

INDUCTION OF A CATIONIC SHIFT IN IgG ANTI-DNA AUTOANTIBODIES

Role of T Helper Cells with Classical and Novel Phenotypes in Three Murine Models of Lupus Nephritis

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Spontaneously produced anti-DNA autoantibodies play a major role in the pathogenesis of glomerulonephritis and other autoimmune lesions of systemic lupus erythematosus (SLE)¹ (1). However, B cells from normal individuals and even normal fetal or newborn mice can produce such autoantibodies (2–4). Those natural anti-DNA antibodies are usually of IgM class (4). By contrast, pathogenic anti-DNA antibodies in SLE are IgG in isotype and cationic in charge (5, 6). Glomeruli in the kidney have anionic sites and a net negative charge (7, 8); therefore, cationic antibodies that form immune complexes bearing a net positive charge are preferentially deposited in the kidney to cause glomerulonephritis (9). Pathogenic anti-DNA autoantibodies can also be distinguished from nonpathogenic anti-DNA antibodies by their antigenic specificity patterns and idiotypic markers (10–12). Therefore, the origin and regulation of pathogenic autoantibodies may be different from natural autoantibodies.

Here we investigated the cellular mechanisms controlling the production of pathogenic anti-DNA autoantibodies that are of IgG class and cationic in charge, in three apparently different murine models of SLE. The (NZB × NZW)F₁ (B/WF₁) and (NZB × SWR)F₁ (SNF₁) hybrids studied here resemble human SLE; early and severe lupus nephritis occurs spontaneously and uniformly in the females by 5–8 mo age (13–15). The SWR parents of the SNF₁ hybrids do not have any autoimmune abnormalities (13, 14), whereas the NZW parents of the B/WF₁ mice develop autoantibodies and nephritis later in life (16). We also studied the MRL-+/+ mice that produce anti-DNA autoantibodies by 5 mo (17) and begin to die from lupus nephritis between 12 and 18 mo of age (15, 17). In the MRL model, the primary mechanism of autoimmunity lies in the genetic background of the MRL-+/+ strain (18). A recessive lymphoproliferation gene, *lpr*, exerts a secondary accelerating effect on the disease in the MRL-*lpr/lpr* strain, which is the congenic partner of the lupus-prone MRL-+/+ strain (15, 18). Each of these strains manifests distinct and multiple abnormalities of T and

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¹ *Abbreviations used in this paper:* CRI, crossreactive idiotype; dsDNA, double-stranded DNA; SAS, saturated ammonium sulfate; SLE, systemic lupus erythematosus; ssDNA, single-stranded DNA.

B lymphocytes, suggesting that the cellular bases of lupus in the NZB and MRL (MRL/*lpr*) models are different (15, 19). However, genetic studies have demonstrated that several generalized nonspecific abnormalities of T and B cells found in these lupus strains are not primarily required for the development of autoimmune disease (14, 19, 20). For instance, the polyclonal hyperactivity of B cells of NZB mice that occurs due to an expansion of the Ly-1⁺ B cell subset (21, 22) can be dissociated from the development of autoimmune hemolytic anemia (20) and lupus nephritis (14). These observations are consistent with the properties of antierythrocyte autoantibodies; the fine antigenic specificity and isotype of the pathogenic variety of antibodies (23) are entirely different from the natural antierythrocyte antibodies produced by Ly-1⁺ B cells of NZB and normal mice (22, 23). Similarly, the anti-DNA autoantibodies produced by the hyperactive B cells of NZB mice are predominantly of IgM class, anionic in charge, and are nonpathogenic (10, 22). Severe lupus nephritis occurs only when the NZB mice are crossed with the NZW or SWR strains and when highly cationic nephritogenic anti-DNA autoantibodies of IgG class with distinct immunochemical and idiotypic profile are produced in the F₁ hybrids (10–12). Therefore, the production of pathogenic autoantibodies is determined by discreet cellular and genetic defects that are distinct from the generalized abnormalities associated with overproduction of natural autoantibodies.

In the present study we found that all three non-*lpr* lupus strains (B/WF₁, SNF₁ and MRL-+/+) manifested a common cellular defect specifying the production of pathogenic (cationic) IgG anti-DNA autoantibodies at about the age they began to develop glomerulonephritis.

Materials and Methods

Mice. NZB, SWR, (NZB × NZW)_{F1} (B/WF₁), MRL-+/+, C3H, AKR, and BALB/c mice were obtained from The Jackson Laboratory, Bar Harbor, ME. (NZB × SWR)_{F1} or (SWR × NZB)_{F1} (SNF₁) hybrids were bred by us. Maternal direction of the cross does not affect the incidence of autoimmune disease in these mice (13, 14).

Isolation of Lymphocyte Subpopulations. T lymphocytes were separated from spleen cell suspensions by two cycles of panning on goat anti-mouse Ig-coated plates as described (24). 97–99% of the nonadherent cells were Thy-1.2⁺ positive and <1% were surface Ig⁺, as shown by immunofluorescence (cytofluorograph, Ortho Diagnostic Systems Inc., Westwood, MA). B cells (plus macrophages) were separated by treating the spleen cells twice with an anti-Thy-1.2 mAb (New England Nuclear, Boston, MA) and rabbit complement (C) (H2; Pel-Freeze Biologicals, Rogers, AR). The Lyt-2⁻ (L3T4⁺) T cell subset was prepared by treating the purified T cells with anti-Lyt-2.2 mAb AD4(15) (reference 25; a gift of Dr. David Raulet, Massachusetts Institute of Technology, Cambridge, MA) and C, in the case of the NZB hybrid T cells, or anti-Lyt-2.1 mAb 116-13.1 (HB-129; American Type Culture Collection, Rockville, MD) and C, in the case of the MRL-+/+ T cells. The L3T4⁻ (Lyt-2⁺) subset was prepared by treating the T cells with the anti-L3T4 mAb GK1.5 (TIB207, ATCC) and C, as described (26). To deplete Lyt-1⁺ cells, two anti-Lyt-1.2 mAbs plus C were used with similar results; one mAb was from clone 3-3.1, a gift of Dr. F. W. Shen (Sloan-Kettering Memorial Cancer Center, New York), and the other was C3PO.7, from Dr. Jan Klein (Max Planck Institute, Tubingen, Federal Republic of Germany). All antibody treatments were done by incubating the cells (20 × 10⁶/ml) with an optimum dilution of the mAb for 40 min at 4°C followed by C (10⁷ cells/ml) for 45 min at 37°C. Where cells were treated with two or three antibodies, sequential treatments were done with each mAb, then they were treated with complement followed by washing before incubation with the next mAb. Unfractionated T cells were treated with C alone

for use in the cultures. Efficacy of the antibody plus C treatments were determined by functional assays and immunofluorescence analysis as described (25, 26).

Culture of Fractionated Lymphocytes. Cells were cultured in Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY), supplemented with 5×10^{-5} M 2-ME, 10% FCS, and antibiotics in multi-well trays (24-well cluster; No. 3524; Costar, Cambridge, MA). Each well contained a combination of 2.5×10^6 B cells (plus macrophages) in 1 ml medium plus 2.5×10^6 T cells or T cell subsets in another milliliter of medium. In preliminary studies, we found the above ratios to be optimum for antibody production. Each culture combination was set up in six replicate wells. These cells were cultured for 5 d in 6% CO₂, humidified atmosphere at 37°C.

Quantitation of Total Ig, IgG, and IgG Class Anti-DNA Antibodies Produced in the Culture Supernatants. On the day 5 of culture the supernatants were harvested after centrifugation of the cells, filtered (0.22 μ m), centrifuged (10,000 g for 20 min), and frozen in aliquots in the presence of protease inhibitors. Total Ig and IgG produced in the culture supernatants were quantitated by ELISA methods as described (12, 27). Three serial dilutions of culture supernatants were tested in duplicate. Standard curves were obtained with BALB/C-derived myeloma Ig (Litton Bionetics, Kensington, MD) of appropriate isotypes (12).

IgG class antibodies to single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) were also measured by ELISA as previously described (10–12). Appropriately blocked DNA-poly-L-lysine-coated plates were incubated with serial dilutions of culture supernatants followed by washing; the bound antibodies were then detected with goat anti-mouse IgG conjugated to alkaline phosphatase (1:2,000 dilution, Boehringer Mannheim Diagnostics, Inc., Houston, TX). The rest of the assay was the same as described (10, 12). The culture supernatants were also incubated in duplicate poly-L-lysine-coated wells that did not contain DNA and the values of binding to these sham wells were subtracted from the binding values in the DNA coated wells. Besides this specificity control, competitive inhibitions of the antibodies in the culture supernatants by incubation with ssDNA or dsDNA (10) before addition to the DNA-coated wells were also done. Finally, aliquots of saturated ammonium sulfate (SAS)-precipitated proteins from each culture supernatant after treatment with immobilized deoxyribonuclease (see below) were also assayed for anti-DNA antibodies and the results were comparable to those for untreated culture supernatants. Eight serial twofold dilutions of a IgG anti-DNA mAb (No. 205; reference 10) that binds equally well to ssDNA and dsDNA, was used to generate a standard curve in each assay. The concentrations of the anti-DNA mAb 205 in the dilutions ranged from 5.4 μ g/ml to 0.042 μ g/ml. The A₄₀₅ value of DNA binding activity of the mAb at a concentration of 1 μ g/ml was taken arbitrarily to be equivalent to 1 U of anti-DNA antibody per milliliter. In all the assays with each 5-d culture supernatant the values for duplicate day 0 (5-min incubation) culture supernatant were subtracted to eliminate measurement of any antibodies produced in vivo.

Isoelectric Focusing and Immunoblots of IgG Produced in the Culture Supernatants. Aliquots of the 5-d culture supernatants from each cell culture combination were pooled (1.5-ml aliquot from each well equals a total of 9 ml from six replicate wells), then were precipitated by 47% SAS, resuspended, and dialyzed against PBS using a pro-Dicon concentrator dialyzer (Bio-Molecular Dynamics, Beaverton, OR). The SAS-cut proteins of the different culture supernatants were thus concentrated to equal volumes (0.9 ml). Protein and total IgG in the concentrated samples were monitored by a protein assay (Bio-Rad Laboratories, Richmond, CA) and ELISA, respectively. The concentrations of IgG in the different SAS-cut samples remained the same relative to each other as in the original uncut supernatants. Aliquots of each sample were then loaded onto 1% agarose gels containing 10% sorbitol, 3 M urea, and a mixture of ampholytes to undergo IEF according to previously described methods (12). The ampholine concentrations were slightly different from the previous gels (12) to provide a sharper resolution of the cationic bands; 0.6 ml of pH 3–10 ampholines and 0.4 ml each of pH 8–10.5 and pH 6.5–9 ampholines were added to the gel mixture. The focused proteins were then blotted onto nitrocellulose. After blocking and washing, the blots were reacted with rabbit anti-mouse IgG (1:1,500 dilution; Cappel

Laboratories, Malvern, PA) followed by biotin-conjugated donkey anti-rabbit IgG. The immunoblots were then developed with streptavidin-peroxidase as described (12).

Affinity Chromatography on DNA-Cellulose Columns Followed by IEF Immunoblots to Detect IgG Class Anti-DNA Antibodies with Cationic Charge. Aliquots (0.6 ml) of the SAS-precipitated proteins from the culture supernatants described above were next incubated individually with immobilized Sepharose-bound deoxyribonuclease (5 U in a 25- μ l volume; Worthington Biochemical Corp., Freehold NJ) to remove any DNA bound to the anti-DNA antibodies produced in the cultures (28, 29). After digestion, the reaction was stopped with 10 mM EDTA and the Sepharose-DNase beads were removed by centrifugation. The supernatants were dialyzed and concentrated in PBS/10 mM EDTA to 0.4-ml volumes using Centricon microconcentrator dialyzer tubes (Amicon Corp., Danvers, MA) and then fractionated by DNA-affinity column chromatography as described (3, 27). We used DNA-cellulose (28, 29) columns for these experiments; 0.125 gm of each of ssDNA- and dsDNA-cellulose (Sigma Chemical Co., St. Louis, MO) were mixed and swollen to yield a 1-ml packed down column. The conditions for washing, sample application and elution of the columns were the same as described (3, 27) except, the flow-through and eluate fractions were monitored for protein content by a spectrophotometer since these samples were not radioactively labeled. All fractions with an A_{280} above 0.001 were collected. The capacity and specificity of the affinity columns were determined with standard anti-DNA mAbs that were either unlabeled or metabolically labeled with [35 S]methionine (3, 27).

The flow-through and eluate fractions from the DNA-affinity columns were next pooled separately, then concentrated and dialyzed against PBS using the Centricon tubes to 100 μ l volumes each. Thus the original SAS-precipitated proteins (600 μ l) from the culture supernatants were further concentrated threefold (100 μ l flow-through and 100 μ l eluate) after the DNA-affinity chromatography. In some cases (see Results), the eluates were concentrated further to 50 μ l volumes. Protein content (Bio-Rad Laboratories assay), total Ig, IgG, and IgG class anti-DNA antibody (by ELISA) in the flow-through and eluates from each sample were next measured. The pooled flow-through fractions had no DNA-binding activity (background levels), and from 80–90% of the DNA-binding IgG in the original unfractionated samples could be recovered in the eluates. Aliquots of each flow-through and eluate samples underwent IEF and blotted onto nitrocellulose, and then IgG in the focused proteins were detected as described above.

Flow Cytometry Analysis. Single cell suspensions were stained for analyses with a FACS as described (30) using a cytofluorograph from Ortho Diagnostic Systems Inc., Westwood, MA. Saturating concentrations of antibodies were used. In the case of staining with anti-Thy-1.2, anti-Lyt-2.1 or anti-Lyt-2.2, and anti-Lyt-1.2 antibodies, an FITC-rabbit anti-mouse Ig (anti-IgM plus IgG plus IgA) was used as the second-step antibody. For staining the cells with anti-L3T4, the second step antibody used was FITC-rabbit anti-rat IgG. Both second-step antibodies were affinity purified and absorbed to remove cross-species reactivity (Nos. 64-6411 and 61-9611, respectively; Zymed Laboratories, San Francisco, CA) and they were diluted in 10% absorbed normal rabbit serum containing FACS buffer (30). Background control staining of the cells was done by reacting with an isotype-matched BALB/c-derived myeloma or normal rat IgG (in the case of L3T4 control) followed by incubation with the respective second-step antibodies. Direct immunofluorescence staining was done for Ly-5 (B220) antigen by using FITC-conjugated mAb 6B2 (31), kindly provided by Herbert C. Morse (National Institutes of Health, Bethesda, MD); 10 μ l was used to stain 10^6 cells in a 50- μ l volume. An FITC-rat IgG (Zymed Laboratories) was used for background control staining.

Results

T Cells of Old Lupus Mice are Essential for Inducing B Cells of Old Lupus Mice to Produce Cationic Anti-DNA Antibodies of IgG Class. In preliminary studies, spleen cells of old B/WF₁ (5–6 mo old), SNF₁ (5–6 mo old), and MRL-+/+ (~11 mo old) mice spontaneously produced highly cationic IgG class autoantibodies to

TABLE I
Spontaneous Production of Igs and IgG Anti-DNA Antibodies by B Lymphocytes of Old (NZB × NZW)_F₁ (B/WF₁) Mice in Different Culture Combinations

Groups	Types of cells cocultured with old B/WF ₁ B cells*	Total Ig <i>μg/ml</i>	Total IgG <i>μg/ml</i>	IgG anti-DNA antibody [‡]		Cationic shift [§]	
				Anti-ssDNA <i>U/ml</i>	Anti-dsDNA	Total IgG	Anti-DNA
A	None ^d	5.65 ± 1.08	0.31 ± 0.09	0.085 ± 0.06	0 [¶]	—	—
B	Old (B/WF ₁) T**	14.10 ± 1.61	2.68 ± 0.25	0.675 ± 0.09	0.109 ± 0.008	+++	+++
C	Lyt-2 ⁻ old T	17.08 ± 2.31	3.30 ± 0.39	0.892 ± 0.12	0.186 ± 0.011	+++	++++
D	L3T4 ⁻ old T	13.59 ± 1.94	2.71 ± 0.28	0.649 ± 0.04	0.113 ± 0.007	+++	+++
E	L3T4 ⁻ ,Lyt-2 ⁻ , old T	14.24 ± 1.75	2.59 ± 0.27	0.602 ± 0.07	0.116 ± 0.009	+++	+++
F	L3T4 ⁻ ,Lyt-2 ⁻ ,Lyt-1 ⁻ old T	9.35 ± 1.43	1.03 ± 0.43	0.215 ± 0.15	0.043 ± 0.010	++	++
G	Young T**	6.24 ± 1.20	0.73 ± 0.18	0.166 ± 0.08	0	—	—
H	Lyt-2 ⁻ young T	7.23 ± 2.02	0.76 ± 0.15	0.205 ± 0.10	0	+	—
I	L3T4 ⁻ young T	6.35 ± 1.28	0.63 ± 0.19	0.151 ± 0.04	0	—	—
J	Young T + old T ^{‡‡}	8.60 ± 1.20	1.72 ± 0.24	0.440 ± 0.07	0.075 ± 0.008	++	++
K	Dead old T + old T ^{‡‡}	7.25 ± 1.40	1.70 ± 0.22	0.385 ± 0.09	0.082 ± 0.005	++	++
L	L3T4 ⁻ ,Lyt-2 ⁻ old T cultured alone, without old B cells	0.29 ± 0.13	0	0	0	—	—

* T cells and B cells (plus macrophages) were fractionated from spleen cells of old (5–6 mo) and young (1–2 mo) (B/W)_F₁ mice and were cultured in different combinations for 5 d, after which the culture supernatants were assayed for antibody production. The optimum ratios were 2.5×10^6 T cells of different phenotypes with 2.5×10^6 B cells per 2 ml of culture and six replicate cultures for each combination (group) were done. The results represent mean ± SD of three experiments. For each experiment, spleen cells from 15 old and 12 young mice were pooled separately and fractionated for use. Old B, anti-Thy-1 + C-treated spleen cells of old mice. Old T and young T, anti-Ig plate nonadherent spleen cells of old mice and young mice, respectively.

[‡] 1 U is equivalent to the amount of DNA-binding activity of an IgG anti-DNA mAb 205, at a concentration of 1 μ g/ml. The mAb 205 binds strongly to both ssDNA and dsDNA (10), and was used as a standard in all assays.

[§] —, No cationic bands on IEF even when several-fold more of the samples (relative concentration and volume) were loaded on the IEF gels. +, Intensity and degree of cationicity of the protein bands (see Fig. 1).

[¶] Similar results were obtained when T cells from old B/WF₁ mice were irradiated (3,000 rad) and 2.5×10^6 dead cells were washed and added to the cultures.

[¶] 0, Not detectable (<0.003 U/ml).

** These T cells were treated with C alone.

^{‡‡} In groups J and K, 1.25×10^6 of each type of T cell were used in the cultures.

both ssDNA and dsDNA in vitro, at or just before the age they began to develop lupus nephritis. In contrast, the IgG anti-DNA antibodies produced by the spleen cells of young preautoimmune mice (1–2 mo) of the same strains were much less in amount, anionic in charge, and were mostly specific for ssDNA (data not shown). The spleen cell populations were then fractionated and cultured for 5 d in different combinations (Tables I, II, and III). In the case of the old B/WF₁ cells in culture (Table I), T cells augmented the production by B cells (group B vs. A) of total Ig by 2.5-fold ($p < 0.1$), total IgG 8.6-fold ($p < 0.005$), and IgG anti-ssDNA antibodies by 7.9-fold ($p < 0.001$), on the average. IgG anti-dsDNA antibodies were detectably produced only with the help of the T cells. Highly cationic polyclonal IgG and IgG class anti-DNA antibodies were produced by the B cells only when they were cultured with the T cells of old B/WF₁ mice (Table I and examples in Fig. 1A, lane 1; B, lanes 1 and 2; and C). Neither cationic anti-DNA antibodies nor DNA non-binding IgG with cationic charge were detectable when the B cells (Table I, group A) were cultured alone (Fig. 1B, lanes 3 and 4), even though the focused antibodies from such cultures were concentrated severalfold more (3-fold after DNA-affinity chromatography and 4.5-fold more volume of the sample was loaded for IEF) than the unfractionated IgG samples from old T plus old B cultures (Fig. 1A, lane 1 vs. B, lanes 3 and 4).

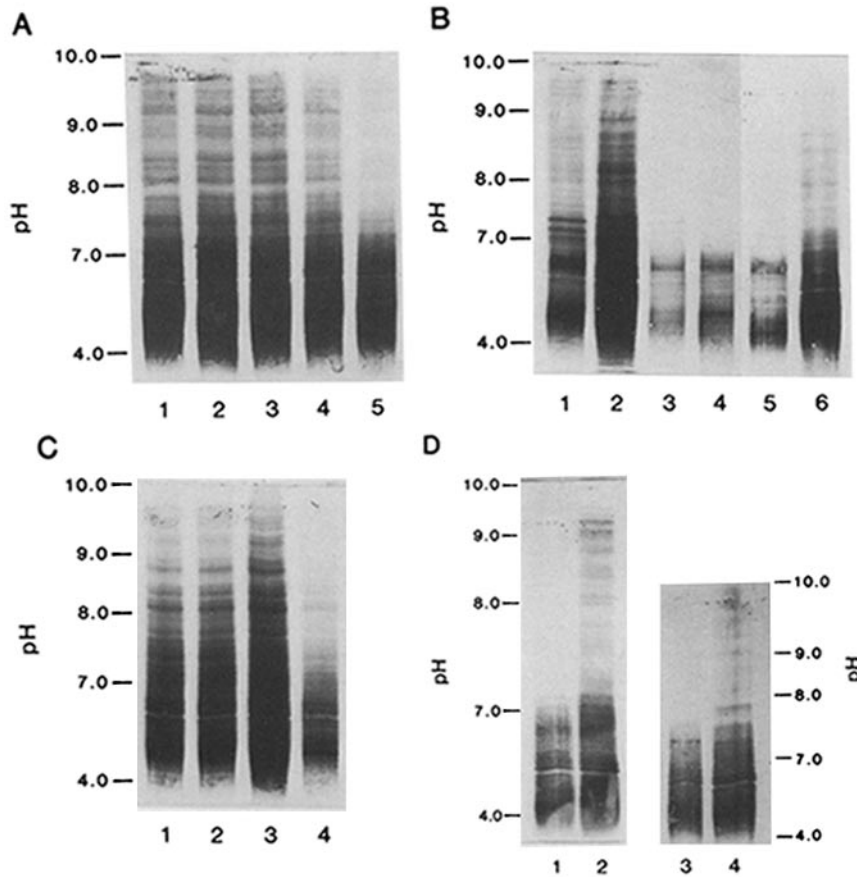


FIGURE 1. Examples of immunoblots of isoelectrically focused IgG antibodies produced in culture supernatants. (A) Total polyclonal IgG produced by B/WF₁ cells in different culture combinations. SAS-precipitated proteins from the culture supernatants underwent IEF followed by blotting and then were reacted with anti-mouse IgG-specific antibody. (1) Sample from old T + old B; (2) Lyt-2⁻ old T + old B; (3) L3T4⁻ old T + old B; (4) L3T4⁻ Lyt-2⁻ old T + old B; and (5) Lyt-2⁻ young T + old B cultures. Volumes of the samples loaded on the IEF gel were 2 μl in lanes 1-4 and 4 μl in lane 5. (B) IEF immunoblots showing IgG in eluates (DNA binder: lanes 1, 3, and 5) and flow-through (DNA nonbinder: lanes 2, 4, and 6) fractions after DNA-cellulose column chromatography of B/WF₁ cell culture supernatants. (1 and 2) Samples from old T + old B; (3 and 4) old B cells cultured alone; and (5 and 6) Lyt-2⁻ young T + old B culture combination. Sample volumes loaded were 3 μl each in lanes 2, 4, and 6 and 9 μl each in lanes 1, 3, and 5. (C) IgG in eluates (DNA binder) obtained from DNA-affinity column chromatography of another set of B/WF₁ cell culture supernatants. (1) Sample from L3T4⁻ old T + old B; (2) L3T4⁻ Lyt-2⁻ old T + old B; (3) Lyt-2⁻ old T + old B; and (4) L3T4⁻ Lyt-2⁻ Lyt-1⁻ old T + old B culture combinations. Eluate samples were loaded in 15-μl volumes in each lane. (D) IgG in DNA-affinity column eluates (DNA binder) of SNF₁ (1 and 2) and MRL-+/+ (3 and 4) cell culture supernatants. (1) Young T + old B; (2) old T + old B SNF₁ cultures; (3) young T + old B; and (4) old T + old B MRL-+/+ cultures. Sample loading volumes: 7 μl in lanes 2 and 4 and 18 μl in lanes 1 and 3. The young T + old B eluates in both cases (SNF₁ and MRL-+/+) were concentrated to 50-μl volumes, 2 fold more than the corresponding old T + old B eluates and ~2.6-fold more of the former samples were loaded on the gels (lanes 1 and 3) to achieve approximately equal IgG concentrations in the IEF samples from both types of culture combinations.

TABLE II
Spontaneous Production of Igs and IgG Anti-DNA Antibodies by B Lymphocytes of Old (NZB × SWR)_F₁ (SNF₁) Mice in Different Culture Combinations

Groups	Types of cells cocultured with old SNF ₁ B cells*	Total Ig	Total IgG	IgG anti-DNA antibody [‡]		Cationic shift [§]	
				Anti-ssDNA	Anti-dsDNA	Total IgG	Anti-DNA
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	U/ml			
A	None ¹	5.15 ± 1.13	0.49 ± 0.12	0.094 ± 0.06	0	-	-
B	Old (SNF ₁)T**	13.10 ± 2.03	2.18 ± 0.21	0.568 ± 0.10	0.157 ± 0.009	+++	+++
C	Lyt-2 ⁻ old T	16.50 ± 2.80	2.30 ± 0.31	0.643 ± 0.14	0.162 ± 0.013	+++	++++
D	L3T4 ⁻ old T	11.60 ± 2.23	2.27 ± 0.18	0.534 ± 0.12	0.150 ± 0.006	+++	+++
E	L3T4 ⁻ ,Lyt-2 ⁻ old T	12.05 ± 2.51	2.23 ± 0.24	0.513 ± 0.17	0.149 ± 0.010	+++	+++
F	Young T**	6.27 ± 1.82	0.87 ± 0.19	0.147 ± 0.12	0 [†]	-	-

**†§§ See footnotes to Table I, except SNF₁ mice were used in these experiments. Old SNF₁ mice were 5-5½ mo in age and young SNF₁ were 1-2 mo old.

TABLE III
Spontaneous Production of Immunoglobulins and IgG Anti-DNA Antibodies by B Lymphocytes of Old MRL-+/+ Mice in Different Culture Combinations

Groups	Types of cells cocultured with old MRL-+/+ B cells*	Total Ig	Total IgG	IgG anti-DNA antibody [‡]		Cationic shift [§]	
				Anti-ssDNA	Anti-dsDNA	Total IgG	Anti-DNA
		$\mu\text{g/ml}$	$\mu\text{g/ml}$	U/ml			
A	None	1.47 ± 0.40	0.57 ± 0.13	0.097 ± 0.05	0	-	-
B	Old (MRL-+/+) T**	3.65 ± 0.58	1.55 ± 0.33	0.712 ± 0.12	0.219 ± 0.04	+++	+++
C	Lyt-2 ⁻ old T	3.85 ± 0.76	1.73 ± 0.36	0.794 ± 0.24	0.285 ± 0.07	+++	++++
D	L3T4 ⁻ old T	3.42 ± 0.63	1.16 ± 0.25	0.645 ± 0.15	0.164 ± 0.05	+++	+++
E	L3T4 ⁻ ,Lyt-2 ⁻ old T	3.31 ± 0.54	1.10 ± 0.21	0.596 ± 0.17	0.158 ± 0.05	+++	+++
F	L3T4 ⁻ ,Lyt-2 ⁻ ,Lyt-1 ⁻ , old T	2.45 ± 0.65	0.83 ± 0.23	0.239 ± 0.19	0.055 ± 0.02	++	++
G	Young T**	3.40 ± 0.46	0.81 ± 0.34	0.175 ± 0.07	0	-	-
H	Lyt-2 ⁻ Young T	3.59 ± 0.60	0.94 ± 0.24	0.192 ± 0.11	0.007 ± 0.002	-	-
I	L3T4 ⁻ Young T	2.09 ± 0.47	0.78 ± 0.21	0.143 ± 0.07	0	-	-

**†§§ See footnotes to Table I, except MRL-+/+ mice were used in these experiments. Old MRL-+/+ mice were ~11 mo old and the young mice were 1-2 mo old.

Very similar results were obtained with the cells of old SNF₁ mice (Table II); when T cells were cultured with B cells (group B vs. A) total Ig production increased 2.5-fold ($p < 0.01$), total IgG 4.4-fold ($p < 0.001$), and IgG anti-ssDNA antibodies 6-fold ($p < 0.005$). The T cells also induced a pronounced cationic shift in the population of total IgG, and IgG anti-DNA antibodies produced by the B cells and antibody to dsDNA were detectable only in these cultures with old T cells (Table II and Fig. 1D, lane 2). The cells of old MRL-+/+ mice behaved similarly in culture (Table III); T cells helped B cells to produce 2.5-fold more total Ig ($p < 0.01$), 2.7 more total IgG ($p < 0.02$), and 7.3 times more IgG anti-ssDNA antibodies ($p < 0.01$, groups B vs. A). Again, cationic anti-DNA antibodies and antibodies to dsDNA were induced by the old T cells (Table III and Fig. 1D, lane 4).

Phenotype of the Th Cells of Old Lupus Mice That Induce a Cationic Shift in Anti-DNA Antibodies. When cultured at a 1:1 ratio with the B cells of the old mice, anti-Lyt-2.2 plus C-treated T cells (Lyt-2⁻ old T), which are enriched for L3T4⁺ T cells, augmented the helper effect further. Total Ig ($p < 0.02$), total IgG ($p < 0.02$), IgG anti-ssDNA ($p < 0.01$), and IgG anti-dsDNA ($p < 0.001$) antibody

production were all significantly increased (Table I; group C vs. B), and the cationic shift in anti-DNA antibodies was more pronounced (Fig. 1C, lane 3). Surprisingly, when anti-L3T4 plus C-treated T cells (L3T4⁻old T) were cultured with the B cells at a 1:1 ratio, there was no significant reduction in the helper effect as compared with untreated old T cells, and the cationic shift plus anti-dsDNA antibody induction occurred to a similar extent (Table I, group D vs. B and Fig. 1A, lane 3 and C, lane 1). The T cells were also treated sequentially with anti-L3T4 plus C followed by anti-Lyt-2 plus C and the remaining cells were cultured with B cells at a 1:1 ratio. The augmenting and inducing effect of such double negative (L3T4⁻,Lyt-2⁻) T cells were not significantly different from the untreated old T cells (Table I, group B, D, and E and Fig. 1A, lane 4 and 1C, lane 2). When L3T4⁻,Lyt-2⁻ T cells prepared as above were further treated with anti-Lyt-1 antibody and C there was a significant but not complete reduction of the helper-inducer effect. When group E (or B and D) in Table I is compared with group F, total Ig ($p < 0.01$), total IgG ($p < 0.01$), IgG anti-ssDNA ($p < 0.02$), and IgG anti-dsDNA ($p < 0.001$) production all decreased significantly. However, the helper-inducer effect was still significant when compared with antibody production by B cells alone (Table I; group F vs. A), and the cationic shift of anti-DNA antibodies was still detectable, although to a lesser degree (Table I, group F and Fig. 1C, lane 4). There was very little contamination by antibody-producing cells in the L3T4⁻,Lyt-2⁻ T cell population (Table I, group L). Similar results were obtained with the fractionated T cells of old SNF₁ and MRL-+/+ mice (Tables II and III); both L3T4⁺ and L3T4⁻,Lyt-2⁻ T cells could augment antibody production and induce the cationic shift in IgG anti-DNA antibodies when cultured with Thy-1.2⁻ spleen cells (B cells and macrophages).

FACS analysis of the L3T4⁻,Lyt-2⁻ T cells revealed that they contained on the average, 79.5% Thy-1.2⁺ cells (Fig. 2A), 26.6% Lyt-1⁺ cells (both dull and bright; Fig. 2B), and 16.8% Ly-5⁺ (B220) cells (dull-positive, Fig. 2D). Staining of these cells with anti-L3T4 and anti-Lyt-2 antibodies were at background levels (Fig. 2C).

T Cells of Young Lupus Mice Fail to Induce B Cells of Old Lupus Mice to Produce Cationic IgG Anti-DNA Antibodies. When T cells from young B/W F₁ mice (young T) were cultured with the B cells of the old B/WF₁ mice, they provided significantly less help than the old T cells (Table I, group B vs. G). Total Ig ($p < 0.001$), total IgG ($p < 0.001$), and IgG anti-ssDNA ($p < 0.01$) production were significantly less and no anti-dsDNA antibody was detectable in such cultures. Moreover, cationic anti-DNA antibodies or cationic IgG were not induced by the young T cells (Table I, group G). When L3T4-enriched (Lyt-2⁻) young T cells were added to the old B cells, antibody production was still significantly less (Table I, group B vs. H) and anti-dsDNA antibodies were not induced. However, some cationic polyclonal IgG were produced, but no cationic anti-DNA antibodies were detectable (Table I, group H and Fig. 1A, lane 5 and B, lanes 5 and 6) in this culture combination. In comparison to old B cells cultured alone (Table I, group A), a significant increase in total Ig and total IgG production occurred when the young T or Lyt-2⁻ young T cells were added (groups G and H vs. group A; $p < 0.01$), but there was no significant increase in anti-ssDNA antibody production. There was no evidence of suppression by the young T cells even

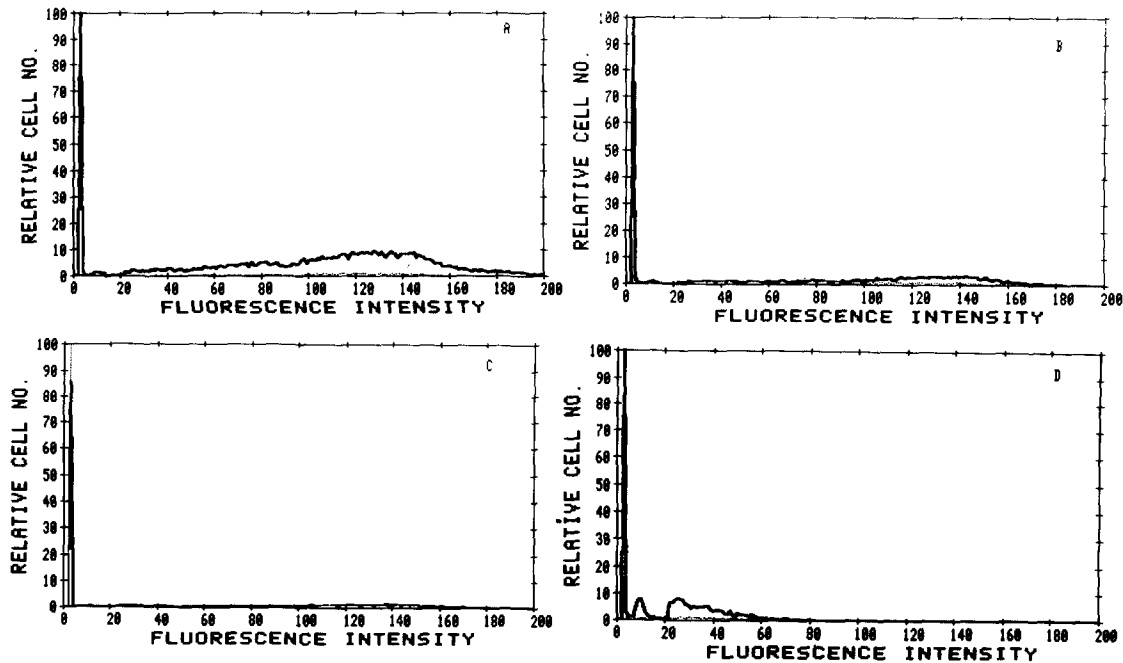


FIGURE 2. Example of cytofluorogram profile of $L3T4^+$, $Lyt-2^-$ T cells. In this case purified T cells of 6-mo-old B/WF₁ mice were treated successively with anti-L3T4 + C and anti-Lyt-2.2 + C. The remaining viable cells were stained with experimental (—) or control (·····) antibodies (Materials and Methods). (A) Stained with anti-Thy-1.2; (B) anti-Lyt-1.2; (C) anti-L3T4; and (D) anti-Ly-5 (B220). Results similar to those shown in C were obtained with anti-Lyt-2.2.

when Lyt-2 enriched ($L3T4^+$) young T cells were cultured with the old B cells (Table I, group A vs. I). Moreover, when the young T cells were admixed with old T cells and then cultured with the old B cells, no suppressor effect was detected (Table I; groups J and K). There was some decrease in the helper effect of the old T cells in these combinations (groups J and K vs. group B) because only 1.25×10^6 viable old T cells instead of 2.5×10^6 were added in these cultures.

Similar results were obtained with the SNF₁ cultures (Table II). The young T cells in comparison to old T cells provided very little help in the production of the antibodies by old B cells (group B vs. F, $p < 0.001$). No anti-dsDNA antibodies or cationic antibodies were induced by the young T cells (Table II, group F and Fig. 1D, lane 1). Cationic anti-DNA antibodies of IgG class were not detectable even when the concentration and IEF volume of the DNA-affinity column eluate sample from young T plus old B cultures were deliberately increased to achieve IgG concentrations approximately equal to the old T plus old B eluate (Fig. 1D, lanes 1 and 2). Results with the MRL-+/+ cells were also similar, and in this strain, even $L3T4$ -enriched ($Lyt-2^-$) young T cells failed to induce any cationic IgG (compare B/WF₁) or cationic anti-DNA antibodies (Table III, groups G, H, and I and Fig. 1D, lanes 3 and 4). Again there was no evidence of any suppressive effect by the young T cells in both the SNF₁ and MRL-+/+ cultures.

TABLE IV
Spontaneous Production of Igs and IgG Anti-DNA Antibodies by B Lymphocytes of Young Lupus Mice in Different Culture Combinations

Groups	Types of cells cocultured with B cells of young mice*	Total Ig <i>µg/ml</i>	Total IgG <i>µg/ml</i>	IgG anti-DNA antibody [‡]		Cationic shift [§]	
				Anti-ssDNA <i>U/ml</i>	Anti-dsDNA	Total IgG	Anti-DNA
(NZB × NZW) _{F1}							
A	None [†]	2.02 ± 0.37	0.22 ± 0.05	0.007 ± 0.004	0	-	-
B	Young (B/WF ₁) T**	3.69 ± 0.68	0.36 ± 0.14	0.031 ± 0.007	0	-	-
C	Old (B/WF ₁) T**	7.20 ± 1.07	1.20 ± 0.21	0.107 ± 0.01	0	+	-
MRL-+/+							
A	None [†]	0.35 ± 0.08	0.08 ± 0.02	0	0	-	-
B	Young (MRL-+/+) T**	0.76 ± 0.13	0.19 ± 0.07	0.017 ± 0.005	0	-	-
C	Old (MRL-+/+) T**	1.92 ± 0.25	0.80 ± 0.13	0.091 ± 0.02	0	+	-

*†‡§ See footnotes to Table I and III, respectively, for (NZB × NZW)_{F1} and MRL-+/+ strains.

We also performed biosynthetic labeling experiments with the old T plus old B and young T plus old B culture combinations (Table I, groups B and G) in some cases. On the fifth day of culture, the supernatants of the unlabeled cultures were harvested and tested as described above. The cells from the Costar culture wells were separately harvested and pooled from each culture combination. The cells were then washed and labeled with [³⁵S]methionine for 6 h as described (3, 27). Supernatants containing metabolically labeled proteins synthesized in the 6-h cultures were applied to DNA-cellulose affinity columns and the eluate and flow-through fractions were immunoprecipitated with rabbit anti-mouse IgG antibodies (3, 27). The radioactivity in the immunoprecipitates were quantitated and they were analyzed by SDS-PAGE. The results confirmed the observations with the unlabeled 5-d culture supernatants. Old T cells markedly augmented the production of IgG class anti-DNA antibodies by the old B cells, in contrast to the young T cells (not shown).

T Cells of Old Lupus Mice Cannot Induce B Cells of Young Lupus Mice to Produce Cationic IgG Anti-DNA Antibodies. When T cells of old B/WF₁ mice were cocultured with B cells from young B/WF₁, they significantly augmented the production of total Ig (*p* < 0.01), total IgG (*p* < 0.01), and IgG anti-ssDNA (*p* < 0.005) (Table IV, group C vs. A). The old T cells also induced the young B cells to produce cationic polyclonal IgG (Fig. 3, lane 1), but no cationic anti-DNA antibodies or anti-dsDNA antibodies of IgG class were induced in such cultures (Table IV, group C and Fig. 3, lanes 5 and 6). Results with the MRL-+/+ cells in culture gave results that were similar to B/WF₁ (Table IV).

Spleen Cells from Old Nonautoimmune Strains and NZB Mice Do Not Produce IgG Anti-DNA Antibodies with Cationic Charge. Spleen cells from 1-yr-old mice of four nonautoimmune strains and the NZB strain produced low amounts of IgG antibodies to ssDNA but no anti-dsDNA antibodies were detectable in the cultures (data not shown). Moreover, none of the normal mouse cells produced cationic IgG (Fig. 4A) or anti-DNA antibodies with cationic charge (data not shown). The NZB spleen cells did produce cationic IgG that was not specific for DNA, but they produced very little anti-DNA antibodies of IgG class and those antibodies were not cationic in charge (Fig. 4A, lane 5 and B, lanes 1 and 2).

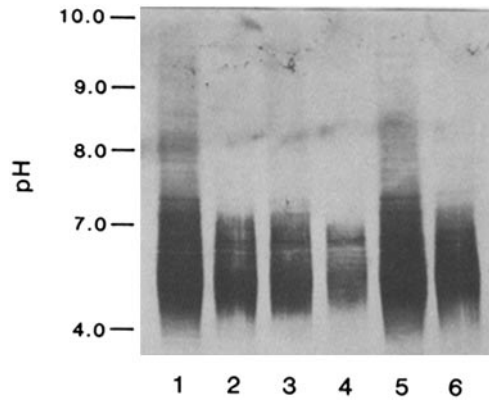


FIGURE 3. IEF-immunoblots showing IgG produced by B/WF₁ cells in culture. (1 and 2) Total unfractionated IgG: old T + young B (1) and young T + young B (2) culture samples. (3-6) IgG after DNA-cellulose column chromatography: young T + young B, flow-through (3) and eluate (4); old T + young B, flow-through (5), and eluate (6). Sample volumes loaded on the IEF gels were 4 μ l in lane 1, 6 μ l in lane 2, 4 μ l in lanes 3 and 5, and 16 μ l in lanes 4 and 6.

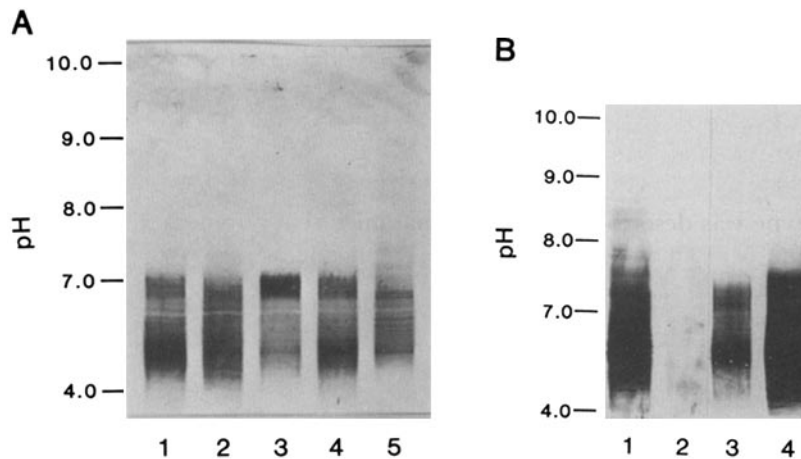


FIGURE 4. (A) IEF-immunoblots of total IgG produced by SWR (1), C3H (2), AKR (3), BALB/c (4), and NZB (5) spleen cells in culture. Sample loading volume was 4 μ l per lane. (B) IEF of IgG after DNA-affinity column chromatography. NZB spleen cell culture sample: DNA-nonbinder IgG (flow-through, lane 1), DNA-binder IgG (eluate, lane 2). Young B/WF₁ spleen cells stimulated by LPS for 5 d: DNA-binder IgG (3) and DNA-nonbinder IgG (4) produced in the culture supernatants. Sample loading volumes in the IEF gel were 6 μ l in lane 1, 12 μ l in lane 2, 18 μ l in lane 3, and 4 μ l in lane 4.

Mitogen-stimulated Spleen Cells from Young B/WF₁ Mice Fail to Produce Cationic IgG Antibodies to DNA. Spleen cells ($5 \times 10^6/2$ ml/well) from 1-2-mo-old B/WF₁ mice were stimulated with LPS (50 μ g/ml culture, *S. Typhosa*; Difco Laboratories, Inc., Detroit, MI) in culture (3). On the day 5 the culture supernatants were harvested and analyzed as described above. On the average the cultures produced 20.44 μ g of total Ig/ml, 4.64 μ g/ml of total IgG, 0.098 U/ml of IgG anti-ssDNA, and 0.012 U/ml of IgG anti-dsDNA antibodies. No cationic IgG or anti-DNA antibodies with cationic charge were produced in these cultures despite the abundant production of Igs (Fig. 4B, lanes 3 and 4).

Discussion

The underlying mechanisms of systemic autoimmune disease can be analyzed in those murine models in which the natural history of SLE is not secondarily

modified by superimposed disease-accelerating genes. Here we investigated three such strains, namely the MRL-+/+ and the (NZB × NZW)_{F1} and (NZB × SWR)_{F1} hybrids, specifically for the cellular mechanisms regulating the spontaneous production of pathogenic anti-DNA autoantibodies that are of IgG class and cationic charge. All three strains manifested a common immunoregulatory defect at or just before the age they began to develop lupus nephritis. Spleen cells of the mice at this age contained Th cells that were essential for inducing their B cells to produce highly cationic IgG class autoantibodies to both ssDNA and dsDNA in vitro. By contrast, syngeneic T cells from younger preautoimmune mice could not provide this help. We found two populations of cationic anti-DNA antibody-inducing Th cells in the older lupus mice; one set belonged to the classical Th category with L3T4⁺,Lyt-2⁻ phenotype, whereas the other surprisingly belonged to a double-negative (L3T4⁻,Lyt-2⁻) population. The latter set of Th cells might have expressed both high and low levels of Lyt-1 antigen, since they were partially resistant to anti-Lyt-1 plus C treatment. In addition, cells within this Th population might have expressed the Ly-5 (B220) antigen. T cells of a similar phenotype are expanded in the massive lymphoproliferation of MRL-*lpr/lpr* mice (32), but we did not expect to find them in the three strains studied here since they lack the *lpr* gene. The double-negative T cells were thought to be nonexistent in the mature peripheral T cell population of normal mice (26), but while our work was in progress, a Th population of a similar phenotype was described (33) in normal mice that augmented Ig production by MOPC-315 plasmacytomas. In addition, another study suggested the presence of *lpr*-type T cells in non-*lpr* mice, although the role of those cells in autoantibody production and their L3T4 and Lyt-2 phenotypes were not investigated (34). Immature thymocyte precursors also have a dull Lyt-1⁺,L3T4⁻,Lyt-2⁻ phenotype, but those cells do not express the mature α/β T cell receptor, nor are they known to have any direct helper effect on B cells (35). The MRL-*lpr/lpr* T cells on the other hand express mature T cell receptors and produce B cell differentiation factors (32, 36). Thus, the cationic autoantibody inducing double-negative Th cells found here in the non-*lpr* lupus mice may resemble the MRL-*lpr/lpr* T cells. However, the L3T4⁻,Lyt-2⁻ helper cells studied here consisted of a mixed population since they were purified by negative selection. We did not attempt any further rigorous purification steps since activated Th cells may be lost in such procedures (37). Nevertheless, it is clear that the double-negative helper cells do belong to a Thy-1⁺ population since Thy-1⁻ cells could not produce cationic anti-DNA antibodies when cultured alone. Moreover, the L3T4⁻,Lyt-2⁻ T cells produced negligible amounts of Ig when cultured by themselves (Table I, group L) and they were surface Ig⁻ (Fig. 2, background control staining with FITC-rabbit anti-mouse Ig). All these observations rule out the possibility that Ly-1⁺ B cells might have contaminated the L3T4⁻,Lyt-2⁻ T cell population and contributed to the cationic shift to any significant degree. We believe both types of cationic anti-DNA antibody-inducing Th cells were preactivated and expanded in vivo in these lupus mice due to some unknown stimulus or due to some intrinsic abnormality of those cells. The demonstration here of the existence of Th cells that can induce pathogenic anti-DNA antibodies is supported by the observation that anti-L3T4 treatment decreases the severity of lupus nephritis

in B/WF₁ mice (38). However, the studies here show that several Th cell subsets are involved.

The Th cells from the older lupus mice also augmented the production of polyclonal IgG that were cationic in charge but not specific for DNA. Moreover, this phenomenon could be dissociated from cationic anti-DNA autoantibody production. For example, Th cells from the older mice induced polyclonal cationic IgG when cultured with B cells from younger preautoimmune mice, but no cationic anti-DNA antibody was produced. Conversely, T cells from the younger lupus mice could not induce the B cells of the older mice to produce cationic anti-DNA antibodies although they could induce the production of cationic IgG that were not specific for DNA. Similarly, cells of NZB mice that rarely develop nephritis produced cationic IgG but no cationic anti-DNA antibodies of IgG class, consistent with our previous studies with monoclonal anti-DNA autoantibodies from these mice (10). Moreover, anionic or neutral anti-DNA antibodies were also produced in all types of cultures combinations from the old lupus mice (Fig. 1), and highly cationic IgG that did not bind DNA were produced as well (Fig. 1B, lanes 2 and 6). Taken together, these results indicate that the DNA-binding property of antibodies was not simply due to a cationic shift in charge of all IgG produced in the cultures. The production of cationic anti-DNA autoantibodies was a selective phenomenon that emerged in the older lupus mice at the late prenephritic stage. B cells from only the older lupus mice and not the young preautoimmune mice contained a population specifically committed to the production of cationic anti-DNA autoantibodies and these B cells were selectively induced by a specific subset of Th cells from the older mice.

Antibody H chain variable regions (V_H) determined the highly cationic charge of a major set of nephritogenic anti-DNA autoantibodies produced by the SNF₁ hybrids and those cationic autoantibodies also shared a distinct crossreactive idiotype (CRI) that was not found among nonpathogenic autoantibodies (10–12). Therefore, the B cell clones that could be induced to produce cationic anti-DNA antibodies in the older lupus mice studied here were probably distinct from the B cells of the younger mice producing anionic or neutral anti-DNA antibodies. The cationic autoantibody-producing B cells were probably expanded under the influence of the specific Th cell subsets that also emerged in the older nephritis-prone mice. The expansion of those particular types of B cells might have been facilitated by an age-dependent intrinsic defect or other unknown stimuli. Indeed, we have found that these cationic anti-DNA antibody-producing B cells are markedly expanded at a later age when the mice are suffering from florid glomerulonephritis (data not shown). The role of multiple cellular components in the selective immunoregulatory defect specifying the spontaneous production of pathogenic anti-DNA antibodies is consistent with the development of lupus nephritis being a complex multi-step and multi-gene process (39). Several studies have previously demonstrated that spontaneous anti-DNA antibody production by lupus mice is T-cell dependent (37, 40–42). None of those studies discriminated between pathogenic and nonpathogenic anti-DNA antibodies, nor were the T cell subsets involved in autoantibody production analysed. For instance, one study (42) focused on the production of anti-DNA autoantibodies by NZB lymphocytes; however, those autoantibodies are not

pathogenic and most of them are of IgM class, and even the IgG autoantibodies produced by the NZB are anionic in charge (reference 10, Fig. 4B). These features explain the low incidence (1%) of nephritis in 1-yr-old NZB females (14). In other cases, polyclonal mitogens, such as LPS, were used to induce anti-DNA antibody production (41). However, LPS stimulates only an immature subset of B cells that are different from those induced by activated Th cells (43), and indeed, cationic anti-DNA antibodies could not be induced by LPS stimulation (Fig. 4B). Another noteworthy feature of our culture system is that no antigen (such as DNA) was deliberately added to the cultures.

An understanding of the mechanisms in SLE that specifically induce the production of the entire population of pathogenic anti-DNA autoantibodies may also be of therapeutic importance. For instance, suppression of even a dominant anti-DNA CRI may not affect the total pathogenic autoantibody response. Minor pathogenic idiotypes will probably escape and replace the dominant CRI⁺ antibodies upon such manipulation, as has been the experience in other CRI systems (44). Furthermore, our results indicate that these are unique sets of T and B cells within the lymphocyte subpopulations of lupus mice that are specifically responsible for pathogenic anti-DNA autoantibody production. Further characterization of these lymphocyte subsets will permit the design of specific therapy.

Summary

We investigated the underlying mechanisms of systemic autoimmune disease in MRL-+/+, (NZB × NZW)_{F1}, and (NZB × SWR)_{F1} mice, since these strains develop glomerulonephritis without the superimposition of any secondary lupus-accelerating genes. All three strains manifested a common immunoregulatory defect specific for the production of pathogenic anti-DNA autoantibodies that are of IgG class and cationic in charge. At or just before the age they began to develop lupus nephritis, spleen cells of the mice contained a subpopulation of Th cells that selectively induced their B cells in vitro to produce highly cationic IgG autoantibodies to both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). By contrast, T cells from younger preautoimmune mice were incapable of providing this help. Moreover, only B cells of the older lupus mice could be induced to secrete cationic anti-DNA antibodies of IgG class. B cells of young lupus mice could not produce the cationic autoantibodies even with the help of T cells from the older mice, nor upon stimulation with mitogens. In the older lupus mice we found two sets of Th cells that spontaneously induced the cationic shift in autoantibodies; one set belonged to the classical Th category with L3T4⁺,Lyt-2⁻ phenotype, whereas the other surprisingly belonged to a double-negative (L3T4⁻,Lyt-2⁻), Lyt-1⁺ subpopulation. The latter set of unusual Th cells were unexpected in these lupus mice since they lacked the *lpr* (lymphoproliferation) gene. Thus three apparently different murine models of systemic lupus erythematosus possess a common underlying mechanism specific for the spontaneous production of pathogenic anti-DNA autoantibodies.

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