Clonal analysis of the anti-DNA repertoire of murine B lymphocytes

(autoimmunity/autoantibodies/Ly-1 B cells/antibody-forming cell clones/lupus)

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ABSTRACT The present studies characterize at the clonal level the repertoire of lipopolysaccharide-responsive murine B lymphocytes committed to the production of antibodies reactive with denatured DNA. This repertoire is vast in normal mice as 1–5% of total mitogen-induced antibody-forming cell clones secreted denatured DNA-reactive antibodies when the splenocyte donors were BCA (Igβ, BALB/c (Igβ), C57BL/6 (Igβ), CBA nu/nu, and C57BL/6 nu/nu athymic mice. The autoimmune NZB (Igβ) strain did not display elevated proportions of anti-denatured DNA antibody-forming cell precursors. Cross-reactions shown by BCA anti-denatured DNA antibodies suggest that many antibodies might derive significant binding energy from interaction with the bases or similar hydrophobic moieties. Cross-reactions with other tested polynucleotides were frequent, but cross-reactions with phospholipids and phosphocholine were undetectable. Most anti-DNA antibodies bound preferentially or exclusively to single-stranded denatured DNA as compared to double-stranded native DNA. The frequency of anti-denatured DNA antibody-forming cell precursors among BCA peritoneal cells was not elevated. Fluorescence-activated cell sorter-selected Ly-1-positive NZB splenic B cells were not enriched, and Ly-1 negative B cells were not depleted of anti-DNA antibody-forming cell precursors. These results show that antibody-forming cell precursors specific for denatured DNA are not restricted to the Ly-1 positive B-cell subset.

Elevated serum levels of anti-DNA antibodies are characteristic of human systemic lupus erythematosus and of autoimmune syndromes of certain inbred mouse strains, e.g., (NZB × NZW)F1, MRL-lpr/lpr, and BXSB (1, 2). The generation of monoclonal antibodies (mAbs) by hybridoma technology from B cells of such humans and mice has allowed detailed characterization of the anti-DNA antibodies. A remarkable range of cross-reactions with, for example, multiple polynucleotides, cardiolipin and other phospholipids, proteoglycans, haptenes, and some intracellular proteins has been displayed (3–5). To understand whether a selective process is operative, it is necessary to compare the cross-reactivity patterns of monoclonal anti-DNA antibodies from normal and “autoimmune” donors. Other studies of the normal B-cell repertoire of mice (6–9) show a surprisingly high incidence of cells producing antibodies that can bind to self-antigens including DNA.

Experiments of Hayakawa et al. (10) suggest that the subset of Ly-1-positive (Ly-1+) B cells are of special importance in autoantibody production, including anti-DNA and anti-erythrocyte autoantibodies. The present experiments use in vitro clonal analysis for the identification of potentially anti-DNA antibody-forming cells (AFC) from normal, autoimmune, and congenitally athymic mouse strains and among cell populations enriched for Ly-1+ B cells.

MATERIALS AND METHODS

Mice. Specific pathogen-free male and female CBA/Ca-H-Wehi (CBA +/+), BALB/c AnBradley-Wehi (BALB/c +/+), C57BL/6J Wehi (C57BL/6 +/+), NZB/B12 Wehi (NZB +/+), and the congenic athymics CBA nu/Wehi (CBA nu/nu) and C57BL/6J nu/Wehi (C57BL/6 nu/nu) were used at 6–12 weeks of age, except NZB +/+ mice, which were 4–12 months of age.

Antigens. DNA (herring testes DNA, type XIV, Sigma) was used as native DNA or as denatured DNA. The denatured DNA was prepared as described by Serban et al. (11), treated with DNase-free RNase, then stored at −70°C until use. High molecular weight genomic DNA extracted from the ST4 murine T-cell hybridoma was provided by I. van Driel of this institute. The following materials were obtained from the Sigma: poly(t-Lysine) [polylysine, M_r >47,000]; poly(A); poly(dT); chondroitin sulfate; dextran sulfate (type 500-S). Cardiolipin (bovine heart) and L-α-phosphatidic acid (egg yolk lecithin) were prepared as described by Lafer et al. (12). The solution of polyvinyladenine (13) had an OD260 of 106. Bovine serum albumin (BSA) coupled to azophenylphosphorylcholine or with fluorescein (Flu) isothiocyanate was also used.

Cell Populations and Fluorescence-Activated Cell Sorter Procedures. Spleen cell suspensions, peritoneal exudate cells, and Flu-gelatin binding B-cell populations depleted of erythrocytes and damaged cells were prepared as described (14–17). For analysis and sorting with the fluorescence-activated cell sorter (FACS) (FACS II; Becton Dickinson), NZB splenocytes were sequentially labeled with a fluoresceinated sheep F(ab')2 anti-mouse immunoglobulin reagent, a biotinylated rat mAb to mouse Ly-1, then phycoerythrin-streptavidin (Serotec; Blackthorne Biester, U.K.). The anti-Ly-1 staining step was omitted for a fraction of cells that served as controls for autofluorescence. An initial two-parameter analysis was used to determine appropriate gating for red and green fluorescence for each individual spleen preparation to enable separation of the surface immunoglobulin-positive, dull Ly-1+ B cells and Ly-1− cells with an equivalent intensity of surface immunoglobulin.

B-Cell Cloning System. The filler cell-supported cloning system was used as described (18). B-cell populations were stimulated with Escherichia coli lipopolysaccharide (LPS) at 50 μg/ml (Difco) at an average of from 20 to 600 cells per well in the presence of 500 3T3 fibroblast-filler cells in 60-well Terasaki trays in 10 μl of RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum and 100 μM 2-mercaptoethanol. Trays were held at 37°C in a humidified atmosphere of 10% CO₂/90% air.

Abbreviations: AFC, antibody-forming cell; AFCp, AFC precursor(s); Flu, fluorescein; LPS, lipopolysaccharide; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; BSA, bovine serum albumin.

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Assay for AFC Clones. An ELISA was performed essentially as described (18, 19). For detection of all AFC clones, irrespective of specificity, flexible 96-well U-bottomed polystyrene microtiter plates (Dynatech, Alexandria, VA, or Costar, Cambridge, MA) coated with affinity-purified sheep anti-mouse immunoglobulin antibody (Silenus Laboratories, Dandenong, Australia) were used. Culture supernatants were individually transferred into wells of the assay tray with 40 μl of PBS (0.02 M, pH 7.2) containing 0.3% skim milk powder, 0.05% Tween 20, and 1% fetal calf serum (M-PBS). For antigen stimulation, cultures were fluo-

Inappropriate to make interstrain comparisons based on this data but a substantial fraction, albeit generally less than one-half, of the total B-cell repertoire was surveyed. For estimation of the frequency of AFC clones producing antibody reactive with either denatured DNA or Flu, supernatants were assayed on denatured DNA, Flu-BSA, and poly-

RESULTS

AFC, Anti-Denatured DNA, and Anti-Flu Clone Formation by Murine Splenocytes. An assessment of the frequency of activation by LPS of splenic B cells to AFC clone-forming status, was always performed parallel to the determination of the frequencies of anti-denatured DNA and anti-Flu AFC precursors (AFCp) so that specific precursor frequencies could be expressed in terms of total input splenocytes as well as total resultant immunoglobulin-secreting clones. Supernatants from cultures containing from 1 to 30 splenocytes, stimulated with LPS for 6 days in the presence of 3T3 filler cells were assayed for AFC clones. Plots of the number of input cells vs. the logarithm of the fraction of nonresponding wells gave consistent linearity (data not shown) as expected according to the Poisson equation (20). The frequency of AFCp of a variety of mouse strains is shown in Table 1. The high SEM seen with some strains is a reflection of the fact that these experiments were performed over a lengthy period with unavoidable changes in the system. It would, therefore, be in

Table 1. Frequencies of AFC, anti-denatured DNA, and anti-Flu precursors among splenocytes from various mouse strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>% AFC clones*</th>
<th>Precursor frequency, no. per 10⁶ splenocytes†</th>
<th>Precursor frequency, no. per AFCp‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-denatured DNA</td>
<td>Anti-Flu</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CBA +/+</td>
<td>8.08 ± 1.41</td>
<td>3.54 ± 0.36 (1/282)</td>
<td>1.26 ± 0.11 (1/794)</td>
</tr>
<tr>
<td>BALB/c +/+</td>
<td>3.40 ± 0.76</td>
<td>0.93 ± 0.16 (1/1075)</td>
<td>0.27 ± 0.07 (1/3704)</td>
</tr>
<tr>
<td>BS7BL/6 +/+</td>
<td>8.49 ± 0.89</td>
<td>3.33 ± 0.98 (1/300)</td>
<td>1.42 ± 0.50 (1/704)</td>
</tr>
<tr>
<td>CBA nu/nu</td>
<td>15.2 ± 5.33</td>
<td>5.06 ± 2.85 (1/198)</td>
<td>2.45 ± 1.49 (1/408)</td>
</tr>
<tr>
<td>BS7BL/6 nu/nu</td>
<td>29.8 ± 18.9</td>
<td>2.70 ± 2.03 (1/370)</td>
<td>1.64 ± 1.45 (1/610)</td>
</tr>
<tr>
<td>NZB +/+</td>
<td>18.7 ± 2.17</td>
<td>3.22 ± 0.13 (1/310)</td>
<td>1.84 ± 0.62 (1/543)</td>
</tr>
</tbody>
</table>

*Percent of input splenocytes forming AFC clones per se, irrespective of specificity. Values represent mean ± SEM for four or five experiments.
†Frequency of AFC clones reactive against Flu-BSA, polysine only, or polysine-denatured DNA in a split-assay procedure. Rare clones with similar OD values on both polysine and polysine-denatured DNA trays taken as false positives. Values in parentheses represent the incidence of AFCp.
‡Average value of the proportion of AFC clones reactive against either denatured DNA and/or Flu as determined for each individual experiment.
no crossreactions with denatured DNA, were noted in these studies. To address the question of antibody cross-reactions between Flu and denatured DNA more definitively, affinity-enriched, Flu-specific B cells (14-16) were stimulated at ≤1 AFCp per well. The fraction of AFC clones secreting anti-Flu antibody also reactive with denatured DNA was 13 ± 2% (three experiments, data not shown).

Anti-Denatured DNA AFCp Among Cell Populations Enriched or Depleted for Ly-1⁺ B Cells. To test whether anti-denatured DNA AFCp are more frequent in the Ly-1⁺ B cells, AFC clones generated from populations enriched or depleted for such cells were examined. Ly-1⁺ B cells reportedly (17) constitute up to 50% of peritoneal B cells. In three experiments (Table 3), peritoneal cells from CBA donors showed no enrichment for anti-denatured DNA AFC clones. The spleens of many adult NZB mice contain a sizeable, discrete population of dull Ly-1⁻ B cells (Fig. 3A), as reported by others (10). Populations highly enriched for Ly-1⁺ or Ly-1⁻ B cells (Fig. 3B) were selected using the FACs, then assessed for AFC-clone formation following LPS stimulation in vitro. The selected Ly-1⁺ B cells were slightly but not significantly lower in total AFCp than were Ly-1⁻ B cells (Table 3). Interestingly, less cellular proliferation was evident in the Ly-1⁺ B cells, and there was some evidence to indicate that the mean absorbance per AFC clone was also lower (data not shown). There was no indication that the Ly-1⁻ B cells were depleted of anti-denatured DNA AFCp relative to the Ly-1⁺ B cells. The comparative anti-

Table 2. Cross-reactions of anti-denatured DNA AFC clones

<table>
<thead>
<tr>
<th>Test antigen</th>
<th>% anti-denatured DNA clones cross-reactive*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse genomic DNA</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>Poly(A)</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>Poly(dT)</td>
<td>54 ± 20</td>
</tr>
<tr>
<td>Polyvinyladenine</td>
<td>ND</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>ND</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>Dextran sulfate</td>
<td>5 ± 3</td>
</tr>
<tr>
<td>Flu-BSA</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Phosphocholine-BSA</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

*Mean ± SEM based on analysis of from 45 to 270 AFC clones reactive against denatured DNA generated from CBA splenocytes. Polysine-pretreated trays were coated with polynucleotides (5 μg/ml) and the polyanions chondroitin sulfate and dextran sulfate (both 20-500 μg/ml). The following concentrations were tested for inhibition: polyvinyladenine, 1:100 stock; cardiolipin at 20 and 50 μg/ml; phosphatidic acid at 50 μg/ml; chondroitin sulfate at 25, 50, 100, and 1000 μg/ml; dextran sulfate at 100 and 1000 μg/ml. As there were no clear differences, results for each inhibitor at different concentrations were pooled. ND, not done.

A decrease in OD of ≥50% was considered significant inhibition. High molecular weight genomic DNA was extracted from murine T-cell hybridoma cells.

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Fig. 1. Limiting dilution analysis of anti-denatured DNA AFC clones generated among splenocytes from various mouse strains following LPS stimulation. Results from four separate experiments are shown. For CBA +/+ , 1/148 splenocytes was an anti-denatured DNA AFCp (A); for C57BL/6 +/+ , 1/382 splenocytes was an anti-denatured DNA AFCp (B); for NZB +/+ , 1/295 splenocytes was an anti-denatured DNA AFCp (C); and for C57BL/6 nu/nu, 1/771 splenocytes was an anti-denatured DNA AFCp (D).

Fig. 2. Preferential binding of normal CBA anti-DNA mAbs to denatured DNA as compared to native DNA. Supernatants from 21 clones were split-assayed on ELISA trays coated with either denatured DNA or native DNA.
denatured DNA/AFC clone ratios for the Ly-1+/Ly-1- B cells varied widely between experiments.

**DISCUSSION**

These studies were undertaken to characterize more fully the anti-DNA AFCp repertoire primarily in mice not noted for the spontaneous development of autoimmunity. The first clear conclusion is that AFCp committed to production of antibodies reactive with denatured DNA are remarkably frequently represented in the repertoire of normal mice constituting 1–5% of all LPS-responsive AFCp among splenic B cells of four mouse strains representing different immunoglobulin heavy-chain haplotypes. This could be an underestimation as some small clones might have produced too little antibody to score as positive on denatured DNA-coated trays while still being detectable on a tray coated with high-affinity sheep anti-mouse immunoglobulin antibodies. A high frequency of anti-DNA AFCp in the normal repertoire has been indirectly suggested by some reports (23–26). Our results confirm those of Pisetsky and Caster (27), who performed a similar quantitative clonal analysis and concluded that anti-DNA AFCp are frequent in the B-cell repertoires of two normal mouse strains.

Why are anti-denatured DNA AFCp so common in the normal repertoire? One possibility is that the phenomenon has been evolutionarily selected; that is, the anti-DNA clones may have an important role to play in normal mice (28). The explanation we favor is simpler and based primarily on physicochemical factors. The relevant physical property of denatured DNA that renders it "attractive" to many IgMs could be its many appropriately spaced similar or identical hydrophobic epitopes. The simultaneous occupation of two or more antigen-binding sites on an antibody molecule by a multiepitope antigen permits detectable antibody binding even when the intrinsic affinity of each individual binding site is relatively low (29, 30), and multipoint binding is more readily achieved by IgMs than by IgGs. It has also been reported (27, 31, 32) that AFCp specific for relatively hydrophobic epitopes, such as the trinitrophenyl hapten, are more frequent in the B-cell repertoire than are those specific for hydrophilic determinants. These principles may also explain the results of Andersson et al. (33), who noted that a strikingly high percentage (up to 10%) of IgM AFCp in the normal murine B-cell repertoire showed apparent specificity for a nitrophenyl hapten when indicator erythrocytes employed in their hemolytic plaque assays were densely coupled with the hapten.

Does the presence in high numbers in normal mice of AFCp the IgM product of which can bind a self-antigen with detectable affinity rule out the theory that self-tolerance may be based in part on the purging of immature self-reactive B cells from the repertoire (34)? We believe that this is not necessarily the case. First, it would be necessary to determine the frequency of AFCp with specificity for a nonself-antigen of complexity comparable to denatured DNA to know whether the frequency estimated for denatured DNA is relatively high or low. Second, free DNA may not be present in a sufficiently high concentration in body fluids (35) to effect tolerance in the B-cell compartment, i.e., DNA may represent a sequestered self-antigen. Third, even if the B-cell repertoire modification is significant only a small fraction of the highest-affinity anti-DNA clones may be eliminated or silenced.

In the present studies, athymic CBA and C57BL/6 mice showed slightly but not significantly lower proportions of anti-denatured DNA and anti-Flu clones than did their euthymic counterparts, consistent with reports of no thymic influence on the B-cell idiotype repertoire (36) and the frequencies of AFCp specific for nonself-antigens (32). The frequency of anti-denatured DNA AFC clones from NZB donors was, surprisingly, the lowest of all euthymic strains analyzed. Pisetsky and Caster (27) reported no apparent increase in the frequency of anti-DNA AFCp in a different autoimmune strain, MRL-lpr/lpr.

The cross-reactivities displayed by "normal" anti-denatured DNA clones demonstrated certain similarities to those reported for anti-DNA lupus mAbs. Thus, the frequent
cross-reactions of CBA anti-denatured DNA mAbs with poly(A) and poly(dT) accords with the finding by others (3–5) that lupus mAbs to DNA often display reactivity with multiple polynucleotides and is in contrast to the strict specificity noted to be shown by intentionally induced antibodies for the particular immunizing polynucleotide antigen. A preference in binding to primarily single-stranded denatured DNA as opposed to largely double-stranded native DNA has been noted for the majority of lupus anti-DNA antibodies (37) and was found here to be true also for those deriving from the normal murine repertoire. Our finding of significant antibody cross-reactions between denatured DNA and the polycyclc aromatic Flu hapten parallels the binding of some lupus anti-DNA mAbs to the hydrophobic trinitrophenyl group (11). In contrast, anti-denatured DNA clones from normal mice in our studies lacked reactivity with cardioliopin and chondroitin sulfate, cross-reactions that have sometimes, but not always, been noted with lupus anti-DNA mAbs (11, 12, 21).

We interpret the specificity studies described herein as suggesting that interactions with the bases of DNA may be important for many of the denatured DNA-reactive mAbs from normal mice. Thus, the preferential binding displayed by most normal mAbs to denatured DNA as compared to native DNA may be a consequence of the greater exposure of bases in the former. Also, the fact that the anti-denatured DNA mAbs showed no detectable reaction with two phospholipids or phosphocholine indicates that the mere presence of phosphate esters does not necessarily suffice to mimic the relevant epitopes of denatured DNA. Furthermore, polyvinyladenine, which lacks a sugar-phosphate backbone, significantly inhibited the binding of a fraction of the mAbs to denatured DNA. Finally, the Flu hapten could be said to “resemble” the aromatic bases more than the backbone. However, the idea that the anti-denatured DNA mAbs react largely with either the bases alone or the backbone alone is undoubtedly overly simplistic as the spatial configuration of the polynucleotide is likely to be critical to the definition of many actual epitopes.

Studies of Hayakawa et al. (10) indicate that most AFCp committed to production of antibodies reactive with blemain-treated mouse erythrocytes in either normal or autoimmune mice are confined to the Ly-l+ B-cell subset. In contrast, our results show that the Ly-l+ B cells contain plentiful numbers of anti-denatured DNA AFCp. In most experiments involving FACS-sorted B cells, the Ly-l+ B cells appeared to be somewhat depleted of anti-denatured DNA and anti-Flu AFCp compared with the unsorted or Ly-l- populations. The two following possible explanations for how such a result could be reached artifically are: (i) the mean clone size could be lower for AFC clones derived from Ly-l+ as compared to Ly-l- B cells, or (ii) many AFC clones originating from the Ly-l- subset could secrete lower-avidity IgGs rather than IgMs. This requires further analysis. Our results deal only with the situation at the level of the repertoire and do not in any way exclude the hypothesis that Ly-l+ B cells are the actual secretors of most autoantibodies in some autoimmune syndromes.

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