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Ly-1 B cells: Functionally distinct lymphocytes that secrete IgM autoantibodies

(fluorescence-activated cell sorter/B-cell subpopulation/NZB mice/autoimmunity)

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ABSTRACT **Studies presented here introduce another perspective on the mechanisms responsible for IgM autoantibody production. A unique subpopulation of B lymphocytes (Ly-1 B) that concomitantly expresses IgM, IgD, Ia, and Ly-1 membrane glycoproteins is present at higher frequencies in NZB and NZB-related mice. The Ly-1 B subpopulation in these autoimmune animals is responsible for the "spontaneous" IgM secretion demonstrated with cultured NZB spleen cells and contains the cells that secrete typical NZB IgM autoantibodies to single-stranded DNA and to thymocytes. In addition, the Ly-1 B population in normal mouse strains (and in NZB) contains virtually all of the spleen cells that secrete IgM autoantibodies reactive with bromelain-treated mouse erythrocytes. Since a different B-cell subpopulation (IgM^+, IgD^-, $Ly\text{-}1^-$) secretes most of the IgM antibodies produced in responses to exogenous antigens, we conclude that Ly-1 B cells constitute a functionally distinct B-cell population important in certain kinds of autoimmunity.**

NZB mice have characteristically increased serum IgM levels and produce relatively large amounts of IgM autoantibodies to cell surface determinants (e.g., on erythrocytes and thymocytes) and to DNA (1–4). In addition, these mice have splenic B cells that spontaneously secrete IgM when cultured in the absence of exogenously added mitogens. BALB/c mice, in contrast, have low serum IgM levels and do not normally produce very much IgM (or IgG) autoantibody. Furthermore, splenic B cells from these animals do not spontaneously secrete IgM *in vitro*.

The hyperactive behavior of NZB B cells is approximated by BALB/c B cells that have been stimulated with LPS (a lipopolysaccharide commonly used *in vitro* as a polyclonal B cell activator). BALB/c spleen cells cultured with LPS, for example, divide and differentiate to IgM-secreting cells. Similarly, BALB/c mice injected with LPS rapidly initiate production of IgM autoantibodies that NZB mice produce constitutively—e.g., those that detect a cryptic antigen revealed on mouse erythrocytes by proteolytic treatment with the enzyme bromelain (5–10). Thus, with respect to B-cell activation and functional differentiation, the NZB autoimmune disorder would appear to mimic the effects of LPS stimulation on normal B cells (1, 2, 10).

Recent fluorescence-activated cell sorter (FACS) studies suggest that this hypothesis applies more accurately to a relatively rare B cell subpopulation rather than to B cells in general. Using two-color FACS analysis and sorting, we have shown that the NZB B cells that spontaneously secrete IgM *in vitro* belong to a distinct splenic B cell subpopulation that carries the Ly-1 surface marker previously considered unique to T cells (11). This Ly-1 B subpopulation (11, 12), which is also marked by characteristic levels of surface IgM and IgD, is present at increased frequencies in NZB and NZB-related autoimmune mice (e.g., 5–10% in NZB spleen compared with 1–2% in BALB/c spleen). Furthermore, it tends to be more active in NZB in the sense that Ly-1 B cells sorted from these animals spontaneously secrete much more IgM *in vitro* than BALB/c Ly-1 B cells sorted and cultured at the same density (11).

Continuing these studies, we present evidence here defining the role that Ly-1 B cells play in autoimmunity. We show (*i*) that FACS-sorted Ly-1 B cells from LPS-stimulated BALB/c mice are responsible for essentially all of the IgM autoantibody produced to bromelain-treated mouse erythrocytes; (*ii*) that the spontaneously secreted IgM from FACS-sorted NZB Ly-1 B cells contains typical NZB autoantibodies reactive with DNA and with thymocyte cell surface antigen(s); and (*iii*) that although Ly-1 B cells produce most of the IgM autoantibodies produced to the above antigens, they produce only minimal IgM responses to exogenous antigens such as sheep erythrocytes, 2,4,6-trinitrophenyl-conjugated keyhole limpet hemocyanin (TNP-KLH), and TNP-Ficoll.

Finally, to complete the identification of Ly-1 B cells as a functionally unique subpopulation, we show that the IgM antibodies produced to exogenous antigens are mainly produced by cells whose surface marker characteristics (IgM^+, IgD^-, $Ly\text{-}1^-$) distinguish them clearly from Ly-1 B cells and most other (resting) B cells in spleen. This evidence, taken in conjunction with data presented showing that *in vitro* LPS stimulation does not induce the expression of Ly-1 on activated B cells, suggests that a high level of autoantibody production in NZB animals reflects a defect in the regulation of Ly-1 B frequency, activation, and differentiation rather than a general defect in the regulation of B-cell function.

MATERIALS AND METHODS

Mice. BALB/cN, NZB/BinJ, and C3H.SW (CSW) mice were bred in the Stanford Genetics Department facility and were used at 2–3 months of age. NZB/N mice were bred at the animal facility maintained by one of us (A.D.S.) (National Institutes of Health). Female mice 2–5 months old were used.

Antigens and Mitogens. TNP-conjugated *Brucella abortus* (TNP-BA) was kindly provided by J. J. Mond (National Institutes of Health). TNP_{48}-AECM-Ficoll was purchased from Biosearch Research Chemicals (San Rafael, CA). TNP-LPS was prepared by the method of Jacobs and Morrison (13) and the amount was determined by weighing lyophilized

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Abbreviations: TNP, 2,4,6-trinitrophenyl; BA, *Brucella abortus*; SRBC, sheep erythrocytes; LPS, lipopolysaccharide; FACS, fluorescence-activated cell sorter; KLH, keyhole limpet hemocyanin; TR, Texas red; brmRBC, bromelain-treated mouse erythrocytes; PFC, plaque-forming cells; ss DNA, single-stranded DNA; Fl, fluorescein; Bi, biotin.

product. TNP-KLH was prepared by incubating trinitrobenzenesulfonic acid with KLH (Pacific Biomarine, San Rafael, CA) in sodium bicarbonate solution for 3 hr at room temperature. Sheep erythrocytes (SRBC) were obtained from Flow. LPS from *Salmonella typhosa* was purchased from Difco.

Immunizations. For immunization, 50 μl of TNP-BA solution, 50 μg of TNP-Ficoll, 10 μg of TNP-LPS, 100 μg of TNP-KLH, or 2×10^7 SRBC were injected intraperitoneally. To induce autoantibody, 10 μg of LPS was injected in the same way.

Cell Staining and Two-Color Sorting with the FACS. For separation of Ly-1 B cells and other B cells, spleen cells from antigen- or mitogen-injected mice (depleted of erythrocytes) were simultaneously stained with fluorescein (Fl)-conjugated monoclonal rat anti-IgM (clone 331.12) and biotin (Bi)-conjugated monoclonal rat anti-Ly-1 (clone 53-7.3) in conical centrifuge tubes (0.5 μg of each antibody per million cells at 2×10^7 cells per ml), washed, and further incubated with Texas red (TR)-conjugated avidin. The method has been described in detail (11). In this particular experiment, either bi-conjugated monoclonal rat anti-Lyt-2 (53-6.7) or mouse anti-Igh-5^a(10-4.2) was also used together with Fl-anti-IgM. Sorted cell fractions were always reanalyzed to check the purity in each expected region.

Plaque-Forming Cells (PFC). Bromelain-treated mouse erythrocytes (brmRBC) were prepared by the method of Cunningham (5). A 50% suspension of erythrocytes from peripheral blood of 2-month-old BALB/c mice was incubated in a bromelain (Sigma) solution in phosphate-buffered saline at a final concentration of 10 mg/ml, at 37°C for 30 min. TNP coupling to SRBC has been described elsewhere (14) and was used for anti-TNP PFC. Both PFC were developed by using guinea pig complement in Cunningham chambers (15).

Cell Culture and Assays for Autoantibody in the Secreted Culture Supernatant. Spontaneous secretion of IgM was obtained from the 6-day culture of cells from NZB spleen in RPMI 1640 medium (Irvine Scientific) supplemented with 10% fetal calf serum and 50 μM 2-mercaptoethanol. The secreted IgM level was determined by a competitive solid-phase radioimmunoassay (RIA) (11). Autoantibodies in the culture supernatants were measured by an enzyme-linked immunosorbent assay (ELISA) described previously (16) for single-stranded DNA (ss DNA) and modified slightly to allow measurement of anti-T cell antibodies.

RESULTS

Detecting and Sorting Ly-1 B Cells from BALB/c Mice. The increased frequency of Ly-1 B cells in NZB spleen (11, 12) makes the splenic NZB Ly-1 B population relatively easy to visualize on two-color FACS-VAX contour plots that display analyses of cells stained with anti-IgM and anti-Ly-1. The frequency of Ly-1 B cells in BALB/c spleen, in contrast, is too low to visualize with analysis conditions that clearly reveal the NZB population (see Fig. 1). Thus to measure the Ly-1 B-cell frequencies and to sort Ly-1 B cells from BALB/c (and other normal mouse strains), we define a set of FACS gates that encompass the splenic NZB Ly-1 B-cell population, and we use these gates for sorting and analysis of BALB/c and NZB cell suspensions in the experiments described below.

BALB/c Ly-1 B Cells Secrete IgM Autoantibodies. BALB/c mice normally have small numbers of splenic PFC that produce IgM autoantibodies to cryptic determinant(s) revealed on brmRBC. The frequency of these PFC, however, increases rapidly after *in vivo* LPS stimulation and reaches a maximum within 1–3 days (6, 17). Since we suspected that the cells responsible for this IgM autoantibody response in BALB/c might be related to the Ly-1 B cells that spontaneously secrete IgM in NZB mice, we sorted the Ly-1^+ and Ly-1^- B cells from BALB/c spleen 1 day after LPS stimulation and tested the sorted populations for the presence of direct (IgM) anti-brmRBC PFC.

These FACS sorting studies demonstrate clearly that the BALB/c anti-brmRBC PFC response is mediated by the Ly-1 B-cell subpopulation (see Fig. 2). That is, anti-brmRBC PFC activity is highly enriched in the sorted Ly-1 B-cell population and essentially depleted from the IgM^+, Ly-1^- population. Furthermore, very little anti-brmRBC PFC activity is recovered in a control population stained with a comparably prepared monoclonal antibody of the same isotype (anti-Lyt-2) and sorted with the same gates as the Ly-1 B population (see sort 2, lower half of Fig. 2). Thus, insofar as the anti-brmRBC response can be taken as representative of IgM autoantibody production in BALB/c mice, Ly-1 B cells are uniquely responsible for this function.

NZB Ly-1 B Cells Secrete IgM Autoantibodies. Young NZB mice (2–5 months of age) commonly produce IgM autoantibodies to thymocyte surface antigens and to ss DNA. Data presented in Table 1 (and in similar experiments not presented) show that NZB spleen cells spontaneously produce these autoantibodies *in vitro*. In addition, data from two experiments presented in this table show that FACS-sorted Ly-1 B cells are responsible for most (if not all) of this autoantibody production—i.e., Ly-1 B populations were enriched for autoantibody-producing cells and Ly-1^- B populations were depleted of these cells.

Of the two example experiments shown in Table 1, the first is typical of previous Ly-1 B sorts in which essentially all of the cells capable of spontaneous IgM secretion sorted with the Ly-1 B population (11). The second experiment is atypical in that the Ly-1^- B cells produced nearly as much IgM as the Ly-1^+ B cells. This latter experiment was explicitly selected for autoantibody studies because it provided an

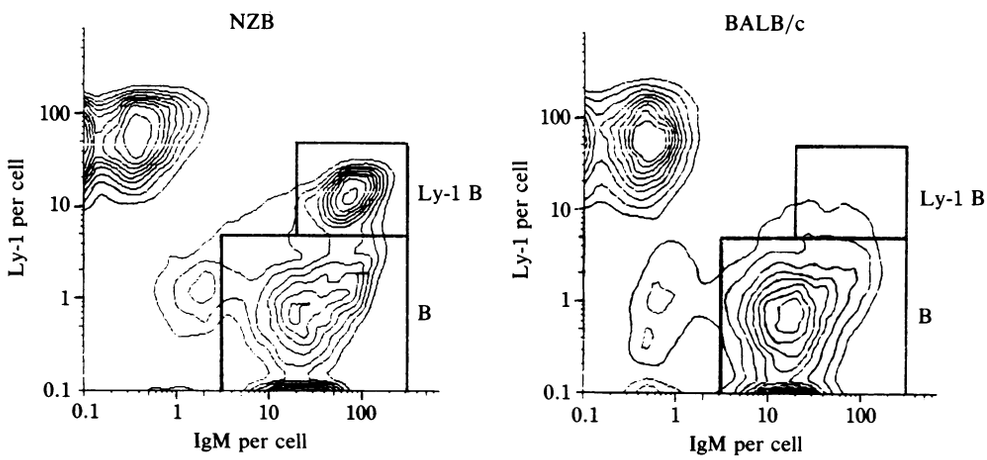


FIG. 1. Ly-1 B cells in NZB and BALB/c spleens. Spleen cells from 2-month-old NZB and BALB/c mice were simultaneously stained with Fl-anti-IgM and Bi-anti-Ly-1, followed by TR-avidin. The upper box corresponds to the Ly-1 B-cell population (bright IgM and dull Ly-1 cell region) and the lower box corresponds to the Ly-1^- B-cell population.

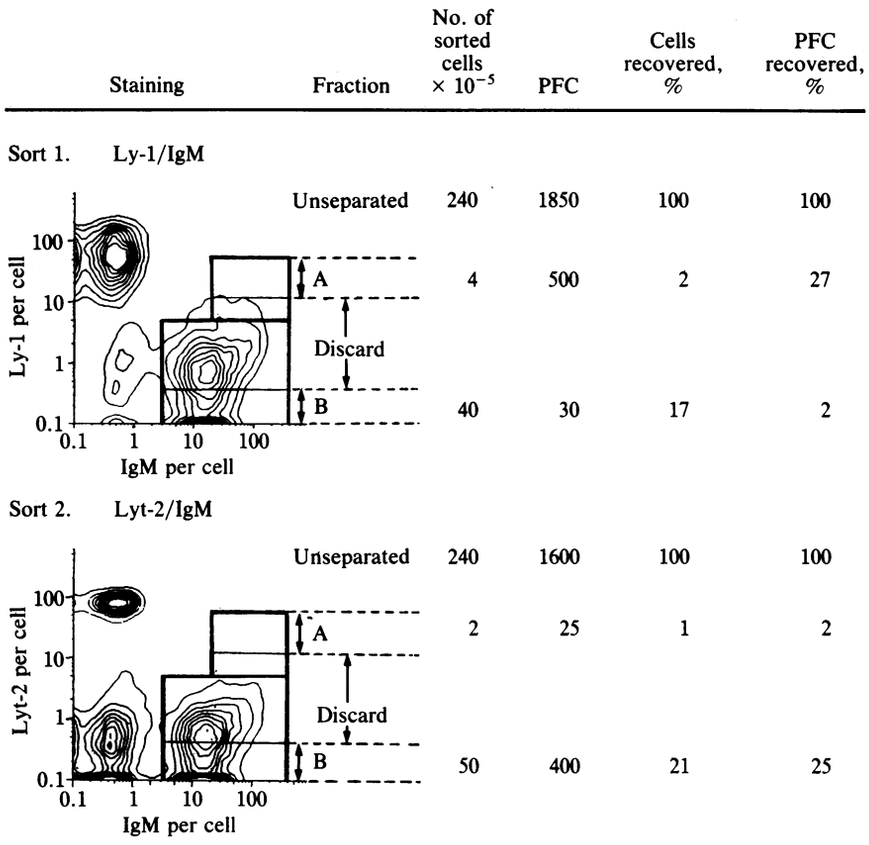


FIG. 2. Ly-1 B cells contain the cells (PFC) producing IgM autoantibodies to brmRBC. Portions of a pool of two spleens from BALB/c mice (2 months old) injected with 10 μg of LPS 1 day before were stained either with Fl-anti-IgM plus Bi-anti-Ly-1 or with Fl-anti-IgM plus Bi-anti-Lyt-2, followed by TR-avidin, and sorted on the FACS. To minimize contamination, 50% of cells (fraction A) in the Ly-1 B region and 25% of cells (fraction B) in the Ly-1⁻ B-cell region were sorted in sort 1; in sort 2, yields were 60% and 30%, respectively. Under these conditions, approximately half of the cells sorted into fraction A fell within the Ly-1 B gates on reanalysis (60% and 35% in sorts 1 and 2, respectively). The remainder were IgM⁺ but negative for Ly-1 (or Lyt-2 in the control sort). Fraction B, in contrast, showed minimal contamination (<0.3%) with cells that fell within the Ly-1 B gate. Correcting the data shown in the figure for contamination and losses in sorting indicates that virtually all PFC in the original population belong to the Ly-1 B population.

internal control for the specificity of the ELISA used to measure supernatant IgM autoantibody levels. That is, the demonstration that Ly-1⁺ B cells produce substantially more IgM autoantibody than Ly-1⁻ B cells in this experiment is based directly on comparisons between supernatants that should yield equivalent levels of nonspecific IgM binding.

Table 1. Ly-1 B cells from NZB mice secrete autoantibodies

Exp.	NZB spleen cell population cultured*	Total IgM,† μg/ml	IgM autoantibodies‡ Anti-T cell, units	IgM autoantibodies‡ Anti-ss DNA, μg/ml
1	Unseparated	35	10	4
	Ly-1⁺ B	150	50	18
	Ly-1⁻ B	4	<1	<1
2	Unseparated	150	ND	34
	Ly-1⁺ B	230	ND	71
	Ly-1⁻ B	120	ND	9

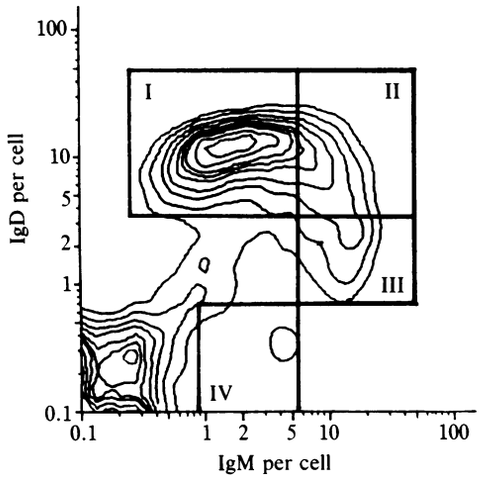
*Spleen cells from NZB/N mice were stained (Fl-anti-IgM and Bi-anti-Ly-1 + TR-avidin) and sorted on the FACS. Ly-1⁺ B cells (Ly-1 B) and other B cells (Ly-1⁻ B) respectively constituted 5% and 45% of the unseparated spleen. Sorted cells were cultured at 5 × 10⁶ per ml. Cells from a 2-month-old mouse were used for Exp. 1 and cells from a 4-month-old mouse were used for Exp. 2.
†Determined by competitive RIA.
‡IgM autoantibodies measured by an ELISA assay (16). Antibodies to ss DNA are expressed as μg/ml of culture supernatant. Antibodies to T-cell determinants on thymocytes (anti-T cell) are expressed in units based on the reciprocal of the supernatant dilution required to achieve a fixed binding level. ND, not determined.

Most Exogenous Antigens Barely Stimulate Antibody Production by Ly-1 B Cells. Previous studies have shown that SRBC stimulate very little antibody production by NZB Ly-

Table 2. Antibody responses to exogenous antigens are mainly produced by Ly-1⁻ cells

Antigen*	Direct PFC recovered in spleen cell populations† Unseparated	Ly-1⁻ B	Ly-1 B
SRBC	39,000	38,000	1000
SRBC	30,000	29,000	700
TNP-Ficoll	43,000	42,000	830
TNP-Ficoll	10,000	10,000	70
TNP-KLH	2,000	2,000	<60
TNP-LPS	10,000	10,000	300
TNP-BA	8,000	7,000	640
TNP-BA	35,000	30,000	5100

*NZB donors were immunized with the indicated antigens 4 days prior to staining and sorting.
†Direct PFC were enumerated with TNP-SRBC except in the case of SRBC immunization, in which SRBC were used for detection. Recovered spleen cells were within the range of 1–2 × 10⁸ per spleen. Data are presented as PFC per spleen or PFC recovered in the sorted fractions (corrected according to the percentage of the sorted fraction in the unseparated spleen). About 5–8 × 10⁵ Ly-1 B cells and 1–3 × 10⁶ Ly-1⁻ B cells were usually sorted and assayed for PFC. The frequency of Ly-1 B was always 5–10% of spleen cells and the Ly-1⁻ B contamination was less than 20% in the Ly-1 B fraction.



Splenic B cells	Nucleated cells, % of total	IgM anti-TNP PFC, % of total
Unseparated	100	100
All IgM$^+$ cells	65	100
Population I	39	2
II	12	2
III	6	2
IV	3	93

FIG. 3. IgM anti-TNP PFC generated by TNP-Ficoll immunization do not carry surface IgD (or Ly-1). Spleen cells from CSW mice immunized 4 days previously with TNP-Ficoll were stained with Fl-anti-IgM and Bi-anti-IgD plus TR-avidin and separated according to the gates shown in the figure. Population III in the figure contains virtually all Ly-1$^+$ B cells (11).

1 B cells. Nearly all of the anti-SRBC PFC in SRBC-immunized NZB mice were recovered in the Ly-1$^-$ splenic B cell population and the PFC found in the Ly-1 B population were attributable to contamination with Ly-1$^-$ cells (11). Data presented in Table 2 confirm and extend these findings. Ly-1$^-$ B cells sorted from NZB animals stimulated with TNP-Ficoll, TNP-KLH, or TNP-LPS contain virtually all of the direct anti-TNP PFC found in unseparated spleen. A few anti-TNP PFC (1–3% of the total response) are recovered in the Ly-1 B cell population, but the frequency of these PFC is commensurate with the contamination of the Ly-1 B population with Ly-1$^-$ B cells.

These data, however, should not be interpreted as indicating that Ly-1 B cells cannot be stimulated to produce antibodies to exogenously introduced antigens. As Table 2 also shows, TNP presented on *Brucella abortus* stimulates substantial numbers of IgM anti-TNP PFC in the Ly-1 B population. Thus Ly-1 B cells are quite capable of producing antibodies to TNP (and presumably to other epitopes) but apparently do not do so unless presented with the epitope on certain carriers.

IgM PFC Produced in Response to Exogenous Antigens Belong to a B-Cell Subpopulation That Does Not Express Ly-1 or IgD. Studies with splenic B cells sorted from CSW (C3H.H-2^b) mice immunized with TNP-Ficoll yield results comparable to those obtained with NZB. That is, most of the IgM anti-TNP PFC stimulated by this immunization are recovered among B cells that do not carry Ly-1, and very few are found in the subpopulation enriched for Ly-1 B cells. In these experiments, however, we used an alternative staining and sorting strategy that enriches for Ly-1 B cells in one sorted subpopulation and enriches for TNP-Ficoll-stimulated IgM anti-TNP PFC in another.

Thus, to enrich for Ly-1 B cells, we sorted the subpopulation of IgM$^+$, IgD$^+$ B cells (labeled III in Fig. 3) that has high levels of surface IgM and low levels of surface IgD. This subpopulation (18) contains virtually all of the Ly-1 B cells in spleen (11). It does not increase significantly in frequency after immunization (data not shown) and does not contain significant numbers of IgM anti-TNP PFC (see Fig. 3).

The subpopulation enriched for anti-TNP PFC, in contrast, is usually too rare to detect in nonimmunized animals

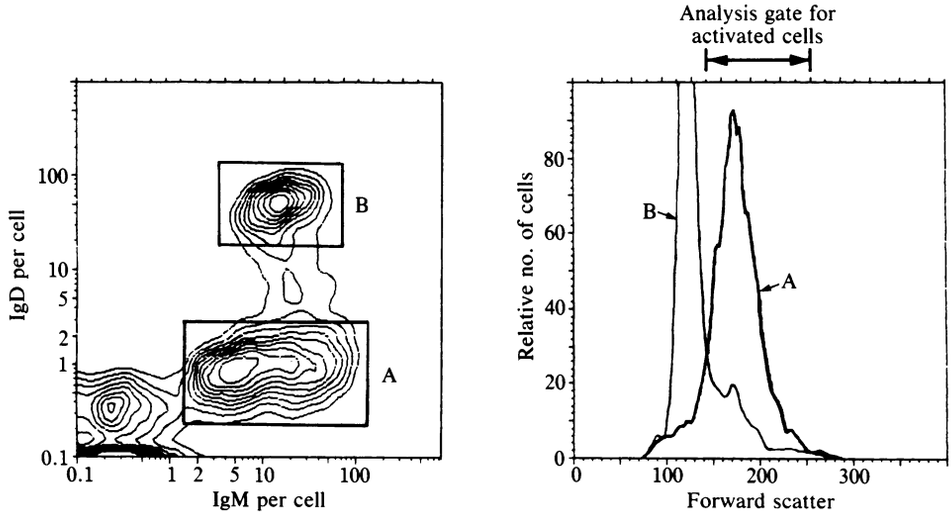


FIG. 4. FACS profiles for LPS-activated spleen cells. Unseparated spleen cells were cultured with LPS for 5 days and then stained with Fl-anti-IgM and Bi-anti-IgD, followed by TR-avidin. The region marked A in the FACS contour plot shown at *Left* contains a population of cells (low IgD, IgM$^+$) that is very rare in spleen cell suspensions (see Fig. 3). Cells in this region show the size increase (measured by forward scatter) typically associated with activated B cells (curve A at *Right*). Cells in the region marked B in the contour plot show surface IgM and IgD levels typical of the predominant splenic B cell subpopulation (I) and are correspondingly smaller by forward angle scatter (curve B at *Right*). None (<1%) of the cells in this culture showed detectable levels of surface Ly-1.

but tends to be detectable several days after immunization in spleen and in lymph nodes that drain the immunization site. This subpopulation (labeled IV in Fig. 3) contains more than 90% of the IgM anti-TNP PFC found in spleens from TNP-Ficoll-immunized donors. It expresses intermediate levels of IgM and does not express detectable amounts of IgD or Ly-1 (i.e., no Ly-1-bearing cells were detected when cells from the sorted population were stained and analyzed with anti Ly-1).

Ly-1 Expression Is Not Induced on Activated B Cells. As indicated above, cells (PFC) that produce IgM antibodies to exogenous antigens do not express Ly-1. Thus Ly-1 clearly does not represent a simple activation marker that appears whenever B cells are stimulated to divide and differentiate to antibody-forming cells. However, because the appearance of Ly-1 B cells that secrete antibodies is more easily stimulated by LPS than by exogenous antigens, we investigated the possibility that LPS induces the appearance of Ly-1 on typical splenic B cells. Thus we cultured unseparated BALB/c spleen cells and sorted Ly-1$^-$ B cells in the presence of LPS and analyzed for the appearance of Ly-1 on the cultured cells.

Data from these experiments demonstrate clearly that LPS activation does not stimulate the appearance of Ly-1 on cells that persist in culture. After 5 days in culture with LPS, the unseparated spleen cell cultures and the cultures containing sorted Ly-1$^-$ cells showed substantial numbers of activated cells (see Fig. 4). However, less than 1% of the activated cells (that fell within the "gates" shown in Fig. 4) or of the total cells recovered from the culture had detectable surface Ly-1. Similar results were obtained on day 2 and day 3 of culture although fewer activated cells were present. Thus if Ly-1 marks activated B cells, it must mark only an activated subpopulation that does not survive and expand under the conditions normally used to generate activated B cells.

We belabor this point because earlier studies (e.g., refs. 1, 2, and 10) attributed the IgM secretion and autoantibody production in NZB mice to the presence of increased numbers of B cells that were considered essentially equivalent to the activated B cells that arise and differentiate to IgM-secreting cells after immunization or LPS stimulation. Our findings, in contrast, indicate that this autoantibody production is due to a specialized population of B cells (Ly-1 B) that is increased in frequency and is activated to differentiate to secrete IgM in NZB mice. Thus, although we challenge the current paradigm, the hypothesis we offer in its place basically modifies previous concepts to bring them into line with contemporary understanding of the complexity of B-cell subpopulations.

DISCUSSION

Studies presented here introduce the idea that a small subpopulation of Ly-1-bearing B cells that constitutes less than 2% of BALB/c spleen and less than 10% of NZB spleen are responsible for the production of much of the IgM autoantibody found in normal and autoimmune mice. As we have shown, FACS-sorted IgM$^+$, Ly-1$^+$ (Ly-1 B) cells from young NZB mice constitute the main source of the IgM anti-DNA and anti-T-cell autoantibodies produced *in vitro* by spleen cells from these mice. Furthermore, FACS-sorted Ly-1 B cells from (LPS-stimulated) BALB/c mice contain essentially all of the autoantibody-producing cells that form plaques when spleen cells from these mice are plated on brmRBC. Thus Ly-1 B cells clearly account for the production of several commonly studied IgM autoantibodies.

The production of all autoantibodies is probably not due to the activity of this B-cell subpopulation. IgG autoantibodies, for example, are commonly produced in older NZB mice and in other autoimmune mice (16). These antibodies are likely to be produced by IgG-bearing B cells rather than by Ly-1 B cells, which express IgM and IgD. Such IgG-producing cells could be related to Ly-1 B cells, but we have no reason to think that they are. Therefore, we suggest that Ly-1 B cells are responsible for producing certain (perhaps most) IgM autoantibodies but that other B cells also contribute to autoimmunity.

These limitations do not detract from the potential importance of the data presented here vis-à-vis the development of new therapies for autoimmune diseases in humans. In essence, the concentration of certain IgM autoantibody-producing functions into a small, phenotypically distinct B cell population in both autoimmune and normal mice opens the possibility that an analogous B cell subset might be found in humans with particular autoimmune diseases. If so, then finding methods to selectively eliminate this subpopulation might allow the treatment of such diseases without compromising other B-cell functions in the treated individual.

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