ANTIGEN RESPONSIVENESS OF THE MATURE AND GENERATIVE B CELL POPULATIONS OF AGED MICE*

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The decline in humoral immunity with advancing age has been well documented (1-12). A major contribution to the decline of the response to T-dependent antigens is due to reduced T cell help (7) or increased T cell suppression (8, 9). Nevertheless, the reduced response to T-independent antigens and mitogens (10-12) strongly suggests that a functional decline in the B cell compartment as well, occurs with age. The total number of B cells in the spleen or in lymph nodes as determined by the number of Ig-bearing cells, does not decrease with age (6), implying that B cell depletion per se cannot account for the decreased B cell responsiveness with age. The reduced B cell response after antigenic stimulation might, however, be caused by a reduced number of antigen-responsive B cells in aged mice or by a defect in the capacity of existent B cells to divide after stimulation and/or produce substantial amounts of antibodies, or both.

We have studied the response of B cells of aged mice to a T-dependent antigen (dinitrophenylated hemocyanin [DNP-Hy]) at the clonal level to investigate the origin of the impaired humoral response in aged mice. The study was performed using the splenic fragment culture technique with limiting numbers of spleen cells from aged mice (24 mo) injected into young adult carrier-primed recipients thus providing the B cells with maximum help. The findings reveal that DNP-responsive B cells in aged individuals yield antibody-producing cell clones that are normal in both the amount and relative affinity of the antibody produced. These clones, however, appear less likely to produce IgG antibodies. Most important, the proportion of B cells responsive to DNP is reduced in the spleen of aged mice. To further investigate this difference between the mature splenic B cell population of old and young mice, the prereceptor B cell population of the bone marrow of these mice was characterized. No differences were found within these immature “generative” populations derived from old vs. young mice.

Materials and Methods

Mice. 24-mo-old BALB/c male mice and their 3-4-mo-old cohorts were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, MA. 2-mo-old BALB/c mice to be used as recipients for cell transfer were obtained from the Scripps Clinic Breeding Colony. Recipient mice were immunized intraperitoneally with 100 µg of hemocyanin (Hy) in complete

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Abbreviations used in this paper: BSA, bovine serum albumin; DNP, dinitrophenyl; FACS, fluorescence-activated cell sorter; Hy, hemocyanin; RIA, radioimmunoassay; slg, surface immunoglobulin.
Freund's adjuvant, followed 4 wk later by an intraperitoneal injection of 100 μg of Hy in saline. Mice were used as recipients 6–8 wk after secondary immunization.

Antigens. The proteins limulus polyphemus Hy and bovine serum albumin (BSA) and the antigens 2,4-dinitrophenyl (DNP)-Hy (12 mol of DNP/100,000 g Hy) and DNP-BSA (10 mol of DNP/65,000 g BSA) were prepared as previously described (13–15).

Depletion of Ig-bearing Cells from Bone Marrow. Bone marrow cells were obtained from both femurs and tibias of old and young donors. Depletion of Ig-bearing (sIg) cells was achieved as described by Walker et al. (16). Briefly, the cells were washed twice in Hanks' balanced salt solution containing 0.02% sodium azide and mixed in a 1:5 ratio with glutaraldehyde-fixed sheep erythrocytes (Flow Laboratories, McLean, VA) coated with affinity-purified goat anti-mouse IgM + IgG heavy and light chain antibody (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD). The cell mixture was pelleted (7 min at 200 g) resuspended, and repelleted. The pellet was left in the cold for 20 min, after which the cells were resuspended and layered on a Ficoll-Hypaque density gradient (1.09 g/cm³) and centrifuged for 20 min (1,500 rpm). The cells at the interface were collected and considered depleted of sIg-bearing cells. The remaining antibody-forming cell precursors appear to be immature by several criteria: (a) They lack surface immunoglobulin by fluorescence-activated cell sorter (FACS) analysis (<2% Ig*). (b) The vast majority are susceptible to in vitro tolerance induction (17). (c) Antibody-producing clones derived from such stimulated cells in fragment culture yield IgM or IgA and not IgG (Fig. 1), which is characteristic of immature fetal and neonatal B cells (18).

Splenic Fragment Culture Technique. Anti-DNP monoclonal antibodies were produced in the splenic fragment system as previously described (13–15, 17). Briefly, limiting numbers of donor cells from old or young mice were injected intravenously into Hy-primed young BALB/c recipients that had been irradiated at 1,300 rad using a cesium source 4 h before injection of donor cells. 4 × 10⁶ spleen cells, 5 × 10⁶ bone marrow cells, or 10 × 10⁶ sIg- bone marrow cells were transferred per recipient. Fragments were prepared from recipient spleens 18 h after cell transfer and incubated individually with DNP-Hy at a concentration of 10⁻⁶ M for DNP for 3–4 d. Culture fluids were changed every 3–4 d and collected individually beginning on day 10.

Radioimmunoassay and Isotype Analysis. Collected culture fluids were assayed for anti-DNP antibody content by solid phase radioimmunoassay (RIA) as described previously (13–15, 17).

Fig. 1. Anti-DNP-specific monoclonal antibodies were analyzed for the isotypes present as described in Materials and Methods. The relative frequency of each isotype or combination of isotypes present is expressed as the percent of total clones analyzed.
The RIA supernatants that were found positive for anti-DNP antibody were reanalyzed for heavy chain isotype(s) and light chain class produced. This was done by the same RIA except that the rabbit anti-mouse Ig reagent was substituted with rabbit anti-mouse heavy chain isotype specific or anti-light chain specific antibodies: (a) anti-α, (b) anti-α, (c) anti-γ (anti-γ, anti-γ, anti-γ, anti-γ, anti-γ), (d) anti-κ, and (e) anti-λ. All reagents were purchased from Litton Bionetics, Kensington, MD, and adsorbed where needed to ensure specificity (19).

**Affinity Assay.** Relative affinity of the IgM, IgG, and IgA monoclonal antibodies was assayed by hapten inhibition of antibody binding (20). Binding of anti-DNP antibodies to DNP-BSA coated plates was determined in the presence of varying concentrations (10^-6 M–10^-8 M) of DNP-L-lysine (Sigma Chemical Co., St. Louis, MO). Inhibition was calculated as follows: percent inhibition = [(cpm, absence of inhibitor – cpm, presence of inhibitor)/(cpm, absence of inhibitor – cpm, background)] × 100. The concentration of inhibitor causing 50% of antibody binding was determined from log plots of the data (percent inhibition vs. log-hapten concentration).

**Results**

**Frequency of DNP-specific B Cells.** The frequency of DNP-specific B cells within the spleen and bone marrow of young and aged BALB/c mice was assessed by limiting dilution cell transfer into young Hy-primed, irradiated syngeneic mice. The anti-DNP specific B cell frequencies obtained are shown in Table I. The proportion of Ig^+ cells among spleen cells of both old and young mice was ~50% by FACS analysis. However, it can be seen that the DNP-specific B cell frequency was significantly lower (P < 0.05) in spleens of old mice compared with young mice. Anti-Thy-1 + complement treatment of spleen cells before transferring them into recipients did not increase DNP specific B cell frequencies in spleens of either old or young mice on a per B cell basis. This suggests that suppressor T cells that might be present in high numbers in old spleens (8) were not responsible for the reduced B cell frequency observed.

Because the frequency of mature responsive B cells was reduced in aged mice, we tested whether this was caused by an impaired generative capability of pre-B cells in aged mice. For this purpose, the frequency of DNP-specific precursors in bone marrow depleted of s-Ig^+ cells was determined. As shown in Table I, there was no significant difference between young and old mice in the frequency of DNP specific cells found

| Table 1 |
| Frequency of DNP-specific B Cells in BALB/c Mice |

|          | Number of clones analyzed | Number of cells injected | Average frequency per 10^6 cells injected |
|----------|---------------------------|--------------------------|-----------------------------------------|
| Young    |                           |                          |                                         |
| Spleen   | 55                        | 24 × 10^6                | 2.3 ± 0.28                              |
| Bone marrow | 48                      | 32 × 10^6                | 0.94 ± 0.1                              |
| sIg^- bone marrow | 59                 | 112 × 10^6               | 0.57 ± 0.13                             |
| Old      |                           |                          |                                         |
| Spleen   | 92                        | 93 × 10^6                | 1.1 ± 0.28                              |
| Bone marrow | 94                     | 82 × 10^6                | 0.84 ± 0.19                             |
| sIg^- bone marrow | 109              | 285 × 10^6               | 0.4 ± 0.06                              |

4 × 10^6 spleen cells, 5 × 10^6 bone marrow cells, or 10 × 10^6 sIg^- bone marrow cells were transferred to each recipient mouse. Antibody-producing clones were detected by RIA of culture fluids collected from days 10–21 of culture.

* Number represents mean ± SE.
in either sIg-depleted bone marrow cells or in unseparated bone marrow cell preparations.

Amount of Antibody Produced per Clone. The amount of antibody produced by each clone of antibody-producing cells is presumably a function of the rate of cell division (number of antibody-producing cells) after stimulation and of the amount of antibody secreted by the cells in the clone. In Table II the amount of antibody produced by clones derived from spleens of young and aged mice is summarized. High and low antibody-producing clones were found in both young and aged B cell populations, with no significant difference in the distribution and average amount between the two. This indicates that although there were fewer responding cells in spleens of aged mice, those that did respond were as efficient with respect to antibody productivity as cells from young mice.

Isotypes Secreted by DNP-specific Clones. All of the DNP-specific monoclonal antibodies obtained in the above experiments were analyzed for immunoglobulin isotype(s) using class-specific rabbit anti-mouse Ig isotype specific antibodies. The isotype distribution of clones obtained from spleen, bone marrow, or sIg-depleted bone marrow of young and aged mice is shown in Fig. 1. It can be seen that in spleens of old mice a higher proportion of the clones produced only μ (33%) compared with young mice (21%), whereas clones from young mice tended to switch classes and produce multiple isotypes. The difference in isotype production with age was particularly marked with respect to the number of clones producing IgG. As seen in Table III, which summarizes the percent of clones producing each isotype, there was no difference in IgA production, whereas IgG production was decreased significantly.

No significant difference between young and aged mice in the distribution of isotypes was seen in the total bone marrow. The sIg-depleted bone marrow (Fig. 1, Table III) of both young and old mice produced mainly IgM and or IgA, which is typical of immature B cells (19). It should be mentioned that no age-related changes were observed in light chain production. About 90% of the clones derived from spleen, bone marrow, or sIg-depleted bone marrow from young and aged mice produced the κ light chain, whereas 10% produced the λ light chain.

Affinity Distribution of Monoclonal Anti-DNP Antibodies. Relative affinities of the IgM, IgG, and IgA antibodies produced by the clones obtained, was assessed by hapten inhibition of antibody binding to DNP-BSA coated plates using DNP-L-lysine as the inhibitor. The hapten concentration causing 50% inhibition of binding (I-50) was considered as a measure of relative antibody affinity for DNP. The range of affinities (I-50) found was very broad for each of the isotypes but there was no significant difference between clones derived from young and aged spleens in the distribution of

| Table II |
|----------|
| **Average Antibody Production per Clone** |
| Donor cells (spleen) | Number of clones | ng/d |
| Young | 64 | 20.6 |
| Old | 87 | 18.1 |

Culture supernatants that were collected on days 10, 13, 16, and 21 and found to be positive for anti-DNP antibody were combined, and the total amount of antibody produced was determined.
**Table III**

*Immunoglobulin Isotype Composition of Monoclonal Antibodies*

| Isotype | Young | %  | Old | %  |
|---------|-------|----|-----|----|
| Spleen  | μ     | 89 | 71  |
|         | γ     | 71 | 35  |
|         | α     | 47 | 44  |
| Bone marrow | μ | 63 | 57  |
|          | γ    | 50 | 45  |
|          | α    | 38 | 53  |
| slg bone marrow | μ | 95 | 99  |
|          | α    | 6  | 15  |

Anti-DNP-specific monoclonal antibodies were analyzed for the isotypes present by RIA with rabbit anti-mouse heavy chain isotype-specific antibodies.

* Numbers represent the percentage of total clones analyzed producing that isotype.

**Table IV**

*Relative Affinity of DNP-specific Antibodies*

|       | Old       | Young      |       |       |
|-------|-----------|------------|-------|-------|
|       | Number of clones | Hapten concentration for 50% inhibition (I-50) | Number of clones | Hapten concentration for 50% inhibition (I-50) |
| IgM   | 38        | 16.0 × 10^{-4} M (± 3.1)* | 16    | 19.1 × 10^{-4} M (± 4.6)* |
| IgG   | 25        | 76.1 × 10^{-6} M (± 52)  | 20    | 76.6 × 10^{-6} M (± 43)  |
| IgA   | 17        | 23.3 × 10^{-6} M (± 6.3) | 4     | 40.3 × 10^{-6} M (± 29) |

Relative affinity of the anti-DNP specific monoclonal antibodies derived from splenic B cells was determined in a RIA assay by hapten inhibition of antibody binding to DNP-BSA coated plates using DNP-L-lysine as the inhibiting hapten.

* Results are expressed as mean ± SE.

those affinities ($P < 0.05$). High and low affinity antibodies were found in both young and aged populations. Moreover, no difference between young and aged mice in the average affinity of the IgM, IgG, or IgA monoclonal antibodies was seen (Table IV).

**Discussion**

Previous studies on the humoral immune response in aging mice have shown that this response decreases with age as assessed by the amount of antibody secreted (1, 2) or by the number of specific plaque-forming cells (5). These types of quantitation, looking at the “end-product” of the immune response, cannot discriminate between possible reduction in the number of antigen-specific B cells as opposed to intrinsic defects in the responding B cells per se such as reduced ability to proliferate after antigenic stimulation or to secrete considerable amounts of antibody. The present study was designed to investigate the B cell response at the clonal level looking for quantitative and qualitative differences between cells of young and aged mice. To study only the B cell response of aged mice, cells were removed from their natural aged environment, which has been reported to be deficient in T cell help (7) or to
have environmental suppression (8, 9), and injected into young, carrier-primed, irradiated recipients. Using this procedure, B cells were provided with maximum help by the young host T cells.

No abnormalities were observed with respect to the amount of antibody produced by clones derived from B cells of aged mice at any time after stimulation. To the extent that this is a measure of the rate and extent of cell division and the amount of antibody secreted by the cells within clones, these findings suggest that with advancing age, B cells that can be triggered, respond with the same efficiency as do the B cells of young mice. Studies on the proliferative capacity of lymphocytes from young and aged mice in response to T and B cell mitogens have led others to similar conclusions as to the efficiency of responding senescent cells (12).

In addition to producing normal quantities of antibody, clones derived from B cells of aged mice produced antibodies whose relative affinities for DNP did not differ significantly from that of monoclonal antibodies derived from B cells of young mice. At the serum level, the production of antibody with lower affinity by old animals than young animals has been well documented (9, 21, 22). Goidl et al. (9) have also reported a lack of high affinity IgG-antibody-producing plaque-forming cells in spleens of old BALB/c mice, a finding which one of us has confirmed in C57BL/6J mice (D. Zharhary and H. Gershon, unpublished results). Since, in the present study our results suggest that high affinity antibodies can be produced by B cells derived from aged mice, the lack of high affinity antibodies in the serum or splenic plasma cells of intact mice is probably due to experimental or T cell effects as has been suggested and demonstrated by others (9, 23).

Antibody-forming cell clones derived from splenic B cells of aged vs. young mice do differ in the distribution of secreted Ig isotypes. While in aged mice, more clones produced solely IgM, B cells from young mice tend to switch classes and produce multiple isotypes (Fig. 1). This difference is preferentially seen in IgG production. Whereas 71% of the clones derived from young spleens secrete IgG, only 35% of the clones from aged spleens produce IgG. The preferential loss of IgG production, which has been previously found in aged mice (2, 9), was thought to be caused by a T cell defect (9). Our findings suggest that this IgG reduction may be caused, in part, by a B cell dysfunction as well.

By far the most significant difference between B cell populations of old vs. young mice is the decrease in the proportion of B cells responsive to DNP in the spleens of aged mice. As the number of antigen-responsive B cells is reduced in spleens of aged mice, it was of interest to determine whether this is a consequence of defective generative capability of the pre-B cells in the aged bone marrow. No difference between young and aged mice in the frequency of DNP-specific cells was found in bone marrow depleted of sIg-bearing cells or even in the whole bone marrow population (Table I). As the yields of cells from bone marrow after depletion of Ig-bearing cells was comparable in young and aged mice (30–50%), it is unlikely that a selective enrichment of sIg− B cells in the aged Ig-depleted bone-marrow preparations had occurred. These results imply that pre-B cells in the aged bone marrow have normal generative capability, but that in the process of B cell clonal maturation in the aged environment and subsequent migration to the spleen, a loss in their capacity to respond to antigen occurs.

The fact that while the total number and representation of B cells is not reduced in
spleens of aged mice (6), the number of responding B cells decreases, raises questions as to the function of these existing B cells. One possibility is that there is a shift in the repertoire of the B cells in aged mice, perhaps towards antigens which those mice have been repeatedly exposed to during their lifetime. If that were the case, one might expect to find antigens to which B cells of aged mice would respond better than young mice. Nevertheless, the reduced B cell response to polyclonal activators like lipopolysaccharide (12) implies that there is an overall decrease in B cell function with age. Alternatively a reduction in the frequency of responsive cells with no concomitant reduction in the number of B cells (Ig" cells) can be due to the presence of B cells which have been tolerized or inactivated in some way but still exist in the aged spleen. The question of whether induction of tolerance is accompanied by an actual elimination of lymphocytes ("clonal deletion") or rather by modification of their function, has not yet been resolved. Nossal and Pike (24) have shown that antigen-binding B cells, incapable of responding to antigen or mitogen, can exist in tolerized animals in a functionless state ("clonal anergy"). Supporting the existence of "anergic" cells in old mice are the findings described by Callard et al. (6) showing that the proportion of SIII-antigen binding cells in spleens of unimmunized old mice is comparable to that of young mice while the antibody response to the same antigen is greatly reduced. Finally, it has been shown that aged mice exhibit a markedly enhanced level of spontaneously acquired antiidiotype reactivity (13). If such reactivity, like tolerance induction, can result in clonal anergy, then it would be an excellent candidate for diminishing the frequency of responsive B cells within the mature B cell population of aged individuals.

Summary

The deficit of humoral immune responsiveness associated with aging was investigated at the level of individual antigen-specific B cells. It was found that mature dinitrophenyl (DNP)-responsive B cells isolated from the spleen of aged mice gave rise to clones of antibody-forming cells that were normal with respect to both the amount and relative affinity of anti-DNP antibody produced. However, although the proportion of immunoglobulin-bearing cells in the spleen of aged mice was normal, the proportion of cells that responded to T cell dependent DNP-specific stimulation (1.1 per 10⁶ injected cells) was significantly lower than the proportion that responded when cells were obtained from the spleen of young mice (2.3 per 10⁶ injected cells). To examine the origin of this diminution in antigen-responsive B cells, the responsiveness of precursor cells from the B cell generative pool isolated as the surface immunoglobulin negative (sIg- ) cells within the bone marrow was evaluated. The frequency of DNP-responsive cells in both intact bone marrow cell suspensions and the sIg- subpopulation was not significantly different when such cells were isolated from aged vs. young individuals. Thus, it would appear that among the immunologic deficits associated with aging is a decrease in the proportion of antigen-responsive B cells, which is associated with maturation of B cell clones in the aged environment and occurs during the migration of cells from the bone marrow to the spleen.

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