IMMUNOCOMPETENT AUTOREACTIVE B LYMPHOCYTES ARE ACTIVATED CYCLING CELLS IN NORMAL MICE

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Autoreactive, immunocompetent B lymphocytes in peripheral lymphoid organs have long been considered "abnormal," as it was believed that such "forbidden clones" were normally deleted or inactivated in mature repertoires (1–3). In fact, for the last 10 years autoreactive B cells were shown to exist in available repertoires by the appearance of autoantibodies in graft vs. host disease and upon systemic injection of B cell mitogens (4–8). It was believed, however, that such autoreactive B cells were resting lymphocytes in physiological conditions. More recently, the repeated finding of auto-antibodies in the sera of normal individuals led to the suggestion that some autoreactive B cells could be activated in the internal environment (9–11).

Independent approaches have provided considerable evidence for autonomous activities in the immune system. Even in germ-free mice maintained with low molecular weight, chemically defined diets, there is a considerable level of internal activation within the various lymphocyte compartments (12–14). In what concerns B lymphocytes, these mice show normal frequencies of activated blasts and of effector high-rate Ig-secreting cells. As the activation of immunocompetent lymphocytes is dependent upon stimulations and interactions with their immediate environment, which are driven by clonally distributed surface receptors, we could expect that the repertoire of these internally activated B cells would not be a random sample of the available antibody repertoire, but rather would be biased to components of the organism.

Here we investigate the repertoire of naturally activated B cells, as compared with that of small resting B lymphocytes, in the same individuals. Taking as criteria for activation the size of cells ("large cells"), their effector function (i.e., their ability to produce antibodies), and their mitotic activity in vivo, we show that most autoreactive B cells reacting with autologous thyroglobulin and erythrocytes are activated.

Materials and Methods

Animals. C57BL/6 mice, 6–12 wk old, and Fisher strain rats, 3–4 weeks of age, were obtained from our animal facilities.

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Hydroxyurea (HU)[1] *Treatment of Mice.* In vivo treatment of C57BL/6 mice was performed by intraperitoneal injection at a dose of 1 mg/g weight per injection. In some experiments mice received four cycles of two injections at a 7 h interval, and were killed the day after. In other experiments, mice received one cycle of two injections and were killed 2 d later.

Cell Suspensions. Murine spleen cells and growth-supporting rat thymus cells were prepared as previously described (15).

Antigen and Antibodies. Murine thyroglobulin (TG) was prepared according to McLachlan et al. (16) and purified by exclusion chromatography on an ACA 34 1 liter column. KLH and beef hemoglobin (beef Hb) were purchased from Sigma Chemical Co. (St. Louis, MO). Syngenic mouse erythrocytes were treated with a saturated solution of bromolein (Sigma Chemical Co.) in PBS (BMRC), according to Cunningham (17). Purified sheep anti-mouse Ig was a gift from Dr. L. Forni (Basel Institute for Immunology, Basel, Switzerland). Goat anti-mouse Ig coupled to horse radish peroxidase was obtained from Nordic Immunology Laboratories (London). LPS was a Westphal extract obtained from *S. abortus equi* (Difco Laboratories, Inc., Detroit, Michigan).

Spleen Cell Fractionation and Cell Size Analysis. Spleen cells from normal, unmanipulated C57BL/6 mice were fractionated in discontinuous Percoll gradients (18); the cell fractions with densities $1.060 \leq \rho < 1.070$ are referred to as large cells, while those with $\rho > 1.070$ are referred to as small cells. Cell sizes were controlled in a cell size analyzer (Coulter Counter 2M with a Coulter Chanalyser 256, Coultronics France, Margency).

Cultures and Limiting Dilution Analysis. These were done as previously described (15). Briefly, limited numbers of spleen cells were distributed in 0.2 ml cultures containing growth-supporting thymus cells (1.5×10^6/ml) in RPMI medium containing 5×10^{-5} M 2-ME, glutamine, antibiotics, and 10% FCS (selected batch), and 25 μg/ml of LPS. For each determination of the frequency of clonal precursor B cells, at least four different cell concentrations were set up, with 48 replicate identical cultures per point. Another set of 48 cultures received rat thymus filler cells and LPS, but no responding murine spleen cells, and these cultures were used in all assays as the negative standard.

Plaque Assays. Direct anti-BMRC PFC were assayed at day 4 of culture, while direct anti-SRBC PFC were assayed at day 5. The total number of LPS-reactive B cells was determined by the protein A–plaque assay at day 5 (19). Wells containing a number of PFC exceeding the mean of the negative standard by at least 3 SD were scored as positive.

Enzyme-linked Immunoassay (EIA). Briefly, the test was carried out as follows: 100 μl of purified sheep anti-mouse Ig (5 μg/ml), mouse TG (100 μg/ml), KLH (5 μg/ml), or beef Hb (5 μg/ml) were adsorbed to polystyrene plates by incubation. Culture supernatants to be tested were added to the plates (50 μl/test), and after appropriate washing, the bound Ig was detected by addition of peroxidase-labeled goat antibodies to mouse IgM. Finally, H_2O_2 and orthophenyldiamine (OPD) as a chromogen were used to develop the reaction. OD at 450 nm were assayed in a photometer (Titertek Multiscan, Poly Labo Paul Block and Co., Strasbourg Meineau, France). Mean OD and SD were calculated from culture supernatant from negative standard cultures. Mean OD plus 3 SD was considered as the threshold for positivity.

Derivation of "Natural" Antibody-secreting Hybridomas. Spleen cells from normal adult mice were separated by velocity sedimentation at unit gravity (20) in small and large cell fractions. These were separately cultured with LPS for 48 h and hybridized with SP 2/0 cells, as previously described (21).

Affinity Determinations with "Natural" mAbs to TG. Culture supernatants containing natural mAbs were assayed for affinity to mouse TG by a modified EIA method as described by Friquet et al. (22). In brief, a fixed amount of antibody was incubated to equilibrium together with a titration of free mouse TG. The fraction of nonbound antibody at equilibrium was then measured in EIA by transfer of these supernatants to

[1] *Abbreviations used in this paper:* Beef Hb, beef hemoglobin; BMRC, bromolein-treated syngeneic mouse erythrocytes; d, density; EIA, enzyme-linked immunoassay; HU, hydroxyurea; LDA, limiting dilution analysis; OPD, orthophenyldiamine; TG, thyroglobulin.

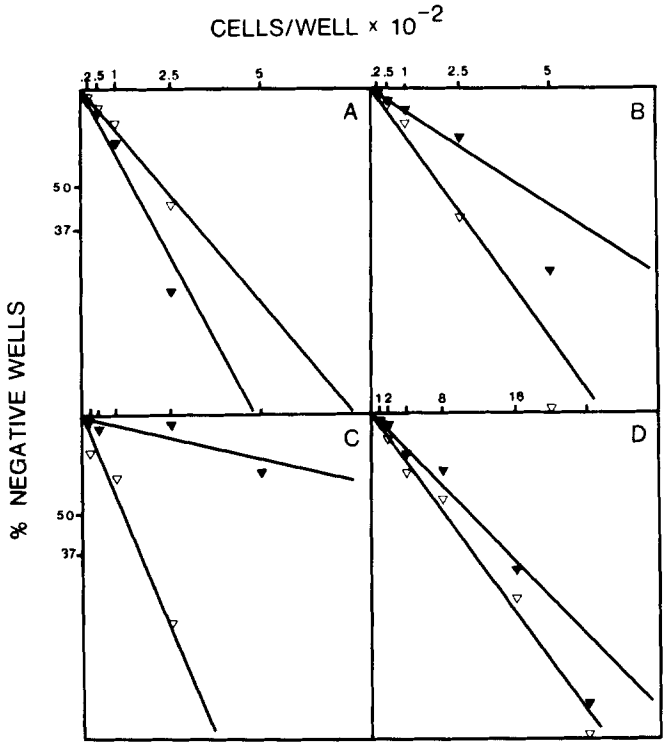

FIGURE 1. LDA for frequencies of clonal precursor B cells in large (▽) and small (▼) spleen cells (culture supernatants assayed in EIA). (*A*) anti-beef Hb; (*B*) anti-KLH; (*C*) anti-TG; (*D*) frequencies of total LPS-reactive B cells.

TG-coated plates. Dissociation constants (K_d) were then calculated from the ratio of total to unbound antibody as a function of reciprocal TG concentration expressed in molarity.

Results

Frequency of Clonal Precursor Cells Producing Anti-Mouse TG Antibodies among Large and Small B Cells. In a first set of experiments, we have taken the size of freshly collected spleen cells as a criterion for in vivo activation and investigated the distribution of autoreactive B cells among large and small lymphocytes separated by density on Percoll gradients. The frequencies of anti-TG clonal precursor B cells were determined by limiting dilution analysis (LDA) in small and large cells from normal, unmanipulated C57BL/6 mice. Frequencies of anti-KLH and anti-beef Hb B cells were also determined as controls for external antigens, as well as the frequencies of total LPS-reactive B cells.

Fig. 1 shows a representative experiment. Anti-TG clonal precursor B cell frequency was 1:200 in large cells, as compared with 1:1600 in small cells (Fig. 1 *C*). In contrast, no clear-cut differences were found for beef Hb (1:300 large cells vs. 1:200 small cells, Fig. 1 *A*) nor for KLH (1:300 large cells vs. 1:600 small cells, Fig. 1 *B*). Total frequencies of LPS-reactive B cells were 1:11 large cells vs. 1:16 small cells (Fig. 1 *D*). These findings were consistent in several other experiments. Thus, while total frequencies of LPS-reactive B cells among large

TABLE I

Affinities of mAbs to Autologous Thyroglobulin Isolated from Normal Mice

mAbs*	K_d‡
10 A 11	10^{-6}/M
1 D 1	$8.7.10^{-7}$/M
5 G 3	$5.6.10^{-7}$/M

* Randomly selected from hybridoma collections isolated from normal, adult BALB/c mice. The frequency of anti-TG binding in these collections was 1/43, after the screening of some 250 independent hybridomas. All three antibodies are IgM, K.

‡ Determined as described in Materials and Methods.

and small lymphocytes were roughly similar, as well as the frequencies of anti-KLH and anti-beef HG B cells, a clear-cut difference was reproducibly found for anti-mouse TG B cells, which were 7–10-fold higher in large than in small cells.

Affinity of Natural Antibodies to TG. In view of the high frequency of anti-TG clonal precursors detected in EIA, we sought another method to detect TG-specific, antibody-secreting clones, as it could be argued that our observations would apply to low-affinity antibodies only. By coupling mouse TG to indicator red cells, we could, in preliminary tests, detect TG-specific PFC. LDA for anti-TG clonal B cell precursors assayed on PFC revealed somewhat lower frequencies, as expected, but there was the same nearly 10-fold difference between large and small spleen cells. Thus, absolute frequencies of anti-TG among all LPS-reactive B cells were 1:214 in large and 1:1,909 in small spleen cells, respectively.

The direct approach to this question, however, would involve the determination of affinity to the anti-TG antibodies. This, in turn, required the preparation of antibody-secreting hybridomas from normal mice. We have randomly chosen three of them, with anti-TG activity, to perform these experiments.

It has already been reported that natural mAbs display high affinity for the respective autoantigen (reference 23 and T. Ternynck et al., manuscript submitted for publication). The method used in these determinations was recently developed by Friguet et al. (22) and allows affinity measurements in crude culture supernatants containing the mAbs. These authors compared this particular method with other current and more laborious techniques for measurements of antibody affinity, and found it to give very similar results.

The three anti-TG IgM antibodies chosen from a collection of hybridomas isolated from normal, adult BALBb/c mice were studied by this method, and the respective K_d are shown in Table I. Dissociation constants range from 10^{-6} to $5–6 \times 10^{-7}$ M^{-1}.

Frequency of Clonal Precursor B Cells Producing Anti–Mouse Erythrocyte Antibodies among Large and Small Lymphocytes. We next investigated whether the above finding could be extended to other autoantigens. Anti-BMRC PFC have been used as a model system for the study of autoreactive B cells (17). Thus, we carried out LDA of small and large spleen cells from normal mice, and assayed for clones with anti-BMRC specificity vs. those with anti-SRBC specificity. These,

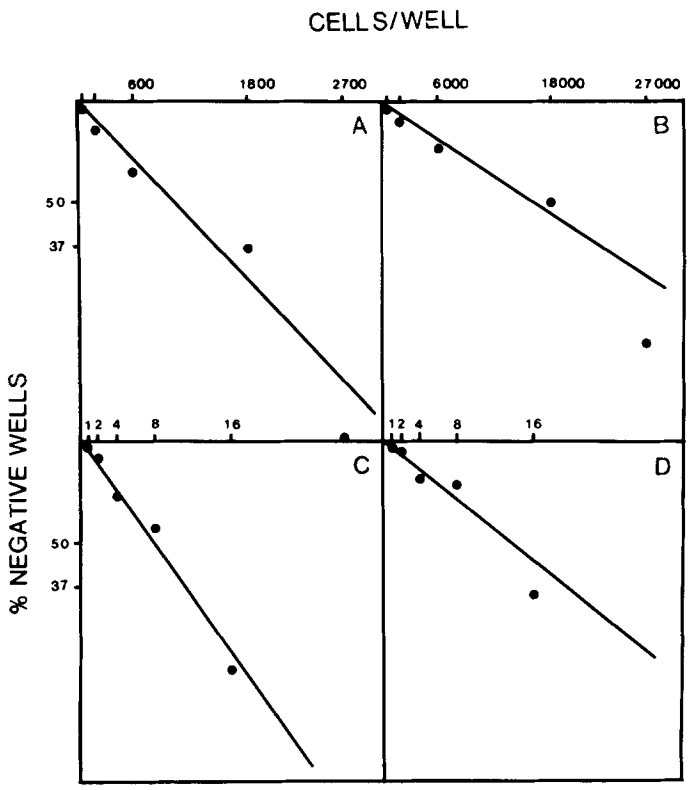

FIGURE 2. LDA for frequencies of anti-BRMC precursor B cells in large (*A*) and small (*B*) spleen cells, assayed by direct anti-BRMC PFC. Total frequencies of large (*C*) and small (*D*) LPS-reactive B cells were assayed by the Protein A–plaque method.

as well as the total frequency of LPS reactive B cells, were determined in plaque assays detecting IgM-secreting clones, exclusively.

As shown in Fig. 2, a major difference was found again between large and small cells with regard to the frequency of anti-BMRC clonal precursor B cells. In large cells this frequency was 1:1,500 cells (Fig. 2*A*), while in small cells the frequency was 1:25,000 cells (*B*). Total frequencies of LPS reactive B cells were only slightly different: 1:10 in large cells (*C*) vs. 1:18 in small cells (Fig. 2*D*). Thus, the absolute frequencies of anti-BMRC clonal precursor B cells was 1:150 in large cells and 1:1,400 among small cells. For comparison, the frequencies of anti-SRBC B cells assayed in the same conditions were 1:40,000 large (Fig. 3*A*) and 1:35,000 small cells (Fig. 3*B*). The total frequencies of LPS-reactive B cells were 1:30 in large cells and 1:15 in small cells (Fig. 3, *C* and *D*). Thus, the absolute frequencies of anti-SRBC clonal B cell precursors were 1:1,300 in large and 1:2,300 in small cells, a result that is in agreement with previous determinations (24).

We conclude from these two experimental systems that autoreactive clonal precursor B cells are overtly more frequent in large than in small cells, while clonal precursor B cells directed against external antigens are equally distributed in these two cell populations. Actually, some extent of cross-contamination cannot

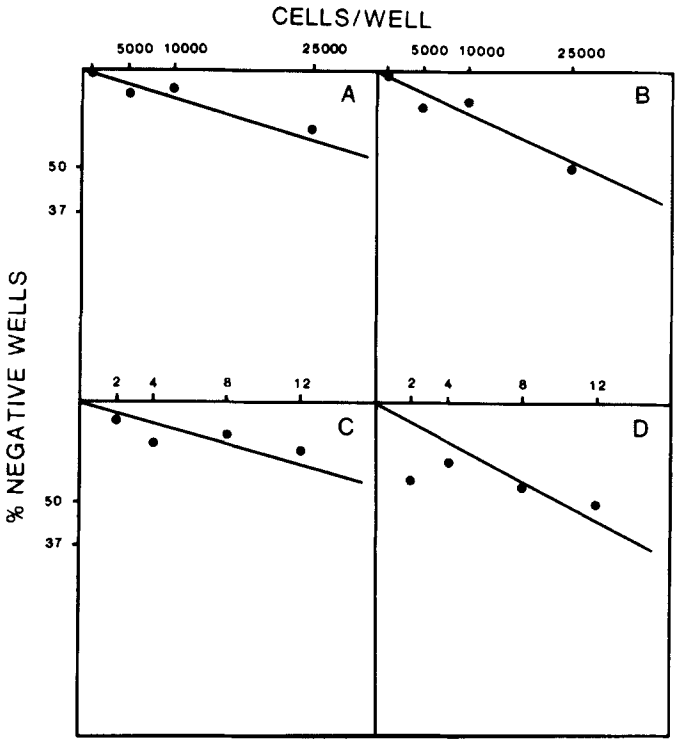

FIGURE 3. LDA for frequencies of anti-SRBC precursor B cells in large (*A*) and small (*B*) spleen cells, assayed by direct anti-SRBC PFC. Total frequencies of large (*C*) and small (*D*) LPS-reactive B cells were assayed by the Protein A–plaque method.

be avoided in Percoll separations. According to cell size analysis performed in the Percoll-separated fraction used in LDA (Fig. 4*A*), contamination with large cells in the pool of small cells could account for the totality of autoreactive B cells found in small cells. Our results, therefore, are compatible with the possibility that all autoreactive B cells are in fact large cells.

Killing of In Vivo Cycling Cells Drastically Reduces the Frequency of Cells Producing Anti-TG Autoantibodies. HU treatment of mice at appropriate doses kills all cells in S phase and synchronizes other cycling cells. A second injection 7 h later will kill all cycling cells that were not in S at the time of the first injection (25). We used this method to test the hypothesis that autoreactive B lymphocytes are activated cycling cells. It has already been reported that most large lymphocytes naturally activated in normal mice are cycling cells (13).

Thus, we first confirmed that in vivo HU treatment of mice reduces the proportion of large cells in the spleen, as shown in Fig. 4*B*. We next performed LDA with total spleen cells from normal or HU-treated C57BL/6 mice. The frequencies of autoreactive anti–mouse TG B cells and, as a control, of anti-KLH B cells, were determined using the same cell suspensions.

As shown in Table II, in vivo HU treatment of mice drastically reduces the frequency of anti–TG B cells. In contrast, such a treatment only slightly affects the frequency of anti-KLH B cells (Table II, Exp. 2).

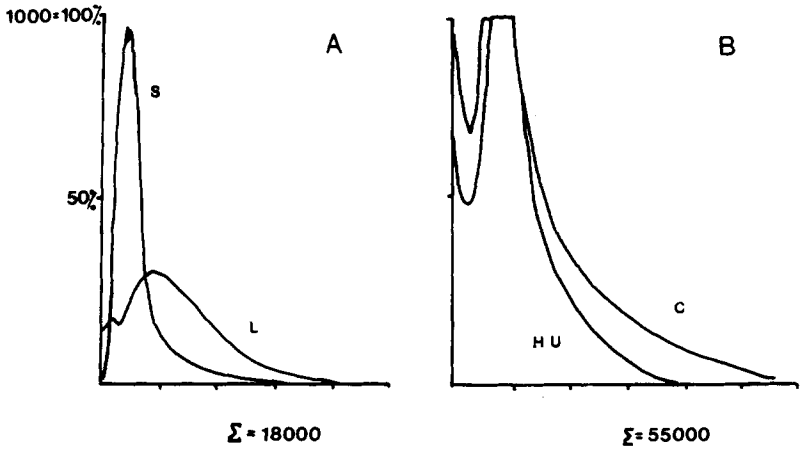

FIGURE 4. Cell size analysis of spleen cells. (*A*) cell fractions used as large (*L*) and small (*S*) lymphocytes after Percoll separation; (*B*) total spleen cells from normal (*C*) or HU-treated mice. These had been given two injections of HU, with a 7 h interval, 2 d before.

TABLE II

Effects of In Vivo HU Treatment of C57BL/6 Mice on Frequencies of Anti-KLH, Anti-Thyroglobulin, and LPS-reactive B Cell Precursors

Exp.	Treatment with HU*	LPS-reactive per total spleen‡	Anti-KLH per total spleen‡	Anti-TG per total spleen‡
1	–	1 in 11	ND	1 in 300
	+	1 in 14	ND	1 in 5,000
2	–	1 in 7	1 in 500	1 in 1,700
	+	1 in 5	1 in 350	1 in 7,000

* Spleen cells were taken 2 d after HU treatment, as indicated.
‡ Determined by assay of the culture supernatants in EIA.

Discussion

The present results show that autoreactive anti-TG and anti–BRMC B lymphocytes in normal individuals are not only present in peripheral tissues and competent to proliferate and secrete antibodies, but also that a large majority of them are already activated in physiological in vivo conditions. We show here that these lymphocytes are blast, cycling cells, and previous observations have already shown that they secrete autoantibodies into the natural Ig pool (9–11, 26–28).

Many observations on autoantibodies in healthy individuals have shown that the titer and/or affinity of these molecules do not correlate with clinical disease (29–31). Obviously, therefore, autoaggression is not a necessary consequence of auto-immunity, and the frontier between physiology and pathology of autoreactivities in the immune system should be studied at a level of complexity that goes beyond current views on the presence or absence of activated, self-reactive cells. If our results can be extended to other systems, the rule in physiology

would be the continuous activation of self-reactive B cells, which would, therefore, radicalize the above argument.

The network theory (32) already implies internal recognition, therefore, it is incompatible with the classical point of view of "self-not-self discrimination." In its original formulation, however, the theory postulates internal recognition that is restricted to immunoglobulin determinants, "idiotopes," and excludes structural and functional interactions of other components of "self" outside the immune system. If, as we believe, internal activation reflects a network of interactions at the cellular level (33), then the "idiotypic network" has to be extended to nonimmunoglobulin components of self. The results shown here support these postulates.

Internally activated B cells also contain appreciable frequencies of clones reacting with antigenic molecules not present in the internal environment. This is readily explained by the characteristic "multispecificity" of natural antibodies (9–11). In this case, the *actual* antibody repertoire, although biased to self components because they are induced upon interactions with "self," is in fact complete because of degeneracy. Alternatively, natural antibodies reacting with foreign molecules could be thought to be induced upon interactions with "internal images" of external antigens, as postulated in the network theory. We find this less likely, however, as a very considerable frequency of clones with the same nominal specificities is found among small resting cells, in contrast with autoreactive B cells, at least in the two antigenic systems that were analyzed here. It could be argued that these "forbidden clones" are actually depleted from the pool of small cells, as originally proposed in the clonal selection theory, the remaining autoreactive clones being activated by the same mechanism as the "alloreactive" B cells found in the pool of large cells. In such a case, frequencies of autoreactive B cells in total spleen cells should be considerably lower than the frequencies of B cells reacting with foreign molecules. This, however, is clearly not true (Table II), even if the frequencies of B cell clonal precursors to the same antigenic molecule in autologous and heterologous forms are compared (34).

It should be pointed out that the set of "natural antibodies," that is, the repertoire of activated B cells partly analyzed here, has been previously characterized by three major differential properties, as compared with the available repertoire of small resting B lymphocytes: (*a*) a very high degree of idiotypic connectivity (21, 35, 36), that is, each molecule interacts "specifically" with a high percentage of all others in the set; (*b*) a high degree of degenerate reactivity with ligands (antigens, idiotypes, and antiidiotypes), that is, each molecule specifically binds to a large number of ligands (9–11, 21, 35); and (*c*) a very high frequency of expression of V_H genes from the two most D-proximal families, namely, 7183 and QUPC 52 (Holmberg, D. et al., manuscript submitted for publication) in young mice. These characteristics are important to consider in the context of the present observations. It could be hypothesized that physiologic auto-antibodies have no pathogenic effects, precisely because they are part of a highly connected network of naturally interacting and mutually neutralizing antibody molecules. The same argument could be extended to regulation of lymphocyte activity in the set of activated cells (13). Thus, high connectivity

would impose considerable "inertia" in this set of cells, clonal expansion of each member necessarily drawing hundreds or thousands of other connected clones. In this case, natural antibodies themselves, with exactly these properties, could be expected to be an important component in the regulation of autoreactivity, as they actually are (37–40).

In essence, these findings of productive auto-reactivity go well with in vivo lymphocyte physiology, where 10–20% of all splenic B and T cells are internally activated, and with the characteristic properties of "natural antibodies." In turn, they suggest the necessity of enlarging network perspectives of the immune system to all other components of the internal environment.

Summary

Frequencies of B cell clonal precursors producing antibodies that react with mouse thyroglobulin, mouse erythrocytes, beef hemoglobulin, KLH, and sheep erythrocytes were determined by limiting dilution analyses among small, resting lymphocytes, and among large activated cells from normal adult mice. While frequencies of clones reacting with external antigens were equally distributed in large and small B cells, most, if not all, autoreactive B lymphocytes were found in the large cell fraction. Analysis of antithyroglobulin hybridomas isolated from normal mice revealed dissociation constants ranging from 10^{-6} to $5–6 \times 10^{-7}$. Treatment of normal donors with antimitotic drugs dramatically decreases the frequencies of autoreactive B cells, but not those of B lymphocytes reacting with external antigenic molecules. Taken together, these experiments show that immunocompetent, autoreactive B lymphocytes are activated and cycling cells in the peripheral lymphoid tissues of normal individuals.

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