A Transgenic Model of Autoimmune Hemolytic Anemia

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Summary

We made double transgenic mice bearing immunoglobulin heavy and light chain genes encoding an autoantibody against the mouse erythrocyte by the cross of C57BL/6 mice carrying the transgene for each chain of the immunoglobulin. Although no obvious disorders were found in the single-chain transgenic mice, severely anemic symptoms were found in some of the double transgenic mice, in which most B cells express, at least on their surface, the autoantibody reactive to self-antigens on the erythrocyte. Individual double-transgenic mice showed a wide variation of phenotypes between severe anemia and no symptoms. Both deletion and anergy of autoreactive B cells were seen in each individual mouse, but their relative contribution to self-tolerance was variable and not directly related to the severity of anemia or the amount of the autoantibody produced. This transgenic system provides a good autoimmune disease model for exploring its onset mechanism, and means of its treatment and prevention.

Functional Ig genes are created by somatic DNA rearrangement of sub-exon DNA segments: V-D-J rearrangement for an H chain gene and V-J rearrangement for an L chain gene (1). Such a random genetic event on one hand contributes to amplification of limited germ-line repertoire of the Ig genes, but, on the other produces B cell antigen receptors that recognize and potentially react with self-components. Without proper selection mechanisms, the immune system could not serve as an effective defense mechanism for the organism. Autoimmune diseases are, in a way, unwelcome byproducts of the sophistication of our defense system.

Clonal anergy and deletion of self-reactive B cells are two major mechanisms that have been postulated for clonal selection of self-reactive B lymphocytes (i.e., self-tolerance). These mechanisms were demonstrated to be invoked for tolerance to self antigens in vivo using transgenic mouse models (2–4). Interestingly, however, the three transgenic models gave rise to apparently contrasting results. Goodnow et al. (2) established two lineages of transgenic mice. The first lineage has a transgene encoding an artificial self-antigen, hen egg lysozyme (HEL),[1] which is constitutively expressed under the control of the mouse metallothionein I promoter. The other line contains the Ig H and L chain genes encoding a high-affinity antibody specific for HEL (both IgM and IgD). Splenic B cells from mice of this lineage predominantly express transgene-derived IgM and/or IgD, the majority of which bind HEL. Double transgenic mice, derived by mating the two lineages, are indistinguishable from their littermates in the number and frequency of splenic B cells bearing the transgene-allotype–positive IgM or in the number of HEL-binding B cells. However, splenic B cells from the double-transgenic mice are functionally silent. Similar self-tolerance mechanism, i.e., anergy of B cells without clonal deletion, was found in a transgenic line expressing an IgM antibody specific to single-stranded DNA (3).

By contrast, Nemazee and Bürki (4) found that clonal deletion of autoreactive B cell precursors takes place in another transgenic model, in which the H-2^d homozygous mice carrying the Ig transgenes (both H and L chain genes) encoding an IgM antibody specific to the murine H-2^k MHC class I molecule are mated with H-2^k homozygous animals. These heterozygous animals (H-2^k × H-2^d) lack B cells bearing the anti–H-2^k idiotype and contain no anti–H-2^k antibody in the serum.

The contrasting observations in the three transgenic lines were explained by difference of the nature of the antigens used as tolerogens (5). Cell surface antigens like H-2^k molecules would facilitate extensive receptor crosslinking on developing B cells, resulting in clonal deletion. On the other

[1] *Abbreviations used in this paper:* AIHA, autoimmune hemolytic anemia; HEL, hen egg lysozyme; MFI, mean fluorescence intensity.

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hand, HEL and single-stranded DNA which are soluble and relatively low in concentrations would trigger anergic state of mature B cells. In addition to this discrepancy, the previous transgenic models for B cell tolerance lack an essential feature for understanding autoimmune diseases: breakage of tolerance for the antigens and resultant pathological symptoms.

We addressed this issue using transgenic mice that carry the Ig genes encoding an anti-murine erythrocyte autoantibody derived from the hybridoma 4C8 (6). As the expression of this autoantibody in animals causes autoimmune hemolytic anemia (AIHA), we can easily correlate the onset of the autoimmune disease with deregulation of relevant autoreactive B cells (6, 7). Interestingly, the individual transgenic mice showed variable phenotypes ranging from severe AIHA to complete tolerance. Furthermore, the majority of the transgenic mice showed partial tolerance ascribed to both deletion and anergy with variable relative contributions.

Materials and Methods

DNA Constructions. The H chain transgene was constructed using DNA fragments described as follows. The 1.3-kb XhoI-NcoI fragment containing the promoter region and the initiation site was isolated from pSV-Vμ1 (8). The 0.4-kb NcoI-EcoT14I fragment was isolated from cDNA encoding the H chain of the 4C8 mAb (9). The 1.8-kb EcoT14I-EcoRI fragment containing the J_H3, J_H4 and enhancer sequences, and the 12-kb EcoRI-XhoI fragment containing the major intron and the C_μ region derived from the genomic clones Ch·M·Ig·μ-18 (10) and λgtWES·IgH714 (11), respectively. The pSP72/73- (Promega Corp., Madison, WI) based plasmid containing the complete H chain gene (illustrated in Fig. 1 *A*) was designated pMO-μ4C8. For construction of the L chain transgene, the 5.6-kb BamHI fragment extending from the promoter to the C_κ region was recloned into Bluescribe13(−) Stratagene Inc., La Jolla, CA). This plasmid was designated pMO-κ4C8.

Generation of Transgenic Mice. To generate H chain transgenic mice, the 15.5-kb XhoI fragment of pMO-μ4C8 was prepared and microinjected into fertilized eggs of C57BL/6 mice, followed by transfer of viable eggs into the oviducts of the pseudopregnant ICR mice according to Hogan et al. (12). L chain transgenic mice were generated similarly using the 5.6-kb BamHI fragment of pMO-κ4C8 as described above. Animals were obtained from Japan SLC Inc. (Hamamatsu, Japan). The presence of the transgenes was detected by Southern blot analysis (13, 14) with the probes presented in Fig. 1. Unless otherwise stated, experimental data were generated using age-matched transgenic and wild-type control animals 5–8 wk of age.

RNase Protection Analysis. To generate the antisense probe for detection of microinjected H chain transgene-specific mRNA, the 485-bp HindIII fragment derived from pMO-μ4C8 was subcloned into pSP72 and designated pMO-$V_{H\text{-}4C8}$. As with L chain transgene, the 222-bp KpnI-RsaI fragment derived from pMO-κ4C8 was subcloned into pGEM-4 (Promega Corp.) and designated pMO-$V_{K\text{-}4C8}$. Total RNA was prepared by the procedure of Chirgwin et al. (15). Experimental procedure was performed as described (16).

Flow Cytometry. Single-cell suspensions from spleen, lymph node, bone marrow, thymus, or peritoneal cavity were deprived of erythrocytes by lysis in 0.83% NH_4Cl solution, stained with the appropriate antibodies, and analyzed by flow cytometry on FACScan® (Becton Dickinson & Co., Mountain View, CA) after exclusion of dead cells by propidium iodide gating. The percentage of positively stained cells was calculated on the basis of cutoffs set by control experiments. Indirect fluorescence staining was done as follows: (*a*) staining with unconjugated rat anti-mouse B220 (RA3-6B2) (17) and anti-mouse MAC-1 (M1/70.15.11.5.HL ATCC TIB 128) mAbs was followed by the addition of FITC-mouse anti-rat κ mAb (MARK1; Immunotech S.A., France); (*b*) staining with biotinylated goat anti-mouse IgM (Zymed Laboratories Inc., San Francisco, CA) and anti-Id mAb (S54) was followed by the addition of PE-streptavidin (Becton Dickinson & Co.); (*c*) staining with biotinylated anti-mouse IgM^a mAb (DS-1) (18) was followed by the addition of PE-streptavidin. Biotinylation was done using sulfosuccinimidyl-6-(biotinamido)-hexanoate (Pierce Chemical Co., Rockford, IL) according to Harlow and Lane (19). FITC-goat anti-mouse IgM (Cappel Laboratories, West Chester, PA) and FITC-anti-mouse IgM^b mAb (MB86) (20) were used for the direct staining procedure. Each staining was performed in much excess of mouse monoclonal IgG1 and IgG2b (Cappel Laboratories) to block Fc receptors on cells. To estimate autoantibodies on autologous erythrocytes, blood was collected by puncture of the retro-orbital venous plexus of the eye with a heparinized capillary tube and washed in PBS solution twice. Erythrocytes were stained with FITC-goat anti-mouse IgM (Cappel Laboratories) and analyzed on FACScan®.

In Vitro Antibody Secretion and ELISA System. Spleen cells were cultured in the medium described before (16) in the presence of 50 μg/ml LPS (*Salmonella minnesota*), 2 μg/ml Con A, or 10 μg/ml PHA (Sigma Chemical Co., St. Louis, MO) for 3 or 7 d in flat-bottomed microculture plates at 37°C in 5% humidified CO_2. Secreted antibodies in the medium of duplicate wells were measured by solid-phase ELISA using goat anti-mouse IgM (Cappel Laboratories) or anti-Id as the coating antibody and alkaliphosphatase-conjugated goat anti-mouse IgM (Tago, Inc., Burlingame, CA) as the second antibody according to Harlow and Lane (19).

Generation of Anti-Id mAb. Anti-Id mAbs to the 4C8 mAb were obtained as described previously (21) with slight modification. In brief, spleen cells from BALB/c mice immunized with the 4C8 mAb coupled to KLH were fused with myeloma X63Ag8.653 cells. Hybridoma supernatants were tested for the production of mAb to the 4C8 mAb and polyclonal IgM from NZW sera by ELISA. Three hybridomas secreting mAbs that reacted to the 4C8 mAb but did not react to polyclonal NZW IgM were selected. These mAbs were tested for staining of spleen cells from H3 mouse, L1 mouse, or the hybridoma 4C8 to identify a mAb that can recognize the idiotope formed by the combination of both the H and the L chains of the 4C8 mAb. Among these mAbs only the anti-Id mAb (S54) could react with the 4C8 hybridoma to the same extent as goat anti-mouse IgM (Cappel), but stained no more than 0.7% of spleen cells from H3 and L1 mice.

Results

Production of Transgenic Mice Containing the Ig Genes of the 4C8 mAb and Their Expression. The H and L chain genes represented in Fig. 1 were injected separately into C57BL/6 fertilized eggs for fear that expression of the antierythrocyte autoantibodies might cause severe AIHA, resulting in stillbirths. Seven lines of H chain and two lines of L chain transgenic mice were established. We extensively investigated, as representative lines, ARH3 (or H3) and ARL1 (or L1) of H and L chain transgenic mice, respectively. But all other lines of each transgene showed similar properties. Line H3

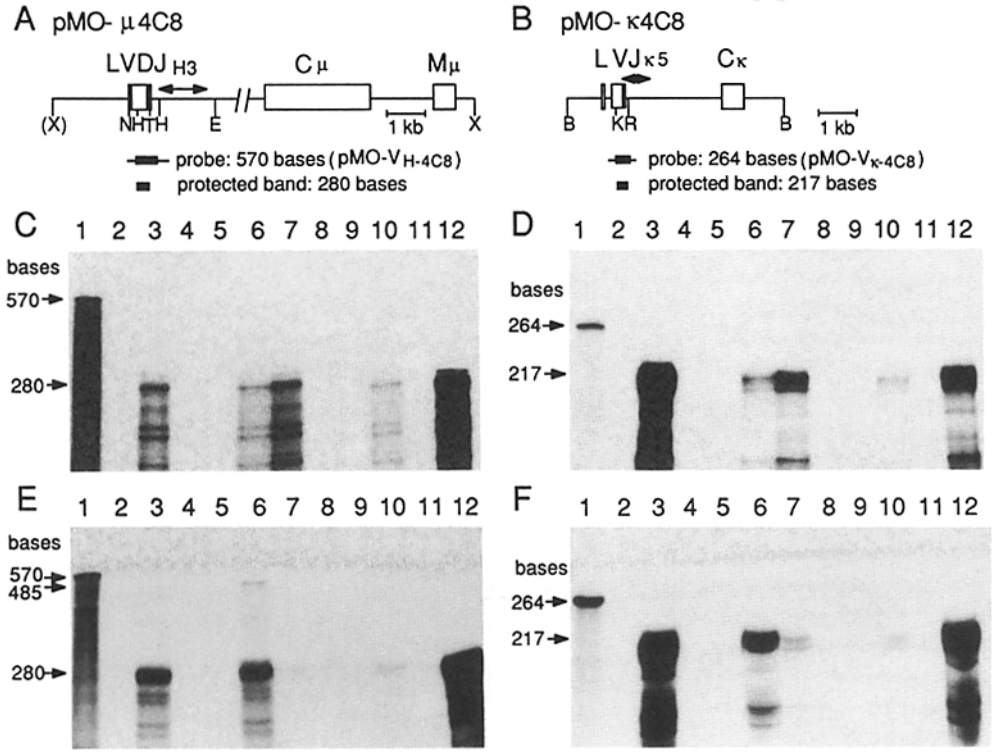

Figure 1. Structures of microinjected Ig genes and their expression. (*A*) 4C8 H chain gene. (*B*) 4C8 L chain gene. Coding regions and untranslated regions are represented by open boxes. Probes for RNase protection are shown below with expected protection bands. Arrows indicate regions of probes used for Southern blotting analyses. Relevant restriction sites are shown: B, BamHI; N, Nco I; E, EcoR I; T, EcoT14I; X, Xho I; K, Kpn I; H, Hind III; R, Rsa I. A site in a linker is shown with a bracket. RNase protection assay in (*C*) H3 mouse; (*D*) L1 mouse; and (*E* and *F*) H3L1 mouse. Each lane contained 2.5 μg of total RNA. The 485 base-band is due to unspliced mRNA. Origin of RNA: lane *1*, no RNA without digestion; lane *2*, brain; lane *3*, lymph node; lane *4*, heart; lane *5*, lung; lane *6*, thymus; lane *7*, spleen; lane *8*, liver; lane *9*, kidney; lane *10*, bone marrow; lane *11*, muscle; lane *12*, 4C8 hybridoma.

was bred from a female founder carrying about 10 copies of the H chain transgene at a single locus in an autosome, while line L1 was bred from a male founder bearing about twenty copies of the L chain transgene at a single locus in an autosome. These transgenes were stably inherited as long as we checked by Southern blotting analysis (data not shown).

RNase protection analyses were performed to test the tissue-specific expression of the transgenes. We detected transgene-specific expression by using riboprobes complementary to the V_H and V_L sequences (Fig. 1, *C* and *D*). As expected, both H and L transgenes were expressed only in lymphoid tissues such as lymph nodes, thymuses, spleens and bone marrows. Expression in the thymus was weaker than that in the spleen and lymph node in both transgenic mice. No detectable protected bands were detected in lymphoid RNA from normal C57BL/6 mice (data not shown). FACS® analysis clearly showed that no surface Ig were found on T cells (data not shown).

Both H and L Chain Genes of the Endogenous Loci Are Excluded. To determine whether expression of endogenous Ig H and L chain genes is excluded by the transgenes, we analyzed the spleen cells of H3 and L1 mice by FACS®. To stain the H chain we used anti-IgM antibody and antiallotypic mAbs (DS-1 for IgM^a and MB86 for IgM^b). IgM^+, IgM^{a+} (transgenic), and IgM^{b+} (endogenous) B cells were 39, 38, and 0.9%, respectively, of the total spleen cells derived from an H3 mouse (data not shown). This means that only about 2.5% of B cells express endogenous H chain genes, indicating that the endogenous H chain genes are almost completely excluded by expression of the H chain transgene.

As the allotypic specificity for the L chain is not known, we cannot estimate the degree of allelic exclusion in L1 mice.

However, strong allelic exclusion appeared to be accomplished for the endogenous H and L chain genes since almost all surface IgM on spleen B cells of H3 × L1 double-transgenic (H3L1) mice expressed the transgene Id, which is formed by the combination of the H and L chains of the 4C8 mAb and recognized by the S54 mAb (Fig. 2 *A*). Very few spleen

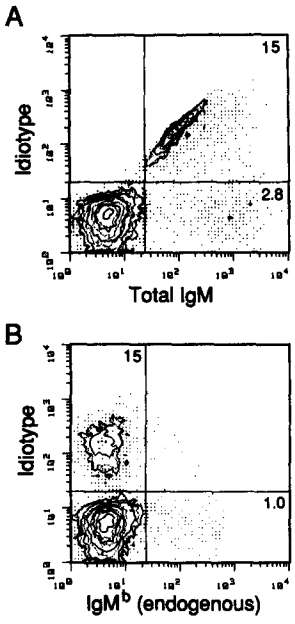

Figure 2. Allelic exclusion in H3L1 mouse. Spleen cells were stained doubly with either (*A*) anti-IgM or (*B*) MB86 mAb, and anti-Id mAb (S54). Contour maps show correlated fluorescence in arbitrary relative fluorescence units. Percentages of positively stained cells were indicated in each compartment.

B cells expressed endogenous allotype-specific IgM which did not carry the idiotype of the transgenes (Fig. 2 *B*). Serum IgM of normal C57BL/6 mice was 2,400 ± 853 (μg/ml) (mean ± SD of 10 mice), whereas that of H3L1 mice was 143 ± 71 (μg/ml) (mean ± SD of 18 mice) by ELISA in agreement with strong allelic exclusion.

AIHA in H3L1 Mice. We examined whether H3L1 mice which should carry B cells reactive to autologous erythrocytes suffer from AIHA. Items of (heterozygous H3 × L1)F_1 428 mice examined were as follows: H3L1, 91 mice; H3, 119 mice; L1, 101 mice; nontransgenic, 117 mice. This distribution excludes the possibility that we analyzed a selected population of H3L1 mice that had escaped from stillbirths due to overexpression of the autoantibodies. Expression of the transgenes in the H3L1 mice was limited to lymphoid organs as described for the single-chain–transgenic mice, but the relative pattern of expression was different (Fig. 1, *E* and *F*). Expression of the transgenes in the spleen and bone marrow was greatly reduced probably because of clonal deletion and downregulation of surface IgM expression of autoreactive B cells as described below.

We quantitated the amount of the autoantibody in H3L1 mice, and found little free form in serum but considerable amounts associated with erythrocytes. We measured the hematocrit values and the amounts of the autoantibody bound to erythrocytes by surface staining of erythrocytes with anti-IgM antibodies. To our surprise, wide variations of the hematocrit values and autoantibody amounts existed among H3L1 individuals (Fig. 3 *A*). The mean fluorescence intensity (MFI), which is proportional to the amounts of the autoantibodies associated with erythrocytes, spread in almost two-log range. Reverse correlation of the hematocrit values with MFI indicates that the total amount of the autoantibody produced directly correlates with AIHA. The result also implies that IgM associated with erythrocytes retains its original pathogenicity as well as bindability, and argues against the hypothesis that a large amount of IgM exists because these molecules have lost their pathogenicity probably by somatic mutation. In fact, some of these mice died of typical AIHA. We analyzed a number of H3 or L1 mice and did not find any signs of AIHA. Their hematocrit values were around 45% at the age of 5–8 wk, and no autoantibodies were detected throughout their life in agreement with requirement of both chains of the antierythrocyte antibody for binding to erythrocytes.

Based on the hematocrit values, we classified the H3L1 transgenic mice into the three groups: the anemic type with hematocrit <30%; the tolerant type with hematocrit >40%; and the intermediate type with the intermediate hematocrit. Items of 79 mice analyzed were the anemic type, 5% (four mice); the intermediate type, 44% (35 mice); and the tolerant type, 51% (40 mice). Their typical staining patterns are shown in Fig. 3 *C*. The pedigree of H3L1 mice did not show any genetic or sexual bias among the three types (Fig. 4). Our preliminary data show that even a single mouse often changed the tolerance state, giving rise to an acute onset of AIHA from the state of complete tolerance, and vice versa (data not shown). The results clearly indicate that the efficiency of tolerance to the antierythrocyte autoantibody is variable among individuals in spite of the same genetic background.

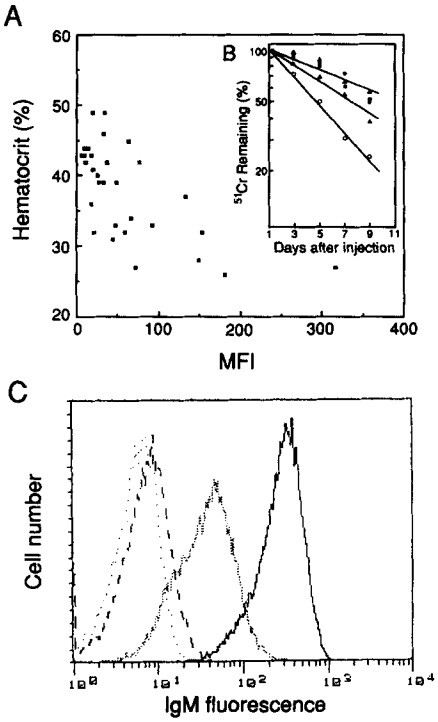

Figure 3. Various phenotypes of H3L1 mice. (*A*) Reverse correlation of hematocrit values with amounts of autoantibodies on erythrocytes. 32 H3L1 mice were analyzed by quantitating hematocrit values (*vertical axis*) and MFI of FITC–anti-IgM antibodies bound to erythrocytes (*horizontal axis*). Each square represents one mouse. (*B*) Survival of ^{51}Cr-labeled syngeneic erythrocytes in H3L1 mice. Erythrocyte survival study was done according to Cox et al. (22). The first sample was collected 24 h after injection and this value was taken as 100%. Subsequently, samples were collected at a 2-d interval. Closed circles, triangles, and squares represent normal C57BL/6, H3, and L1 mice, respectively. Open circles and triangles show the anemic and the intermediate types mice, respectively. (*C*) Erythrocytes were stained and analyzed as described in experimental procedures: solid line, the anemic type; fine stippled line, the intermediate type; broken line, the tolerant type; rough stippled line, normal C57BL/6.

The half-life of ^{51}Cr-labeled erythrocytes from the anemic type mice was half of those from the H3, L1, and normal C57BL/6 mice (Fig. 3 *B*). In addition, histological analysis

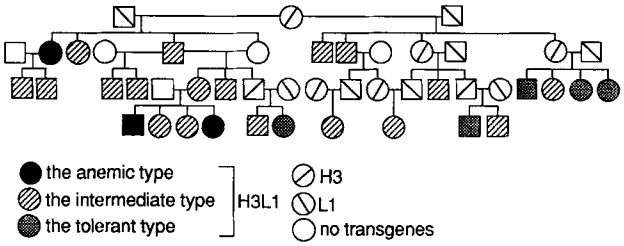

Figure 4. A pedigree of H3L1 mice. Four generations of H3L1 mice were shown in a partial pedigree. The squares and circles represent males and females, respectively. All the H3L1 mice were described. Littermates other than H3L1 were included only when they were used for subsequent breeding. Genotypes and phenotypes of mice are as indicated.

of the anemic mice showed an enormous accumulation of agglutinated erythrocytes in the entire spleen as described previously (7). The half-life of erythrocytes from the intermediate type mice was slightly shortened. The results indicate that the anemic phenotype is not due to fluctuation of the hematopoietic capacity of individual animals but rather to reduction of the half-life of erythrocytes, which is caused by the increase of the autoantibody. These results also indicate that destruction of erythrocytes is due to the autoantibodies bound to erythrocytes and that this severe anemia comes from AIHA.

Individual H3L1 Mice Showed Both Deletion and Anergy of Autoreactive B Cells. To investigate how autoreactive B cells are regulated, the numbers of pre-B and B cells in the spleen, lymph node, and bone marrow of each H3L1 mouse were

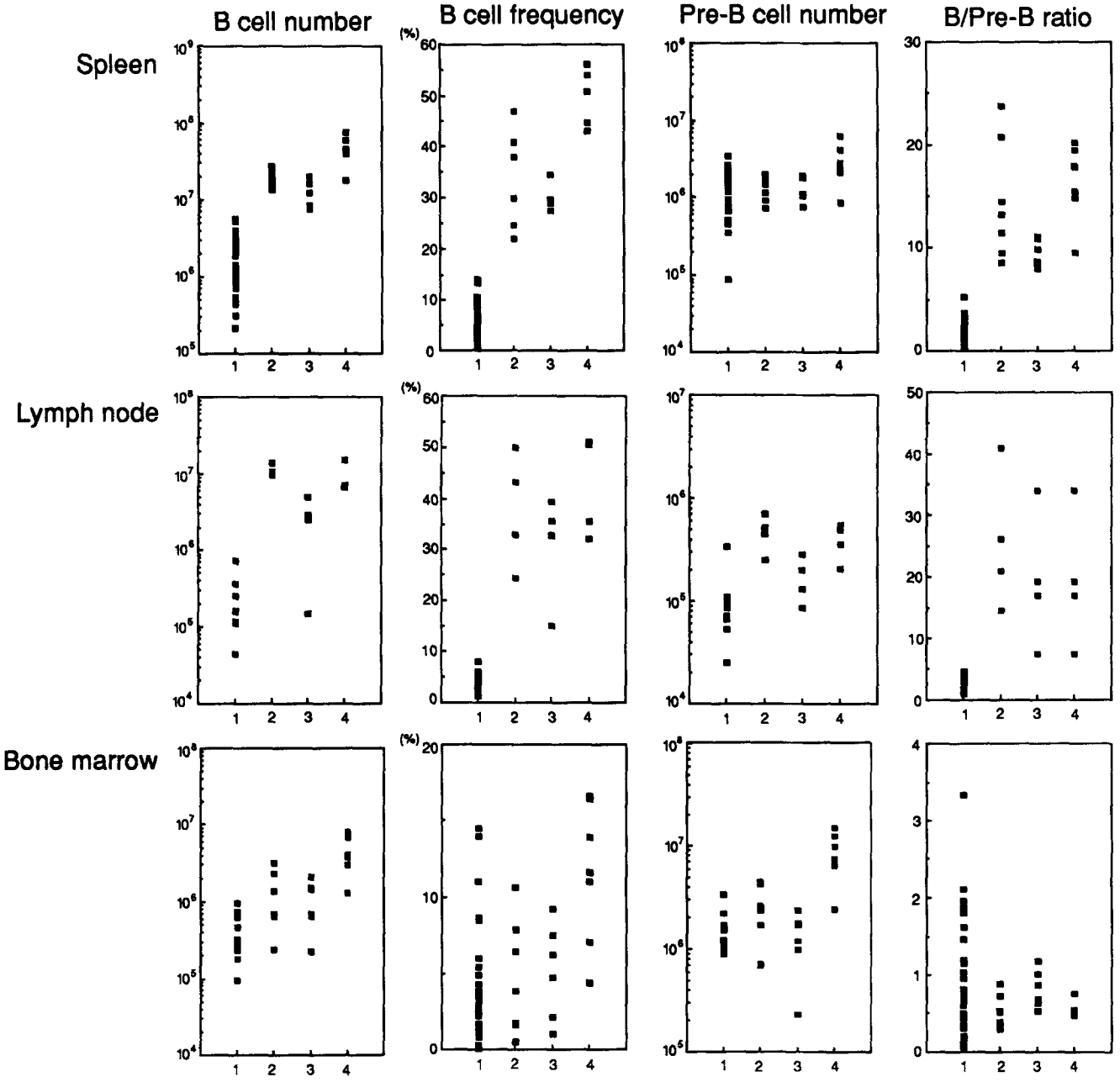

Figure 5. Deletion of autoreactive B cells in H3L1 mice. Spleen, lymph node, and bone marrow cell suspensions of H3L1, H3, L1, and normal C57BL/6 mice were stained and analyzed by two color FACS® with anti-B220 (RA3-6B2), and either one of S54 and anti-IgM antibodies. Numbers of B, Id^+ B or pre-B cells were calculated by (% positive cells) × (cells/organ). Id^+ B cell number was shown in H3L1 mice. Pre-B cells were detected as $B220^+$ cells without surface IgM and IgG. Total bone marrow cell suspensions were obtained from two femurs and total lymph node cell suspensions were obtained from the inguinal, axillary, and submandibular regions for all mice. Each square represents one individual. Column *1*, H3L1 mice; column *2*, H3 mice; column *3*, L1 mice; column *4*, normal C57BL/6 mice.

estimated and compared with those of H3, L1, and normal C57BL/6 mice. In the spleen and lymph nodes of H3L1 mice, Id^+ B cells decreased generally and ratios of B cells to pre-B cells were also greatly reduced regardless of the hematocrit values as compared with H3 or L1, as well as normal mice (Fig. 5). This indicates that autoreactive B cells are deleted through recognition of the self-antigen but not through simple expression of the rearranged Ig transgenes. In fact, the numbers of pre-B cells in spleens and lymph nodes of H3L1 mice are comparable with those of H3, L1, and normal mice. The majority of the remaining B cells were not stained with the anti-MAC-1 mAb, indicating that they are not Ly-1 B cells (23). It should be noted, however, that the degree of deletion is very different among individuals. The ratios of the number of B cells of the H3L1 mice to that of H3 or L1 mice varied between 0.01 and 1.0. On the other hand, in the bone marrow we cannot find any apparent differences in either the number of B cells or the ratio of B to pre-B cells between H3L1 mice and the other control mice. Fig. 6 shows the representative staining patterns of B cells in the spleen and bone marrow with the anti-IgM, anti-Id, and anti-B220 antibodies. In H3L1 mice the staining patterns with anti-IgM antibody were almost identical to those with the anti-Id mAb (S54). It should be noted that B cells in H3L1 mice are confined to the cells expressing low levels of surface antigen receptors. This suggests that autoreactive B cells with high levels of surface antigen receptors are preferentially deleted. Resultant B cells with the low density of surface antigen receptors are often observed in an anergic state (2, 24, 25).

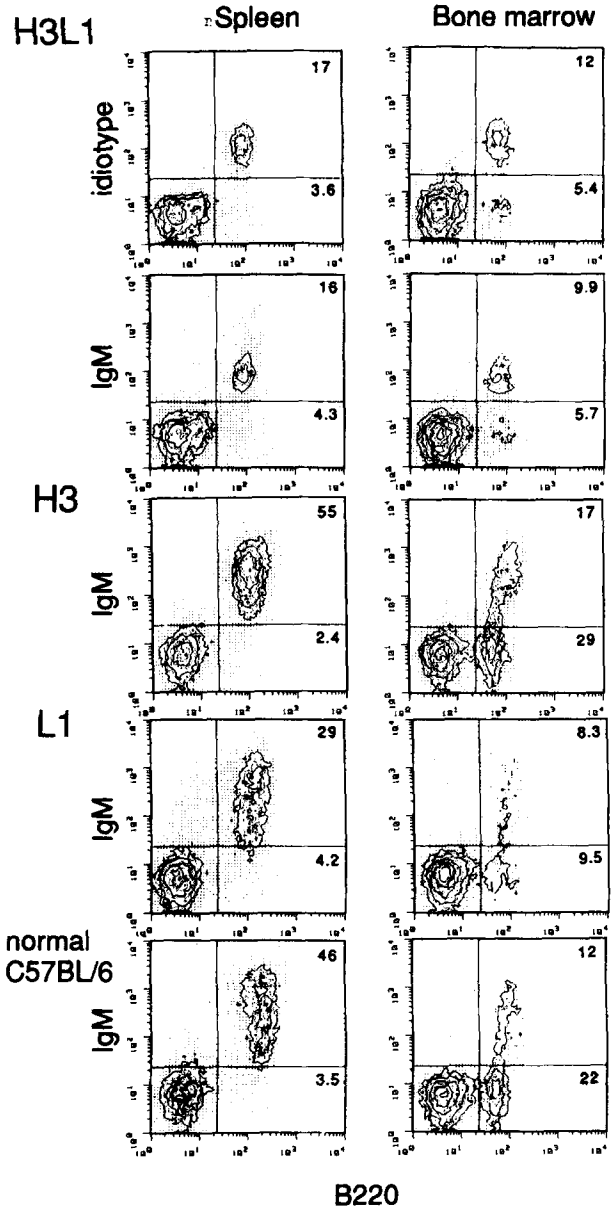

Figure 6. Two-color FACS® analyses of transgenic and normal mice. Spleen and bone marrow cell suspensions were stained and analyzed as described in Fig. 5. Contour maps are constructed as Fig. 2. The percentage of positively stained cells was indicated in each compartment.

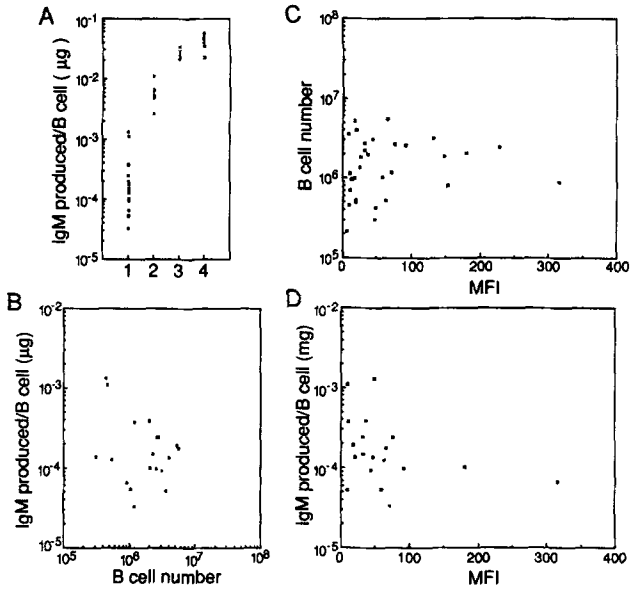

Figure 7. Poor correlation among deletion, anergy, and autoantibody amounts. Each square represents one individual. (*A*) Reduced LPS-response of autoreactive B cells of H3L1 mice. Total spleen cells were cultured in vitro in the presence of 50 μg/ml LPS at three different cell densities: 10^5/ml, 10^6/ml, and 10^7/ml for 3 or 7 d. The highest Ig production by spleen cells of normal, H3, L1, and H3L1 mice were found in cultures containing 10^5, 10^5, 10^5, and 10^6 cells/ml, respectively, for 7 d. Amount of secreted IgM in the supernatants of LPS-induced in vitro cultures was divided by the number of B cells in the culture which gave the most efficient Ig production. In the case of H3L1 mice Id^+ IgM/Id^+ B cell and in other cases total IgM/total B cell are shown. Column 1, H3L1 mice; column 2, H3 mice; column 3, L1 mice; column 4, normal C57BL/6 mice. (*B*) No correlation between the extent of deletion and anergy of autoreactive B cells in H3L1 mice. LPS-induced Id^+ IgM per Id^+ B cell was plotted against remaining Id^+ B cell number in the spleen. (*C* and *D*) Amount of the autoantibody in H3L1 mice is not directly related with either B cell number, LPS-responsive capacity of spleen cells. Id^+ B cell number (*C*) and LPS-induced Id^+ IgM per Id^+ B cell (*D*) were plotted against MFI of FITC-anti-IgM bound to autoantibodies associated with erythrocytes.

To confirm the anergic state of the remaining B cells, we investigated whether B cells in the spleen can be activated to produce the autoantibody by LPS stimulation in vitro (26–28). Total spleen cells were used because it was very difficult to purify B cells enough for the culture from an individual mouse. Secreted total IgM and Id^+ IgM in the supernatant were quantitated by ELISA. The IgM production value per B cell was calculated as an index of the reactivity to LPS (Fig. 7 *A*) and these values of H3L1 mice were reduced on the average to less than one-tenth of those of H3 or L1 mice. The results indicate that remaining splenic B cells of H3L1 mice are anergized. By contrast, the same set of cells cultured with Con A (2 μg/ml) or PHA (10 μg/ml) did not produce any significant amounts of the autoantibody as compared with normal C57BL/6 mice (data not shown). The numbers of total T cells, $CD4^+$ T cells or $CD8^+$ T cells did not correlate to the amounts of the autoantibody (data not shown). In addition, administration of LPS in vivo rendered tolerant mice severely anemic (our unpublished data). These results demonstrate that T cells do not secrete the autoantibody enough to give rise to AIHA.

Finally, we could not find any correlation between the extent of clonal deletion and the degree of B cell anergy in each individual H3L1 mouse (Fig. 7 *B*). Taken together, these results show that both deletion and anergy are invoked to get a tolerant state of potentially autoreactive B cells in H3L1 mice, but the relative contribution of the two mechanisms differs greatly in each individual. We failed to find a strong correlation between the amount of the autoantibody in blood and the degree of tolerance as assessed by clonal deletion and anergy of potentially autoreactive B cells in the spleen (Fig. 7, *C* and *D*).

Escape of Ly-1 B Cells from Clonal Deletion. We investigated whether Ly-1 B cells expressing the transgenes are rendered tolerant because they are considered distinct B lineage cells from the conventional B cell (29). We recovered peritoneal cells in which Ly-1 B cells are rather enriched and enumerated Ly-1 B cells by surface staining with both anti-IgM (or anti-Id) and anti-MAC-1 antibodies (23). The number of Id^+ Ly-1 B cells was almost equal to that of IgM^+ Ly-1 B cells in H3L1 mice (data not shown). The percentage and number of the Ly-1 B cells in H3L1 mice were not reduced as compared with those in H3, L1, and normal C57BL/6 mice. In contrast, conventional B cells in the peritoneal cavity were deleted as those in other peripheral lymphoid organs, resulting in relative enrichment of Ly-1 B cells (Table 1). We could not find a significant correlation of the amount of the autoantibody in blood in H3L1 mice with the number of Ly-1 B cells (data not shown).

Discussion

Previous transgenic models demonstrated that deletion and anergy of autoreactive B cells were actually invoked in vivo for the mechanism of B cell tolerance (2–4). However, apparently either of the two mechanisms played a predominant role in each model system: anergy for the anti-HEL (2) and anti-single-stranded DNA (3) antibodies; and deletion for the anti-MHC class I antibody (4). In contrast, both deletion and anergy were invoked in the same individual of our transgenic mouse system, and furthermore, their relative contribution varied among the individuals with the same genetic background. These findings indicate that these mechanisms are not mutually exclusive even in an individual mouse for the combination of the same antigen and antibody.

Why do tolerance mechanisms between previous transgenic systems and ours differ? Different antigen properties may be one possible cause. Two previous contrasting results, i.e., clonal deletion versus anergy, were explained by different concentrations of self-antigens or by different forms, i.e., soluble versus membrane-bound (5). The high concentration of allotypic H-2 class I antigen expressed on the cell surface as

Table 1. *Ly-1 B Cells in the Peritoneal Cavity Escape from Deletion*

Mice	Percent Ly-1B	Percent conventional B	Ly-1B/conventional B
H3L1	33 ± 4.8	6 ± 2.3	5.5
	(5.3 ± 1.3)	(1.2 ± 0.6)	
H3	7 ± 1.3	12 ± 0.8	0.6
	(1.6 ± 0.5)	(2.9 ± 0.7)	
L1	21 ± 4.5	15 ± 3.1	1.4
	(7.3 ± 0.2)	(5.2 ± 0.1)	
Normal	24 ± 4.3	23 ± 4.1	1.1
C57BL/6	(3.9 ± 0.5)	(3.7 ± 0.2)	

Peritoneal cells were stained and analyzed by two-color FACS® with anti-Id (S54) or anti-IgM and anti-MAC-1 antibodies to discriminate Ly-1 B cells from conventional B cells. Actual cell numbers × 10^{-5} of each category are also shown in parentheses. Cell number was calculated as described in the legend to Fig. 5. Id^+ B cell numbers are shown in H3L1 mice. Results are expressed as the mean ± SD of eight mice.

a multivalent antigen may lead to clonal deletion, whereas the relatively low concentrations of HEL and single-stranded DNA in a soluble form may cause clonal anergy. The concentration of the antigen recognized by the 4C8 mAb on an erythrocyte in our system can be estimated roughly from the amount of the autoantibodies bound on erythrocytes in the severely anemic H3L1 mice (Fig. 3 *C*). This concentration does not seem less than that of the H-2 antigen on cells, suggesting that the concentration of the 4C8-recognized antigen may be high enough to cause deletion. It is hard, however, to assess the effective or average concentration of the 4C8-recognized antigen and compare it with that of the H-2 class I antigen because expression of the 4C8-recognized antigen is rather restricted to the erythroid lineage whereas that of the class I antigen is ubiquitous.

Another aspect of the antigen expression that may trigger different mechanisms of self-tolerance is the onset of antigen expression during embryogenesis or timing of contact between antigens and B lymphocytes. It is possible that B cells that had interacted with self-antigens in early stages of differentiation tend to be eliminated by clonal deletion, whereas relatively mature B cells may be anergized by the same self-antigens (24). Although no definite characterization of the 4C8-recognized antigen is known, it is hard to believe that its expression, i.e., appearance of erythrocytes or their progenitors, is much later than that of B lymphocytes, and such delay would allow B cells to escape tolerance. The timing and/or effective concentration of the antigen to encounter an individual B lymphocyte might vary in our transgenic system because of the restricted expression of the antigen, and the compartmental development of B lymphocytes and erythrocytes in the bone marrow.

In contrast to the previous model systems, our transgenic model showed breakage of tolerance and wide variation of the phenotypes among the individuals with the same genetic background. Some of the H3L1 transgenic mice actually came down with severe AIHA. This is the first case of an autoimmune disease in the transgenic animals carrying the Ig genes encoding the autoantibody. One of the reasons why we have been able to observe such breakage of tolerance might be that we had chosen an appropriate autoantibody that has a strong pathogenicity (6, 7). In fact, no pathogenic symptoms were found even through significant amounts of the anti-HEL antibody were found in blood in the double-transgenic mice of Goodnow et al. (2). The autoimmune symptoms correlate well with the amount of autoantibody bound to the autologous erythrocytes. This indicates that most of the autoantibody produced is actually pathogenic and not mutated to escape from tolerance. It is important to stress that our transgenic model system offers a more reasonable model for clinical autoimmune disease in variation among individuals, fluctuation during life (suggesting participation of environmental factors), and typical pathological symptoms. We will be able to assess the screening method for agents that treat or prevent AIHA and general autoimmune diseases using our model mouse.

It is not likely that all B cells produce small amounts of the autoantibody in our transgenic mice because the total number of B cells in the spleen or peritoneal cavity is not related to the amounts of the autoantibody associated with erythrocytes, although remaining B cells are anergic with downregulation of surface IgM. It is more likely that only a small fraction of B or plasma cells that escaped from the tolerance mechanism produced a relatively large amount of the autoantibody. It is interesting that the numbers of the Ly-1 B cells in the peritoneal cavity were not reduced in the H3L1 mice. This finding suggests that the Ly-1 B cells might have left the bone marrow rather early and developed separately from the conventional B cells. Ly-1 B cells may not have seen erythrocytes until they became mature B cells. Alternatively, Ly-1 B cells might have proliferated extensively after selection. Since the presence of Ly-1 B cells in the peritoneal cavity alone does not cause the autoimmune symptoms, not all Ly-1 B cells appear to produce the autoantibody. Most of them are likely to be anergized and only a fraction of them may produce the autoantibody.

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