Conventional B Cells, Not B-1 Cells, Are Responsible for Producing Autoantibodies in *lpr* Mice

By Elizabeth A. Reap, Eric S. Sobel, Philip L. Cohen, and Robert A. Eisenberg

From the Departments of Medicine and Microbiology/Immunology, University of North Carolina, Chapel Hill, North Carolina 27599-7280

Summary

Mice homozygous for the *lpr* gene develop autoantibodies and polyclonal B cell activation similar to what is seen in human systemic lupus erythematosus patients. We have previously shown that an *lpr*-specific intrinsic B cell defect was necessary for autoantibody production in this model. In the current study, we have further defined these autoantibody-producing B cells. Two major subsets of B cells have been described. B-1 cells ($CD5^+$ B cells) can be distinguished from conventional B cells on the basis of phenotype, cytokine secretion, gene expression, anatomical location, and function. In addition, B-1 cells have been implicated in autoimmunity in several murine and human studies. To address the question of which B cell subset produces autoantibodies in *lpr* mice, we used immunoglobulin heavy chain (Igh) allotype-marked peritoneal (B-1 cell source) and bone marrow (conventional B cell source) cells from *lpr* mice to establish B cell chimeras. We used two general approaches. In one, we reconstituted sublethally irradiated mice with B-1 cells of one allotype and bone marrow cells of another allotype. In the second method, we suppressed endogenous B cells in neonatal mice with allotype-specific anti-IgM antibody, and injected peritoneal cells of another allotype. After antibody treatment was stopped, the mouse's conventional B cells recovered, but the B-1 subset was only reconstituted by the donor. In both types of chimeras, antichromatin, rheumatoid factor, and anti-single stranded DNA (ssDNA) autoantibodies were produced by the conventional B cell bone marrow source. In addition, an age-related decrease in peritoneal B-1 cells was seen, even in unmanipulated *lpr* mice. These data show that *lpr* B-1 cells are not important producers of autoantibodies. Conventional B cells are the source of autoantibodies directed at chromatin, ssDNA, and IgG.

Distinct subsets of B cells are recognized on the basis of function and cell surface phenotype (1). Those B cells initially characterized by their surface expression of the CD5 antigen, provisionally called B-1 cells (2), have recently received attention as important autoantibody-producing cells in several human and murine experimental models (3). Cell transfer studies using fetal omentum have shown independent development of B-1 and conventional B cells consistent with separate lineages (4), although other evidence suggests some ongoing interchange between the B-1 and conventional subsets based on phenotypic markers (5). In addition, B-1 cells can be distinguished from conventional B cells by anatomical localization, gene usage, function, and phenotype (3). B-1 cells are most prominent early in ontogeny and have a restricted tissue distribution and cellular origin (6–10). The antibodies produced by B-1 cells are generally of the IgM isotype, lack somatic mutation, and have few to no N regions (3, 11–15). Their frequent reactivity with the autoantigens single stranded (ss)[1] DNA, bromelain-treated mouse red blood cells (BrMRBC), and autologous IgG has raised the possibility that autoantibody production in systemic autoimmunity is mainly due to the B-1 cells (16, 17). In support of this notion, it has been reported that B-1 cells are primarily responsible for the production of RF and polyreactive autoantibodies (13, 14, 17). Some autoimmune mice, such as motheaten, NZB, and related strains, have elevated levels of this subset (11, 13), and several autoantibody specificities are secreted by NZB B-1 splenic cells (13). Autoantibodies to BrMRBC are secreted by splenic and peritoneal B-1 cells

[1] *Abbreviations used in this paper:* B6, C57BL/6; B6/*lpr*, C57BL/6-*lpr*/*lpr*; B6/*lpr*-Igh^a, C57BL/6-*lpr*/*lpr*-Igh^a; B6-Thy-1.1, B6.PL-$Thy\text{-}1^a$/Cy; BrMRBC, bromelain-treated mouse red blood cells; EDF, equivalent dilution factor; FALS, forward angle light scatter; IgH, immunoglobulin heavy chain; MRL/*lpr*, MRL-MpJ-*lpr*/*lpr*; PenStrep, penicillin streptomycin; PerC, peritoneal washout cells; ssDNA, single stranded DNA.

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in nonautoimmune mice as well (13). Recently, mice with Coombs autoantibody transgenes have provided the first direct evidence that B-1 cells can produce potentially pathogenic autoantibodies in vivo (18).

Mice homozygous for the autosomal recessive gene *lpr*, recently demonstrated to code for a mutant form of the apoptosis-inducing surface receptor, Fas (19), develop generalized lymphoproliferation (20). They also produce a spectrum of autoreactivity that resembles that found in human SLE. Whether their autoantibodies are produced by a discrete B cell subset is unknown. In the current studies, we have investigated the role of B-1 cells in SLE-like systemic autoimmunity in Igh allotype-congenic *lpr* mouse strains. Taking advantage of the capability of B-1 cells to self-renew after transfer in vivo (6), we prepared double chimeric *lpr* mice in which B-1 and conventional B cells and their antibody products could be readily distinguished using allotype-specific reagents. We found that the autoantibodies were produced almost entirely by bone marrow-derived conventional B cells. Furthermore, there was an unexpected age-related disappearance of peritoneal B-1 cells both in *lpr* chimeras and in unmanipulated *lpr* mice.

Materials and Methods

Mice. C57BL/6 (B6), B6.C20, C57BL/6-*lpr*/*lpr* (B6/*lpr*), B6.PL-Thy-1^a/Cy (B6-Thy-1.1), and C57BL/6-*lpr*/*lpr*-Igh^a (B6/*lpr*-Igh^a) mice were raised in our breeding colony. The B6/*lpr* strain was originally obtained from The Jackson Laboratory (Bar Harbor, ME), and the B6/*lpr*-Igh^a strain was developed in our laboratory by crossing B6.C20 (obtained from Dr. Gayle Bosma, Institute for Cancer Research, Philadelphia, PA) and B6/*lpr*. By 5 mo of age, the B6/*lpr*-Igh^a mice develop autoantibodies to chromatin and to IgG, lymphadenopathy, and splenomegaly (21). Mice used for peritoneal cell donors were 4–8-wk-old. Bone marrow donors were 6–10-wk-old, and recipients for irradiation chimeras were 6–8-wk-old. Animals of one sex were used in individual experiments.

Preparation of Cells. Femurs and tibias were used as sources of bone marrow cells. Cold medium (RPMI 1640 with 15 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin (PenStrep) from the University of North Carolina Cancer Center Tissue Culture Facility) was injected into the marrow cavity, and cells were washed three times. Peritoneal washout cells (PerC) were obtained by injecting 4–6 ml of chilled media containing 10% newborn calf sera into the peritoneal cavity. The recovered cells, ~60% lymphocytes, were washed twice with serum-free medium.

Preparation of Radiation Chimeras. 1 wk before cell transfer, recipients and donors were bled. Recipients were moved to autoclaved cages in an isolation cubicle, and water was treated with neomycin (0.2% wt/vol). 1 d before transfer, mice received 750 rad of gamma-radiation in a ^{137}Cs gamma cell 40 apparatus (Atomic Energy of Canada, Ltd., Ottawa, Canada) or from a ^{60}Co source (Atomic Energy of Canada, Ltd.). Cells were resuspended in serum-free medium, and 10^7 bone marrow cells mixed with 10^7 PerC were injected into the tail vein of irradiated recipients (6).

Neonatal Chimeras. B6/*lpr* mice were injected within 1 d of birth with 0.1 mg of anti-IgM^b mAb (AF6–78.25), followed by twice weekly injections of 0.2 mg for 1 mo. On day 2 or 3 after birth, treated mice were injected intraperitoneally with 3×10^6 PerC from 4–8-wk-old B6/*lpr-Igh*^{a} donors (22, 23).

Antibodies. Anti-Igh-6b (anti-IgM^b; AF6–78.25, mouse IgG1) was obtained from Dr. A. Stall (Columbia University, New York) (24) and was purified from mouse ascites by 50% ammonium sulfate precipitation for neonatal injections. For immunofluorescence, the antibody was further purified over a protein G column (Genex, Gaithersburg, MD), or a directly fluoresceinated preparation was used (PharMingen, San Diego, CA). Anti-Igh-5a (anti-IgD^a; Hδ^a/1, mouse IgG2b) was obtained from Dr. F. Finkelman (Uniformed Services, University of Health Sciences, Bethesda, MD) (25). Anti-Igh-5b (anti-IgD^b; AF3.33.3.2, mouse IgG2a) was obtained from Dr. V. Oi (Becton Dickinson & Co., Mountain View, CA) (24). Anti-Ly-1 (anti-CD5; 53–7.313, rat IgG2a) was obtained from Dr. G. Haughton (University of North Carolina, Chapel Hill, NC) (26) and purified from serum-free culture supernatant (HB102; New England Nuclear Research Products, Boston, MA). Fluoresceinated anti-Igh-6a (anti-IgM^a; DS-1, mouse IgG1) was from PharMingen (27). Cell lines for anti-Fc-γ receptor antibody (2.4G2, rat IgG2b) and anti-Mac-1 (M1/70.15.11.5.HL, rat IgG2b) were obtained from American Type Culture Collection (Rockville, MD) (28, 29). Anti-IL-5 receptor antibody (R52.120, rat IgG1) was a gift from Dr. R. Palacios (Basel Institute for Immunology, Basel, Switzerland) (30), and biotinylated anti-CD23/IgE FcR (B3B4, rat IgG2a), was a gift from Dr. T. Waldschmidt (University of Iowa, College of Medicine, Iowa City, IA) (31). Biotinylated anti-CD45R/B220 (RA3–6B2, rat IgG2a) was obtained from PharMingen (32).

Immunofluorescence. Allotype-specific two-color flow cytometric analysis of IgD^a vs. IgM^a, IgD^b vs. IgM^b, CD5 vs. IgM^b, and CD5 vs. IgM^a was routinely performed on peritoneal cells and splenic lymphocytes at the time of killing. PerC were obtained as described above. Splenic lymphocytes were isolated by disrupting the splenic capsule between frosted ends of glass slides and washing twice with RPMI 1640 supplemented with 3% FCS, Hepes, 0.1% NaN_3, and PenStrep. Erythrocytes in spleen and peritoneal cells were lysed with ammonium chloride for 5 min at 4°C. All samples were treated with anti-Fc-γ receptor antibody for 20 min at 4°C to block any nonspecific binding. For the allotype-specific IgM vs. IgD staining of the *a* allotype, fluoresceinated DS-1, and biotinylated Hδ^a/1 were added for 30 min at 4°C. The cells were washed three times between steps, PE-coupled avidin (Fisher Scientific Co., Pittsburgh, PA) was added, and the cells were incubated on ice for 30 min. They were washed once in staining medium and twice in PBS and fixed with an equal mixture of PBS and 2% paraformaldehyde in PBS. The IgM vs. IgD staining for the *b* allotype was essentially the same except fluoresceinated AF6–78.25 and biotinylated AF3–33.3.2 were added for the first step. For the CD5 vs. IgM^a or IgM^b staining, biotinylated 53.7.313 was added along with the allotype-specific fluoresceinated anti-IgM reagents described above. After washing, PE-coupled avidin was added, and the procedure was continued as described above. Two-color analysis with biotinylated anti-Mac-1 or anti-IL-5R and allotype-specific FITC-conjugated anti-IgM reagents was performed on some samples to confirm B-1 cell staining. For each assay, age-matched B6, B6.C20, B6/*lpr*, and B6/*lpr*-Igh^a splenic lymphocytes and PerC were prepared as controls. Analysis was performed on a flow cytometer (Epics V; Coulter Electronics, Hialeah, FL) with size gating on the lymphocyte population. B-1 and conventional B cell populations were defined by gates set on the control samples in each experiment. In most cases, backgating was performed on the two-color histograms for size and granularity analysis to confirm staining of the B-1 cell population. Initially, cells were also stained with isotype-matched biotinylated anti-Ly-2 (53–6.72, rat IgG2a [26]), obtained from Dr. L. Arnold (University of North Carolina, Chapel Hill, NC) to establish specificity of the anti-CD5 antibody reagent. Data

were plotted on a three-decade logarithmic scale, except forward angle vs. 90° light scatter, which is linear. 20,000 events were collected per sample in most experiments.

ELISA. Allotype-specific total serum IgG2a and IgM, IgM RF, IgM and IgG2a anti-ssDNA, and IgG2a antichromatin were measured as previously described (21). RF and IgG2a anti-ssDNA concentrations were expressed in equivalent dilution factors (EDF), defined by the formula: EDF = (Dilution of standard reference sera which gives the equivalent OD of the test serum) × 10^6 (21).

Results

Conventional B Cells Were Responsible for Autoantibody Production in lpr Irradiation Chimeras. Equal numbers of B6/*lpr* (Igh^b) bone marrow cells and B6/*lpr*-Igh^a peritoneal cells were transferred to irradiated (750 R) B6/*lpr* recipients (*irradiation chimera I*, Table 1). In this model, the bone marrow donor should reconstitute the conventional B cell subset, and the peritoneal cells should reconstitute the B-1 subset (6). Adequate chimerism was established by 3 mo after transfer, as both *a* and *b* allotypes significantly contributed to total serum IgM (Table 2). B-1 cells (*a* allotype) made only a minor contribution to total IgG2a. Two-color flow cytometry showed that virtually all B cells in the peripheral blood were derived from the bone marrow (data not shown). All of the antichromatin autoantibody at 3 mo was from the conventional (*b* allotype) B cell source, as well as most of the IgM RF (Table 2). At 5 mo after transfer, B-1 cells still contributed significantly to total serum IgM, but essentially all of the autoantibodies came from the conventional B cells.

A reciprocal set of irradiation chimeras gave similar results. Irradiated B6/*lpr*-Igh^a mice received B6/*lpr*-Igh^a bone marrow and B6/*lpr* peritoneal cells (*irradiation chimera II*, Table

Table 1. *Chimeras Established to Determine the Role of B-1 Cells in Autoantibody Production in lpr Mice*

Chimera	Type	Host	Cell Transfer	Conventional B Cell Allotype	B-1 Cell Allotype	Fas
I	Irradiation	B6/*lpr*	B6/*lpr* bone marrow and B6/*lpr*-Igh^a peritoneal cells	*b*	*a*	*lpr*
II	Irradiation	B6/*lpr*-Igh^a	B6/*lpr*-Igh^a bone marrow and B6/*lpr* peritoneal cells	*a*	*b*	*lpr*
III	Neonatal	B6/*lpr*	B6/*lpr*-Igh^a peritoneal cells	*b*	*a*	*lpr*
IV	Irradiation	B6	B6 bone marrow and B6.C20 peritoneal cells	*b*	*a*	+
V	Irradiation	B6.C20	B6.C20 bone marrow and B6 peritoneal cells	*a*	*b*	+
VI	Neonatal	B6	B6.C20 peritoneal cells	*b*	*a*	+
VII	Irradiation	B6/*lpr*-Igh^a	B6/*lpr*-Igh^a bone marrow and peritoneal cells and B6-Thy1.1 bone marrow and peritoneal cells	*a* and *b*	*a* and *b*	+ and *lpr*

Table 2. *Conventional, Bone Marrow-derived B Cells Produced Autoantibodies in Chimera I**

	Serum Ig				Serum autoantibody					
							RF specificity			
					Antichromatin		IgG1^a(EDF)		IgG2b^b(EDF)	
Age after transfer	IgM^a	IgM^b	IgG2a^a	IgG2a^b	IgG2a^a	IgG2a^b	IgM^a	IgM^b	IgM^a	IgM^b
	μg/ml				*ng/ml*					
3 mo (16)‡	840(78)§	206(57)	47(8)	495(24)	–‖	516(59)	18(5)	105(42)	28(9)	238(38)
5 mo (16)	509(106)	886(35)	35(10)	3,000(260)	–‖	8,672(4,845)	10(7)	96(27)	9(2)	97(24)

* B6/*lpr* bone marrow and B6/*lpr*-Igh^a peritoneal cells were transferred into irradiated B6/*lpr* recipients.
‡ Number of animals tested.
§ Data represent means (SE) of ELISA results.
‖ Below lower limits of detection of assay, i.e., <37 ng/ml.

Table 3. *Conventional, Bone Marrow-derived B Cells Produced Autoantibodies in Chimera II**

	Serum Ig				Serum autoantibody					
							RF specificity			
					Antichromatin		IgG1^a(EDF)		IgG2b^b(EDF)	
Age after transfer	IgM^a	IgM^b	IgG2a^a	IgG2a^b	IgG2a^a	IgG2a^b	IgM^a	IgM^b	IgM^a	IgM^b
	$\mu g/ml$				ng/ml					
2 mo (4)‡	325(50)§	380(60)	2,960(831)	156(53)	1,627(654)	–‖	–¶	–**	28(18)	–**
3 mo (7)	1,300(440)	370(60)	2,385(433)	150(60)	6,807(2,517)	–‖	30(25)	–**	40(12)	–**

* B6/*lpr*-Igh^a bone marrow and B6/*lpr* peritoneal cells were transferred into irradiated B6/*lpr*-Igh^a recipients.
‡ Number of animals tested.
§ Data represent means (SE) of ELISA results.
‖ Below lower limits of detection of assay, i.e., <32 ng/ml.
¶ Below lower limits of detection of assay, i.e., <5 EDF.
** Below lower limits of detection of assay, i.e., <3 EDF.

1). Chimerism was established by 2 mo after transfer, as shown by quantitation of IgM of the *a* and *b* allotypes (Table 3). A minor contribution by the B-1 cells to total serum IgG2a was seen. The allotype distribution of autoantibodies measured 2 and 3 mo after transfer indicated that conventional B cells (*a* allotype) were responsible for the specificities assayed (Table 3). In this set of chimeras, consistent with the previous experiment, the B-1 cells (*b* allotype) made no contribution to antichromatin or RF.

Peritoneal Cell Populations in lpr Irradiation Chimeras Did Not Show Persistence of B-1 Cells. In B6 and B6.C20 control mice, CD5-bearing peritoneal B-1 (subset B-1a) cells were first identified by double staining for CD5 and IgM (Fig. 1 *A*, *top*). This population is also IgM^{hi}IgD^{lo}, as is the CD5$^-$ B-1 (subset B-1b) "sister population" (Fig. 1 *A*, *bottom*) (14, 22, 23). B6 mice averaged 25% CD5$^+$IgM$^+$ and 33% IgM^{hi}IgD^{lo} peritoneal B-1 cells (n = 5; age, 3 mo). Control mice of the *a* allotype, B6.C20, showed 33% CD5$^+$IgM$^+$, and 55% IgM^{hi}IgD^{lo} B-1 peritoneal cells (n = 5; age, 3 mo). In contrast, however, peritoneal cells from a representative irradiation chimera I (B6/*lpr* host), killed 5 mo after transfer, showed no B-1 cells of either allotype (Fig. 1 *B*).

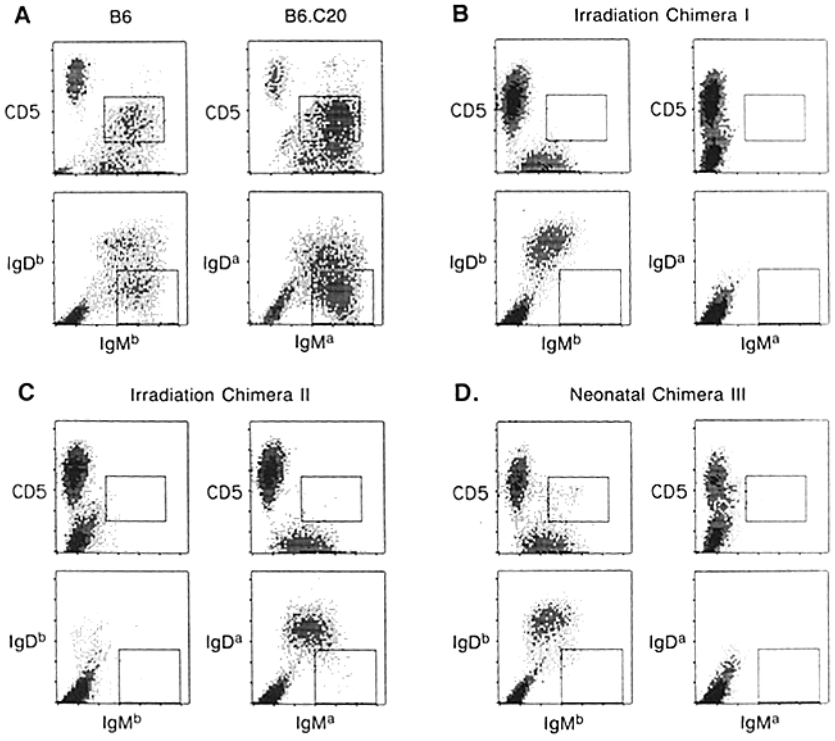

Figure 1. *Lpr* chimeras had few peritoneal B-1 cells. Representative data for two-color flow cytometric analysis of peritoneal cells stained with anti-CD5 and appropriate allotypic markers for unmanipulated control mice (*A*), and chimeras killed 5 mo after cell transfer (*B–D*) are shown. B-1 cells are identified by the indicated gates. Refer to Table 1 for description of chimeras.

Table 4. *Conventional, Bone Marrow-derived B Cells Produced Autoantibodies in Chimera III**

	Serum Ig				Serum autoantibody					
							RF specificity			
					Antichromatin		IgG1[a](EDF)		IgG2b[b](EDF)	
Age after transfer	IgM[a]	IgM[b]	IgG2a[a]	IgG2a[b]	IgG2a[a]	IgG2a[b]	IgM[a]	IgM[b]	IgM[a]	IgM[b]
	μg/ml				*ng/ml*					
5 wk (12)‡	217(44)§	–‖	29(19)	123(13)	ND	ND	ND	ND	ND	ND
2 mo (11)	698(87)	590(67)	81(12)	1,536(1,180)	–¶	309(100)	–**	15(6)	5(1)	16(4)
4 mo (12)	421(56)	2,850(180)	–‖	5,048(2,210)	–¶	12,900(6,770)	–**	216(22)	–**	127(20)
5 mo (5)	84(15)	3,000(200)	82(43)	8,706(2,780)	–¶	6,741(3,400)	–**	182(33)	–**	117(20)

* B6/*lpr*-Igh[a] peritoneal cells were transferred into anti-IgM[b]-treated B6/*lpr* neonatal recipients.
‡ Number of animals tested.
§ Means (SE) of ELISA results.
‖ Below detection of assay i.e., <29 μg/ml.
¶ Below lower limits of detection of assay, i.e., <37 ng/ml.
** Below lower limits of detection of assay, i.e., <5 EDF.

Similarly, peritoneal cells from a representative irradiation chimera II (B6/*lpr*-Igh[a] host) killed 5 mo after transfer showed few B-1 cells of either allotype by both criteria used to identify B-1 cells (Fig. 1 *C*).

Neonatal Chimeras Paralleled the Results of the Irradiation Chimeras. In a second approach to establish B cell chimeras (22, 23), neonatal B6/*lpr* mice were treated with anti-IgM[b] and injected with B6/*lpr*-Igh[a] peritoneal cells from young adults (*neonatal chimera III*, Table 1). In this model, the injected antibody suppresses host B cells. The conventional B cells recover after the treatment is stopped, and the B-1 cell subset is reconstituted by the injected peritoneal population (22, 23). Suppression of the host IgM-bearing neonatal B cells by anti-IgM[b] was successful, as assayed by ELISA at 5 wk of age (Table 4). At 2 mo, both B-1 and conventional B cells contributed to total serum IgM, but thereafter the contribution of the B-1 cells (*a* allotype) decreased. The IgG2a antichromatin and IgM RF directed against IgG1[a] and IgG2b[b] were made exclusively by the *b* allotype, conventional B cells at all time points (Table 4). Two-color flow cytometric analysis demonstrated that the B-1 cell population was nearly absent from the peritoneal cavity 3 mo after transfer (Fig. 1 *D*). Subsequent killing at 8 mo after transfer showed that B-1 cells did not return (data not shown).

Conventional B Cells Produced IgM and IgG2a ssDNA in All lpr Chimeras. Since B-1 cells were previously reported to secrete authoantibodies specific for ssDNA (13), sera from *lpr* chimeras were assayed for IgM and IgG2a anti-ssDNA (Table 5). In all cases, these autoantibodies were made only by conventional B cells.

Normal Chimeric Mice Demonstrated Persistence of the B-1 Cells After Transfer. When chimeric mice were set up using normal congenic mouse strains in the same way as *lpr* strains described above, transferred peritoneal cells were found to reconstitute a persistent B-1 lineage, as has been reported (6, 9, 22, 23, 33). Shown in Fig. 2 are representative flow cytometric anal-

Table 5. *Conventional, Bone Marrow-derived B Cells Produced Anti-ssDNA Autoantibodies in lpr Chimeras**

	OD		EDF	
Chimera	IgM[a]	IgM[b]	IgG2a[a]	IgG2a[b]
Irradiation I (10)‡	**0**‖(0)§	.14(.06)	**0**(0)	18(13)
Irradiation II (8)	.06(.02)	**.01**(.01)	7.6(3.4)	**.04**(.03)
Neonatal III (12)	**.03**(0)	.24(.07)	**.08**(.03)	43(22)

* Refer to Table 1 for description of chimeras; data obtained from bleeds done 4–5 mo after transfer.
‡ Number of mice.
§ Mean (SE).
‖ Data for B-1 cells are in bold.

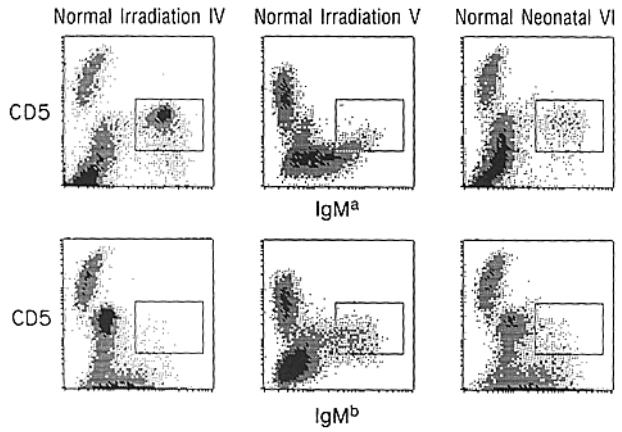

Figure 2. Normal chimeras had significantly more B-1 cells of the transferred allotype than *lpr* chimeras. Peritoneal cells stained for B-1 cells using CD5 and IgM allotypic markers are shown from representative chimeras killed 4–6 mo after cell transfer. B-1 cells are outlined in the gates. Refer to Table 1 for description of chimeras.

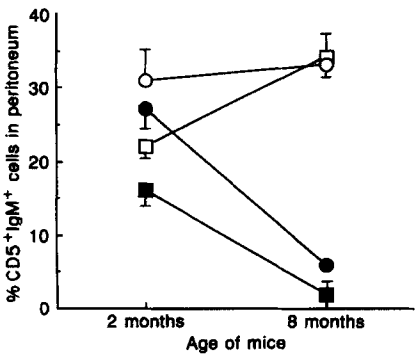

Figure 3. *Lpr* mice showed a decrease in B-1 peritoneal cells with age. Age-matched B6/*lpr*, B6/*lpr*-Igh^a, and control B6 and B6.C20 mice were analyzed by flow cytometry for B-1 cells in the peritoneal cavity by double staining for IgM and CD5. Data represent an average of 3–5 mice per time point ± SE. (□) B6; (■) B6/*lpr*; (○) B6.C20; (●) B6/*lpr*-Igh^a.

yses of each type of normal chimera (*Types IV–VI*, Table 1) killed at ~4–6 mo after transfer, and stained for CD5 and IgM. Double staining with IgD and IgM showed higher numbers of B-1 cells (data not shown). The transferred B-1 cells also were persistently functional in all three types of normal chimeras, as evidenced by their contribution to total serum IgM at 3–5 mo after transfer (Table 6).

Intact B6/lpr and B6/lpr-Igh^a Mice Showed a Loss of Peritoneal B-1 Cells with Time. The chimera results raised the possibility that *lpr* mice might undergo spontaneous loss of B-1 cells with time. Unmanipulated *lpr* and control mice were therefore stained for B-1 cells at different ages. Figs. 3 and 4 show that B6/*lpr* and B6/*lpr*-Igh^a mice had an age-associated decrease in the proportion of B-1 cells in the peritoneum, when compared with age- and allotype-matched normal congenic B6 and B6.C20 controls. Shown in Fig. 4 are peritoneal cells from a 4-mo *lpr*, a 2-mo *lpr*, and a 2-mo control mouse. *Lpr* mice of the approximate age previously used as peritoneal cell donors (2-mo-old) had a normal population of B-1 cells. However, a relative decrease in the B-1 cell population was apparent already in the 4-mo-old *lpr* mouse. Even fewer B-1 cells could be identified in the peritoneum of 8-mo-old unmanipulated *lpr* mice using four additional markers (IL-5R, Mac-1, B220^{lo}, and absence/low expression of CD23) for B-1 cells in conjunction with anti-IgM (Fig. 5) (34, 35). The small population of cells defined by anti-CD23 and IL-5R in the *lpr* mouse within the B-1 gates (Fig. 5) may represent activated conventional B cells (34, 35). Shown also is the forward angle light scatter vs. linear 90° scatter, since B-1 cells have increased forward angle and 90° scatter.

The age-related relative decrease in peritoneal B-1 cells in *lpr* mice shown in Figs. 3–5 reflects a comparable absolute diminution of this population. For example, in the animals from whom the data in Fig. 5 were obtained, 5,000 peritoneal B-1 cells were recovered from the *lpr* mice, compared with 900,000 from the normal one. In contrast, both *lpr* and normal 2-mo-old animals, such as the ones shown in Fig. 4, had ~250,000 peritoneal B-1 cells. In general, the total number of cells recovered from the peritoneal washouts of *lpr* mice did not differ systematically from what we obtained from normal mice, although there was a high degree of individual variability. The conventional B cells also decreased with age in *lpr* peritoneal populations, but to a much lesser degree (data not shown). Thus the apparent loss of B-1 cells in the *lpr* strain was not merely due to dilution by the expansion of other cell populations.

Table 6. *B-1 Cells Are Present at 3–5 mo after Transfer in Chimeras Using Normal Congenic Strains of Mice**

Chimera	No. mice	Serum Ig			
		IgM^a	IgM^b	IgG2a^a	IgG2a^b
			μg/ml		
Irradiation IV	2	**585**(15)‡	365(35)	**31**(19)	2,200(300)
Irradiation V	5	598(100)	**352**(47)	3,200(1,400)	**42**(17)
Neonatal VI	5	**490**(140)	486(106)	**0**	2,400(370)

* Refer to Table 1 for description of chimeras; data obtained from chimeras 3–5 mo after transfer. Data for B-1 cells are in bold.
‡ Mean (SE).

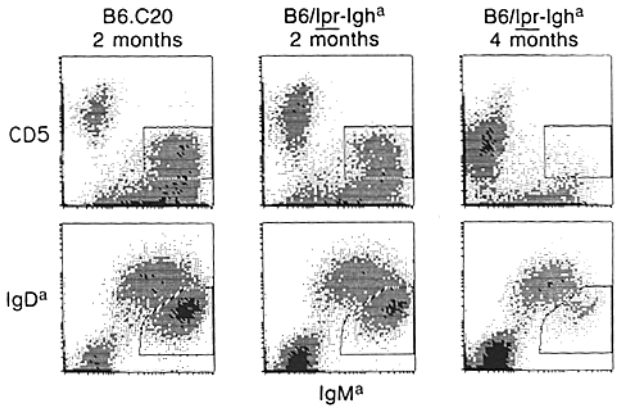

Figure 4. The *lpr* peritoneal B-1 cell population decreased with age. 2-mo-old B6.C20 and B6/*lpr*-Igh^a, and 4-mo-old B6/*lpr*-Igh^a peritoneal washouts were stained with appropriate reagents to delineate B-1 cell populations.

The lpr Peritoneal Environment Was Not Able to Support Development of Normal B-1 Cells. To investigate whether the loss of *lpr* B-1 cells was due to an intrinsic defect in the *lpr* B-1 cells, chimeras were created in which normal B-1 cells were transferred into an *lpr* environment. Equal numbers of B6/*lpr*-Igh^a and B6-Thy-1.1 bone marrow and peritoneal cells were transferred into irradiated (750 R) B6/*lpr*-Igh^a recipients (*irradiation chimera VII*, Table 1). No B-1 cells of the normal *b* allotype (B6-Thy-1.1) and few B-1 cells of the *lpr a* allotype (B6/*lpr*-Igh^a) were found in the peritoneal cavity 3 mo after transfer (Fig. 6). Five chimeras killed 1–3 mo after transfer averaged 0% *b* allotype normal B-1 cells, and 3% *a* allotype *lpr* B-1 cells.

Discussion

Our results demonstrate that B-1 cells were not responsible for producing autoantibodies to chromatin, ssDNA, and IgG in *lpr* mice. Two different methods were used to make B cell chimeras, such that B-1 and conventional B cell subsets were differentially marked by allotype. In all situations, we found that the conventional B cells were nearly entirely responsible for producing the autoantibodies measured. As autoantibody and Ig production by the conventional B cells increased with age, B-1 cell contribution to IgM decreased or remained constant. The occasional small contribution to RF from B-1 cells at two mo after transfer decreased further with time. B-1 cells in the peritoneal cavity were substantially depleted in all types of *lpr* chimeric mice 5 mo after transfer. Our data also showed a time-dependent loss of B-1 cells in unmanipulated *lpr* mice.

Certain evidence had already suggested that B-1 cells might not be involved in autoimmunity in *lpr* mice and related models. Monoreactive, high affinity autoantibodies, generally IgG, are produced by the conventional subset, and low affinity, generally IgM polyreactive autoantibodies come from the B-1 subset (36, 37). Autoantibodies characteristic of *lpr* systemic autoimmunity are in most cases IgG and undergo extensive somatic mutation and addition of N regions (38–40). Autoantibodies produced by the B-1 cells, in contrast, generally do not have somatic mutations or N regions (3, 15). Furthermore, irradiated *lpr* mice reconstituted with autologous marrow generate high titers of autoantibodies (21), despite the fact that B-1 cells are thought not to originate from adult marrow. The numbers of splenic B-1 cells in MRL/MpJ-*lpr*/*lpr* (MRL/*lpr*) mice were not found to be increased (8). In addition, autoantibodies (nonpathogenic) characteristic of the B-1 cell subset have previously been shown to decrease in unmanipulated MRL/*lpr* mice (41).

In contrast to these studies, a recent paper showed that transgenic mice carrying genes encoding antierythrocyte an-

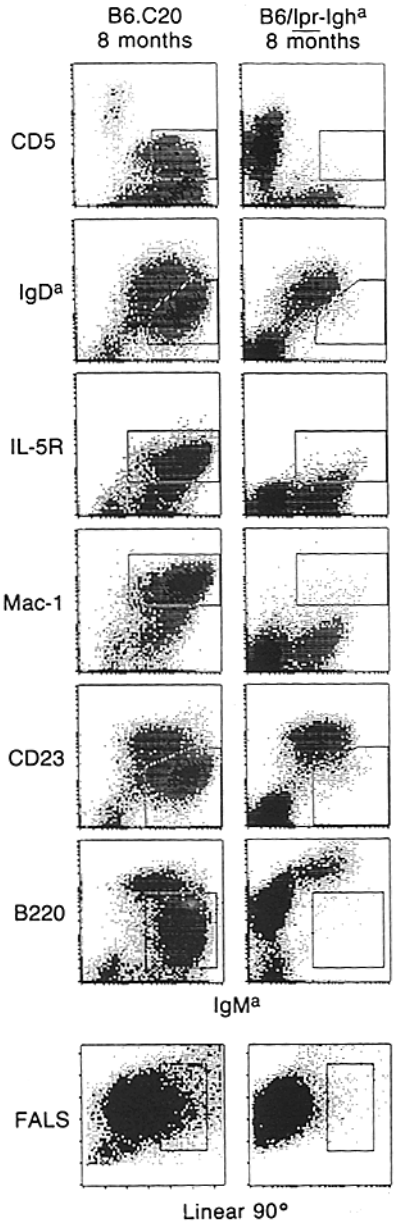

Figure 5. Failure to detect B-1 cells in old *lpr* mice. 8-mo-old control B6.C20 (*left*) and age-matched B6/*lpr*-Igh^a (*right*) peritoneal cells were stained for B-1 cells with mAbs specific for CD5, IgD^a, IL-5R, Mac-1, CD23, B220, and IgM^a. (*Bottom*) Linear 90° light scatter vs. forward angle light scatter (FALS). B-1 cells are outlined for each stain by the gates shown.

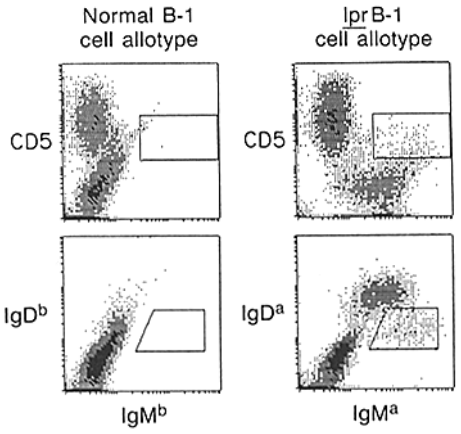

Figure 6. Normal B-1 cells, represented by *b* allotype, did not persist in peritoneal cavities of *lpr* mice. A representative chimera VII killed 3 mo after cell transfer is shown. The peritoneal cells were stained with reagents to identify B-1 cells, which are outlined in the gates shown for each stain. Refer to Table 1 for description of chimera.

tibodies had B-1 cells that apparently produced pathogenic autoantibodies (18). Although this model points to the direct involvement of B-1 cells in autoimmune disease, the transgenes encoded IgG anti-self antibodies that are not typical of B-1 cells. These autoantibody-producing B-1 cells may thus not exist naturally and may therefore represent disease-causing cells only in these special circumstances. Also, the model employed and the specificity of autoantibodies were different from those studied in the present report. In other experiments, a 10–100-fold increase in the B-1-related specificities anti-BrMRBC and anti-T cell was seen, again using a different technique and measuring different specificities than we employed in the present studies (42). An alternative possibility to implicate B-1 cells in the pathogenesis of disease was suggested by Shirai et al. (43). These investigators postulated that IgM B-1 cells in autoimmune NZB mice and related strains switch to pathogenic autoantibody producing cells and lose their CD5 antigen. This scenario, however, can be ruled out in our chimeras, since allotype marked cells were used.

The age-associated disappearance in *lpr* mice of peritoneal B-1 cells, seen in chimeras and in unmanipulated mice, is consistent with the lack of significant contribution to autoantibody production by this subset. On the other hand, in the irradiation chimeras (chimeras I and II) but not the neonatal chimera (chimera III), B-1 cells must have persisted somewhere, since their contribution to serum IgM was relatively stable. The failure of *lpr* B-1 cells to produce autoantibodies thus cannot be entirely a result of their disappearance from the peritoneum, but rather also implies a basic inability of this subset to participate in the *lpr* syndrome.

The loss of peritoneal B-1 cells could be due to several mechanisms. We did not find a massive increase in other peritoneal cell populations in *lpr* mice, so a trivial dilution effect cannot be the major explanation. Our experiments with the cotransfer of normal B-1 cells into an *lpr* environment point to an extrinsic influence rather than an intrinsic *lpr* B-1 cell defect. The *lpr* T cells could thus suppress the B-1 cells. Alternatively, the regulation of the B-1 cells could be under the control of the conventional *lpr* B cell. Recently, the disappearance of B-1 cells after treatment with anti-IL-10 in normal mice was shown to be due to increases in γ-interferon, since cotreatment with anti-γ-interferon prevented B-1 cell loss (44). We are investigating these as possible mechanisms for loss of B-1 cells in *lpr* mice, particularly since some autoimmune strains have increased levels of γ-interferon (45).

SJL is another mouse strain in which a deficit of B-1 cells correlates with autoimmunity. SJL mice do not produce anti-BrMRBC plaque-forming cells in vitro (46), and phenotypic B-1 cells represent only 1–5% of their peritoneal cells (47). Furthermore, the SJL strain is unusually susceptible to the induction of certain autoimmune diseases, such as experimental autoimmune encephalomyelitis (48).

It is tempting, therefore, to speculate that B-1 cells may play some role in maintaining homeostasis of the organism and that the loss of B-1 cells in *lpr* mice might in fact contribute to the autoimmune syndrome. Since B-1 cells have been deemed important in idiotypic networks and B cell ontogeny, and because they are increased in neonates (10, 49, 50), the disappearance of B-1 cells could disrupt the network and release autoreactive clones that are normally held in check by idiotypic-antiidiotypic interactions. It was also recently found that B-1 cells are important producers of IL-10, a cytokine that inhibits production of several T cell cytokines and monokines (51). Previously, it has been shown that certain B-1 cells produce a helper B cell factor, assigning an additional regulatory function to this subset (52). B-1 cells may thus participate in the immune response as active, regulatory cells (53–57), and may not merely represent a vestigial subset of a more primitive, first line defense. B-1 cells themselves, however, are not responsible for autoantibody production in *lpr* mice.

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Address correspondence to Dr. Robert A. Eisenberg, CB #7280, 932 FLOB, University of North Carolina, Chapel Hill, NC 27599–7280.

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