SPLENIC IMMUNOGLOBULIN-SECRETING CELLS AND THEIR REGULATION IN AUTOIMMUNE MICE*

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(NZB × NZW)F_1, MRL, and BXSB mice, and to a lesser extent NZB mice, develop a progressive autoimmune disease that simulates the human disease systemic lupus erythematosus (SLE).[1] The murine syndrome is marked by B-lymphocyte hyperactivity manifested by hypergammaglobulinemia, spontaneous polyclonal antibody production, and secretion of various autoantibodies (1–4).

The mechanisms responsible for the B-cell hyperactivity in these mice remain unexplained. The cause may be one defect or combinations of defects such as primary genetic or acquired B-cell malfunction, the presence of endogenous or exogenous B-cell activators, the lack of a negative influence by suppressor T cells, an enhanced positive influence by helper T cells, defects in subsets of intra-T regulatory cells (i.e., Ly 123^+), and defects in other elements of the immune system such as macrophages. Evidence for functional or numerical inadequacies in antigen-nonspecific suppressor T cells (5), lack of regulatory Ly 123^+ T cells (6, 7), and lack of acceptor sites for suppressor messages on the surfaces of B cells (8) has been reported in one or another of the susceptible strains.

Because Ig secretion represents an indicator of the B-cell maturational stage and activity, we have investigated in vitro the magnitude, nature, and regulation of the spontaneous and mitogen-induced secretion of Ig by splenic lymphocytes from these autoimmune mice. Our results indicate that all of the above autoimmune murine strains do in fact have an increased frequency of mature, Ig-secreting B cells (IgSC) in their spleens at one time or another as compared to age-matched, immunologically normal strains. The enhanced B-cell maturity, although apparent in the early life of NZ mice (NZB, NZB × W), is somewhat delayed in MRL/l and BXSB mice in spite of an earlier onset of disease in the latter two strains. Antigen nonspecific T-helper cell activity appears to be heightened only in older MRL/l mice and T-nonspecific suppressor cell activity in all autoimmune strains is within normal limits.

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[1] *Abbreviations used in this paper:* C, complement; Con A, concanavalin A; FBS, fetal bovine serum; FACS II, fluorescen-activated cell sorter; FITC, fluorescein isothiocyanate; IgSC, immunoglobulin-secreting B cells; LPS, lipopolysaccharide; PBM, peripheral blood mononuclear cells; PWM, pokeweed mitogen; SLE, systemic lupus erythematosus; SRBC, sheep erythrocyte(s); ss-DNA, single-strand DNA.

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Materials and Methods

Mice. All murine strains used in this study were bred and maintained in the Scripps Clinic and Research Foundation colony, La Jolla, Calif. The immunopathologic characteristics of the autoimmune mice have been detailed elsewhere (1, 9). For this report, it suffices to state that their 50% mortalities are: NZB of both sexes, 16–17 mo; NZB × W females, 8.5 mo and males, 15 mo; MRL/l of both sexes, 5–6 mo; MRL/n, 17 and 23 mo for females and males, respectively; BXSB males, at 5–6 mo, BXSB females, 15 mo. The MRL/l and MRL/n mice are substrains of the MRL strain; the MRL/l has the *lpr* (lymphoproliferation) genes and the MRL/n lacks the *lpr* gene. The proliferating cells in older MRL/l mice are mostly of T-cell type (10). In these experiments, all mice tested were females except for the BXSB strain for which, unless otherwise indicated, males were used.

Mitogens. Concanavalin A (Con A) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Lipopolysaccharide (LPS) from *Escherichia coli* K235 (phenol extract) was a generous gift from Dr. D. Morrison (Scripps Clinic and Research Foundation).

Antisera. Anti-mouse IgM, κ serum was prepared in goats immunized with mouse FII. The serum obtained was affinity purified on Sepharose- (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) bound APBC22 myeloma protein (IgM, κ). This antiserum reacted with mouse IgG and IgM in double immunodiffusion. A polyvalent anti-mouse Ig (IgG + IgM + IgA) was prepared by immunizing a rabbit with mouse Ig precipitated from serum with 50% ammonium sulfate. Rabbit anti-mouse IgG, rabbit anti-mouse IgM, and rabbit anti-mouse C3H brain antibody specific for T cells were all purchased from Litton Bionetics, Kensington, Md. Fluorescein isothiocyanate (FITC) $F(ab')_2$ rabbit anti-mouse Ig (IgG + IgM + IgA) was obtained from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.).

Cell Preparation. Cell suspensions were made aseptically by passing the murine spleen cells through a stainless steel screen in minimum essential medium (MEM; Grand Island Biological Co., Grand Island, N. Y.) supplemented with 2 mM glutamine, 10 mM Hepes buffer, pH 7.2, penicillin and streptomycin, and 5% fetal bovine serum (FBS) (referred to hereafter as medium). The erythrocytes were lysed with 0.17 M NHCl (4°C, 10 min); mononuclear cells were washed twice in ice-cold medium and viable cells were counted by trypan blue dye exclusion. Peripheral blood mononuclear cells (PBM) were enriched by passing peripheral blood through Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) -Hypaque (Winthrop Laboratories, New York) gradients.

Preparation of Enriched B- and T-Cell Populations. B-cell enriched populations were obtained by treating whole murine spleen cell populations with mouse anti-Thy-1.2 serum plus complement (C). In brief, portions of 1×10^8 spleen cells were first incubated (30 min, 4°C) with an appropriate amount of anti-Thy-1.2 serum (AKR anti-C3H), kindly provided by Dr. D. Katz (Scripps Clinic and Research Foundation), prepared as described (11). After 30 min, cell suspensions were washed once in medium without FBS and incubated for 45 min at 37°C in guinea pig serum (source of C) diluted 1:4 in medium without FBS. Cells were washed again twice with medium and subjected to Ficoll-Hypaque centrifugation. Live cells recovered from the interface were washed and brought to 2×10^6 cells/ml medium. More than 97% of these cells bore surface Ig as evidenced by staining with FITC-$F(ab')_2$ rabbit anti-mouse Ig.

T cell-enriched populations were obtained by using the fluorescence-activated cell sorter (FACS II; Becton, Dickinson & Co., Mountain View, Calif.) as follows: Spleen cells were first subjected to Ficoll-Hypaque centrifugation to remove dead cells. Thereafter, cells at the interface were washed and stained with an optimum amount of FITC-$F(ab')_2$ rabbit anti-mouse Ig antibody, washed three times in medium containing 0.1% NaN_3 and brought to 2×10^6 cells/ml medium before being subjected to cell sorting. FITC-stained cell suspensions were sorted on the basis of bright fluorescence as previously described (12). Bright cells were contaminated with up to 1% of dull cells, and dull cells were contaminated with up to 0.2% of bright cells as judged by repeat analysis of sorted cells in the regular fluorescence microscope. The dull cells (>95% stained when incubated with FITC rabbit anti-mouse brain antibody) were considered to represent T cells.

The Reverse Hemolytic Plaque Assay. IgSC were detected by a reverse hemolytic plaque assay that has been described in detail (13). Briefly, 50 μl of a 10% suspension of sheep erythrocytes (SRBC) previously coated by the chromium chloride method (14) with an optimum amount of

a rabbit anti-mouse IgM, κ antibody and 100 μl of a lymphocyte suspension in medium (1–2 × 10^5 cells depending on the strain used) were pipetted into 10- × 75-mm glass test tubes (Scientific Products Div., American Hospital Supply Corp., McGraw Park, Ill.) containing 0.5 ml of a 0.5% solution of agarose (Indubiose A.37, L'Industrie Biologique Francaise SA, Gennevilliers, France) in medium. The contents were mixed and poured on microscope slides and allowed to gel. After incubation for 1 h at 37°C, the slides were floated in predetermined optimum dilutions of developing rabbit anti-mouse Ig, anti-mouse IgM, or anti-mouse IgG antiserum and incubated for 1 h at 37°C. The developing antiserum was replaced with 20% guinea pig serum (Pel Freeze, Rogers, Ark.) as a source of C and then incubated for 1 final h at 37°C. IgSC were then detected as hemolytic plaques by using a dissecting microscope (Bausch and Lomb, Rochester, N. Y.).

To determine the in vitro roles of T cells in spontaneous Ig secretion, we assessed the frequency of IgSC in 2 × 10^5 cells of a whole spleen cell population compared to that of 2 × 10^5 cells in a population from which T cells had been depleted as described above. On the basis of the results obtained and the predetermined proportion of T cells in the whole spleen cell population, the predicted frequencies of IgSC in the depleted population were determined. The percent reduction in IgSC by T-cell removal was determined by the formula:

$$\text{Percent reduction} = 1 - \frac{\text{Net IgSC}/10^6 \text{ T cell depleted population}}{\text{Net IgSC predicted}/10^6 \text{ T cell depleted population}} \times 100.$$

The same procedure, adapted from Bell et al. (15) and Roder et al. (16), was used for enumerating anti-single-strand DNA (ss-DNA)-secreting cells, the only difference being that SRBC were coupled with ss-DNA by the chromic chloride method. Calf thymus DNA (Worthington Biochemical Corp., Freehold, N. J.) was rendered single stranded by heating to 100°C for 12 min, followed by rapid cooling in an ice bath. The binding of DNA to erythrocytes was assessed by a microhemagglutination assay using a human SLE serum with anti-DNA activity. The net numbers of anti-DNA plaques were determined by subtracting the plaques obtained with uncoupled SRBC. The net PFC obtained were inhibited in a dose-dependent manner if free ss-DNA was added in the agarose.

Culturing of Cells for the Generation of IgSC. Apart from determining the frequency of IgSC present in freshly obtained cell populations (spontaneous IgSC), we also determined the frequency of IgSC after culturing in vitro with and without bacterial LPS. Unseparated spleen cells, as well as T and B cells, and combinations thereof (Results), were suspended at a density of 1 × 10^6 cells/ml in RPMI-1640 medium (Microbiological Associates, Bethesda, Md.) supplemented with 5% FBS (from a lot predetermined to have low mitogenic activity), penicillin, streptomycin and fungizone, and 2 mM L-glutamine. Portions of 200 μl (1–2 × 10^5 cells) were cultured 3 d at 37°C in 5% CO_2 in the presence or absence of LPS. At the termination of culture, the cells were washed twice in medium and assayed for the number of IgSC.

Suppression of Spontaneous and Mitogen-induced IgSC. To study the effect and state of suppressor T cells and their products on the spontaneous IgSC, spleen cells were cultured for 48 h either in RPMI-1640 medium alone or in medium to which various amounts (0.5–20 μg/well) of concanavalin A (Con A) had been added. To study the effect of suppressor T cells on the LPS-induced IgSC, we used direct and indirect assays as described (3). For the direct assay, spleen cells were cultured for 3 d with 20 μg/well of LPS and with Con A in amounts ranging from 0.5 to 10 μg/well or without Con A and washed twice, after which the frequency of IgSC was determined. In the indirect assay, spleen cells (2 × 10^5) were incubated with various amounts (2–20 μg/well) of Con A for 48 h along with untreated controls in RPMI-1640 supplemented as above. These Con A-preactivated cells were then washed twice and added to fresh cultures containing 2 × 10^5 cells of the indicated splenocytes together with 20 μg/well of LPS. IgSC were enumerated at day 3. Controls with LPS-stimulated cells that did not receive Con A-preactivated cells were also included.

Detection of Cells Staining for Intracytoplasmic Ig. Aliquots of erythrocyte-depleted spleen cells were analyzed for cytoplasmic Ig after fixation in a 5% acetic acid, 95% ethanol solution at −20°C followed by washing and staining with FITC-F(ab')$_2$ anti-mouse Ig and subsequent processing in the FACS II.

Results

Spontaneous IgSC in Spleens of Autoimmune and Normal Mice. For assessing the state of spontaneous Ig secretion, spleen cells were plaqued immediately after they were obtained. The results of this experiment are depicted in Fig. 1 and indicate that at the stage of clinically obvious disease, four of the five autoimmune strains (NZB, NZB × W, MRL/l and BXSB) had four- to sixfold higher frequencies of IgSC (0.4–0.6% of the whole spleen cell population) than the three immunologically normal H-2-matched strains (BALB/c, C3H/St, C57BL/6). The MRL/n autoimmune strain, which develops the disease in the second year of life, although showing some tendency to increase in frequency of IgSC with age, was within the normal limits at the latest time tested (7 mo of age). Similarly, BXSB females, which develop delayed autoimmune disease, were within the normal limits at the latest time tested (6 mo of age). In the NZB and NZB × W mice, the high frequency of splenic IgSC was detectable as early as 1 mo of age and increased somewhat thereafter. In contrast, in BXSB male and MRL/l female mice the high frequency of IgSC was first observed at or a little before the clinical onset of the disease (3 mo of age).

The number of IgSC in freshly prepared peripheral blood was also determined. Although the frequency of IgSC in peripheral blood was lower than that in syngeneic spleens, the trends were similar, i.e., autoimmune mice had severalfold higher frequency of IgSC than normal strains (Table I).

IgSC Represent Active Ig Secretion. Because the presence of thymocytotoxic and cytophilic antibodies on cell surfaces may lead to false-positive plaques, we performed two types of experiments to demonstrate that the spontaneous plaques reflected active Ig secretion by lymphoid cells rather than release of Ig passively bound to cells. First, spleen cells taken from five NZB mice at 6 mo of age were preincubated for 2 h at 37°C in medium containing 5% FBS to allow for release of cytophilic antibodies. After the cells were washed three times, the number of IgSC was determined and

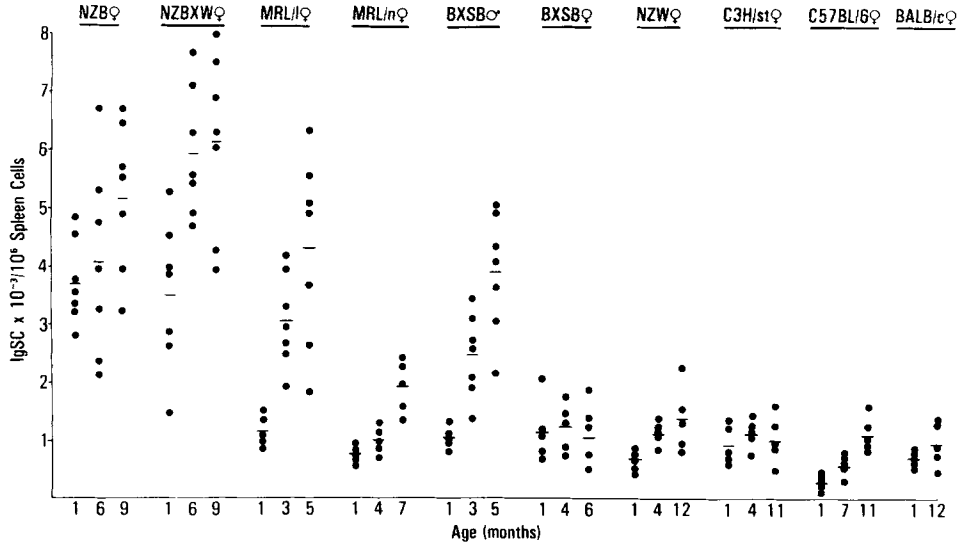

FIG. 1. Frequencies of spontaneous IgSC in spleens of autoimmune and normal murine strains at various ages. Five to seven individual mice were tested in each group.

TABLE I

Circulating IgSC in Autoimmune and Normal Murine Strains

Strain	Age	IgSC/10^6 PBM cells
	mo	
NZB	1	511 ± 213*
	8	780 ± 254
NZB × W	1	406 ± 61
	9	660 ± 205
MRL/l	1	50 ± 13
	5	395 ± 102
MRL/n	1	29 ± 12
	8	73 ± 18
BXSB	1	61 ± 16
	6	161 ± 102
C3H/St	2	20 ± 7
	7	49 ± 9
BALB/c	2	55 ± 12
	8	69 ± 12

* Numbers represent the mean ± SD of five individual mice.

compared with the number of IgSC found when the same cells were assayed immediately after isolation. There was no significant fall in the number of IgSC (<3.4 ± 1.1%) as a result of incubation as would be expected if passive carry-over of Ig was a factor in the assay. However, spontaneous formation of IgSC was reduced significantly upon further incubation in vitro. A representative time course for spontaneous IgSC in NZB and BALB/c spleen cells cultured in vitro is shown in Fig. 2. After 24 h of culture, there was a 60% reduction and at 48 h there was a 76.5% reduction in the number of IgSC compared to the starting value in NZB spleen cells.

Second, the action of cycloheximide, an inhibitor of protein synthesis, on the number of IgSC was also examined. Some of the pooled spleen cells from three NZB mice were assayed immediately after they were obtained, and others were incubated with medium or with medium containing cycloheximide (100 μg/ml) for 2 h at 37°C and then assayed for the number of IgSC. Spleen cells incubated for 2 h at 37°C in medium alone had almost the same frequency of IgSC as cells that were not incubated (4, 328 versus 4,180 IgSC/10^6 cells). However, incubation for 2 h with cycloheximide decreased the number of IgSC to 112/10^6 spleen cells. Cell viabilities, as determined by trypan blue exclusion, were identical in treated and untreated populations.

The number of spontaneous IgSC detected by the hemolytic plaque assay correlated with the number of Ig-synthesizing cells found by staining for intracytoplasmic Ig with FITC-anti-mouse Ig (Table II). Older NZB, NZB × W, and MRL/l strains had higher frequencies and numbers of intracytoplasmic Ig-containing cells than the normal strains; ~6–7% of the total spleen cells in the former vs. 1.3% in the latter. In older BXSB mice, the frequency of Ig-containing cells was only two to three times higher than that of older normal mice. With advanced clinical disease, NZ mice had 5- to 10-fold and MRL/l mice had a 30-fold higher number of Ig-containing cells than younger, syngeneic animals.

Isotypes of Ig Secreted Spontaneously by Splenic Lymphocytes. By developing the plaques with antisera specific for the two major murine isotypes, IgG and IgM, we found that all autoimmune and normal strains tested during youth spontaneously formed con-

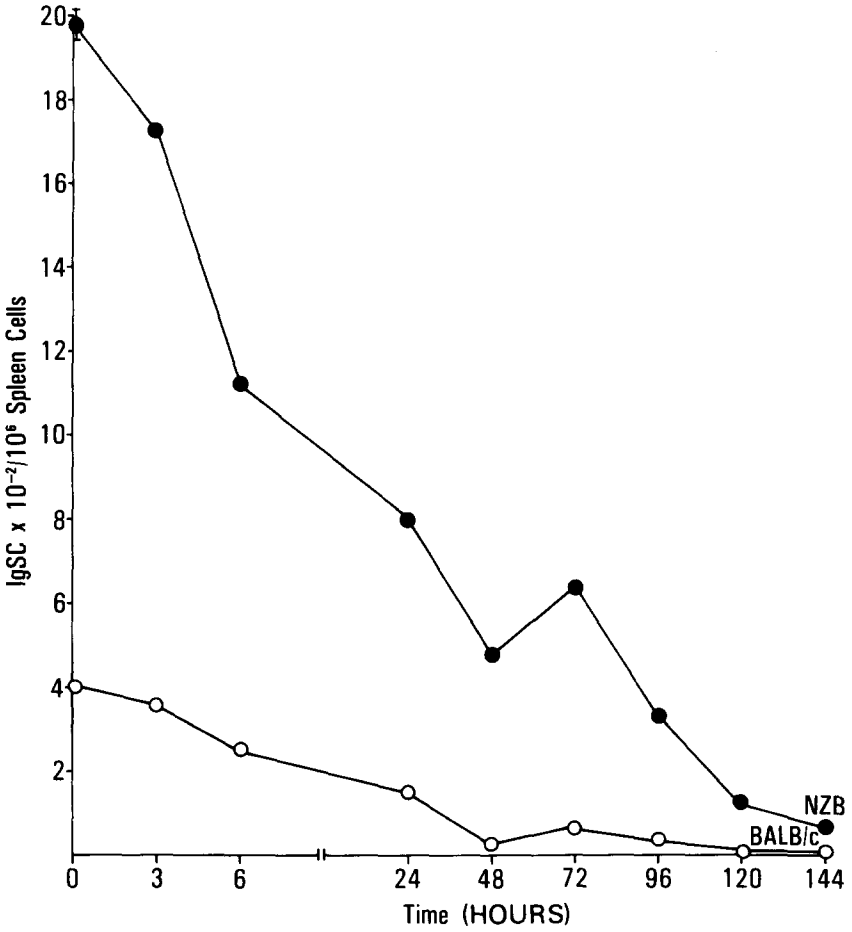

FIG. 2. Time-course of spontaneous Ig secretion. Each point represents the mean of three individual NZB or BALB/c mice. 3-mo-old female mice were used.

TABLE II

Cytoplasmic Ig-containing Cells in Spleens of Autoimmune and Normal Murine Strains

Strain	Age	Percentage of cytoplasmic Ig^+ cells	No. spleen cells	No. of cytoplasmic Ig^+ cells
	mo	%	$\times 10^{-6}$	$\times 10^{-6}$
NZB	1	2.20 ± 0.65*	52.8 ± 23.2	1.16 ± 0.15
	9	6.67 ± 2.40	79.5 ± 30.8	5.30 ± 0.74
NZB × W	1	1.94 ± 0.83	49.6 ± 23.3	0.96 ± 0.19
	9	6.83 ± 2.31	141.6 ± 19.5	9.67 ± 0.45
MRL/l	1	1.16 ± 0.26	102.8 ± 25.7	1.19 ± 0.06
	4.5	6.06 ± 2.02	546.7 ± 136.3	33.13 ± 2.7
BXSB	1	1.08 ± 0.40	74.8 ± 12.8	0.80 ± 0.05
	5	2.12 ± 0.80	153.5 ± 12.0	3.25 ± 0.09
BALB/c	1	1.21 ± 0.50	79.2 ± 11.1	0.95 ± 0.02
	8	1.23 ± 0.45	62.4 ± 18.2	0.76 ± 0.08
C57BL/6	1	1.24 ± 0.37	41.6 ± 16.2	0.51 ± 0.06
	12	1.13 ± 0.11	57.1 ± 14.4	0.64 ± 0.01

* Numbers represent the mean ± SD of five individual mice.

TABLE III

Isotypes of Ig Secreted Spontaneously by Spleen Cells of Autoimmune and Normal Murine Strains

Strain	Age	IgSC/10^6 Cells		
		Total Ig	IgG	IgM
	mo			
NZB	1	2,319 ± 822*	424 ± 203	1,495 ± 719
	7	4,351 ± 1,230	2,193 ± 338	1,718 ± 660
NZB × W	1	2,754 ± 1,019	518 ± 174	1,509 ± 619
	7	4,660 ± 507	2,686 ± 902	1,855 ± 383
MRL/l	1	529 ± 180	181 ± 54	377 ± 104
	4	3,481 ± 1,117	1,992 ± 427	1,282 ± 724
BXSB	1	933 ± 178	253 ± 99	491 ± 186
	5	2,353 ± 685	1,172 ± 329	820 ± 283
BALB/c	1	785 ± 109	117 ± 25	603 ± 113
	7	1,410 ± 319	354 ± 83	1,068 ± 297
C3H/St	1	1,128 ± 328	266 ± 38	858 ± 152
	11	1,404 ± 160	392 ± 107	884 ± 87
C57BL/6	1	894 ± 79	168 ± 111	503 ± 66
	10	1,203 ± 310	274 ± 153	708 ± 204

* Numbers represent the mean ± SD of five individual mice.

siderably higher frequencies of IgM-secreting cells than IgG-secreting cells (Table III). However, in contrast to immunologically normal strains, which show almost the same magnitude and isotype profile of Ig secretion in youth and advanced age, the older autoimmune mice not only had higher frequencies of total IgSC as well as IgG- and IgM-secreting cells than the younger mice, but also they switched from predominantly the IgM isotype at youth to predominantly the IgG isotype. The increase in frequencies of IgGSC in older as compared to young autoimmune mice was approximately fivefold for the NZ and BXSB mice and 11-fold for the MRL/l strain. The sum of the IgG- and IgM-secreting cells in all mice tested approached the total number of spontaneous IgSC.

Frequency of Spontaneous Autoantibody Secreting Cells. Because one of the major autoimmune responses in all SLE mice is that directed against DNA, we assessed the proportion of anti-ss-DNA-secreting cells of the total spontaneous IgSC cells of young and old mice (Table IV). Young mice, both autoimmune and normal, had very low frequencies of anti-ss-DNA-secreting cells in their spleens. As expected, spleens of the older autoimmune mice contained a significantly higher number of anti-ssDNA-secreting cells than spleens of the normal mice; these numbers were highest in older NZB × W and MRL/l mice followed by NZB and BXSB mice. In older MRL/l and NZ mice, ~1 of 5–10 of spontaneous IgSC secreted anti-ss-DNA antibodies (0.05–0.09% of the total spleen cell population). In BXSB mice, 1 of 29 IgSC secreted anti-ss-DNA antibodies (0.009% of the total spleen population).

Effect of T cells on the Frequency of Spontaneous IgSC. The influence of T cells on the spontaneous synthesis and secretion of Ig by murine spleen cells was then investigated in vitro. As shown in Table V, removal of T cells resulted in a significant reduction of the frequency of IgSC in both autoimmune and normal spleen cell populations. The reduction was greater in T-depleted spleen cells of older MRL/l mice than in the

TABLE IV

Frequencies of Spontaneous Anti-ss-DNA-secreting Cells in Spleens of Autoimmune and Normal Murine Strains

Strain	Age	IgSC/10^6 cells	Anti-ss-DNA-secreting cells/10^6 cells
	mo		
NZB	1	3,560 ± 902*	48 ± 18
	7	4,300 ± 819	507 ± 102
NZB × W	1	3,804 ± 705	63 ± 9
	7	4,815 ± 1,004	906 ± 249
MRL/l	1	1,017 ± 417	24 ± 11
	4	3,692 ± 518	539 ± 83
BXSB	1	1,150 ± 203	17 ± 5
	5	2,784 ± 296	96 ± 13
BALB/c	1	1,094 ± 103	12 ± 6
	8	1,226 ± 382	19 ± 6
C57BL/6	1	1,150 ± 117	10 ± 4
	8	1,413 ± 94	23 ± 7

* Numbers represent the mean ± SD of three individual mice.

TABLE V

Effect of T Cells on the Spontaneous In Vitro Ig Secretion of Spleen Cells from Autoimmune and Normal Murine Strains

Strain	Age	Percentage Thy-1.2$^+$ cells	IgSC/10^6 cells		Predicted No. IgSC/10^6 T-cell depleted	Percentage reduction
			Unseparated	T-cell depleted		
	mo	%				%
NZB	1	44	1,750 ± 418*	1,620 ± 84	3,125	48.2
	6	25	3,655 ± 519	1,947 ± 193	4,873	60.0
NZB × W	1	41	2,475 ± 306	1,980 ± 292	4,194	52.8
	6	26	3,930 ± 732	1,819 ± 96	5,310	65.7
MRL/l	1	40	1,100 ± 123	723 ± 79	1,833	60.5
	4	78	2,885 ± 404	2,150 ± 266	13,113	83.6
BXSB	1	52	934 ± 183	949 ± 56	1,945	51.3
	5	39	2,500 ± 477	1,800 ± 313	4,098	56.0
C57BL/6	1	38	756 ± 132	616 ± 103	1,219	49.4
	8	37	875 ± 211	687 ± 88	1,388	50.5

* Numbers represent the mean ± SD of three experiments. Pools of three spleens for each age point were used in each experiment.

young ones. It should be emphasized that the lymphocytes used in these experiments were not triggered with mitogens and were cultured only long enough to complete the assay (3 h).

Effect of Polyclonal B-Cell Stimulators (LPS) on IgSC. The ability of LPS to induce or maintain splenic B-cell differentiation and Ig secretion, as well as the optimum dose for this mitogen, were examined after preliminary studies indicated that the response in both normal and autoimmune mice was highest on the third day of culture. As depicted in Fig. 3, spleen cells from 1-mo-old NZB × W and NZB mice stimulated with LPS (0.02 μg/well) had a 5- to 10-fold higher frequency of IgSC cells than spleen cells from the two other autoimmune strains (MRL/l, BXSB) or several normal strains. However, even cells from NZ mice incubated for 3 d with optimal amounts of

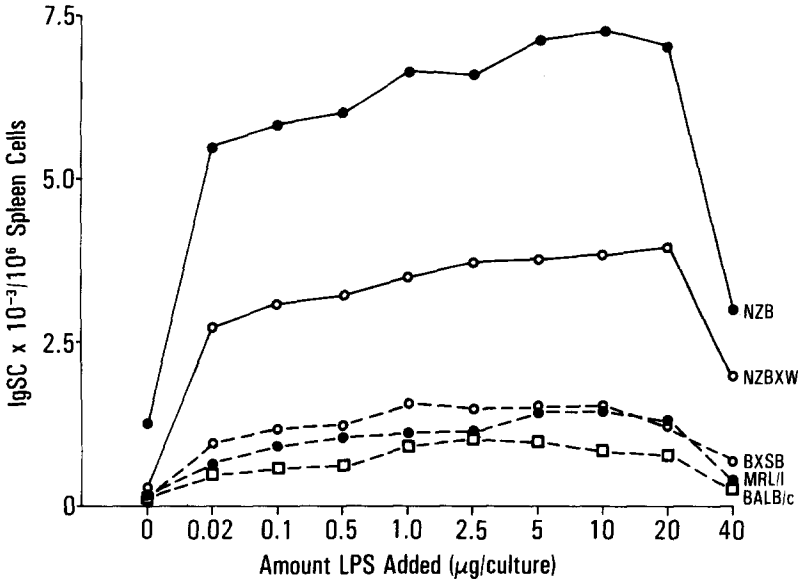

FIG. 3. Dose-response curve of LPS-induced Ig secretion by spleen cells of 1-mo-old autoimmune and normal murine strains. Each point represents the mean of five individual mice. Two additional normal strains tested (C3H/St, C57BL/6), not depicted in the figure, gave curves very similar to that of BALB/c mice.

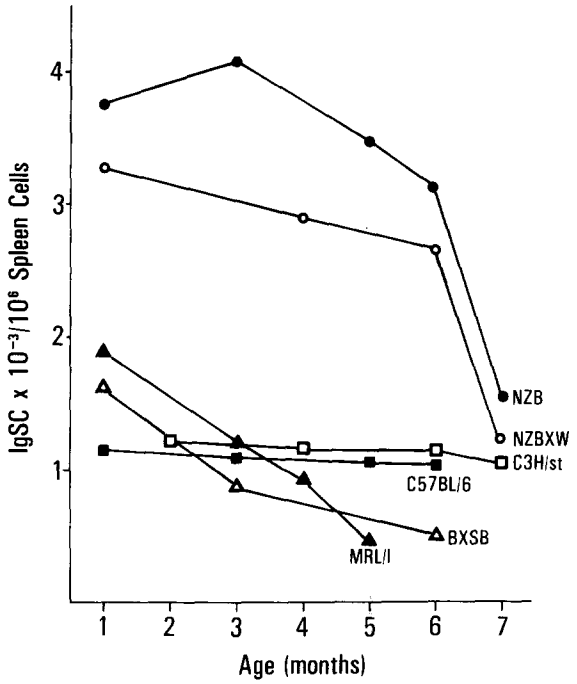

FIG. 4. Age-dependent responsiveness of spleen cells from autoimmune and normal strains to LPS. Each point represents the mean of five individual mice.

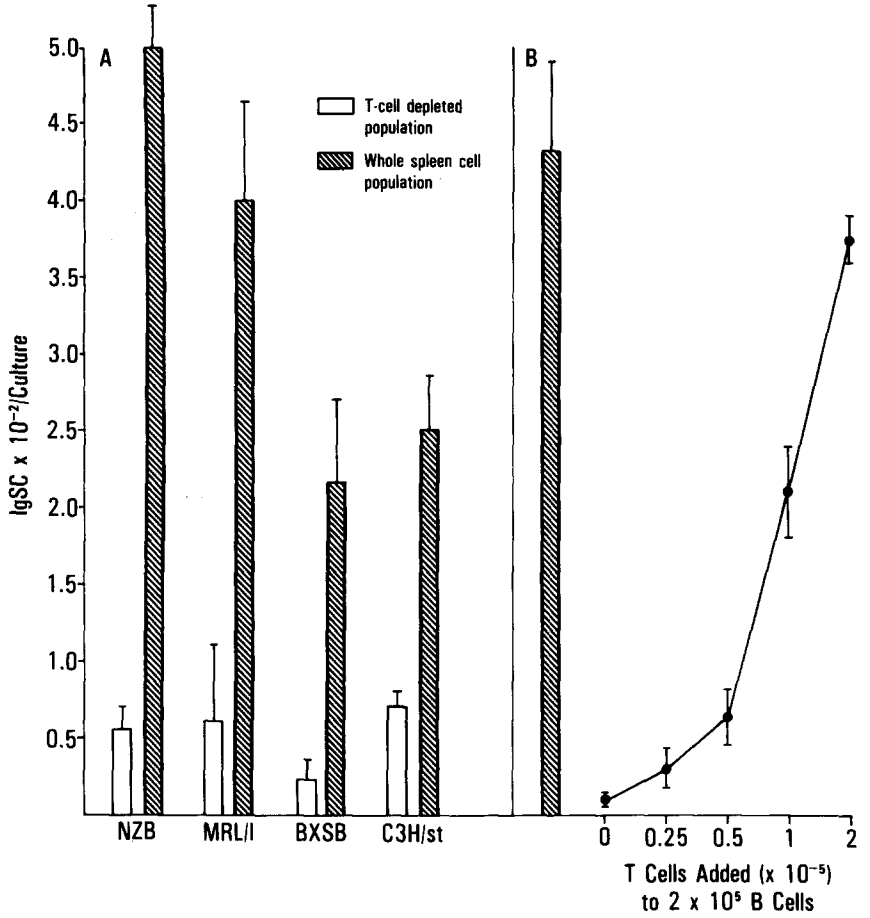

FIG. 5. Dependence of LPS-induced Ig secretion on T cells. (A) Dashed columns represent the mean ± SD of the frequency of IgSC in 2×10^5 whole spleen cell populations and open columns represent the mean ± SD of the frequencies of IgSC in syngeneic T cell-depleted populations. (B) Restoration of the frequency of IgSC in 2×10^5 T cell-depleted NZB spleen cell populations by the addition of increments of isolated syngeneic T cells. Dashed column represents the frequency of IgSC in the whole NZB spleen cell population. Results of three experiments. In each experiment, whole spleen cells, B cells, and T cells were derived from a pool of three spleens of 1-mo-old animals.

LPS did not have a substantially higher frequency of IgSC than isogeneic spleen cells freshly obtained and plaqued immediately (compare with Fig. 1). It should be reiterated that the spontaneous IgSC decreased substantially within 72 h in the absence of LPS.

As shown in Fig. 4, the frequency of IgSC generated by LPS was greatly reduced in all older autoimmune mice as compared to young animals. The reduction in LPS responsiveness was acutely apparent with the onset of the disease. However, no similar reduction occurred in age-matched, immunologically normal strains (C57BL/6, C3H/St).

As shown in Fig. 5, LPS-induced Ig secretion by spleen cells from normal and autoimmune mice required the presence of syngeneic T cells in the culture. The

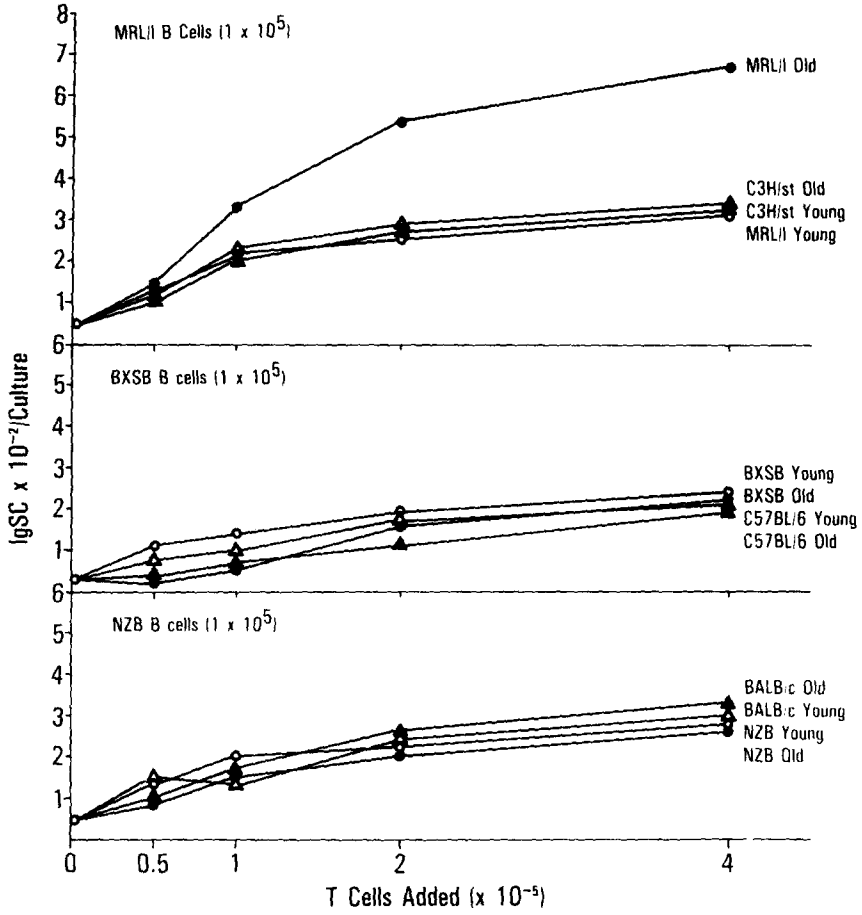

FIG. 6. Frequency of IgSC in 1×10^5 B cell enriched spleen cell populations obtained from young (1 mo old) MRL, BXSB, or NZB mice to which increments of isolated T cells obtained from syngeneic and allogeneic but *H-2*-identical young (1 mo old) and older (7 mo old for NZB, C3H/St, C57BL/6, and BALB/c and 4 mo old for MRL/l and BXSB) animals were added. Each point represents the mean of three experiments. In each experiment, B cells and T cells were obtained from a pool of three spleens.

response of T-depleted cells (with anti-Thy-1.2 + C) was ~10–25% that of whole spleen populations (Fig. 5A). Adding increments of T cells to depleted populations restored the levels of IgSC almost to that obtained with whole spleen cell populations (Fig. 5B). Control cultures of T cells alone gave no (<25) plaques.

Degree of T-Helper Activity in Various Murine Spleen Cell Populations to LPS-induced Ig Secretion. Because Ig hypersecretion is more prominent in older autoimmune mice than young ones, and because such hypersecretion may, at least in part, result from heightened T-helper activity, we examined the degree of help provided by increasing numbers of isolated T cells from young and older animals to a standard number of LPS-stimulated syngeneic and allogeneic but *H-2*-identical B cells isolated from spleens of young animals. The results of these experiments are summarized in Fig. 6. Increments of T cells from young and older NZB and BXSB autoimmune mice added to a standard number of B cells from syngeneic young mice (1 mo old) provided—at

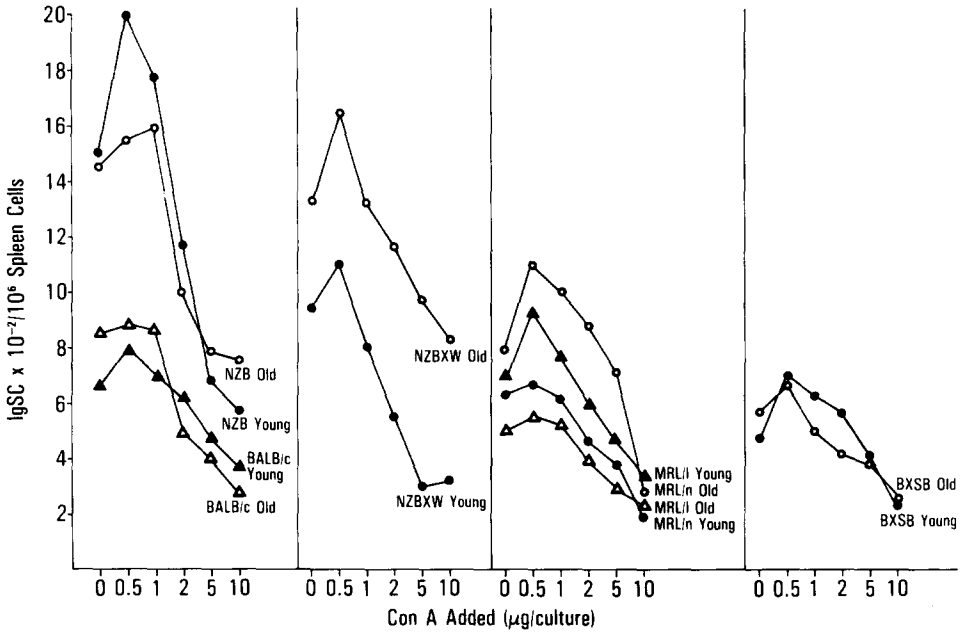

FIG. 7. Suppression of spontaneous IgSC by Con A. Pools of three spleens obtained from young (1 mo old) or older (3 mo old for MRL/l and BXSB, 6 mo old for NZB, NZB × W, MRL/n, and BALB/c) animals were used. Each point represents the mean of three experiments.

all doses and at both ages—equal help in enhancing the frequency of IgSC after LPS stimulation. Moreover, the help provided by T cells from these two autoimmune strains to their own B cells was not significantly different from that provided by T cells from young and older normal mice of the same *H-2* haplotype (BALB/c for NZB, C57BL/6 for BXSB). Similarly, in the reverse situation, when T cells from young and old NZB and BXSB mice were added to B cells from young, normal counterparts, the help was not significantly greater than that from T cells of the normal strains (data not shown). The only notable exception was the MRL/l strain in which T cell-enriched populations from older animals provided twice the help offered by T cells of young syngeneic animals or T cells from young and older normal mice of the same *H-2* haplotype (C3H/St).

Effect of Polyclonal T-Cell Stimulation (Con A). Spleen cell suspensions from young and old normal and autoimmune mice were cultured for 2 d with varying concentrations of Con A (Fig. 7). The frequency of IgSC was significantly suppressed in vitro in all autoimmune and normal murine strains tested when 5–10 μg of Con A was included in the culture; however, these doses of Con A had no apparent effect on cell viability. Much lower concentrations of Con A (0.05–1.0 μg) either had no effect on the frequency of IgSC, or in some experiments, actually enhanced the response in all strains examined.

Effect of Con A on LPS-induced Ig Secretion. Con A-activated spleen cells have been found to suppress the polyclonal response induced by several B-cell mitogens, including LPS (17–19). However, it has been reported that Con A-treated spleen cells from older NZB and NZB × W mice, in contrast to the cells from normal mice of similar age, lack the ability to suppress polyclonal B-cell mitogen-induced Ig secretion (5, 8).

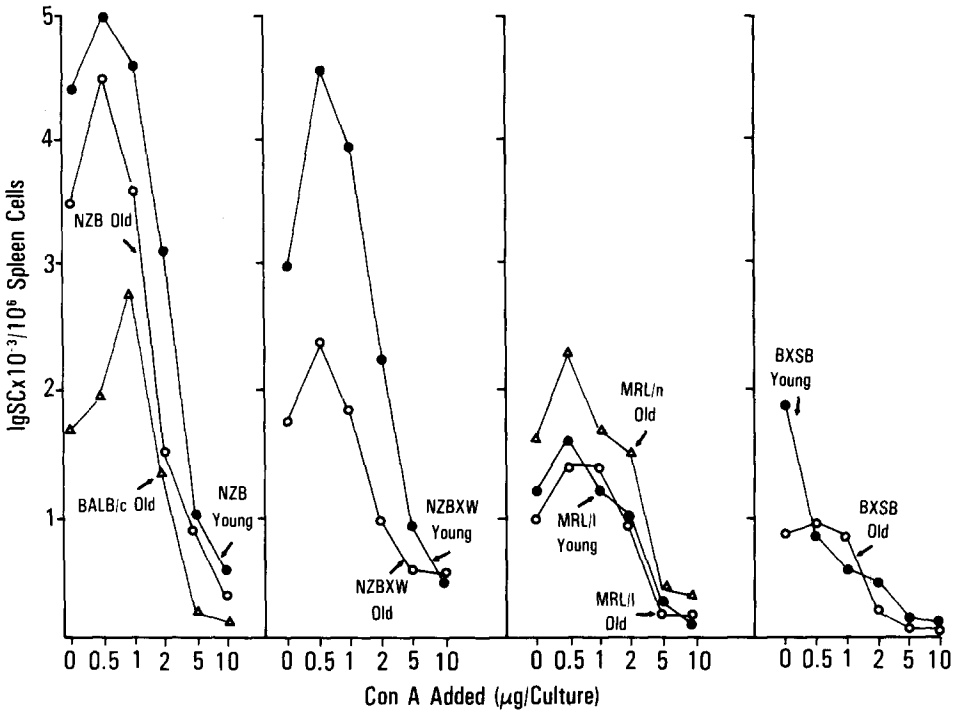

FIG. 8. Direct suppression of LPS-induced IgSC by Con A. Cells from the indicated strain were cultured in the presence of a standard amount of LPS (20 μg/culture) and increasing concentrations of Con A. Each point represents the mean of four experiments. In each experiment a pool of three spleens was used. Young animals were 1 mo old and older animals were 3 mo old for MRL/l and BXSB and 6 mo old for NZB, NZB × W, MRL/n, and BALB/c.

Therefore, we examined the direct effect of Con A on LPS-induced stimulation of Ig synthesis by culturing spleen cells with a standard amount of LPS and increasing amounts of Con A. As shown in Fig. 8, high doses of Con A effectively suppressed LPS-induced Ig synthesis by splenic cells not only from young and older normal mice but also from young and older autoimmune mice, including the NZB and NZB × W strain. It is important to emphasize that in these experiments spleen cells from the older autoimmune mice were chosen only when adequately responsive to LPS.

These results were reassessed by incubating spleen cells from young and older mice for 2 d with varying doses of Con A (0–20 μg/well), washing these cells and then adding them to fresh syngeneic cells derived from both young and older animals to which a standard amount of LPS was added. After 3 d, the cocultures were examined for the frequency of IgSC. As seen in Fig. 9 A and B, spleen cells from both young and older autoimmune animals generated Con A-induced suppressor cell activity, and LPS-responding B cells of young and old syngeneic animals were equally receptive to suppressor messages. That LPS-stimulated B cells of autoimmune mice had no apparent defect in the ability to receive suppressor signals (directly or indirectly delivered to them) was also shown when Con A-preactivated BALB/c spleen cells suppressed spleen cells both from syngeneic young and older animals and from young and older NZB mice (Fig. 9 B, right panel).

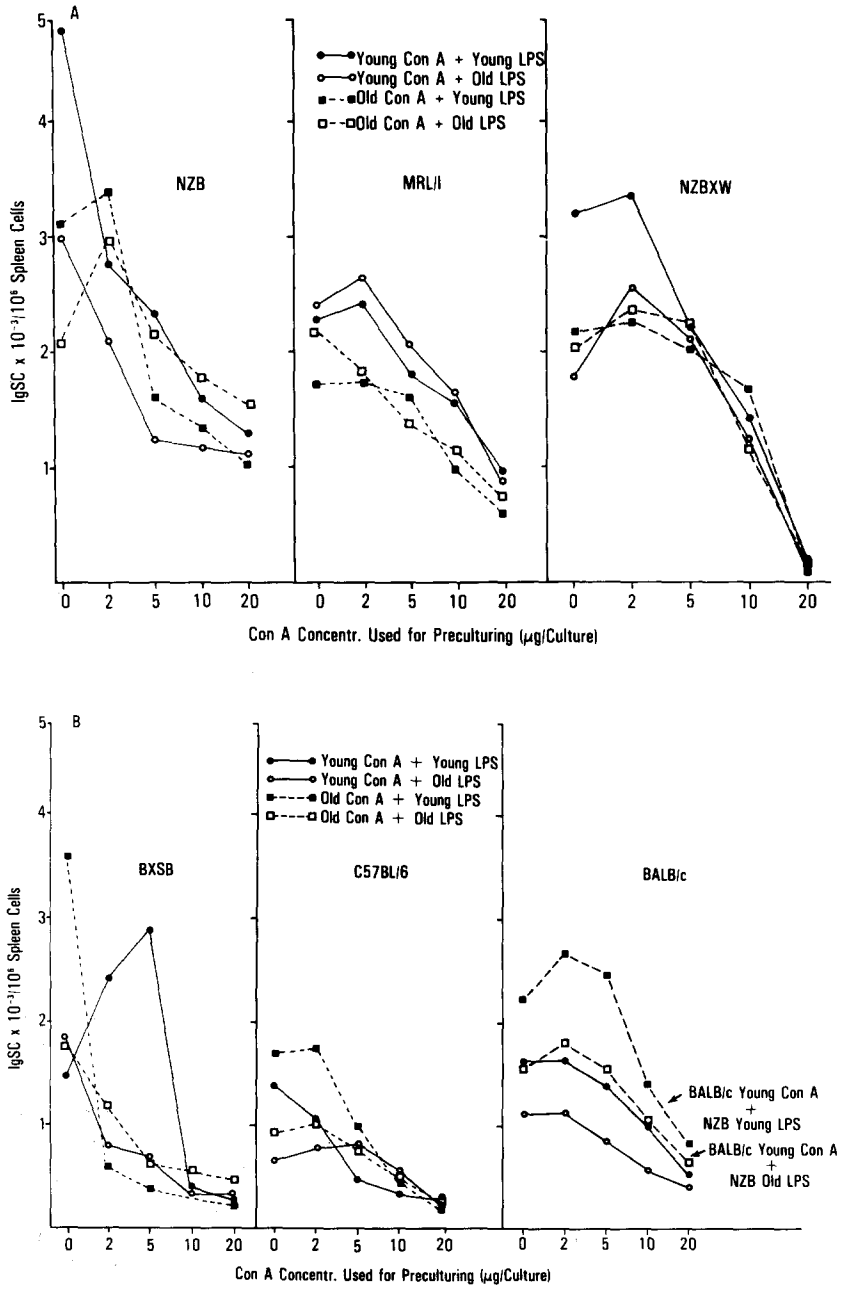

FIG. 9 (A and B). Indirect suppression of LPS-induced Ig synthesis by Con A. Spleen cells from young (1 mo old) or older (3 mo old for MRL/l and BXSB and 6 mo old for NZB, NZB × W, C57BL/6, and BALB/c) mice were cultured with increasing amounts of Con A for 48 h, washed and then added together with a standard amount of LPS (20 μg/culture) to fresh syngeneic spleen cells from young or older mice and cultured for 3 d. Each point represents the mean of four experiments. In each experiment, a pool of three spleens was used.

Discussion

Our work had two principal aims. The first was to examine, by means of spontaneous and mitogen-induced Ig secretion in vitro, the maturational stage of splenic B lymphocytes obtained during youth and advanced age from SLE-prone and immunologically normal murine strains. The second was to analyze the helper and suppressor effect of their T cells on the secretion of Ig by syngeneic and allogeneic but *H-2* identical B cells. Our results indicate that all of the SLE-prone strains have in their spleens at one time or another greater frequencies and numbers of mature B cells than the normal strains. Moreover, the B cell hyperactivity appears not to be the result of a defective T suppressor cell activity or of a lack of acceptor sites for T-suppressor messages on the B-cell surfaces. Several lines of evidence from other studies as well as these suggest that the B-cell hyperactivity in NZ mice may develop from an intrinsic defect, whereas a contributing factor in MRL/l mice may be enhanced T-helper cell activity.

The advanced maturity of the B cells from NZ mice, evidenced by the high frequency of IgSC in their spleens, was detectable at 1 mo of age, the earliest age examined. This finding confirms the results of others who observed hypersecretion of Ig (2, 3) and high frequencies of IgSC (20) within the first days of life among these mice. Moreover, the high frequencies of IgSC in our study and those of Manny et al. (3) and Taurog et al. (20) correlated well with the increased number of spleen cells containing intracytoplasmic Ig. Nevertheless, in NZ mice as well as the other autoimmune and normal strains, the absolute number of cells containing Ig far exceed the number of IgSC.

The accelerated maturation of the B cells from NZ mice is further documented by (*a*) our observation (10) and the report of Cohen et al. (21) describing an increased ratio of surface IgM-:surface IgD-bearing cells and (*b*) our finding and the conclusions of others (22) that LPS responsiveness of lymphocytes is reduced with aging in autoimmune mice. The first finding suggests advanced B-cell maturity in the context of studies showing a decrease of surface IgD after mitogenic (23) or antigenic stimulation (24), whereas the latter finding may be explained on the basis of recruitment and differentiation of virtually all potentially reactive B cells such that few B cells are available to generate antibody when challenged in vitro with a polyclonal B cell activator. Collectively, these findings clearly demonstrate the exceptionally early maturity and hyperactivity of B cells in NZ mice.

The increased frequency of mature B cells, although apparent even at birth in NZ mice, is delayed for several months until just before the clinical onset of the disease in MRL/l and BXSB mice, yet the two latter strains develop disease earlier than NZ mice. The reason for this disparity between onset of B cell hyperactivity and onset of disease is not clear, but the immunopathologic processes may be more dependent upon the amount and/or quality (class, affinity) of specific autoantibodies produced than the time when nonspecific B-cell hyperactivity appears. In fact, isoelectric focusing studies (25) have shown that immediately before the disease begins, all SLE-prone strains exhibit new clonotypes of autoantibodies.

The number of mature B cells increased with the progression of the disease in all autoimmune strains. Of particular interest, at the time when clinical disease began, the IgSC in all these strains switched from a predominantly IgM to a predominantly IgG phenotype. This finding is in agreement with levels of these two isotypes in the

sera of these mice (1), with the isotypes of autoantibodies deposited in their kidneys (1), and with the observation of others that anti-DNA antibodies in sera of NZB × W mice (26) and anti-DNA-containing supernates of cultured lymphocytes from all autoimmune strains (27) eventually switch from IgM to IgG later in the lives of these animals. Moreover, our finding correlates with our previous observation that the frequency of surface IgG^+ cells increases with age in spleens of autoimmune mice (10). Lymphocytes that bear IgG are known to differentiate to IgG-secreting cells and to exert negative influences on IgM secretion (28, 29).

A relatively large proportion (≃15%) of the total IgSC in all older SLE mice spontaneously secreted antibodies against DNA, one of the major autoantigens involved in their disease. A similar frequency of spontaneous anti-ssDNA antibody-secreting cells has been described in NZB mice (15). Although in our study and others (15, 16), serum anti-DNA titers in the various strains correlated with the number of anti-DNA secreting cells, the foregoing analysis of auto-antibody production at the cellular level offers the advantage that one can examine factors affecting production and affinity of antibodies without interference by antigen. What proportion of the total IgSC is engaged in producing antibodies against the plethora of autoantigens involved in this disease was not determined, but preliminary studies (data not shown) indicate that ~1–2% of the IgSC cells secreted autoantibodies against retroviral gp70, another major autoantigen. Approximately 0.001% of the total NZB spleen cells secrete antibodies against thymocytes in the presence of LPS (30).

The spontaneous Ig secretion in vitro of both autoimmune and normal spleen cells was dependent to some degree on the presence of T cells, as others (31) found with human peripheral lymphocytes. The precise mechanism by which helper T cells induce precursor B cells to secrete Ig remains poorly understood. However, reduction of Ig secretion after T-cell removal would suggest that in vivo Ig production is more under the influence of helper than suppressor cells. Reconstitution experiments with isolated T and B cells are needed to assure that Ig secretion depends on T cells in vitro.

Nonspecific activation of lymphocytes by mitogens has long been used as a model for the study of antigen-induced activation. We found that the frequency of IgSC produced by normal and autoimmune mice after LPS stimulation was similar to the frequency of spontaneously produced IgSC, a finding similar to that of Manny et al. (3). However, spleen cells from young NZ mice were much more sensitive to LPS than the cells of other autoimmune and normal strains. It is possible that the enhanced B-cell maturity in NZ mice may result from the presence of low levels of endogenous polyclonal B cell activators.

Polyclonal activation of B cells to secrete Ig is generally thought to depend only upon the direct interaction between LPS and its target B cell. However, we and others (32, 33) have convincing evidence to support the thesis that LPS-induced polyclonal B-cell activation in the mouse is regulated by T cells. Thus, although Ig was secreted in B cell-enriched populations, this phenomenon was markedly enhanced in the presence of T cells. It is not yet clear whether there is only one population of LPS-responsive B cells whose response is amplified by the action of T cells or whether there are two populations of B cells, one activated directly by a B cell-LPS interaction independent of T cells, and another activated after LPS interacts with T cells.

Because LPS-induced Ig secretion was dependent on T cells in our experiments, we

examined the amplifying effects of T cells from young and older autoimmune and normal mice on syngeneic and *H-2*-identical allogeneic LPS-stimulated B cells from young animals. T cells from young and older NZ and BXSB mice provided essentially the same degree of help, and this help was equal to that by T cells from *H-2*-matched normal strains. Thus, the heightened maturity of the B cells from these autoimmune mice apparently did not result from enhanced T-helper cell activity. The very interesting finding, however, was that the splenic T cells of the older MRL/l mice exerted excessive helper activity in vitro on isolated syngeneic B cells, compared to splenic T cells from young MRL/l mice and cells from young and older normal histocompatible mice. As we have demonstrated (10), the ratio of T to B cells is significantly greater in vivo than the maximal ratio employed in the present experiments in vitro. Therefore, one might well expect the helper activity observed in vitro to be greatly magnified in the intact animal. Splenic MRL/l T cells also heightened in vitro production of anti-DNA antibodies by syngeneic and allogeneic cells (34). Therefore, the *lpr* gene in MRL/l mice may hasten autoimmune disease by causing proliferation of helper T cells. However, such enhanced helper T-cell activity may not be necessarily a prerequisite for the development of autoimmunity because the congenic MRL/n substrain of mice that are devoid of the *lpr* gene do develop autoimmune disease, but much more slowly.

The loss of suppressor T-cell function has been proposed (35, 36). Circumstantial and experimental data both support and refute the concept that a numerical or functional defect in suppressor T cells is responsible for the disease of NZ mice. Thus, NZB × W hybrid spleen cells from older mice seem to be enhanced in the ability to mediate graft-versus-host reactions (37) and, after Con A stimulation, reduced in the ability to suppress pokeweed mitogen (PWM)-induced Ig secretion or anti-hapten responses (5). Moreover, an inhibitory Ly 123^+ T-cell subset may be absent or inoperative in NZB (6) and MRL/l mice (38). However, as our studies with NZB, NZB × W, BXSB, and MRL/l mice indicated, nonspecific Con A-induced suppression of Ig synthesis in vitro is within normal levels very early in their lives and shortly before the clinical onset of overt disease. A possible explanation for the discrepancies in regard to antigen-nonspecific suppression in NZ mice may be that other investigators sometimes used cells from quite old animals, which we found in this study—as previously reported for NZ mice (22)—have far less capacity for polyclonal B stimulation than cells from young animals. This could result in an erroneous interpretation that suppression is lacking, whereas in actuality, polyclonal B-cell stimulation fails. Another difference is that we used LPS as polyclonal stimulation, whereas they used PWM (5). Although both mitogens induce polyclonal responses in the presence of T cells, and their effect is suppressed by Con A, the cellular requirements and the responsible factors may be different. Moreover, those investigators examined the suppressive effects of Con A at just one concentration, 2 μg, whereas we used Con A at doses ranging from 0.5 to 20 μg. Concentrations of 2 μg Con A/culture, in most instances did not exert significant suppression for either autoimmune or normal strains in our system. At any rate, in agreement with our findings, others have shown that thymocyte-mediated antigen-nonspecific suppression is not lost with age in autoimmune hereditarily asplenic (Dh/+) NZB mice (39) or in conventional NZB mice (3). Moreover, suppressor cells, as defined with Ly 23 alloantigen-expressing cells in NZB mice (6) or *I-J*-subregion-controlled alloantigen in MRL/l mice (10), are

normal or elevated in numbers. Finally, NZ, MRL/l and BXSB mice are quite normal as far as antigen-specific suppression (40–42) and, with the exception of one study (43), young syngeneic thymocytes repeatedly transferred into NZB and NZB × W mice had no inhibitory effect on autoantibody production or development of glomerulonephritis (44, 45). The combined results prompt us to question the assignment of a suppressor T-cell defect as the major pathogenic mechanism of autoimmune disease. However, none of these arguments exclude the possible presence of subtle abnormalities of suppressor T cells undetectable in the assays employed or the development of late, secondary suppressor T cell or other immunoregulatory cell abnormalities in these animals. The claim that the defect in older NZB mice is an eventual lack of acceptor sites for suppressor messages on B cells (8) is not supported by our studies, because B cells from young and older NZB mice, as well as the other autoimmune strains, when stimulated with LPS to secrete Ig, were equally receptive to Con A-induced suppressor syngeneic and allogeneic T-cell messages.

It is now clear that all SLE strains have hypergammaglobulinemia, many kinds of autoantibodies, and spontaneous polyclonal antibody synthesis by B cells early in life. While still very young, NZB mice and their F_1 hybrids undergo excessive B-cell activation and hypersecrete IgM; hypersecretion of IgM occurs in NZB fetal livers (3) and in NZB mice depleted of T cells (46), all of which suggests an intrinsic defect or stimulation of these cells. According to genetic studies of crosses between NZB and SWR mice, NZB mice may have a subpopulation of hyperactive B cells whose number is determined by a dominant gene(s) and whose Ig production is controlled by a recessive gene(s) (3). Further experiments with crosses of NZB and the B cell-defective CBA/N mouse have shown that the NZB B-cell abnormality resulting in excessive Ig production occurs almost exclusively in that population of NZB cells affected by the CBA/N X-chromosome-linked defect (20, 47). However, a role of T cells in the switch of B cells from IgM to IgG hypersecretion later in life has not yet been elucidated. How B-cell hyperactivity occurs in the other two autoimmune strains (MRL/l, BXSB) is not yet clear, but in MRL/l mice an excessive T-helper cell component could be operative. Certainly the possibility that exogenous or endogenous polyclonal B-cell activators are responsible for initiating or enhancing B-cell activity should also be considered (4). However, our results strongly suggest that B-cell hyperactivity is not caused by numerical or functional defects in nonspecific suppressor T cells.

Summary

We have investigated in vitro the magnitude, nature, and regulation of spontaneous and mitogen-induced Ig secretion by splenic lymphocytes from several autoimmune murine strains (NZB, NZB × W, MRL/l, BXSB) and appropriate, normal mice. All autoimmune strains had increased numbers of mature splenic B lymphocytes, which secreted and/or contained Ig, compared to age-matched normal strains. In NZB and NZB × W mice, the high frequency of mature B cells was apparent early in life, whereas in MRL/l and BXSB mice it was first noted shortly before the clinical onset of disease. Spleen cells from young autoimmune mice of all four strains secreted predominantly IgM, but with aging and the appearance of disease, the cells switched to IgG secretion predominantly. In contrast, spleen cells from normal mice were predominantly IgM secretors throughout the animals' lives. Approximately 15% of

the total Ig-secreting cells in older NZB, NZB × W, and MRL mice were committed to secretion of anti-ssDNA antibodies. In both autoimmune and normal spleen cells, the B-cell population alone contained fewer secreting cells than the total lymphocyte population, indicating that T cells were required to achieve maximal levels of plaque-forming cells.

Spleen cells of NZB and NZB × W mice had a greater response to lipopolysaccharide (LPS) than other autoimmune and normal strains. Responsiveness to LPS, as measured by the frequency of induced Ig-secreting cells, was considerably diminished with age and onset of disease in all autoimmune but not in normal strains. LPS-induced Ig secretion by B cells of autoimmune and normal mice was subject to regulation by splenic T cells. No significant differences were observed between concanavalin-A (Con A) stimulated spleen cells from young and older autoimmune mice and normal control strains in effectively suppressing spontaneous and LPS-induced Ig secretion. Moreover, B cells from autoimmune mice and from normal strains were equally receptive to Con A-induced suppressor signals. T cells from young and older NZB and BXSB mice added to a standard number of B cells from syngeneic young mice provided equal help in enhancing LPS-induced Ig secretion, and this help in turn was equivalent to that provided by T cells from normal mice of the same *H-2* haplotype. The exception was the MRL/l strain; T cells from older animals provided considerably more help than T cells from young MRL/l or T cells from young and older *H-2*-compatible normal mice.

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