Natural Autoantibodies to Thymocytes: Origin, \( V_H \) Genes, Fine Specificities, and the Role of Thy-1 Glycoprotein

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Summary

15 SM/J mouse hybridoma antibodies that show antithymocyte autoantibody (ATA) activity by immunofluorescence staining were studied. Half of these antibodies react with determinants whose expression is associated with Thy-1, as shown by blocking experiments with anti-Thy-1 and loss of reactivity with Thy-1\(^-\) mutant cell lines. The Thy-1 dependence of three of these ATA is further confirmed by their reexpression on a Thy-1 gene transfectant. However, the remaining antibodies exhibited binding that showed little or no dependence on Thy-1. Furthermore, we find that most ATA derives from the Ly-1 B subpopulation, as demonstrated by lipopolysaccharide-induced ATA secretion in vitro and by comparison of ATA hybridoma frequencies. \( V_n \) region gene sequence data of 14 monoclonal ATA from Ly-1 B cell-derived hybridomas reveal the utilization of nine \( V_n \) genes belonging to four different \( V_H \) families (J558, 3609, Q52, and Vgam3.8). While we find that two of these hybridomas arose from a clonal expansion, we also find four examples of a 3609 family \( V_n \) gene utilized in clonally independent lines showing similar specificity. Yet another example of identical \( V_n \) gene usage by clonally unrelated cells is found in two J558 ATA of a distinct fine specificity. These data suggest that the enrichment of ATA B cells in the Ly-1 B subset is primarily due to repeated independent recruitment of B cells by antigen resulting in the expression of a restricted set of \( V_n \) genes.

The existence of spontaneously occurring autoantibodies has been recognized for many years (1-7). Since the serum levels of these antibodies do not show etiologic relevance to disease (8), the pathologic significance of such “natural” autoantibodies is not clear. One of these natural autoantibodies, antibody to a cryptic determinant on erythrocytes (anti-bromelain-treated mouse RBC [anti-BrMRBC])\(^1\), is largely contributed by a unique B cell subset, Ly-1 B (9, 10). Ly-1 B comprises a minor (1-2%) subset in the normal secondary lymphoid organ (spleen) or PBL, whereas it is uniquely enriched in mouse peritoneal cavity (PerC) (11-13). Ly-1 (CD5) expression has also been found on late-developing IgM\(^+\) B lymphomas (14). From our previous data with cell transfer experiments, we suggest that Ly-1 B constitutes a distinct B cell lineage; most Ly-1 B cells present in adult mice are the progeny of cells generated from an early B cell source (fetal liver) (15), are maintained by self-renewal, and poses the potential for disregulated growth later in life (13, 14, 16-19).

Although the biological role of Ly-1 B in the immune system is not clearly understood, this subset appears to be phylogenically conserved since it is found in both mice and humans (13, 20). One fundamental question relating to the biological function of Ly-1 B is the extent of its contribution to autoantibody secretion and the mechanism to allow such expression. Although enrichment of autoantibody specificities in this B cell subset has been suggested previously (11, 13), explicit determination of the antibody specificities (and \( V \) genes) characterizing Ly-1 B would provide help in answering these questions. In murine Ly-1 B, the anti-BrMRBC specificity encoded by \( V_n \)11 (together with \( V_{19} \)) provided the first example demonstrating such bias (9, 10, 21). These \( V \) genes have also been found recurrently (in an unmutated form) in the B lymphomas mentioned above (19).

We report here studies on the cellular origin, specificity, and primary structure of a group of natural antithymocyte autoantibodies (ATA). ATA secretion occurs spontaneously in normal animals, and these antibodies bind to intact thymocytes (3, 4, 7, 8). Although high levels of ATA have been found in animals with autoimmune disease (e.g., NZB mice) (4), they have also been detected at high levels in some strains.

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\(^1\)Abbreviations used in this paper: APC, allophycocyanin; ATA, antithymocyte autoantibody; BrMRBC, bromelain-treated mouse RBC; PerC, peritoneal cavity.
Materials and Methods

Materials. SM/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). BALB/cAnN, AL/N and AKR/J mice, and P384N rats were bred and maintained in the Institute for Cancer Research animal facility. 2-4-mo-old female animals were used in all experiments.

Immunofluorescence Staining and Sorting. Preparation of cells, reagents for immunofluorescence staining, cell sorting using FACS (FACStar plus; Becton Dickinson Immunocytometry Systems, Mountain View, CA), and data analysis were all described previously (10, 31).

Induction of ATA Secretion In Vitro. Spleen cells from SM/J and BALB/c mice and cells from the BALB/c PerC were stained with fluorescein-anti-IgM (331.12) and allophycocyanin (APC)-anti-Ly-1 (53-7). 2-4-mo-old SM/J mice and BALB/c mice consistently show 4-7% and 1-2% Ly-1 IgM+ cells in spleen, respectively (10, 11, 13). Ly-1 B cells represent 10-30% of total cells in BALB/c PerC (13). Ly-1- B cells are identified as Ly-1 IgM+ cells in spleen. Sorting of spleen cells lacking Ly-1 B (Ly-1 B depleted) or Ly-1 B (Ly-1 B depleted) was carried out by using the FACS sort gates set to cancel the selected population. All cell cultures were initiated at 10^6 cells/ml in RPMI 1640 + 10% FCS + 5 μM 2-ME with or without LPS (10 μg/ml).

Production, Screening, and Cloning of ATA Hybridomas. ATA hybridomas were produced and selected at the same time as the anti-BRMBRC hybridomas reported previously (10). Briefly, after staining of spleen cells pooled from five SM/J mice, Ly-1 B and Ly-1- (IgG+* ) B cells were isolated by sorting, cultured for 2 d in the presence of LPS, and then hybridized with a HAT-sensitive MPC11 myeloma cell line (γ2b,κ). Six 96-well plates (estimated three to four clones/well) (10) from the Ly-1 B hybridization were all positive for IgM secretion, and their supernatants were screened for ATA activity by immunofluorescence staining on SM/J mouse thymocytes, as revealed by APC-anti-IgM (331.12). Simultaneously, these supernatants were tested for anti-BRMBRC, anti-mouse RBC (MRBC), anti-splenic B cell, and anti-bone marrow cell activities by staining and for anti-siDNA and anti-mouse IgG activities by an ELISA. Most ATA-positive wells did not show cross-reactivity in these assays, with the exception of one well (showing binding to siDNA, cloned as 1-6D7). Ly-1- B cell hybridomas were screened only for ATA activity. ATA-positive wells were cloned by cell sorter immediately after the screening.

Analysis of Monoclonal ATA Reactivity to Various Cell Types. Hybridomas were maintained in Opti-MEM (Gibco Laboratories, Grand Island, NY) + 4% FCS + 2-ME. Ascites was produced in CB17 scid mice, and 25 μl of 1/100 diluted ascites for 5 x 10^7 cells was used for immunofluorescence staining (revealed by APC-anti-IgM; 331.12). For thymocyte, splenic T cell and bone marrow staining, BALB/c mice (which show comparable staining with SM/J mice) were normally used, except for 1-IG10 (negative with BALB/c, where AKR mice were used. For splenic non-T cell-staining, AL/N mice (negative with 331.12) were used to avoid B cell staining by second-step anti-IgM antibody. AL/N thymocytes were stained by all ATA used here.

Anti-Thy-1 Blocking. To examine the blocking of ATA staining by anti-Thy-1 antibody, 10 μg of rat anti-Thy-1.2 (30-H12) or rat anti-Thy-1.1 (T11D7e2; provided by Dr. M. Dailey, University of Iowa) antibody was used simultaneously with limiting dilutions (1/100-1/3,000) of ATA for BALB/c and AKR thymocyte staining, respectively. Anti-CD45 (30F11) and anti-CD5 (53-7) antibodies were used as negative controls.

Sodium m-Periodate Treatment. Thymocytes were fixed with 3% (wt/vol) paraformaldehyde in PBS, washed, and treated with or without 0.08 M sodium m-periodate in 0.05 M sodium acetate buffer, pH 5.5, at 37°C for 2 h, as described elsewhere (32, 33).

T Lymphoma Cell Lines and Their Mutants. BW5147 (Thy-1- a) and BW5147 (Thy-1- e) are Thy-1- mutant cell lines derived from BW5147 (Thy-1+) wildtype (34, 35). Both class A and E mutants are defective in the anchoring of membrane Thy-1 glycoproteins via the phosphatidylinositol-containing glycolipid anchor of Thy-1 (36), and the class E mutant has a mutation in the gene coding for GDP-mannose: dolichol-P-mannosyltransferase, which presumably affects the biosynthesis of glycolipid tails in addition to the biosynthesis of N-linked high-mannose oligosaccharides (36, 37). The AKR1 (Thy-1- d) cell line is a class D mutant from AKR1 (Thy-1+) wildtype. This mutant has a deletion of the 5' portion of the Thy-1 gene including the first two alternatively used exons and promoters and a portion of the second intron (38). TFX351 is a Thy-1.1+ transfected of AKR1 (Thy-1- d). An 8.1-kb EcoRI genomic fragment containing the Thy-1.1 gene (39) was ligated into the vector pSV2-gp (40) in an inverse orientation to the SV40 enhancer. This construct was introduced into AKR1 (Thy-1- d) by electroporation.

Sequence Determinations of IgH Variable Regions of RNA. Poly-(A)+ mRNA was isolated from hybridomas and sequenced by μ primer extension, as described previously (10), in most cases. The 3609 sequences showed several strong stops in sequencing by primer extension, so an alternate method using PCR was used for these clones. A first-strand cDNA was synthesized from mRNA with reverse transcriptase, then oligos complementary to the 5' region of FW1 (5'-TTTACCTCAAAGAGTCTGGCCCTGCGG-3') of the 3609 gene and to the 5' region of the Cµ sequence (5'-GGGCCTCCT-
Ly-1B Cells Are Responsible for ATA Secretion. Spleen cells from mice of the nonautoimmune SM/J strain, unlike most mouse strains, secrete IgM ATA detectable by immunofluorescence staining after stimulation with LPS in culture (Fig. 1a). We found that this ATA secretion from SM/J spleen was completely eliminated after depletion of Ly-1B cells (comprising 5% of spleen cells; reference 10) without a significant decrease in the total IgM produced (Fig. 1a). Depletion of Ly-1B cells (the majority of B cells in spleen) did not affect ATA secretion (Fig. 1a) whereas, as expected, total IgM and B cell proliferation (data not shown) were greatly reduced. Thus, the presence of Ly-1 B is required for LPS-induced ATA secretion from SM/J mouse spleen cells.

We found that Ly-1 B cells are exclusively responsible for ATA secretion, not only in SM/J mice but also in BALB/c mice. Purified Ly-1 B and Ly-1B cells from SM/J spleen, or BALB/c peritoneal cavity Ly-1 B cells and Ly-1B cells from BALB/c spleen were used for comparing levels of ATA secretion in LPS culture. As Fig. 1, b and c show, both supernatants from Ly-1 B cells contain ATA activity. The ATA binding activity determined by immunofluorescence staining intensity correlates with ATA levels in the secreted IgM, as shown in Fig. 2. Although Ly-1 B cell cultures initially secrete more IgM than Ly-1B cell cultures, no antithymocyte activity was ever detected in the supernatant from Ly-1B (conventional) B cells during 7 d of LPS culture, even after comparable levels of IgM were attained (Fig. 2). The possibility that ATA secretion from Ly-1B cells in the LPS culture system might be dependent on T cells (or some other cell type) could be excluded since Ly-1B-depleted SM/J spleen cells, which include all non-B cells in spleen, did not show ATA activity during the course of culture (similar to results using purified B cell populations). Thus, these data strongly suggest that a distinct B cell subset, Ly-1 B, is responsible

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Figure 1. Ly-1 B cells in SM/J and BALB/c mice secrete ATA after stimulation by LPS. The histogram of the immunofluorescence staining of thymocytes by the culture supernatant is shown. BALB/c mouse thymocytes (5 x 10⁶ cells/20 µl) were incubated with 100 µl of 3-d culture supernatant on ice for 15 min and washed, and then bound IgM was revealed by APC-anti-IgM in a second step. No binding was seen in supernatants of cultures without LPS (data not shown) or from LPS cultures of SM/J Ly-1B (Ly-1B-depleted spleen cells (a). Total IgM levels present in culture supernatant of unseparated, Ly-1B-depleted, and Ly-1B-depleted SM/J spleen cells were 1.4 µg/ml, 1.0 µg/ml, and 0.4 µg/ml, respectively (a). IgM levels of other cultures (using cell sorter-purified B cell fractions; b and c) are given in Fig. 2.

Figure 2. Thymocyte binding activity was only detected in culture supernatant from Ly-1 B cell irrespective of secreted IgM levels or the kinetics. ATA activity was measured in 3- and 7-d LPS culture supernatants from (a) Ly-1 B and Ly-1B B cells in SM/J spleen cells or (b) from BALB/c PerC Ly-1 B and splenic Ly-1B B. Binding activity is expressed as the mean fluorescence intensity of thymocyte staining. IgM concentrations used for staining (on the abscissa) are proportional to the secreted antibody levels; (a) Ly-1 B d5 (4.0 µg/ml) and d7 (9.0 µg/ml), Ly-1B d5 (3.6 µg/ml) and d7 (5.6 µg/ml); (b) Ly-1 B d3 (20.5 µg/ml) and d7 (43.0 µg/ml), and Ly-1B d3 (1.0 µg/ml) and d7 (18.0 µg/ml). Data of 1:10 (a) and 1:3 (b) diluted supernatants from Ly-1 B cell cultures were also included in plots. Saturation of ATA binding was observed at >10 µg/ml of IgM in the Ly-1 B supernatant.
for natural ATA secretion in most strains, and further, that the increased population of Ly-1 B cells in SM/J mice is responsible for the characteristically high levels of serum ATA found in this mouse strain.

The Specificity of ATA Hybridomas Are Diverse. ATA from Ly-1 B cells was characterized further by establishing hybridomas. Hybridomas made from Ly-1 B and conventional Ly-1\(^{(1)}\) (IgD\(^{2+}\)) B cells sorted from a pool of SM/J mouse spleen were plated in limiting dilution to assess the frequency of clone/well, as described previously\(^{(10)}\). Results from the initial screening of 576 IgM secreting wells from both Ly-1 B and Ly-1\(^{-}\) B cell fusions showed that the frequency of clones with antithymocyte binding reactivity from Ly-1 B was higher, 1% (23/1,728-2,304; three to four clones/well; reference 10) compared with 0.1% (3/2,880; five clones/well) from Ly-1\(^{-}\) B cells, consistent with results of the ATA secretion assay described above. From these positive wells, we isolated 17 antithymocyte Ly-1 B hybridoma clones and one Ly-1\(^{-}\) B hybridoma clone. Two ATA Ly-1 B hybridoma clones were obtained in a separate cell fusion experiment\(^{(10)}\). Table 1 lists the 15 monoclonal ATA (14 from Ly-1 B, 1 from Ly-1\(^{-}\) B) that were characterized further.

After isolating hybridoma clones, several distinct ATA binding profiles to different cell types emerged. As Table 1 shows, all monoclonal IgM ATA from Ly-1 B cells selected by thymocyte binding activity showed specific binding to T lineage cells (among hematopoietic cells tested). However, their specificities appear diverse, exhibiting differential reactivity to thymocytes at various maturational stages (data not shown), variable binding to peripheral T cells (CD4\(^{+}\) and/or CD8\(^{+}\)), and different degrees of species crossreactivity (mouse/rat). Most react with thymocytes from all strains of laboratory mice tested, including SM/J (data not shown). However, several show a preferential (or exclusive) binding to certain strains of mice (for example, BALB/c vs. AKR, as shown in Table 1), although the genetic basis for this has not been determined. In contrast to the T lineage specificity shown by these ATA from Ly-1 B, the single ATA obtained from Ly-1\(^{-}\) B cells (3-6A12) bound to both thymocytes and other cell types from BALB/c (and SM/J), but did not react with any cells from AKR mice. These data illustrate that ATA hybridomas exhibit fine specificities whose reactivity is dependent on the cell type, differentiation stage, mouse strain, or species. The grouping of ATA by putative fine specificity is based on these staining results using normal cells and results from experiments described below.

**Half of the ATA Hybridomas Recognize Determinants Largely Dependent on Thy-1 Expression.** The reactivity patterns de-

### Table 1. ATA Hybridomas Derived from Ly-1 B Are T Lineage Cell Specific and Recognize Diverse Determinants

| Group | Clone | IgH,L | Thymus | CD4 | CD8 | non-T | BM | Rat thymus |
|-------|-------|-------|--------|-----|-----|-------|----|-----------|
| I     | 2-2G7 | \(\mu,\lambda\) | ++ + | + | + | - | - | - |
|       | 1-6C10 | \(\mu,\kappa\) | + + + | + + | + | - | - | - |
|       | 1-1B10 | \(\mu,\kappa\) | + + + | + + | + | - | - | - |
|       | 1-1D9 | \(\mu,\kappa\) | + + | - | - | - | - | - |
|       | 1-4F2 | \(\mu,\kappa\) | + + | - | - | - | - | - |
|       | 1-6B9 | \(\mu,\kappa\) | + + * | - | - | - | - | - |
|       | 1-4A2 | \(\mu,\kappa\) | + + * | - | - | - | - | - |
|       | 1-6D7\(^t\) | \(\mu,\kappa\) | + + * | - | - | - | - | - |
| II    | 1-4F5 | \(\mu,\kappa\) | + S | - | - | - | - | - |
|       | 1-3A2 | \(\mu,\kappa\) | + | - | - | - | - | - |
| III   | 2-1C10 | \(\mu,\kappa\) | + + | - | + | - | + | + |
| IV    | 1-1G10 | \(\mu,\kappa\) | + S | - | - | - | - | - |
| V     | 1-3G11 | \(\mu,\kappa\) | + | + + + | + | - | - | + |
|       | 1-3H5 | \(\mu,\kappa\) | + | + + + | + | - | - | + |
| VI    | 3-6A12 | \(\mu,\kappa\) | + * | + | + | + | + | - |

Hybridoma antibodies prefixed with 1, 2, and 3 are from the hybridization of Ly-1 B with MPC11, Ly-1 B with SP2/0, and Ly-1\(^{-}\) B with MPC11. 1-6C10 and 1-3G11 were reported as SM6C10 and SM3G11, respectively, in our previous papers\(^{(31,46)}\). ++ +, + + +, + +, and - = >60%, 20-60%, 1-20%, and <1% positive cells, respectively.

* Preferential binding to BALB/c thymocytes.
* Preferential binding to AKR thymocytes.
* Preferential binding to anti-DNA activity, whereas other ATA are negative.
* Preferential binding to AKR thymocytes.

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scribed above led us to consider the involvement of carbohydrate structures as the antigenic determinants, since carbohydrate components of cell surface molecules are often altered during cell development or differentiation, and can be expressed in a strain- or species-specific manner (42, 43). Supporting this prediction, the reactivity of 11 of these autoantibodies was sensitive to prior treatment of target cells with sodium metaperiodate, which preferentially oxidizes terminal sialic acid groups (32) (Table 2).

Thy-1 is a major glycoprotein of the thymus, so the ability of anti-Thy-1 antibody to block ATA binding was investigated by immunofluorescence staining (Table 2 and Fig. 3). The binding of the eight group I ATA antibodies was nearly completely blocked by prior or simultaneous incubation of thymocytes with anti-Thy-1 antibody, while the binding of two ATA antibodies (group II) was partially blocked. Blocking by anti-Thy-1.1 or anti-Thy-1.2 antibodies was specific to the appropriate Thy-1 allele, and other antibodies (anti-CD45, anti-CD5) that react with determinants present on the majority of thymocytes did not block (data not shown). This result suggests that the determinant detected by these antibodies either resides on the Thy-1 glycoprotein or on a molecule that is present in close proximity to the Thy-1 glycoprotein.

To more directly study the relationship of the determinants defined by the ATA antibodies of groups I and II to the Thy-1 molecule, the staining of these antibodies on a panel of cell lines was examined. BW5147 is a Thy-1+ lymphoma derived from AKR/J (Thy-1.1) mice, while BW5147 (Thy-1-a) and BW5147 (Thy-1-e) are Thy-1+ mutants with defects in genes affecting post-translational steps necessary for cell surface expression of the Thy-1 glycoprotein (and other cell surface molecules anchored in the cell membrane through a glycosphospholipid moiety) (34-37). AKR1 is also a Thy-1+ lymphoma derived from AKR/J mice, while AKR1 (Thy-1-d) is a Thy-1- mutant that has undergone deletion of a portion of the Thy-1 structural gene (38). TFX351 is a Thy-1+ derivative of AKR1 (Thy-1-d) in which a normal copy of the Thy-1.1 structural gene was introduced by transfection.

The staining results of ATA on this panel of cell lines are summarized in Table 2, and examples of FACS profiles obtained are shown in Fig. 3. The three ATA of group I show an absolute concordance with expression of the Thy-1 glycoprotein. Therefore, it seems highly likely that these ATA recognize one or more determinants present on the Thy-1 glycoprotein. The rest of group I ATA stain neither the wild-type

Table 2. Half of ATA Determinants Are Dependent on Thy-1 Expression and Many Are Carbohydrates

| Group | Clone | Periodate sensitivity | Anti-Thy-1 block | BW5147 Wild | Thy-1-a | Thy-1-e | AKR1 Wild | Thy-1-d | TFX351 |
|-------|-------|-----------------------|------------------|-------------|---------|---------|-----------|---------|--------|
| a-Thy-1* | 2-2G7 | Yes | Yes | +++ | - | - | +++ | - | +++ |
| I (1) | 1-6C10 | Yes | Yes | + + | - | - | + | - | + |
| | 1-1B10 | Yes | Yes | + | - | - | - | - | - |
| | 1-1D9 | Yes | Yes | + | - | - | - | - | - |
| | 1-4F2 | Yes | Yes | + | - | - | - | - | - |
| (2) | 1-6B9 | Yes | Yes | + | - | - | - | - | - |
| | 1-4A2 | Yes | Yes | + | - | - | - | - | - |
| (3) | 1-6D7 | No | Yes | + | - | - | - | - | - |
| II (1) | 1-4F5 | Yes | Part | + | - | - | + / - | + |
| | 1-3A2 | No | Part | + | - | - | + / - | + |
| III | 2-1C10 | Yes | No | +++ | + | + | +++ | + | +++ |
| IV | 1-1G10 | No | No | - | - | - | - | - | - |
| V | 1-3G11 | Yes | No | - | - | - | - | - | - |
| | 1-3H5 | Yes | No | - | - | - | - | - | - |
| VI | 3-6A12 | - | No | - | - | - | - | - | - |

+++ + + + : staining intensity.
* Stained with anti-Thy1.1.
† No staining after treatment without periodate.

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Figure 3. FACS profiles of the monoclonal ATA staining on BALB/c thymocytes (and blocking effect by anti-Thy-1), BW5147-derived Thy-1" mutant cell lines, and AKR1-derived Thy-1" mutant and Thy-1" transfectant.

Table 4

| Group | Clone | Thymocyte anti-Thy-1 Blocking | BW5147 Thy-1 loss Variants | AKR1 Thy-1 loss /Transfectant |
|-------|-------|-------------------------------|---------------------------|-------------------------------|
| I     | 2-2G7 |                                |                           |                               |
|       |       |                                |                           |                               |
| II    | 1-4F5 |                                |                           |                               |
|       |       |                                |                           |                               |
| III   | 2-1C10|                                |                           |                               |
|       |       |                                |                           |                               |
| (-)   | none  |                                |                           |                               |

AKR1 cell line nor transfectant TFX351. However, these antibodies do show moderate to weak staining on the wild-type BW5147 cell line but do not stain either BW5147 mutant.

Antibodies of group II show binding, although weakly with a somewhat brighter tail in the FACS profile, to the class D mutant derived from AKR1. Therefore, it is unlikely that the determinant recognized by this antibody resides solely on the Thy-1 glycoprotein. As with the group I antibodies, group II ATA stain the wild-type BW5147 cell lines, but neither Thy-1" mutant derived from this cell line.

Consistent with the failure of anti-Thy-1 antibodies to block ATA of groups III-VI, ATA of these groups either failed to stain any cell line, whether Thy-1" or not (groups IV-VI), or stained all cells, whether wild-type or mutant (group III). The determinants for group V antibodies (1-3G11 and 1-3H5) are present at high levels on mature T cells where their expression is uncorrelated with Thy-1. Recent studies show that group V antibodies (1-3G11 and 1-3H5) react with carbohydrate determinant present on a glycolipid (ganglioside), which is probably the predominant source for this determinant (Greer, J., T. Koerner, R.R. Hardy, K. Hayakawa, and J. Kemp, manuscript in preparation).

These results suggest that most determinants for ATA are carbohydrate structures that can be expressed on glycoproteins, glycolipids, or possibly on both (42). In summary, these ATA determinants can be grouped based primarily on their relatedness to Thy-1 (sensitivity to anti-Thy-1 blocking, and expression on T lymphoma wild and variant cell lines) and on their distinctive distribution on normal cell types. Further subdivision is possible with respect to sensitivity to periodate treatment and preferential expression in certain mouse strains. In cases where the difference of binding activity by ATA to T lineage cells and cell lines could be considered due to antibody avidity, they are classed in the same group; e.g., in group I-1, levels of staining intensity on the thymus with 1-1D9 and 1-4P2 are lower than the others.

Discussion

Numerous studies have demonstrated that carbohydrate structures can serve as differentiation antigens and oncofetal antigens (42, 43). It becomes increasingly apparent that antibodies to specific cell types or tumor cells often react with carbohydrate determinants, and several reports of "natural antibodies" reactive with carbohydrate structures are available (42, 43). Since comparable carbohydrate structures can be expressed on several glycoproteins, glycolipids, or on both, analysis of such specificities often results in obscure data when attempting to ascertain the precise molecular determinants. Another difficulty in the characterization of the target molecules of natural autoantibodies is due to the frequently observed temperature dependence of their binding (better at low temperature), suggesting low avidity. Our approach has been to establish monoclonal ATA and examine their reactivity to normal cell types or to chemically modified thymocytes, their
crossblocking effect with anti-Thy-1, and their reactivity to Thy-1 mutant or Thy-1 gene transfectants. Such analysis has allowed us to conclude that while determinants on thymocytes recognized by natural ATA are diverse, particular specificities predominate, and further, that most of these specificities probably involve carbohydrate structures.

We found that many ATA are specific to determinants associated with the Thy-1 glycoprotein, which is a heavily glycosylated molecule (30% [wt/vol]) (45) and one of the major glycoproteins present on mouse thymocytes. This also confirms previous data of Thy-1-dependent determinant(s) for ATA that were induced during the production of anti-Thy-1 polymorphic antibody after allogeneic thymocyte injection (24, 26). However, while Thy-1 plays a major role, our data also provides several examples of determinants unrelated to Thy-1, and also leaves open the possibility that even the Thy-1-dependent determinants in the thymus might also be expressed on different molecules in other organs or on tumor cells. This may relate to the earlier finding that ATA is detectable in the serum of athymic nude mice (7), and it is interesting to speculate on the relation of some natural ATA to natural anti-tumor cell activity found in sera (6). Nevertheless, our data suggests that natural autoantibodies specifically recognize fine structures on cell surface molecules that can be altered during differentiation and maturation. Our previous studies of 6C10 and 3G11 expression on CD4+ T cell subsets provide a clear case of such alteration (31, 46).

Intriguingly, we found that although the ATA defined by thymocyte binding activity show diverse specificities, the B cells that produce such autoantibodies are not randomly distributed among B cell populations; that is, ATA B cells are enriched in a minor B cell subset, Ly-1 B. Our data depend on LPS responsiveness in vitro, and further work will be necessary to assess the frequency of ATA B cells in unmanipulated cell populations. However, an elevated frequency of ATA B cells in Ly-1 B can be expected since both Ly-1 B cell and conventional B cell fusions generated after LPS culture showed no obvious differences with regard to fusion frequency or production of IgM-secreting wells. Therefore, our results for ATA B cell frequency and ATA secretion lead us to suggest that the ATA naturally found in serum derives solely from Ly-1 B. An increased frequency of the Ly-1 B subset may ac-

Table 3. Utilization of Diverse V\textsubscript{H} Genes in ATA Hybridomas Derived from Ly-1 B Cells

| Group | Clone | V\textsubscript{H} gene | Size | Amino acid residue | Type | J\textsubscript{H} |
|-------|-------|----------------------|------|-------------------|------|-------|
| I     | 1-6C10| 3609\textsuperscript{a} | 18   | REGGRS           | Q52  | J2    |
|       | 1-1B10| 3609\textsuperscript{a} | 18   | RKGKYD           | Q52  | J1    |
|       | 1-1D9 | 3609\textsuperscript{a} | 27   | RGKRYGTW        | SP2-7| J1    |
|       | 1-4F2 | 3609\textsuperscript{a} | 21   | RWNYYGL          |      |       |
| (2)   | 1-6B9 | J558\textsuperscript{a} | 9    | EGS              | -    | J3    |
|       | 1-4A2 | J558\textsuperscript{a} | 9    | EGS              | -    | J3    |
| V     | 1-3G11| J558\textsuperscript{a} | 12   | EDWP             | SP2  | J2    |
|       | 1-3H5 | J558\textsuperscript{a} | 24   | LGWDVPEI         | Q52  | J2    |

* Ambiguous assignment.

Table 4. Identical V\textsubscript{H} Gene Usage in ATA Hybridomas Is Due Both to Clonal Expansion and to Independent Incidence

| D region | Clones | V\textsubscript{H} gene | Size | Amino acid residue | Type | J\textsubscript{H} |
|----------|--------|------------------------|------|-------------------|------|-------|
| NT       | 1-6C10 | 3609\textsuperscript{a} | 18   | REGGRS           | Q52  | J2    |
|          | 1-1B10 | 3609\textsuperscript{a} | 18   | RKGKYD           | Q52  | J1    |
|          | 1-1D9 | 3609\textsuperscript{a} | 27   | RGKRYGTW        | SP2-7| J1    |
|          | 1-4F2 | 3609\textsuperscript{a} | 21   | RWNYYGL          |      |       |
| (2)      | 1-6B9 | J558\textsuperscript{a} | 9    | EGS              | -    | J3    |
|          | 1-4A2 | J558\textsuperscript{a} | 9    | EGS              | -    | J3    |
| V        | 1-3G11| J558\textsuperscript{a} | 12   | EDWP             | SP2  | J2    |
|          | 1-3H5 | J558\textsuperscript{a} | 24   | LGWDVPEI         | Q52  | J2    |

* Ambiguous assignment.
The mechanism(s) that results in such a bias of ATA-specific clones in Ly-1 B has not been elucidated. Perhaps key to this question, we found that among various V gases encoding ATA specificities, nearly identical V genes are used in clonally unrelated hybridomas showing similar specificity. One such example, 1-3G11 and 1-3H5, also appears to have identical L chains (sharing an identically size rearranged band with the pECK probe by Southern analysis with several enzymes; data not shown). Similar L chain analysis of ATA hybridomas utilizing the 3609 V gene show different sized bands, although their sequences need to be determined to definitely answer the question of L chain usage. Nevertheless, the observation of similar binding specificity with identical V gene usage by clonally unrelated cells suggests that ATA-specific B cells were enriched among Ly-1 B cells primarily due to their specificity, likely by an antigen-driven selection mechanism. The origins of such antigens are not clear. However, we presume natural ligands (environmental or auto-antigens) are the most likely candidates for long-term constant selection of such B cells among Ly-1 B, and furthermore, probably lead in part to antibody secretion recognized as spontaneous natural ATA secretion in the serum. We found that clonal proliferation of Ly-1 B cells, as reported elsewhere (16-18), also contributes to the higher ATA frequency in Ly-1 B. We speculate that a similar selection mechanism may have been operating even in the event of clonal proliferation, resulting in the increase of ATA B cells specifically, as has been suggested in the generation of Ly-1 B lymphomas (19). Therefore, if we expanded our ATA panel with more Ly-1 B-derived hybridomas, we would likely find that all V gases we have described in this paper would be observed repeatedly.

Close examination of the D region sequence data (Table 4) reveals several unusual features in the ATA hybridomas: the arginine (R)-rich sequences of Gr I-1 3609 V, ATA, the short D of Gr I-2, and the apparent D-D fusion of 1-3H5. One possible explanation for the common arginine at the start of the 3609-encoded D segments is that the first two nucleotides may derive from the end of the V segment: in every case the arginine is encoded by a codon that begins with AG. Alternatively, the unique characteristics of arginine may play a role in the binding site of Gr I-1 type ATA antibodies. Unfortunately, the absence of germline sequence data precludes any definitive conclusion regarding the origin of this arginine. The short three amino acid residue D sequence in Gr I-2 is reminiscent of the three residue D always found with Vn11-encoded anti-BrMRBC autoantibodies (also derived from Ly-1 B), but numerous examples exist of longer D segments in Ly-1 B-derived autoantibodies, so the significance of such a short D remains equivocal. Finally, the D segment in 1-3H5 appears to be the result of a D-D fusion, a relatively uncommon event in normal rearrangement. It is interesting to note that the most similar antibody, 1-3G11, has a short D (different from 1-3H5) and that every residue found in 1-3G11 is also found in 1-3H5, although arising completely differently. Thus, we might speculate that these four residues could play a role in the selection of ATA activity of the type in Gr V.

Our data show ATA to be the second autoantibody specificity enriched in the Ly-1 B cell repertoire. We have previously shown another natural antibody, which to BrMRBC, originates almost exclusively from Ly-1 B cells (9, 10). Compared with antigenic determinants for ATA, those on BrMRBC are much more restricted: they are recognized by anti-BrMRBC autoantibodies that predominantly utilize either a Vn11 gene together with a V9 gene or else a Vn12 gene together with a V4 gene (10, 47). Our recent data by PCR amplification of DNA from purified B cell populations demonstrates that Ly-1 B overutilize the Vn11 gene, as compared with other (conventional) B cells, due to a selection mechanism rather than any restriction of particular V genes to this subpopulation (21). Reminiscent of anti-BrMRBC antibodies, the finding of repeated usage of the 3609 Vn gene in the most common ATA specificity is of particular interest, considering its large contribution to the enrichment of ATA-specific B cells in Ly-1 B, whereas members of the 3609 Vn family have only infrequently been seen in conventional antigen responses. However, our result also suggests that diverse Vn genes (from several frequently used Vn families) also characterize the Ly-1 B repertoire established in adult mice. We emphasized here the importance of antigen specificity in contributing to positive selection in the Ly-1 B repertoire, but pose the question as to why such selection preferentially occurs in the Ly-1 B population. This question remains the focus of future investigation.

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