PRODUCTION OF AUTO-ANTIIDIOTYPIC ANTIBODY DURING
THE NORMAL IMMUNE RESPONSE

VII. Analysis of the Cellular Basis for the Increased Auto-antiidiotype
Antibody Production by Aged Mice*

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A decline in immune responsiveness with age has been well documented (1-10). It
is known that the B cell function of aged mice is relatively more intact than T cell
function, at least quantitatively. Qualitative differences in B cell function do exist,
including a decreased affinity of antibody produced by cells from old mice (3) and an
enhanced tendency to produce autoantibodies (1). In a previous report (11) we have
suggested that part of the age-associated decline in the immune response is due to an
increased production of auto-antiidiotype antibodies (anti-Id)¹ by old mice (11). A
high auto-anti-Id response by aged animals was also suggested by studies of Klinman
(12). Such an increase in auto-anti-Id has been demonstrated in both thymic-inde-
pendent (13) and thymic-dependent (14) responses. We describe here a series of
experiments that analyze the cellular basis for the increased production of auto-anti-
Id during the immune response of aged mice to trinitrophenylated-lysyl-Ficoll (TNP-
F). We sought to determine whether the elevated production of auto-anti-Id by old
mice is stable upon transfer of spleen cells to irradiated recipients, and whether B or
T cells were primarily responsible for this phenomenon. The results to be reported
support the view that this age-related alteration in the immune response reflects a
change in the peripheral mature B cell population rather than a change in the
immature B cell population in the bone marrow, and that peripheral T cells play an
important role in determining this property of peripheral B cells.

Materials and Methods

Animals. AKR/J female mice were purchased from The Jackson Laboratory, Bar Har-
bor, ME. Male C57BL/6 mice, of various ages, were obtained from a colony maintained by the
National Institute of Aging at the Charles River Breeding Laboratories, Wilmington, MA.

Antigens, Antisera, and Immunization. The preparation of TNP-F and TNP-bovine gamma
globulin (BGG) has been described previously (13, 15). Animals were immunized by the
intravenous injection of 10 μg of TNP-F in saline, or by the intraperitoneal injection of 500 μg

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National Institutes of Health.

¹ Abbreviations used in this paper: anti-Id, antiidiotype antibody; BGG, bovine gamma globulin; C,
complement; CFA, complete Freund's adjuvant; ELISA, enzyme linked immunosorbent assay; Id, idiotype;
PFC, plaque-forming cell(s); TNP, 2,4,6-trinitrophenyl group; TNP-F, 2,4,6-trinitrophenyl-lysyl-Ficoll.

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TNP-BGG emulsified in complete Freund's adjuvant (CFA). The anti-μ was prepared by immunizing a goat with 100 μg MOPC-104E in CFA. The antiserum was rendered immunospecific by absorption with normal mouse IgG. Anti-Thy-1.2 was produced by immunizing a goat with 100 μg MOPC-104E in CFA, obtained from Dr. N. L. Warner, Becton Dickinson & Co., Mountain View, CA, and grown intraperitoneally in pristane pretreated, 350-rad-irradiated BALB/c mice. The antibody containing ascites fluid was made 50% saturated (cold) in (NH₄)₂SO₄. The precipitate was collected, washed, dissolved, dialyzed, and applied to a DE-52 column, and the rat IgG2b anti-mouse Thy-1.2 was eluted with a Tris-phosphate buffer gradient.

*Enzyme-linked Immunosorbent Assay (ELISA) for Auto-anti-Id.* A serological assay for auto-anti-Id has been described in detail elsewhere (16). Briefly, anti-TNP antibody was purified from sera of mice injected with 10 μg TNP-E, 7 d before bleeding by passage over a TNP-BSA-Sepharose 4B affinity column. The affinity-purified antibodies were coupled to alkaline phosphatase (calf intestine Sigma Type VII; Sigma Chemical Co., St. Louis, MO) by a 2-h incubation in 0.2% glutaraldehyde. Serum to be tested for auto-anti-Id was adsorbed to polyvinyl microtiter wells (Cooke-Dynatech, Alexandria, VA) by standing at a 1:50 dilution overnight at 4°C. The presence of auto-anti-Id was detected by a 6-h incubation with enzyme-labeled Id (purified anti-TNP antibody) and a subsequent 1-h incubation with the enzyme substrate paranitrophenyl phosphate at 37°C. Background binding was determined with age-matched normal sera adsorbed to the wells.

*Plaque-forming Cell (PFC) Assays.* Antibody-secreting cells were enumerated by a slide modification of the Jerne PFC assay (16, 17) in agarose using TNP-conjugated sheep erythrocytes as targets as previously described (18).

*Assay of Auto-anti-Id by Hapten-augmentable PFC.* Enumeration of hapten-augmentable PFC as previously described (15, 19) was used as an assay for the presence of auto-anti-Id blocked potential PFC.

*Results*

**Increased Auto-anti-Id Production by Aged TNP-F-immunized Mice.** Young and old C57BL/6 mice were immunized with 10 μg TNP-F and their serum was assayed for direct binding of enzyme-labeled Id on day 7. Binding of anti-TNP Id by old mice was slightly more than twofold that of young syngeneic animals (Table I). Similar observations have been obtained with TNP-F-immunized old and young AKR mice (data not presented). The serological results are thus consistent with our previous observations using hapten-augmentable PFC and hapten-reversible inhibition of plaque formation as assays for auto-anti-Id. It should be noted that since the affinity-
purified Id probes were derived from young animals, the binding by old sera represents a minimum value, as we have previously found (11) that there is only a partial and not a complete sharing of Id between young and old animals.

The Marked Auto-anti-Id Production During the Immune Response to TNP-F by Aged Mice is Stable upon Spleen Cell Transfer. Spleen cells were obtained from 4- or 18-mo-old C67BL/6 male donors. Pools of cells from three to five donors were transferred (5 × 10^7 cells/recipient) into lethally irradiated 8–12-wk-old syngeneic recipients. Cells were administered intravenously along with 10 μg of TNP-F within 3–4 h after irradiation. The splenic anti-TNP PFC response was assayed 7 d later, and hapten-augmentable PFC were enumerated. It should be noted that we have previously published evidence (15, 19) that hapten-augmentable PFC are B cells whose secretion of antibody has been reversibly inhibited by the binding of auto-anti-Id to cell surface idiotype. The results are presented in Table II. All recipients of aged spleen cells show a high percentage of hapten-augmentable PFC, while the recipients of spleen cells from young donors have relatively few hapten-augmentable PFC. Thus, the high percentage of hapten-augmentable PFC, which we have previously shown to be
characteristic of the immune response of aged mice (11), is also seen in mice reconstituted with spleen cells from aged donors.

Significantly, when thymus cells from 8-12-wk-old syngeneic donors were admixed with spleen cells from aged donors, the production of auto-anti-Id remains high. This is true even though the magnitude of the anti-TNP PFC response tends to be slightly increased by the addition of thymus cells from young donors.

**Inability of Bone Marrow Cells from Aged Donors to Transfer Marked Auto-anti-Id Production.** As reported above, aged mice produce unusually high auto-anti-Id responses, and spleen cells from normal aged donors transfer this marked auto-anti-Id production to irradiated recipients. To determine if this age-related change is limited to the peripheral lymphoid system, or also affects the bone marrow, studies were carried out on irradiated mice reconstituted with bone marrow from donors of various ages. Bone marrow cells (5 × 10^7) were injected intravenously, together with 10 μg TNP-F (Table III). Although the bone marrow cells do reconstitute the recipients to produce a modest anti-TNP-F response, they do not produce a significant auto-anti-Id response as assayed by hapten-augmentable PFC. Recipients of bone marrow from old and young donors are indistinguishable.

**Relative Thymic Independence of the Auto-anti-Id Response of Aged Mice.** In previous studies on young mice, we have shown that T cell-deficient animals, immunized with TNP-F, fail to produce auto-anti-Id (22). Nevertheless, the “transfer” of auto-anti-Id production from TNP-F-primed euthymic mice into normal recipients is relatively T cell independent (15). Since nonirradiated recipients were used in these earlier studies, it was uncertain whether donor or recipient cells were producing the anti-Id. To further explore the thymic dependence of auto-anti-Id production, lethally irradiated, thymectomized or euthymic C57BL/6 mice (8-12 wk old) were reconstituted with spleen cells from 18-mo-old syngeneic donors. In some cases the donor spleen cells were treated with anti-Thy-1.2 monoclonal antibody plus complement (C) before transfer to remove T cells. Recipients were given 10 μg TNP-F together with the

| Experiment | Age of bone marrow donors | Direct anti-TNP PFC (PFC/spleen ± SE) | Percent augmentation of PFC by hapten | mo |
|------------|---------------------------|----------------------------------------|--------------------------------------|----|
| 1          | 2-3                       | 1,760 ± 890 (3)                         | 15 ± 15                              | 18 |
|            | 18                        | 1,860 ± 690 (4)                         | 35 ± 40§                             |    |
| 2          | 2-3                       | 4,000 ± 600 (4)                         | 13 ± 3§                              |    |
|            | 18                        | 1,380 ± 220 (4)                         | 7 ± 5                                |    |

* Bone marrow cells (5 × 10^7) from 2-3- or 18-mo-old C57BL/6 male mice were transferred by the intravenous route into lethally irradiated (950 rad) syngeneic 2-3-mo-old male recipients, along with 10 μg of TNP-F. Recipients were killed and their anti-TNP PFC and hapten-augmentable PFC responses were assayed 7 d after antigen administration.

‡ Direct anti-TNP PFC responses are reported as arithmetic means ± SE. The number of mice assayed is indicated in parentheses.

§ The percentages of augmentation of PFC by hapten for the four mice in this group were 0, 20. 0 and 138%.
TABLE IV
Effect of the Removal of T Cells upon the Production of Auto-anti-Id Antibody by Lymphocytes Obtained from the Spleen of Old Mice*

| Experiment | Treatment of cells | Recipients | Direct anti-TNP PFC (PFC/spleen ± SE) | Percent augmentation of PFC by Hapten % ± SE |
|------------|--------------------|------------|--------------------------------------|-----------------------------------------------|
| 1          | C Thymectomized    | Thymectomized | 1,490 ± 250                         | 42 ± 13                                       |
| 2          | Anti-Thy-1.2 + C   | Thymectomized | 2,000 ± 250                         | 43 ± 13                                       |
| 3          | C Nonthymectomized | Nonthymectomized | 1,320 ± 370                        | 146 ± 80                                      |
| 4          | Anti-Thy-1.2 + C   | Nonthymectomized | 1,425 ± 440                        | 68 ± 50                                       |
| 5          | C Nonthymectomized | Nonthymectomized | 3,590 ± 670                        | 299 ± 91                                      |
| 6          | Anti-Thy-1.2 + C   | Nonthymectomized | 10,520 ± 7000                      | 107 ± 7                                       |

* Lethally irradiated, thymectomized, or euthymic C57BL/6 mice were reconstituted with spleen cells from 18-mo-old donors. Where indicated the spleen cells were treated with anti-Thy-1.2 and complement before transfer to remove T cells. Recipients were given 10 μg TNP-F together with the spleen cells and were killed 7 d after cell transfer for assay of direct splenic anti-TNP PFC and hapten-augmentable PFC. The results are presented as arithmetic means ± SE for groups of five mice.

spleen cells. As shown in Table IV, anti-Thy-1.2 and C treatment, while perhaps reducing somewhat the incidence of hapten-augmentable PFC, certainly does not eliminate the ability of the spleen cells from aged donors to transfer the capacity to produce a marked auto-anti-Id response to the irradiated recipients. The results imply that the auto-anti-Id response by the spleen cell population of unprimed aged mice is relatively T independent. Adequate T cell killing by the anti-Thy-1.2 and C was shown by the lack of anti-TNP PFC response after immunization with the T-dependent antigen TNP-BGG (response went from 6,400 ± 400 indirect PFC/spleen in the presence of T cells to 600 ± 200 indirect PFC/spleen after treatment with anti-Thy-1.2 and C).

Failure of Spleen Cells from Aged TNP-F Immune Mice to Transfer More Enhanced Auto-anti-Id Production to Age-matched Recipients. Although the hapten-augmentable PFC component of normal AKR mice is not affected by the transfer of normal spleen cells before injection of TNP-F, normal mice receiving TNP-F immune spleen cells from young mice at the time of TNP-F injection have a high auto-anti-Id component in their response to TNP-F (15). It was therefore of interest to determine whether spleen cells from aged mice, which confer on irradiated recipients the capacity to produce a marked auto-anti-Id response even when the cell donors have not been immunized with TNP-F, would permit old recipients to make a still higher auto-anti-Id response if taken from immunized old donors.

Spleen cells were transferred from old TNP-F immunized or normal C57BL/6 or AKR/J donors into age-matched, nonirradiated, syngeneic recipients together with 10 μg TNP-F. Spleens of recipient mice were assayed for anti-TNP PFC and hapten-augmentable PFC 4 d after cell transfer (Table V). In neither strain does the transfer of day-7 immune spleen cells from old donors result in a higher auto-anti-Id response in the recipients than does the transfer of normal spleen cells from donors of the same age. In every experiment, although the anti-TNP response is somewhat higher in recipients of immune spleen cells, the number of hapten-augmentable PFC per spleen
Failure of Immunization of Donors to Increase Hapten-augmentable PFC in the Recipient of Spleen Cells from Aged Mice

| Strain   | Age | Immune status | Direct Anti-TNP PFC/ spleen ± SE | Percent augmentation of PFC by hapten |
|----------|-----|---------------|----------------------------------|--------------------------------------|
| C57BL/6  | 18  | Immune        | 3,940 ± 880 (4)‡                | 132                                  |
| C57BL/6  | 18  | Naive         | 2,120 ± 520 (5)                 | 372                                  |
| AKR      | 7   | Immune        | 38,400 ± 5,450 (8)              | 92                                   |
| AKR      | 7   | Naive         | 16,700 ± 4,600 (5)              | 89                                   |

* 5 x 10^7 spleen cells from TNP-F immune or naive mice of the age and strain indicated were transferred into nonirradiated syngeneic recipients of the same age together with 10 µg TNP-F. Immune donors had received 10 µg TNP-F 7 d before killing. Recipients were killed for assay of splenic anti-TNP PFC and hapten-augmentable PFC 4 d after cell transfer.

Parentheses indicate number of animals studied.

We have previously reported that old mice produce a marked auto-anti-Id response using hapten-augmentable PFC as an assay for auto-anti-Id (11). This conclusion was confirmed serologically in the present studies, where it was shown that the serum from old mice contained better than twofold higher levels of auto-anti-Id than did serum from young syngeneic mice. We have found a good correlation between anti-Id assayed by ELISA and the number of hapten-augmentable PFC present in the spleen. This is consistent with the possibility that hapten-augmentable PFC can be used as a marker for auto-anti-Id activity. Clearly mechanisms other than auto-anti-Id can potentially lead to the presence of hapten-augmentable PFC (23–26). Regardless of
Influence of Splenic T Cells on the Incidence of Hapten-augmentable PFC*

| Age of cell donors | Bone marrow T cell donor | Direct anti-TNP PFC/ spleen + SE | Percentage augmentation of PFC by hapten |
|-------------------|--------------------------|----------------------------------|----------------------------------------|
| mo                |                          | mo % ± SE                        | % ± SE                                 |
| 2-3               | 2-3                      | 2,260 ± 460 (6)$§               | 12 ± 4                                  |
| 2-3               | 18                       | 1,210 ± 190 (9)                  | 55 ± 12                                 |
| 18                | 2-3                      | 2,280 ± 490 (10)                 | 16 ± 5                                  |
| 18                | 18                       | 1,510 ± 560 (7)                  | 38 ± 12                                 |

* Male C57BL/6 mice were lethally irradiated (850 rad) and were reconstituted by the intravenous injection of 2.5-3.5 × 10⁷ bone marrow cells obtained from donors of the indicated age and 2-4 × 10⁷ B cell-depleted spleen cells from donors of the indicated age. Recipients were immunized by the intravenous injection of 10 μg TNP-F 5 d after cell transfer and were killed for assay of splenic anti-TNP PFC and hapten-augmentable PFC 7 d later. Data are presented as mean ± SE.

$§$ Parentheses indicate number of animals studied.

the mechanism, a high incidence of hapten-augmentable PFC is characteristic of the immune response of aged mice (11). The main thrust of this report is to identify the cellular requirements for the expression of high levels of hapten-augmentable PFC in aged mice. We have shown that this property of the immune response of old mice is stable upon transfer of spleen cells from old donors into young irradiated recipients and therefore appears to be intrinsic to the peripheral lymphoid cell population rather than an effect of the internal milieu of the aged animal. Furthermore, since neither addition of thymocytes from young donors nor elimination of T cells modifies the auto-anti-Id response of transferred spleen cells from old donors, it appears likely, although not formally proven, that the marked auto-anti-Id response of aged mice is an inherent property of their B cell population.

In contrast to splenic cells from aged mice, which produce a marked auto-anti-Id response in a cell transfer recipient, bone marrow cells from aged mice do not exhibit this property. That is, bone marrow cells from aged donors behave, in this respect, like the bone marrow or peripheral B cell population of young mice. Thus, it appears that the production of marked auto-anti-Id by aged mice is a property of the peripheral lymphoid system and not of the bone marrow cell population. It is interesting to note that Harrison et al. (27, 28) have reported that the stem cell activity of the bone marrow from old mice is comparable to that of young animals, provided tumor-bearing animals are excluded as donors and sufficient time is allowed for recovery in the reconstituted host.

Depletion of T cells from the spleen cell population of aged mice does not eliminate its ability to reconstitute irradiated recipients to produce a marked auto-anti-Id response. This was true despite the fact that the T cell depletion was sufficient to essentially eliminate the response to the T-dependent antigen TNP-BGG. Thus, the auto-anti-Id response of the spleen cell population of aged mice appears to be relatively T cell independent. This is in contrast to the T cell dependence of the auto-anti-Id response of young mice as indicated by the failure of nude mice to produce
auto-anti-Id during the immune response to TNP-F (22). Previous studies have shown that spleen cells from TNP-F immune mice produce far more hapten-augmentable PFC upon transfer than do spleen cells from normal mice, and that this increased auto-anti-Id response is transferable with anti-Thy-1 and C-treated spleen cells (15). Thus, operationally, spleen cells from unprimed old donors behave like spleen cells from primed young donors. The interpretation that the “normal” spleen cell population of aged mice behaves like an “immune” spleen cell population with respect to auto-anti-Id production is consistent with our finding that immunization of old donors does not augment the ability of their spleen cells to transfer auto-anti-Id production as it does with young donors (15). This failure to transfer higher anti-Id production after priming can be explained in various ways. (a) A continued cycling of idiotype followed by auto-anti-Id production, such as described by others (29), could be the basis for this phenomenon. (b) An alternative explanation is based upon our previous studies indicating changes in Id repertoire expression at different ages (11) or perhaps more importantly at different times after immunization (E. A. Goidl, G. W. Siskind, and G. J. Thorbecke, manuscript in preparation.) After all, hapten augmentation of PFC can only be expected to occur when the Id of the PFC and the anti-Id production correspond. (c) It is possible that the high anti-Id response is self-limiting, in that it stimulates an anti-anti-Id response that prevents further expansion of the anti-Id response. (d) Another possible explanation is that the anti-Id response is already primed and further boosting does not yield a detectable increase by our assay techniques.

Thus, with respect to its capacity to produce an auto-anti-Id antibody response, the spleen cell population of aged mice behaves in a number of ways as if it had been previously primed: (a) there is a very marked auto-anti-Id response; (b) transfer of this auto-anti-Id response of the aged is relatively T cell independent; (c) priming the aged donor does not augment the ability of its spleen cell population to transfer an auto-anti-Id response as it does with young donors. It is possible that the continual exposure to self-immunoglobulins and immune complexes throughout life leads to a gradual increase in the incidence of auto-reactive clones capable of producing auto-anti-Id. Superficially, this may be viewed as parallel to the well known increase in the incidence and concentration of autoantibodies with age (30–32). While the general mechanisms responsible for increased autoantibody formation in the aged are not understood, the situation with the immune system may be special. It seems likely that, as proposed by Jerne (33), Id-anti-Id interactions represent a normal regulatory mechanism in the immune system. Such interactions, involving both B and T cells as well as serum antibodies, would be expected to occur throughout life in response to shifts in the distribution of idiotypes as a consequence of exposure to environmental antigens.

It is of particular interest that the altered (high) anti-Id response characteristic of the aging animal is not seen in mice reconstituted with bone marrow from old donors. This implies that the B cell population produced by precursor cells in the marrow of old donors is similar in respect to incidence of B cells with anti-Id specificity to that produced by bone marrow of young mice. If the bone marrow of old mice produces a distribution of B cells comparable to that of young mice, what determines the altered distribution of B cells in the peripheral lymphoid system of old animals? We would propose that the distribution of idiotypes among the peripheral T cell popu-
lation is responsible for modifying the distribution of idiotypes in the B cell population from that which arises from the bone marrow stem cells. The peripheral T cell population, which is known to be very long lived, would represent a logical repository for the memory of interactions with environmental antigens and the modifications in idiotype and anti-Id distributions thereby induced. According to such a view, B cells arising in the marrow interact with Id of peripheral T cells. This results in preferential stimulation of those clones capable of interacting with Id (and anti-Id) represented in the long-lived peripheral T cell population and thereby changes the distribution of B cell clones (Id and anti-Id) from that which is produced by the bone marrow. This hypothesis is consistent with our finding that mice reconstituted with bone marrow from either young or old donors together with B cell-depleted spleen cells from old donors produce a marked auto-anti-Id response like that of old mice, whereas mice reconstituted with bone marrow from young or old donors together with B cell depleted spleen cells from young donors produce relatively few hapten-augmentable PFC as is typical of young mice. Thus the non-B cell population of the spleen appears to determine the magnitude of the auto-anti-Id response. While not formally proven, it seems reasonable to suggest that it is the splenic T cell population which is responsible for these regulatory effects since ≥95% of the cells present in the B cell depleted spleen cell preparations are Thy-1+.

Summary

We have previously shown that old mice produce more hapten-augmentable plaque-forming cells (PFC) than do young animals, suggesting a greater auto-anti-idiotypic antibody (auto anti-Id) component in their immune response. In the present studies this is confirmed serologically. The marked auto-anti-Id response of aged mice can be transferred to lethally irradiated young recipients with spleen but not bone marrow cells from old donors, suggesting that it is an intrinsic property of their peripheral B cell population and that the distribution of Id arising from the bone marrow of old and young mice is similar. In contrast with young mice the auto-anti-Id response of old animals is relatively T cell-independent and old donors do not show an increase in their ability to transfer an auto-anti-Id response after priming with TNP-F. These observations suggest that old mice behave as if already primed for auto-anti-Id production. Irradiated mice reconstituted with bone marrow cells from either young or old donors together with splenic T cells from old donors generate a relatively large auto-anti-Id response, whereas mice reconstituted with bone marrow from either young or old donors together with splenic T cells from young donors produce few hapten-augmentable PFC. It is suggested that differences in Id expression and auto-anti-Id production are the consequences of the interaction of Id (and anti-Id) arising from the marrow with anti-Id (and Id) present in the peripheral T cell population which serves as a repository of information about shifts in Id distribution, resulting from lifelong interactions with environmental and self-antigens.

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