IMMUNOLOGICAL STUDIES OF AGING

IV. The Contribution of Thymic Involution
To the Immune Deficiencies of Aging Mice and
Reversal with Thymopoietin32-36*

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Immune responses are impaired in aged animals and humans (1, 2). The immune
deficiencies that develop during aging may be related to the involution of the thymus
gland and the decline in the serum concentration of thymopoietin (3). This thesis is
supported by the observation that thymic-dependent immune reactions are more
severely compromised during aging than are thymic-independent immune reactions,
and that certain age-associated immune defects may be reversed by young thymocytes
(4). We have shown that two immune responses closely related to thymic function are
particularly affected by aging: (a) the generation of the IgG plaque-forming cells
(PFC)1; and (b) the generation of PFC that have high affinity for antigen. We showed
that both of these deficiencies could be remedied by transfusion of thymocytes from
young donors (4). In this report we present evidence that thymectomy accelerates the
age-associated loss of IgG and high affinity PFC, and that spleen cells from old
animals exposed to a young thymus gland or thymopoietin regain immune function.
Thus, these studies provide additional evidence that thymic involution and a decline
in thymic hormone secretion may play an important role in the immune deficiencies
that accompany aging.

Materials and Methods

Animals and Immunization. Male BALB/c mice of various ages were obtained from the
Charles River Breeding Laboratories (Wilmington, Mass.). Mice were immunized by the
intraperitoneal injection of 500 μg of dinitrophenylated-bovine gammaglobulin (DNP-BGG)
emulsified in 0.2 ml of complete Freund's adjuvant (CFA, containing 2 μg/ml Mycobacterium
butyricum). The anti-DNP PFC response in such mice has been shown in our laboratory to be T-
cell dependent. Other mice were immunized with the T-independent antigen DNP-FicolI
by i.p. injection of 50 μg of soluble DNP-Ficol.

Antigens and Haptens. DNP-BGG was prepared by the reaction of 1-fluoro-2,4-dinitrobenzene
(Eastman Organic Chemicals Division, Eastman Kodak Co., Rochester, N. Y.) with BGG

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1 Abbreviations used in this paper: CFA, complete Freund's adjuvant; DNP-BGG, dinitrophenylated-bovine
gamma globulin; EACA, epsilon-amino-caproic acid; HBSS, Hanks' balanced salt solution; PFC, plaque-
forming cells; SRBC, sheep erythrocytes; TNP, trinitrophenol.

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(Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) as described previously (5). DNP-BGG was purified by prolonged dialysis against 0.001 M potassium phosphate buffer (pH 7.4). The concentration of the product was determined by its dry weight, and the degree of derivatization was estimated from its absorbancy at 360 nm (for DNP-lysine this was taken as 17,400). Two preparations of DNP-BGG were used, one with 55, and the other with 51 DNP groups per molecule of protein. DNP-epsilon-amino-N-caproic acid (DNP-EACA) was prepared as described previously (6). DNP-Ficoll was a gift from Dr. William Paul, National Institutes of Health, Bethesda, Md.

Thymopoietin. The thymopoietin preparation used in these studies was the biologically active pentapeptide fragment Arg-Lys-Asp-Val-Tyr. This is the sequence from 32 to 36 of the 49 amino acid protein (7). The pentapeptide was prepared by organic synthesis.

Thymopoietin Treatment. Thymopoietin was given i.p. daily in doses of 1 μg in 0.1 ml of 0.15 M NaCl, for 5 days before immunization and for the 10 working days in the 2 wk after immunization. Approximately 10^8 spleen cells were incubated with 1 μg thymopoietin in 1 ml of Hanks' Balanced Salt Solution (HBSS, Microbiological Association, Bethesda, Md.), for 15 min at 37°C.

Cell-Transfer Studies. Single-cell suspensions from spleens were prepared by teasing spleens from old or young mice in HBSS containing 0.02 mg/ml heparin (Sigma Chemical Co., St. Louis, Mo.) and enough 1 M NaOH to adjust the pH to between 7.2 and 7.4. Lethally irradiated (600 rads), thymectomized 2-mo-old BALB/c mice were reconstituted with ~10^8 spleen cells intravenously. The recipients were immunized with DNP-BGG in CFA 24 h or 7 wk after adoptive transfer.

Assay for Anti-DNP PFC. Spleens were assayed for anti-DNP PFC 2 wk after immunization by the slide method of Dresser and Greaves (8). Sheep erythrocytes (SRBC) were reacted with 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co.) according to the method of Rittenberg and Pratt (9). 50 μl of an 8% suspension of trinitrophenol (TNP) conjugated SRBC were added to 0.5 ml of 0.5% agarose. Spleen cell suspensions from individual animals were prepared by teasing individual spleens in HBSS and filtering them through a thin layer of cotton gauze. The cells were washed once and resuspended in HBSS. 50 μl of each suspension was then added to the TNP-SRBC agarose suspension and immediately poured onto microscope slides previously coated with a 0.1% aqueous solution of agarose. The slides were incubated at 37°C for 45 min and then exposed for 45 min to guinea pig serum (complement) which had been preabsorbed with SRBC and diluted 1:30. Rabbit anti-mouse immunoglobulin was diluted 1:300 (the predetermined optimal concentration) to develop indirect PFC. More than 80% of the indirect PFC were IgG antibody mediated.

Affinity of indirect anti-DNP PFC was assayed by the inhibition of PFC by DNP-EACA according to the method of Andersson (10), as modified by Goidl et al. (11). Nine concentrations of DNP-EACA phosphate saline ranging from 1 × 10^-5 M to 1 × 10^-9 M in half-log increments were added to both the TNP-SRBC agarose suspension and the complement source. This method of estimating affinity has been validated by DeLisi and Goldstein (12).

Statistical Analysis. The Mann-Whitney U test was used to test significance of differences in PFC of control and experimental groups. The heterogeneity index is derived from Shannon's formula, as described previously (13). The maximal heterogeneity of a spleen is represented by the equidisribution of information bits among all of the arbitrarily set states. The minimal heterogeneity would require all bits of information to be contained in a single subpopulation. Data from the histograms for animals in each experimental group were assembled in matrix form. Matrices to be compared were then tested for equality of variances by applying a test of the variance-ratio (F-test) to establish the applicability of the t test. Matrices were then compared by the t test. If the hypothesis of equality of variances was rejected by the F-test, the matrices were compared by Chi-square analysis. This technique permitted statistical evaluation of conclusions drawn from direct inspection of the histograms.

Results

Effect of Age and Thymectomy on the Immune Deficiency that Accompanies Aging. Mice of different ages were immunized with DNP-BGG in CFA and splenic PFC determined 14 days later (Table I). With increasing age, mice show a progressive decline in the
Effect of Age and Thymectomy on the Anti-DNP PFC Response of BALB/c Mice

A group of mice were thymectomized at 2 mo of age. Age-matched nonthymectomized and thymectomized mice were immunized with DNP-BGG in CFA at 4, 12, and 24 mo of age. 2 wk later, the number of anti-DNP PFC/spleen and their distribution with respect to affinity were determined. The data presented are the mean heterogeneity index and PFC/spleen.

Number of PFC with a preferential loss of indirect and high affinity PFC. The loss of high affinity antibody resulted in a reduction in the heterogeneity index. If these immune deficiencies that accompany aging were attributable to a loss of thymic function, thymectomy would be expected to hasten the occurrence of these immune changes. This was observed. Thus, intact 12-mo-old mice show only a 22% decline in indirect PFC and 15% decline in direct PFC whereas thymectomized 12-mo-old mice had a 60% loss of indirect PFC and a 48% loss of direct PFC (Table I). In addition, intact 12-mo-old mice did not show a loss of high affinity PFC whereas thymectomized 12-mo-old mice had lost high affinity PFC which resulted in a highly significant ($P < 0.01$) fall in the heterogeneity index (Table I). 4-mo-old mice which had been thymectomized 2 mo earlier were comparable to intact age-matched animals. This indicates that many months of thymic deficiency is required before the immune deficiencies associated with aging occur.

Effect of Thymus Gland Function on the Immune Response of Old Spleen Cells. As the immune deficiency observed during aging could be accelerated by thymectomy, the capacity of a young thymus gland to reverse the impaired immune response of spleen cells from old animals was determined. Intact or thymectomized, irradiated, syngeneic 2-mo-old mice were reconstituted with spleen cells from 2- or 24-mo-old mice. Young recipients were immunized 7 wk after cell transfer and the PFC response measured 2 wk later (Table II). Intact recipients of spleen cells from aged mice produced more indirect and high affinity anti-DNP PFC than did thymectomized recipients of aged spleen cell preparations. Thus, the presence of a young thymus gland permitted old spleen cells to generate a normally heterogeneous PFC response and an increased number of IgG PFC. The increase in heterogeneity of the PFC response reflected the reappearance of high affinity PFC (Fig. 1). Although the reconstitution of the high

### Table I

<table>
<thead>
<tr>
<th>Age of intact animals</th>
<th>Direct PFC</th>
<th>Indirect PFC</th>
<th>Heterogeneity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mo</td>
<td>4,130</td>
<td>4,935</td>
<td>2.55</td>
</tr>
<tr>
<td>12 mo</td>
<td>3,494</td>
<td>3,872</td>
<td>2.49</td>
</tr>
<tr>
<td>24 mo</td>
<td>1,018</td>
<td>810</td>
<td>1.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age of thymectomized animals</th>
<th>Direct PFC</th>
<th>Indirect PFC</th>
<th>Heterogeneity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mo</td>
<td>4,325</td>
<td>5,185</td>
<td>2.54</td>
</tr>
<tr>
<td>12 mo</td>
<td>2,443</td>
<td>2,109</td>
<td>1.72</td>
</tr>
</tbody>
</table>

A group of mice were thymectomized at 2 mo of age. Age-matched nonthymectomized and thymectomized mice were immunized with DNP-BGG in CFA at 4, 12, and 24 mo of age. 2 wk later, the number of anti-DNP PFC/spleen and their distribution with respect to affinity were determined. The data presented are the mean heterogeneity index and PFC/spleen.
TABLE II

<table>
<thead>
<tr>
<th>Donors</th>
<th>Thymus gland</th>
<th>Indirect Anti-DNP PFC/spleen</th>
<th>Heterogeneity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Present (8)</td>
<td>5,153 ± 523</td>
<td>2.56 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>Removed (7)</td>
<td>3,955 ± 760</td>
<td>2.53 ± 0.33</td>
</tr>
<tr>
<td>24</td>
<td>Present (8)</td>
<td>1,798 ± 275</td>
<td>2.43 ± 0.48</td>
</tr>
<tr>
<td></td>
<td>Removed (7)</td>
<td>941 ± 170</td>
<td>1.39 ± 0.50</td>
</tr>
</tbody>
</table>

*Spleen cells from 2- or 24-mo-old mice were transferred to 2-mo-old irradiated, intact, or thymectomized syngeneic animals. All recipients were immunized with DNP-BGG in CFA 7 wk after transfer and the splenic anti-DNP PFC response measured 2 wk later. The number of animals in each group is given in parentheses. The data presented are the mean ± the SEM.

affinity PFC response was complete, the increase in IgG PFC, though highly significant ($P < 0.03$), did not equal the IgG PFC response of recipients of young spleen cells. It should also be noted that thymectomized recipients of young spleen cells produced fewer IgG PFC than did intact recipients. No change in the distribution of PFC with respect to affinity was found when young spleen cells were maintained in thymectomized recipients for 7 wk.

Anti-DNP Response of Old Mice Treated with Thymopoietin. As a young thymus gland could ameliorate age-associated immune deficiencies, the capacity of thymopoietin to reverse the defects in immune responses of old spleen cells was tested. 24-mo-old mice were given 1 μg per day of the active pentapeptide of thymopoietin by intraperitoneal injection for 5 days before immunization and 10 days after immunization. The anti-DNP PFC response of young, old, and thymopoietin-treated old mice was compared (Table III). Injection of thymopoietin produced a highly significant ($P < 0.001$) increase in the indirect PFC response and a complete reconstitution of the normal heterogeneity with respect to affinity. The normal heterogeneity of the response resulted from an increase in high affinity PFC populations in old thymopoietin-treated mice (Fig. 2).

Anti-DNP PFC Response of Aged Spleen Cells Incubated in Vitro with Thymopoietin before Transfer to Syngeneic Animals. Spleen cells from 24-mo-old animals were incubated with culture medium containing 1 μg/ml of thymopoietin for 15 min at 37°C before their transfer to irradiated thymectomized syngeneic young recipients. Spleen cells from 2- and 24-mo-old syngeneic mice were also incubated in culture medium not containing thymopoietin before adoptive transfer. All spleen cell recipients were immunized 1 day after cell transfer and the number and distribution of splenic PFC with respect to affinity were determined 2 wk after immunization (Table IV). Incubation of aged spleen cells with thymopoietin for 15 min before adoptive transfer produced a highly significant ($P < 0.001$) increase in the number of indirect PFC and a complete restoration of the heterogeneous immune PFC response which characterizes young animals. The marked restriction in heterogeneity observed in old animals results from a loss of high affinity PFC and disappears after the in vitro treatment of old spleen cells with thymopoietin (Fig. 3).

Response of Young and Old Mice to the T-Independent Antigen DNP-Ficoll. A loss of high affinity and IgG anti-DNP PFC response of aged animals immunized with a T-
dependent antigen might reflect a defect in the T- and/or B-lymphocyte population(s). The capacity of aged mice to generate a normal anti-DNP response to the T-independent antigen, DNP-Ficoll, was tested (Table V). Although the number of direct anti-DNP PFC spleen was less in 24-mo-old animals, this was not statistically significant. The range of response in the young animals (3,000–15,000) completely overlapped the response observed in the aged animals (6,000–13,000). Furthermore, there was no difference in the distribution of PFC with regard to affinity in old as compared to young mice (Fig. 4). Thus, in aged mice, the thymic-independent anti-DNP response was less compromised than thymic-dependent anti-DNP response.
Table III

Effect of Thymopoietin Administration on the Anti-DNP PFC Response of Old Mice*

<table>
<thead>
<tr>
<th>Age of mice</th>
<th>Thymopoietin treated</th>
<th>Indirect anti-DNP PFC/spleen</th>
<th>Heterogeneity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>mo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 (9)</td>
<td>No</td>
<td>5.916 ± 213</td>
<td>2.78 ± 0.20</td>
</tr>
<tr>
<td>24 (7)</td>
<td>No</td>
<td>385 ± 79</td>
<td>1.09 ± 0.05</td>
</tr>
<tr>
<td>24 (8)</td>
<td>Yes</td>
<td>977 ± 102</td>
<td>2.48 ± 0.33</td>
</tr>
</tbody>
</table>

* 24-mo-old mice were given 1 µg of thymopoietin by the intraperitoneal route for 5 days before and 10 days after immunization. The thymopoietin-treated 24-mo-old mice and untreated 2- and 24-mo-old mice were all immunized with DNP-BGG in CFA. The number of splenic anti-DNP PFC was determined 2 wk after immunization. The number of animals in each group is given in parentheses. The data presented are the mean ± the SEM.

Discussion

We have found that two immune responses, the production of IgG and high affinity PFC, are preferentially lost during aging. The development of these immune deficiencies with age can be accelerated by thymectomy. A decline in thymic function with age may contribute to the observed immune deficiency in old mice. This thesis was supported by the improved immune response of old spleen cells transferred to irradiated, syngeneic, thymus-intact young mice. This reactivation of immune competence of old spleen cells in young recipients could be first demonstrated 7 wk after cell transfer. Previous studies have shown that old stem cells required ~50 days in young recipients to regain normal immune function (14). We have also shown that old spleen cells exposed to synthetic thymopoietin in vivo or in vitro increased their capacity to generate high affinity and IgG PFC responses. These studies suggest that a decline in thymic hormone known to occur with age (3) may contribute to the age-associated immune deficiencies.

B-lymphocyte function is less severely impaired than T-lymphocyte function in aged mice. Thus, the anti-DNP PFC response to the thymic-independent antigen, DNP-FicolI, was little if at all reduced in old animals despite their severely impaired anti-DNP response to the thymic-dependent antigen, DNP-BGG. The response of aged BALB/c mice to two other T-independent antigens, lipopolysaccharide and pneumococcal polysaccharide SIII, has been reported to be normal (15). In other strains of mice, T-independent responses were less severely impaired than T-dependent responses in aged mice (16). In some studies, T-dependent and T-independent responses were both impaired with age (17).

It is possible that there are two anti-DNP B-cell populations: one that is activated by the DNP determinant in the presence of carrier-activated T-cells; the other activated by the DNP determinant independently of T-cells. However, an age-associated defect in the B-cell population which cooperates with T-cells would not be expected to be corrected by exposure to the thymus gland or thymopoietin. Previous studies suggested that the lymphocyte population which is responsive to thymopoietin contain lymphocytes of the T lineage (18,19). It seems far more reasonable to suggest that a decline in T-cell function occurs during aging and leads to a loss of the high affinity and IgG PFC response. Impaired T-cell function in aged subjects also leads...
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Fig. 2. Each histogram illustrates the distribution of splenic indirect PFC with respect to affinity as described in Fig. 1. 24-mo-old BALB/c mice were given daily intraperitoneal injections of 1 #g thymopoietin for 5 days preceding immunization with DNP-BGG in CFA, and for 10 days after immunization. The number and distribution of splenic PFC were measured 14 days after immunization.

to a deficiency in a number of cell-mediated immune reactions (20).

Certain aged-associated immune defects can be ameliorated or reversed by grafts of young thymus glands or by the administration of thymic factors. Specifically, transplantation of thymus glands has been reported to reverse age-associated autoantibody formation (21) and to prolong life in autoimmune prone NZB/W mice (22). Furthermore, aged spleen cells incubated with a thymic humoral factor regain their in vitro graft-versus-host reactivity (23).
Table IV

Anti-DNP Response of Recipients given 24-Mo-Old Spleen Cells Incubated with Thymopoietin in Vitro*

<table>
<thead>
<tr>
<th>Age of spleen donors</th>
<th>Thymopoietin</th>
<th>Indirect anti-DNP PFC/spleen</th>
<th>Heterogeneity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (12)</td>
<td>Absent</td>
<td>6,634 ± 1,184</td>
<td>2.35 ± 0.43</td>
</tr>
<tr>
<td>24 (13)</td>
<td>Absent</td>
<td>678 ± 70</td>
<td>1.10 ± 0.47</td>
</tr>
<tr>
<td>24 (10)</td>
<td>Present</td>
<td>1,290 ± 131</td>
<td>1.89 ± 0.57</td>
</tr>
</tbody>
</table>

*Spleen cells from 2- or 24-mo-old mice were incubated in HBSS for 15 min at 37°C in the presence of 1 µg/ml of thymopoietin. The spleen cells were washed and transferred to 2-mo-old irradiated, thymectomized, syngeneic mice. All animals were immunized 1 day after transfer and the anti-DNP response was measured 2 wk later. The number of animals in each group is given in parentheses. The data presented are the mean ± the SEM.

Fig. 3. Each histogram illustrates the distribution of splenic indirect PFCs with respect to affinity as described in legend to Fig. 1. Irradiated 2-mo-old BALB/c mice were reconstituted with spleen cells from 2- or 24-mo-old syngeneic mice. All spleen cells were incubated at 37°C for 15 min before transfer either in culture media containing 1 µg/ml thymopoietin or in culture media alone. Animals were immunized with DNP-BGG in CFA 24 h after transfer. The number and distribution of splenic PFCs were measured 14 days after immunization.
Table V
Response of Old and Young Mice to the T-Independent Antigen DNP-Ficoll*

<table>
<thead>
<tr>
<th>Age of mouse</th>
<th>Direct anti-DNP PFC/spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (9)</td>
<td>9,422 ± 1,332</td>
</tr>
<tr>
<td>24 (6)</td>
<td>7,086 ± 813</td>
</tr>
</tbody>
</table>

* 2- and 24-mo-old mice were given 50 μg of DNP-Ficoll by the intraperitoneal route. 2 wk later, the splenic anti-DNP PFC response was assayed. The number of animals in each group is given in parentheses. The data presented are the mean ± the SEM.

Maintenance of Thymic-Independent Immunofunction in Old Mice

Fig. 4. Each histogram illustrates the distribution of splenic indirect PFCs with respect to avidity as described in legend to Fig. 1. 2- and 24-mo-old BALB/c mice were immunized by the intraperitoneal injection of DNP-Ficoll in saline. The number and distribution of PFCs were measured 14 days after immunization.

Our studies provide additional evidence that the thymus gland or its products can ameliorate the immune deficiencies that accompany aging. If thymic hormone replacement was used to prevent the decline in serum thymopoietin concentrations, the immune deficiencies associated with aging might be ameliorated or prevented.
That is, it may be easier to prevent the immune deficiency that accompanies aging than to reverse it.

Summary

Aged mice preferentially lose the capacity to make IgG and high affinity PFC after immunization with the T-dependent antigen DNP-BGG. We have found that thymectomy accelerates the appearances of these immune deficiencies associated with aging. When splenocytes from old mice are transferred to young lethally irradiated, syngeneic mice and the recipients immunized 7 wk later, the number of IgG and high affinity PFC was increased compared to the response of old splenocytes transferred to young thymectomized mice. These immune deficiencies of aged mice were also reversed when old mice were treated with thymopoietin in vivo or splenocytes from old mice were incubated with thymopoietin before adoptive transfer to young irradiated, thymectomized syngeneic mice. The T-cell independent response to DNP-Ficoll was less impaired than the T-cell dependent response to DNP-BGG in old animals. These data suggest that a decline in thymic function that occurs during aging may contribute to the immunological deficiencies of old animals.

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References


