimmunity, the development of a lupus-like renal disease,4,7,11,12 and the results of therapy with immunosuppressive agents4,13-15 and splenectomy6 are all consistent with the autoimmune concept of pathogenesis.

Several studies have suggested a close relationship between the thymus and the autoimmune disease of NZB/B1 mice. Burnet described an abnormal histologic pattern consisting of germinal centers with peripheral crescents of lymphocytes, increase in plasma cells, and lack of the expected atrophy with age.2,7 He drew attention to the similarity between these lesions and those of myasthenia gravis. DeVries and Hjimans noted a reduction in thymic epithelial cells.16 More significantly, transplantation of the NZB/B1 thymus to other mouse strains led to the development of an autoimmune hemolytic disease in the recipients.5 We have confirmed these observations in an unpublished preliminary experiment in which neonatally-thymectomized mice of other strains were transplanted subcutaneously with newborn NZB/B1 thymus. Five of the 11 developed a positive Coombs’ test and other evidence of hemolytic disease.

In this paper we present the results of studies suggesting that the NZB/B1 thymus releases a substance or agent capable of passing through the pores of a Millipore chamber and inducing hemolytic disease in the recipient host. The possible mechanisms involved in these observations and their interpretations are considered in the discussion.

Materials and Methods

Mice from strains C3H/He, A, LCS, and LCSa were thymectomized within 24 hours of birth. Two to 3 weeks postoperatively they received intraperitoneal implants of Millipore chambers containing thymic tissue from newborn NZB/B1 mice. Control neonatally-thymectomized animals received intraperitoneal implants of empty Millipore chambers or Millipore chambers containing thymus tissue from newborn mice of the A strain. Beginning at 3 months following implantation, observations were made at monthly intervals with Coombs’ tests and reticulocyte counts. Microhematocrits were done on only a few of the animals in each group to keep blood loss at a minimum. Observations were terminated at 8 months of age. Studies of pathologic material from animals dying or sacrificed after 8 months will be reported separately.

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NZB/Bl mice were obtained for breeding from three sources: (1) Laboratory Animal Centre, Medical Research Laboratories, Carshalton, Surrey, England; (2) Dr. C. F. Springer's laboratory, Evanston Hospital Association, Evanston, Illinois; and (3) Dr. Richard Siegler's laboratory, The Albert Einstein Medical Center, Philadelphia, Pennsylvania. Dr. Marianne Bielschowsky had originally supplied these sources with breeding animals. Mice of the C3H/He, A, LCS, and LCSa strains were obtained from the Department of Anatomy of the University of Maryland School of Medicine. * Preliminary studies had shown these strains to be free of Coombs' antibodies of all ages. Animals from four strains were used because of the possibility that genetic composition might play a role in their susceptibility to the induction of autoimmune hemolytic disease. All mice were maintained on standard Purina diet.

Thymectomies were performed on animals less than 24 hours old, using the surgical technic described by Sjödin et al. with few modification. A 20-power dissecting microscope gave good visualization of the operative field. Briefly, the procedure included ether anesthesia, a sternum-splitting incision, and aspiration removal with a pipette and suction of the two thymic lobes. The superior mediastinum was inspected carefully for residual thymic tissue before closing the wound. Postoperative care included oxygen inhalation and placement with mothers in total darkness for 3 days. Mothers were given Librium in their drinking water. The darkness and tranquilizer seemed to reduce cannibalism.

Diffusion chambers were constructed of nylon reinforced Millipore membranes with a 0.45 micron pore rating and a thickness of 100 microns (Millipore Filter Co., Bedford, Mass.). Squares of 8 to 10 mm. were cut and two squares used to make a chamber by approximating the edges. Three sides were sealed by dipping the edges quickly into a thin film of acetone. The assembled chambers were then sterilized with formalin vapor for several hours. They were filled with either one whole lobe of the fresh donor thymus or several smaller pieces when an intact lobe was not available. The remaining edge was then sealed with acetone. The filled chambers were kept in sterile Ringer-Locke solution for not more than 5 minutes before implantation.

Chamber implants were placed intraperitoneally in neonatally thymectomized recipient mice at 2 to 3 weeks of age. A simple midline abdominal incision just large enough to admit the chamber was used, and the chamber was inserted among the intestinal coils. The incision was then closed with fine silk.

A minimal amount of blood needed for testing was obtained from the tail vein. Direct Coombs' tests were performed with three different Coombs' sera: (1) a commercial rabbit antimouse globulin serum (Baltimore Biological Laboratories, Baltimore, Maryland); (2) a commercial goat antimouse globulin serum (Microbiological Associates, Bethesda, Maryland); and (3) antisera to mouse serum prepared in our laboratory by repeated immunization of white rabbits and absorption with mouse red cells. Known positive and negative controls were used with each series of tests. Results with the three sera were the same. Reticulocytes were stained with new methylene blue. Microhematocrit determinations were done in the usual manner, using heparinized capillary tubes.

A total of 130 mice of all strains (LCSa, LCS, A, and C3H/He) survived for 2 to 3 weeks after neonatal thymectomy and received implants. Twenty-nine tests and 32 control animals died within 3 months after implantation, leaving 60 test and 9 control animals for the period of study from 3 to 8 months (Table 1).

RESULTS

The results summarized in Table 2 show that 50 of 60 surviving mice that received Millipore chambers containing NZB/Bl thymic tissue developed positive direct Coombs' tests and elevated reticulocyte counts. Observations made according to strain did not suggest any significant differences among the strains tested, although some of the numbers are small. Twenty of 26 LCSa, 19 of 23 LCS, and all of 5 A and 6 C3H/He mice developed Coombs'

*We are indebted to Dr. Frank Figge for supplying these animals.
### Table 1.—Survival of Mice Used in Study

<table>
<thead>
<tr>
<th>Test Mice</th>
<th>Control Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Empty chamber</td>
</tr>
<tr>
<td>Total</td>
<td>89</td>
</tr>
<tr>
<td>Dead before 3 mo.</td>
<td>29</td>
</tr>
<tr>
<td>Survived at least 3 mo.</td>
<td>60</td>
</tr>
</tbody>
</table>

Antibodies. Positive tests first appeared in a few animals (5 per cent) at 3 months after implantation, but most required 5 to 6 months before the test became positive. All reactions were at least 2+ at the time of the most strongly positive test; about half of them became 4+ after being positive for several months. By the time the animals were 8 months of age, the reactions tended to become somewhat weaker, and in 3 they became negative. No difference was observed in the results between the two sexes of recipient animals. Of 31 male recipients, 26 (84 per cent) developed positive Coombs' tests; of 29 female recipients, 24 (83 per cent) were positive.

Animals developing positive Coombs' tests had an average reticulocyte count of 4.8 per cent; those with negative Coombs' tests had reticulocyte counts within the normal range (average 1.8 per cent). Hematocrit determinations taken on a few animals in each group did not change. Blood smears showed the presence of only scattered microspherocytes, some poikilocytosis and moderate anisocytosis. The artificially-induced autohemolysis, therefore, is a relatively mild compensated hemolytic disease in contrast with the more severe process seen naturally in NZB/B1 mice.

Most of the mice showing positive Coombs' tests developed physical characteristics similar to those seen in NZB/B1 mice, including hunched back, ruffled fur, loss of hair and narrowed palpebral fissures. Varying degrees of splenomegaly and hepatomegaly were seen in all. Although it was impossible to quantitate, encapsulation and vascularization around the chamber appeared to be more prominent in those animals with positive Coombs' tests. There was no microscopic evidence of disruption of the integrity of the chambers.

Of 29 control neonatally thymectomized mice (all strains) receiving empty Millipore chambers, all but 4 died of wasting disease and intercurrent infections within 3 months, as might be expected from the well-known defect in immunologic responsiveness which they exhibit. Only 5 of 12 mice receiving transplants of A strain thymus survived at least 3 months. None of the control animals developed a positive Coombs' test or reticulocytosis. Howie and Helyer19 and deVries and Hijnmans16 have reported positive Coombs' tests in some thymectomized mice. In our experience they have remained negative. This difference in observations needs clarification.

**Discussion**

These results suggest that something passes through the membrane of a Millipore chamber carrying NZB/B1 thymic tissue and affects the thymectomized host animal. It prevents the wasting syndrome seen in control animals.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of Animals</th>
<th>Coombs' Positive</th>
<th>Per cent Developing Positive Coombs'</th>
<th>Mean Percentage of Reticulocytes (Positive Coombs')</th>
<th>Mean Percentage of Reticulocytes (Negative Coombs')</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCSa</td>
<td>26</td>
<td>20</td>
<td>77</td>
<td>4.5</td>
<td>1.6</td>
</tr>
<tr>
<td>LCS</td>
<td>23</td>
<td>19</td>
<td>82</td>
<td>5.6</td>
<td>2.3</td>
</tr>
<tr>
<td>A</td>
<td>5</td>
<td>5</td>
<td>100</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td>C57/He</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>4.2</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>60</td>
<td>50</td>
<td>83</td>
<td>4.8</td>
<td>2.1</td>
</tr>
<tr>
<td>Controls</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>1.8</td>
</tr>
</tbody>
</table>

* Range 2.3-11.3 per cent.
† Range 1.2-2.7 per cent.
‡ See text.
with empty chambers and allows them to thrive until they develop a mild but definite autoimmune hemolytic disease, usually at about 5 to 6 months of age. At this point, one can only speculate as to the nature of the membrane-passing agent or factor.

The question of whether or not intact cells can pass through the membranes used in our work and that of others is obviously an important one. The fact that such a small bit of tissue can prevent wasting disease, restore immunologic responsiveness, and cause autoimmune hemolytic disease suggests the existence of a humoral mechanism. If lymphocytes can traverse the membrane, one might expect to find host lymphocytes repopulating the chamber implant as occurs with subcutaneous thymic grafts. However, in the experiments of Osoba and Miller only epithelial-reticular cells were found in the implanted Millipore chambers, and no cells were seen traversing the chamber walls. Lymphocytes present in thymic implants apparently do not survive longer than 3 to 4 weeks at most. Jankovic and Leskowitz have reported finding no cells in empty or Salmonella-vaccine baited chambers placed in the peritoneal cavity for as long as 6 weeks. On the other hand, Capalbo et al. have presented illustrations suggestive that with the membranes they used some cells may get through those with higher pore ratings.

It is probable that viruses could readily pass through the membranes used in these studies. Although the relationship of viral infections to autoimmune hemolytic disease remains speculative, further studies in this direction might explain many observations on NZB/B1 mice. In this connection, it is of interest that tumors of several types have occurred relatively frequently, usually following the appearance of the hemolytic disease. Virus-like particles have been identified in native and in germ-free NZB/B1 mice. The possibility that Coombs' antibodies are produced by the thymic implant itself must be considered but seems unlikely. No direct evidence exists that the thymus can produce antibodies of this type. Thymectomy in young NZB/B1 mice appears actually to speed the development of autoimmune disease; it does not prevent it. Once the disease is established, thymectomy does not affect it. Further studies are being pursued in an attempt to demonstrate the production of erythrocyte antibodies by host cells.

That the thymus may elaborate humoral factors was first suggested by the work of Metcalf, who demonstrated a "lymphocytosis stimulating factor" in thymic extracts. At least 3 consequences of neonatal thymectomy—namely, lymphoid atrophy, the wasting syndrome, and impaired immunologic competence—are alleviated to varying degree by the implantation of thymic tissue in Millipore chambers. Observations of this type have been made in mice and in hamsters. A similar type of experiment in chicks indicates that the bursa of Fabricius may also produce a humoral factor. Comsa and Trainin and Linker-Israeli have reported that thymic extracts suppress the tested consequences of thymectomy. The studies reported herein raise the possibility that thymic humoral factors might direct the type of immune response (in this case an autoimmune one) as well as its quantitative expression. It remains to be seen whether or not this effect of the abnormal NZB/B1 thymus has an equivalent in normal thymic function.
Summary

Mice of four strains were thymectomized neonatally and implanted with Millipore diffusion chambers containing thymic tissue from newborn mice of the NZB/Bl strain. Of a total of 60 surviving animals, 50 developed a positive Coombs' test and other evidences of autoimmune hemolytic disease. Controls remained negative.

Various possibilities concerning the nature of the membrane-passing factor or agent are considered.

SUMMARIO IN INTERLINGUA

Neonate muses de 4 lineas esseva subijicite a thymectomia e providite de implantationes de cameras diffusori Millipore continente tissu thymic ab neonate muses del linea NZB/B1. Un total de 60 animales superviveva. De istos, 50 disveloppava un positive test de Coombs e altere evidentia de autoimmun morbo hemolytic. Le animales de controlo remaneva negative.

Varie possibilitates es discutite quanto al natura del factor o agente que transversa le membrana.

REFERENCES

19. Howie, J. B., and Helyer, B. J.: The influence of neonatal thymectomy and


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