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II. Loss of IgG and High Avidity Plaque-Forming Cells and Increased Suppressor Cell Activity in Aging Mice*

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Immune competence declines as humans and experimental animals grow old. Thymic (T)-dependent immunity is most severely affected by age as manifested by a depressed response to T-dependent antigens and T-dependent mitogens (1, 2) and an impaired capacity to develop delayed hypersensitivity and graft versus host reactions (3, 4). In addition, aging is associated with an increased incidence of autoantibodies (5) and monoclonal gammapathies (6). The response of aged animals to T-independent antigens and polyclonal B-cell mitogens tends to be less impaired (7). While T-lymphocyte function appears defective in aging animals it is not clear to what extent this represents a reduction in helper T-cell activity or an increase in suppressor T-cell activity.

We have studied the response of mice of different ages to dinitrophenylated bovine gamma globulin (DNP-BGG), a highly T-dependent antigen. The splenic plaque-forming cell (PFC) response of old mice was found to be reduced in number with a preferential loss of IgG and high avidity PFCs. Furthermore, spleen cells from old animals suppressed the PFC response of spleen cells from young animals in mixed cell transfer experiments. The results of these studies indicate that aging is associated with changes in both helper and suppressor T-cell function.

Materials and Methods

Animals. C57L/J and LAF mice were obtained from The Jackson Laboratory, Bar Harbor, Maine and used at 2, 12, and 26 mo of age. 2, 24, and 34 mo BALB/c mice were obtained from the Charles River Laboratories, Wilmington, Mass.

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 bovine gamma globulin (Miles Laboratory, Kankakee, Ill.) as described previously (8). DNP-BGG was purified by extensive 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 derivitization was

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† Abbreviations used in this paper: CFA, complete Freund's adjuvant; DNP-BGG, dinitrophenylated bovine gamma globulin; DNP-EACA, dinitrophenylated ε-amino-N-caproic acid; HBSS, Hanks' balanced salt solution; LPS, lipopolysaccharide; PFC, plaque-forming cell.
estimated from its absorbancy at 360 nm (ε for DNP-lysine was taken as 17,400). Two lots of DNP-BGG were used which had 50 and 38 DNP groups per molecule of protein. DNP-α-amino-N-caproic acid (DNP-EACA) was prepared as described previously (9). *Escherichia coli* endotoxin (lipopolysaccharide, LPS) was obtained from Difco Laboratories, Detroit, Mich.

**Immunization.** Mice were immunized by the intraperitoneal injection of 500 μg of DNP-BGG emulsified in complete Freund's adjuvant (CFA, containing 2 mg/ml *Mycobacterium butyricum*) contained in a final vol of 0.2 ml. The anti-DNP response in these animals is highly T dependent. When indicated, 10 μg of endotoxin dissolved in saline was injected intraperitoneally at the time of immunization.

**Cell Transfers.** All transfer studies were carried out by reconstituting lethally irradiated (600 R), thymectomized 2-mo-old BALB/c mice. Cells were prepared for transfer by teasing spleens or thymuses in Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Grand Island, N. Y.) containing 0.35 mg/ml NaHCO₃ and 0.02 mg/ml heparin sodium salt (Sigma Chemical Co., St. Louis, Mo.). The cells were filtered through cotton gauze to remove aggregated cells, washed once, and resuspended in HBSS. Cells of one type were pooled and injected intravenously into 2-mo-old syngeneic mice 2-4 h after irradiation. Recipient animals were immunized with DNP-BGG 24 h after cell transfer. The number of spleen donors equaled one-half the number of recipient animals. The recipients received from 5-7.5 × 10⁷ spleen cells.

**Assay of PFC.** Animals were sacrificed by cervical dislocation and their spleens assayed for PFCs at various times after antigen administration. Anti-DNP PFCs in the spleen were measured using the slide assay of Dresser and Greaves (10). Sheep erythrocytes (SRBC) were reacted with 2,4,6-trinitrobenzene sulfonic acid (Sigma Chemical Co., St. Louis, Mo.) according to the method of Rittenberg and Pratt (11). 50 μl of an 8% suspension of TNP-conjugated SRBC were added to 0.5 ml of 0.5% agarose. Spleen cells were obtained from individual spleens by gentle manual disruption, filtered through a thin layer of cotton gauze, washed once with HBSS, and were resuspended in HBSS. Spleen cells were added to the TNP-SRBC-agarose suspension and immediately poured onto microscope slides previously coated with 0.1% aqueous solution of agarose. Slides were incubated at 37°C for 45 min and then exposed to guinea pig complement for 45 min at 37°C. Lyophilized guinea pig complement, obtained from Grand Island Biological Company was dissolved in sterile deionized H₂O and diluted 1:7 and used as a source of complement. Rabbit anti-mouse globulin antiserum was used at a dilution of 1/300 to develop indirect plaques. The dilution of anti-mouse γ chain antiserum has been shown to bring about maximum development of indirect PFCs and inhibit direct plaque formation. In this method, more than 80% of the indirect PFCs are IgG antibody mediated. Rabbit anti-mouse μ-chain antiserum inhibited 88-100% of direct PFCs.

**Assay of Avidity of Anti-DNP PFC.** The avidity of the indirect anti-DNP PFC was assayed by the inhibition of plaque formation by various concentrations of DNP-EACA according to the method of Andersson (12), as modified by Goidl et al. (13). Nine concentrations of DNP-EACA ranging from 1 × 10⁻⁹ to 1 × 10⁻⁵ M in half-log increments were used. DNP-EACA dissolved in phosphate-buffered saline was added to both the DNP-SRBC-agarose suspension and the complement source. This method for measuring avidity has been validated by DeLisi and Goldstein (14).

**Statistical Analysis.** The Mann-Whitney "U" test has been employed to test significance of difference in PFC numbers between experimental groups. The heterogeneity index is derived from Shannon's formula, as described previously (13).

The maximal heterogeneity of a system is represented by the equidistribution 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 the 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 a chi-square analysis. This technique permitted statistical evaluation of conclusions drawn from direct inspection of the histograms.

**Results**

**Immune Response in Mice of Different Ages.** Three groups of C57L/J mice aged 2, 12 and 25 mo were immunized with DNP-BGG. The number of spleen
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Table I

Number and Heterogeneity of PFCs in C57L/J Mice of Different Ages: Primary Response*

| Age (No. of animals) | Direct PFCs$ | Indirect PFCs$ | Indirect/direct PFCs | Heterogeneity§ index |
|----------------------|--------------|----------------|----------------------|----------------------|
| 2 mo (4)             | 2,448        | 7,659          | 3.129                | 2.788                |
| 12 mo (8)            | 3,213        | 6,387          | 1.988                | 2.302                |
| 25 mo (6)            | 880          | 1,154          | 1.312                | 1.572                |

* Mice immunized with DNP-BGG in Freund’s adjuvant were sacrificed 2 wk later. The number and distribution of splenic PFCs with regard to avidity were measured.
$ Geometric mean of group given.
§ Shannon function of PFC heterogeneity with respect to avidity.

PFCs and the distribution of PFCs with regard to avidity were determined 2 wk after immunization. The number of both direct and indirect PFCs in 25-mo-old animals was significantly ($P < 0.02$) reduced when compared to the number of splenic PFCs in 12- or 2-mo-old animals (Table I). The decrease in PFCs per spleen in 25-mo-old mice was not due to a decrease in spleen size, since the number of spleen cells obtained from 25-mo-old animals averaged 12% more than the number of spleen cells obtained from 2-mo-old animals. During aging there is a preferential loss of indirect PFCs. Thus, as illustrated in Table I, the ratio of IgG to IgM PFCs in the primary response falls progressively with age. The difference between the values at 2 mo and 25 mo of age is statistically significant ($P < 0.02$).

The distribution of PFCs with regard to avidity was determined by hapten inhibition of plaque formation in mice of different ages. The heterogeneity of the PFC response during aging is illustrated in Fig. 1. PFCs from spleens of 2-mo-old mice were most heterogeneous with regard to avidity and were of the highest average avidity. With aging, there was a progressive decrease in the heterogeneity characterized by a progressive loss of high avidity PFC subpopulations in older animals. The distribution of affinity illustrated may be quantitated using the Shannon function (13) as an heterogeneity index, thereby permitting simple statistical evaluation of the data. This index is expressed as a log2 term and increases with increasing heterogeneity of the PFC distribution. The heterogeneity indices calculated from these data show a significant decrease in heterogeneity of splenic PFCs with age (Table I). Thus, indirect PFCs from 12-mo-old animals are significantly ($P < 0.05$) less heterogeneous than PFCs from 2-mo-old animals, and PFCs from 25-mo-old animals are significantly ($P < 0.01$) less heterogeneous than PFCs from 12-mo-old animals.

As the C57L/J is a relatively long-lived strain of mouse, it was felt that age-related changes in the immune responses might be more dramatically seen over this same time period in the shorter-lived BALB/c mouse. Similar studies were therefore carried out in BALB/c mice, and similar results were obtained (Table II). Thus, a decrease in the number of PFCs ($P < 0.004$) in the ratio of IgG/IgM PFCs and in the heterogeneity index ($P < 0.001$) is seen in aged 24-mo-old mice as compared to young 2-mo-old mice.

The secondary immune response of aged BALB/c mice was also studied. The characteristic features of the primary immune response of aged mice were also
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C57 L/J; 500 µg DNP-BGG; indirect PFC

Fig. 1. Each histogram illustrates the distribution of splenic indirect PFCs with respect to avidity. C57L/J mice of different ages were immunized with DNP-BGG in CFA and sacrificed for PFC assay 14 days later. The abscissa represents the log of the inverse of the free hapten concentration used in the inhibitory assay. The ordinate represents the percent of the total population of PFCs present in each subpopulation. The animal identification number and the total indirect per spleen are given in the upper right of each histogram. Avidity increases to the right.

seen in their secondary responses. Thus, the immune response of aged animals was significantly lower in magnitude ($P < 0.005$) and restricted with regard to heterogeneity of avidity ($P < 0.004$). The aged mice failed to develop high avidity PFCs in their secondary response (Table II and Fig. 2). The restriction of
Table II
Number and Heterogeneity of PFCs in Young and Old BALB/c Mice: Primary and Secondary Response*

| Age (No. of animals) | Primary response | Secondary response |
|----------------------|------------------|--------------------|
|                      | Direct PFCs‡     | Indirect PFCs‡    | Indirect/direct PFCs | Heterogeneity index§ |
| 2 mo (6)             | 9,539            | 10,706             | 1.128                | 2.820                 |
| 24 mo (5)            | 1,882            | 1,991              | 1.058                | 1.755                 |
|                      | 7,966            | 9,212              | 1.156                | 2.616                 |
| 24 mo (6)            | 1,452            | 1,280              | 0.882                | 1.749                 |

* Mice were immunized with DNP-BGG in Freund's adjuvant and either sacrificed 2 wk later or boosted 33 days later with 0.5 mg DNP-BGG in saline. The number and distribution of splenic PFCs were measured 5 days after the boost.

‡ Geometric mean of group given.

§ Shannon function of PFC heterogeneity with respect to avidity.

Fig. 2. 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 with DNP-BGG in CFA and boosted 33 days later with 500 μg DNP-BGG in saline intraperitoneally. The number and distribution of splenic PFCs were measured 5 days after the boost.

heterogeneity, the decrease in the magnitude of both the direct and indirect PFC response, and the preferential loss of high avidity indirect PFCs were comparable in the primary and secondary immune response (Table II).

Immune Response of Irradiated Mice Reconstituted with Spleen Cells from Syngeneic Animals of Different Ages. 2-mo-old irradiated mice were reconstituted with spleen cells from 2-mo-old or 24-mo-old syngeneic animals. Some recipients, in addition to spleen cells from 24-mo-old mice, received young thymus cells. All recipients were immunized with DNP-BGG and splenic PFCs determined 2 wk later. Irradiated recipients of spleen cells from young syngeneic animals had an immune response comparable to young intact animals with respect to avidity distribution of PFCs and ratio of indirect to direct PFCs
although the absolute number of PFCs was lower. The characteristic reduction in number of PFCs and restriction in heterogeneity of PFCs which was described for old animals was also seen in young recipients of spleen cells from old donors (Table III). When recipients of old spleen cells were also given $10^8$ young thymus cells, there was an increase in the number of indirect PFCs and a striking increase in heterogeneity of PFCs associated with the appearance of high avidity indirect PFCs, as compared to recipients given old spleen cells alone (Fig. 3). Animals reconstituted with spleen cells from old donors have significantly fewer PFCs ($P < 0.004$) and suffer a preferential loss of IgG ($P < 0.004$) and high avidity PFCs ($P < 0.003$) as compared to recipients of spleen cells from young mice (Table III). Recipients of spleen cells from old donors together with young thymus cells from young mice had a statistically significant ($P < 0.02$) increase in the magnitude of their indirect PFC response, in their ratio of IgG/IgM PFCs ($P < 0.004$), and in their heterogeneity index ($P < 0.01$) when compared to young recipients which received only spleen cells from old donors.

**Suppression of BALB/c Spleen PFCs by Syngeneic Cells from Aged Animals.** Spleen cells from old animals not only transfer the immune response characteristic of aged animals to young recipients but also suppress the expression of the immune response of spleen cells from young donors in mixed cell transfers. Thus, the number of PFCs that are produced by recipients of spleen cells from young donors were significantly depressed (direct $P < 0.05$, indirect $P < 0.02$) when spleen cells from old and young mice were mixed before transfer (Table IV). However, spleen cells from old mice do not suppress preferentially indirect or high avidity PFCs. The reduced PFC response is not simply a consequence of a greater number of spleen cells transferred. When the total number of spleen cells from 2-mo-old donors was increased to equal the number of spleen cells in the mixture cell transfer, there was no decrease in the PFC response. The suppressive activity demonstrable in spleens from aging animals was directly related to the age of the spleen cell donor, as illustrated in Fig. 4. The capacity of spleen cells to suppress the immune response of 2-mo-old spleen cells increased with age from 12 to 34 mo of age.

| Cells used to reconstitute mice (No. of animals) | Direct PFCs | Indirect PFCs | Indirect/direct PFCs | Heterogeneity index |
|------------------------------------------------|-------------|---------------|----------------------|---------------------|
| 2-mo spleen (8)                                 | 2,068       | 3,617         | 1.758                | 2.595               |
| 24-mo spleen (7)                                | 521         | 603           | 1.087                | 1.546               |
| 24-mo spleen and 2-mo young thymus cells (10⁸) (6) | 540         | 1,050         | 1.944                | 2.166               |

* Irradiated mice were reconstituted with spleen cells from young or old syngeneic mice. In one group, $10^8$ thymus cells were administered with old spleen cells. Mice were immunized 24 h after cell transfer and splenic PFCs determined 2 wk later.
† Geometric mean of group given.
Fig. 3. Each histogram illustrates the distribution of splenic indirect PFCs with respect to avidity 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. In one group, 10^8 thymus cells from 2-mo-old donors were administered with old spleen cells. Mice were immunized 24 h after cell transfer and splenic PFCs determined 2 wk later.

TABLE IV

Suppression of Young BALB/c Spleen PFC Response by Old BALB/c Spleen Cells*

| Cells used to reconstitute mice (No. of animals) | Direct PFCs* | Indirect PFCs* | Indirect/direct PFCs | Heterogeneity index* |
|-----------------------------------------------|--------------|----------------|----------------------|---------------------|
| 2-mo spleen (8)                               | 2,616        | 3,386          | 1.295                | 2.394               |
| 2-mo and 24-mo spleen (10)                    | 1,606        | 1,734          | 1.084                | 2.184               |

* Irradiated mice were reconstituted with spleen cells from young syngeneic animals or a mixture from young and old animals. The mice were immunized 24 h later and splenic PFCs determined 2 wk later.

§ Geometric mean of group given.

Effects of Endotoxin on the Immune Response in Mice of Various Ages. Goidl et al. (13) found that irradiated mice reconstituted with fetal B lymphocytes have an immune response of restricted heterogeneity. Injection of these mice with endotoxin at the time of immunization converts their response to a heterogeneous "adult type" response. The possibility that endotoxin might increase the heterogeneity of the PFC response of old animals was tested. 2, 12, and 25-mo-old C57L/J mice were immunized with DNP-BGG in CFA and given
Fig. 4. Irradiated 2-mo-old BALB/c mice were reconstituted with $10^8$ spleen cells from 2-mo-old syngeneic mice with which were mixed an equal number of spleen cells from 2, 12, 24, or 34-mo-old syngeneic donors. The mice were immunized 24 h later and splenic PFCs determined 2 wk later. Two experiments were then performed: in one the effect of spleen cells from 2, 12, and 24-mo-old animals was compared; in the other, the effect of spleen cells from 12, 24, and 34-mo-old mice was compared. Points illustrated represent the geometric mean of 4–6 animals in each group.

10 $\mu$g of endotoxin. Instead of increasing heterogeneity of PFCs, endotoxin accentuated the age-related restriction in heterogeneity (Fig. 5 compared with Fig. 1 and footnote to Fig. 5). Although this effect of endotoxin was seen at every age tested, the most profound restriction in heterogeneity of PFCs was seen in 12- and 25-mo-old mice. As discussed above, normal 25-mo-old mice display restricted heterogeneity of anti-DNP PFCs with two out of six animals having only four low avidity PFC subpopulations present (Fig. 1). However, when endotoxin was given to 25-mo-old mice, a marked reduction in heterogeneity was observed with 3 out of 7 mice tested having only a single subpopulation of PFCs with the lowest avidity demonstrable (Fig. 5).

Discussion

We have studied the immune response of aging mice to a highly T-dependent antigen, DNP-BGG. A progressive decline in the magnitude of the splenic anti-DNP-PFC response occurred in mice between 2 and 34 mo of age. Aged animals exhibited a preferential loss of direct (IgG) PFCs and high avidity PFCs in both their primary and secondary response. The characteristic immune response of aged animals could be transferred to young irradiated syngeneic mice with
spleen cells from old donors. Using this transfer model, we have found evidence for impaired helper and augmented suppressor cell activity in old animals.

As T cells are important in the generation of high affinity and IgG antibody (15, 16), impaired helper activity in old mice might explain their loss of high
avidity and IgG PFCs. This was supported by the capacity of young thymus cells to increase the number of indirect and high avidity PFCs in recipients of spleen cells from old donors. On the other hand, the capacity of spleen cells from old animals to suppress the response of young spleen cells to DNP-BGG demonstrated an increased suppressor cell activity in spleens of old mice. Suppressor activity in the spleen increased directly with the age of the donor. Suppressor activity of spleen cells from old donors did not preferentially inhibit indirect or high avidity PFCs. Thus, the increased suppressor activity cannot account for all of the effects of aging on the immune response, and it appears that altered activity of both suppressor and helper cells contributes to the impaired immune function in aged animals. Increased presumably nonspecific suppressor activity appears to reduce the magnitude of the immune response, while impaired T-cell helper activity contributes to the preferential loss of indirect and high affinity PFCs.

The accentuation by LPS of the age-associated restriction of PFC heterogeneity contrasts with the LPS-induced reversal of restricted heterogeneity when fetal B cells were transferred to young adult mice (13). This implies a different mechanism for the failure of B cells from immature and from aged animals to produce high affinity PFCs.

Evidence suggesting altered helper or suppressor cell activity during aging can be found in the literature. Makinodan and Peterson (17) showed that 7S antibody was more severely depressed than 19S antibody with age. Kishimoto et al. (18) found that the avidity of splenic anti-TNP PFCs generated in vitro was lower when the spleen donor was 18 mo of age, as compared with donors 2 mo of age. Segre and Segre (19) showed that theta-positive spleen cells from immunized 30-mo-old mice suppressed the secondary response of spleen cells from 3 to 4-mo-old mice. However, unprimed spleen cells from old mice did not suppress the secondary immune response of primed young spleen cells. While T-dependent immune reactions appear to be very severely impaired with age, Gerbase-DeLima et al. (7) have shown that the immune response to LPS, a T-independent antigen, was also impaired. This is particularly interesting in that we might have expected to see an increased response to LPS with age if only T-dependent function were impaired in aged animals. Kerbel and Eidinger (20) have shown that T-cell depletion augments the response to LPS. Thus, the major cause of age-related immunologic impairment appears to be thymic dysfunction, although B-cell function is also, but less severely, altered.

The marked loss of T-dependent immunity is manifested by the impaired capacity to respond to T-dependent antigens or mitogens and to decreased delayed hypersensitivity and graft versus host reactions. A role of the thymus gland in the age-related immune defects is suggested by its morphological involution and the decline in serum thymic hormone levels which accompanies aging (21). In addition, thymic hormone can reverse the impaired capacity of spleen cells from aged animals to induce the graft versus host reaction (4). While it was found that thymus cells from young donors could increase the production of high affinity PFCs by spleen cells from old donors, the restoration of high affinity PFC production was clearly only partial when compared with recipients of spleen cells from young donors (Fig. 3). This might, of course, be merely a quantitative difference which could be overcome by further increasing the
helper cell population obtained from young donors. However, the possibility of an independent defect in the B-cell population clearly exists. The failure of LPS to increase the high avidity PFC response may support this interpretation. Thus, the lack of high avidity PFCs in old mice may reflect a deficiency in the B-cell as well as T-cell population.

Summary

The magnitude and heterogeneity of the immune response to dinitrophenylated bovine gamma globulin was measured in aged and young mice at a cellular level using an inhibition of plaque-forming cell assay. The primary and secondary responses of 24-mo-old mice were markedly depressed in magnitude and restricted in avidity for the DNP determinant when compared to 2-mo-old animals. Bacterial lipopolysaccharide given at the time of immunization increased the restriction in heterogeneity seen in 12- and 24-mo-old mice. Indirect PFCs were more severely depressed than direct PFCs in 24-mo-old mice. Syngeneic, lethally irradiated, 2-mo-old mice reconstituted with aged spleen cells exhibit the depressed and restricted response to DNP-BGG seen in old mice. When 10^8 young thymus cells were given together with old spleen cells, the heterogeneity of the response was increased. When 2-mo- and 24-mo-old spleen cells were transferred together into young recipients the magnitude of the response to the young spleen cells markedly reduced. Thus, there appears to be a loss of thymic-helper cells and an increase in suppressor activity in aged animals.

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