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Brief Definitive Report

UNUSUAL PHENOTYPE OF B CELLS IN THE THYMUS OF NORMAL MICE

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The thymus is a central lymphoid organ for the differentiation of T cells. The thymus contains >95% thymocytes and trace numbers of other cells like macrophages, dendritic cells, and epithelial cells (1). Only a small number of Ig^+ B cells are detected in normal thymi (1), but expanded numbers have been reported during disease. B cell follicles and numerous plasma cells have been reported in the thymus of patients with autoimmune diseases like myasthenia gravis and systemic lupus erythematosus (2-4), and similar findings have been noted in autoimmune-prone mice such as (NZB × NZW)F_1, MRL/Mp-*lpr/lpr* (MRL/lpr), and BXSB (5). In these pathologic situations, it has not been elucidated if the B cells infiltrate the thymus from the blood, or if the B cells originally are present in the thymus and expand as part of the disease.

We have begun a study of thymic B cells to determine their contribution to autoantibody formation. We have succeeded in enriching thymic B cells from normal mice and find that most of these lymphocytes have the unusual phenotype of the Ly-1 B cell subset, a subset previously implicated in autoantibody production (6, 7).

Materials and Methods

Mice. Female (BALB/c × DBA/2)F_1 (CD2F_1) and C57BL/6J (B6) mice were purchased from Japan Clea Corp. (Tokyo) and kept under specific pathogen-free conditions until use at 6-8 wk of age.

Antibodies. mAb to Thy-1.2 [F7D5] was purchased from Olac Ltd. (Bicester, United Kingdom). mAb to Thy-1.2 (30-H12, IgG2b), CD4 (GK 1.5, IgG2b), Lyt-2.2 or CD8 (HO-2.2, IgM), B220 or CD45R (RA3-3A1/6.1, IgM; 14-8, IgG2b), Ia (M5/114.15.2, IgG2b), and Mac-1 or CD11b (M1/70, IgG2b) were from the American Type Culture Collection (Rockville, MD). The mAbs were used for depletion or staining. FITC-coupled rabbit anti-mouse

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μ-chain $F(ab')_2$ or FITC-rabbit anti-mouse Ig $F(ab')_2$ fragments were obtained from Cappel Laboratories (West Chester, PA), while biotinated mouse anti-rat Igκ mAb was from Zymed Laboratories (San Francisco, CA). Biotinated anti-Lyt-1 or CD5 (53.7.3, IgG2a), biotin-anti-CD8 (53.6.7, IgG2a), FITC-avidin, and PE-avidin were from Becton Dickinson & Co. (Mountain View, CA). $F(ab')_2$ fragment of goat anti-mouse μ chain from Cappel Laboratories was used to stimulate B cells.

Cell Preparation. The connective tissue attached to thymic lobes was carefully peeled off to remove the parathymic lymph nodes. Thymocytes were prepared by gentle teasing onto a fine steel mesh, were washed twice in RPMI 1640 medium (Nissui, Tokyo, Japan), and suspended in medium with 10% FCS (HyClone Laboratories, Logan, UT). Cell suspensions were layered onto the 65% Percoll solution (Pharmacia Fine Chemicals, Uppsala, Sweden) (1) and centrifuged at 900 *g* for 20 min. The low-density cells (50-60% of the total) at the interface between the medium and the Percoll solution were collected, washed twice in RPMI 1640, and treated with a mixture of mAb to Thy-1.2, CD4, and CD8 (above) in the presence of rabbit complement. The mAb and dead cells were removed by washing twice in alkaline (pH 7.6-7.8) RPMI 1640. The resulting population was B cell enriched and T cell depleted (see Results). B cells from the spleen were prepared by the same T cell depletion procedure, with or without passage through Sephadex G10.

Proliferative Assays. Triplicate cultures of 10^5 cells were incubated in 0.2 ml of RPMI 1640 medium containing 10% FCS, penicillin, streptomycin, and 50 μM 2-ME in flat-bottomed Corning microculture plates. The stimuli were: 25 μg/ml LPS (*Escherichia coli* 055:85 Difco Laboratories Inc., Detroit, MI), 2 μg/ml Con A (Pharmacia Fine Chemicals), or anti-μ (10 μg/ml) plus rIL-4 or purified BSF-1 (generously donated by Dr. T. Honjo, Kyoto University, and Dr. K. Kumagai, Tohoku University). Proliferation was measured on the third day by pulsing with 1 μCi of $[^3H]$thymidine for 10 h.

Cytofluorography. Cells were suspended in PBS with 2% FCS and 0.05% azide and stained according to a triple-layer method using rat mAb, biotin-mouse anti-rat Igκ, and FITC-avidin. The controls were stained with FITC- or PE-avidin without first mAb, or with nonreactive, isotype-matched mAb (see Results). To double label for Ly-1 and surface Ig, the cells were stained first with FITC-rabbit anti-mouse μ chain $F(ab')_2$ fragment followed by normal rat Ig to block the reactivity of anti-μ with the subsequent antibody and to reduce binding to Fc receptors. Then the cells were stained with biotin-anti-Ly-1 and PE-avidin. Nonreactive control biotin-mAb were to Thy-1 and CD8. The cells were analyzed on a FACStar (B-D Automated Immunochemistry, Salt Lake City, UT).

Results

Enrichment of B Cells from Normal Thymus. To enrich the trace population of thymic B cells, suspensions were separated on Percoll density gradients. Most sIg^+ thymic B cells were recovered in the low-density fraction, whereas only a supopulation of splenic B cells floated under similar conditions. After treatment with a cocktail of anti-T cell mAb and complement, the frequency of sIg^+ B cells rose from ∼3% to >90% (below). The yield was ∼1% of the starting thymus suspension.

B cell enrichment was evident by functional assays, i.e., proliferation to LPS and anti-μ plus IL-4. The starting thymocytes, the low-density fraction, and the B cell-enriched population were compared (Table I). The latter responded best to LPS, and to anti-μ plus IL-4, but was depleted of Con A reactivity. The low-density cells (50-60% of the total thymus cells) responded vigorouly to Con A because most immunoincompetent thymocytes sedimented (not shown), but weakly to LPS and IL-4. The high-density population, even when treated with anti-T cell mAb and complement, failed to respond to the B cell stimuli (not shown).

Phenotype of Thymic B Cells. Cytofluorography was used to compare enriched preparations of thymic B cells with spleen B cells (Fig. 1). More than two of three of the

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TABLE I
Enrichment of Thymic B Cells

Cells from thymus	Proliferative responses*						Ig-bearing cells‡
	—	Anti-μ antibody (10 μg/ml)	IL-4 (10 U/ml)	Anti-μ + IL-4	LPS (25 μg/ml)	Con A (2 μg/ml)	
				cpm			%
Total cells	892	573	1,574	1,261	643	13,883	0.6
Low-density cells§	914	577	4,360	3,492	284	71,091	2.7
B cell-enriched cells‖	415	454	2,602	16,541	36,193	2,776	89.9

* Cells (10^5) were cultured with or without various kinds of stimulants for 72 h, and pulsed with [^{3}H]TdR for the last 10 h of the culture period. Numbers in the table represent mean cpm of triplicated cultures.
‡ Cells in each fraction were stained with FITC-conjugated rabbit anti-mouse Ig antibody.
§ Low-density cells were obtained from the upper layer of the 65% Percoll gradient.
‖ B cell-enriched cells were obtained form the low-density cells by treating them with anti-L3T4, anti-Lyt-2.2, and anti-Thy-1.2 mAbs plus complement.

thymic B cells coexpressed Lyt-1 (CD5), whereas most splenic B cells were Lyt-1$^-$. The thymic B cells could also be killed with a cytotoxic anti-Lyt-1 mAb (not shown). Both thymic and splenic B cells had comparable levels of sIg (Fig. 2, *a* and *f*), but most of the thymic B cells had less B220 or CD45R (*b* vs. *g*) and Ia (*c* vs. *h*). Mac-1 [CD11b] and CD5 were found on most thymic B cells, which did not react with two isotype-matched control mAbs, GK1.5 anti-CD4 and 30-H12 anti-Thy-1 (*d* and *e*).

Discussion

We have been able to enrich the small number of thymic B cells (~1% of thymic suspensions) and have found that most have the features of Ly-1 B cells. The latter

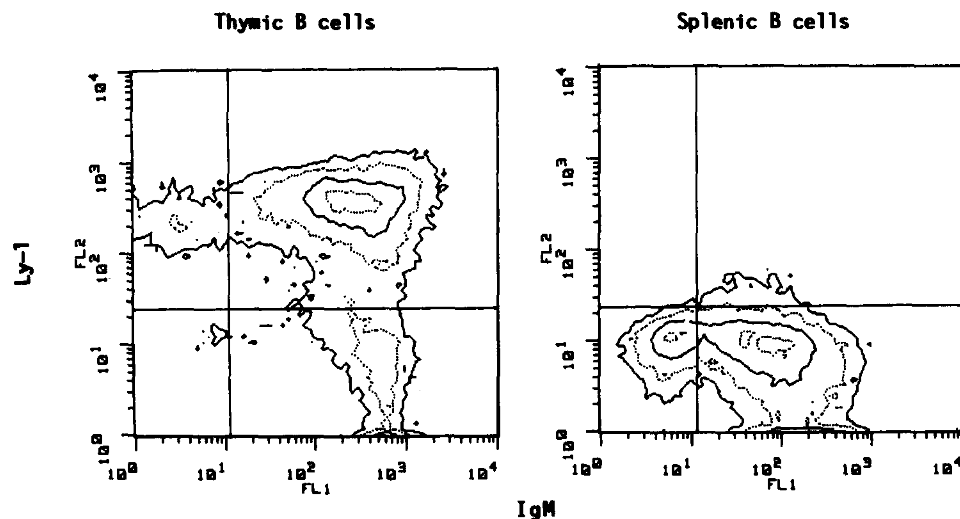

FIGURE 1. Two color FACS analysis of thymic and splenic B cells. Enriched populations of thymic and splenic B cells were treated successively with FITC-rabbit anti-mouse μ chain F(ab')$_2$ fragment, excess normal rat IgG, biotin-anti-Lyt-1 (CD5), and PE-avidin. The stained cells were fixed and analyzed on a FACStar cytofluorograph.

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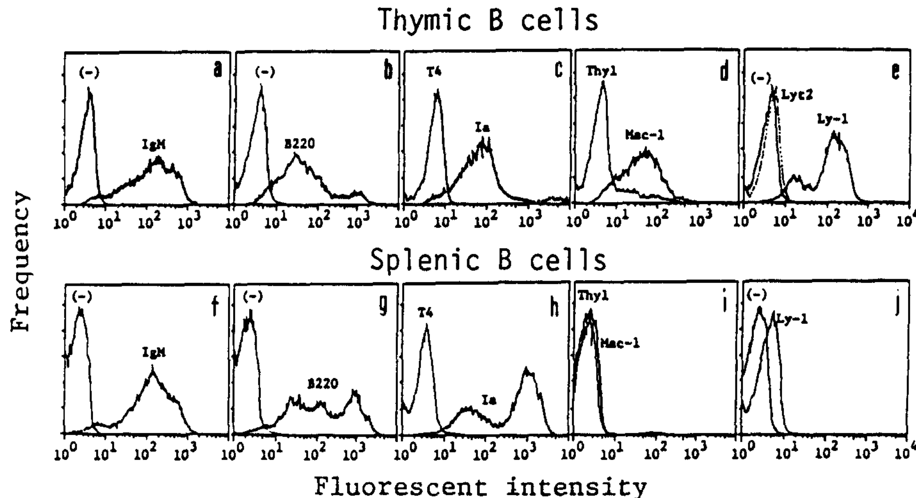

FIGURE 2. Phenotype of thymic and splenic B cells. Enriched populations of thymic (*top*) and splenic (*bottom*) B cells were stained with the different mAbs indicated on the panel and specified in Materials and Methods, followed by biotin-mouse anti-rat Igκ and FITC-avidin. The designation [-] on the panels were controls that were unstained cells (*a* and *f*), FITC-avidin only (*b* and *g*), and biotin-mouse anti-rat Igκ and FITC-avidin (*e* and *j*).

are a distinct population of CD11b$^+$ and CD5$^+$ lymphocytes which are detected in the peritoneal cavity but are hard to find in spleen, blood, and especially lymph nodes (6, 7). Similar results were obtained with thymi of C57BL/6 and young NZB mice, and from C57BL/6 mice varying in age from 3 to 8 wk (not shown). The staining of B cells for CD5 was specific, since no decrease was seen with an excess of rat Ig (500 μg/ml) to block Fc receptors, and there was no staining with other anti-T cell mAbs like anti-Thy-1 CD8 and CD4. Furthermore, the majority of thymic B cells prepared by our procedure could be killed by anti-CD5 (but not anti-Thy-1.2, CD4, or CD8) mAb and complement. Other comparisons with conventional splenic B cells are that the thymic cells had less B220 and Ia antigens, a lower buoyant density in Percoll, and similar amounts of membrane μ. These results are consistent with those reported for Ly-1$^+$ B cells in the peritoneal cavity (8, 9).

Our functional assays demonstrated that thymic B cells responded to B cell stimulants. However, the responses were much lower than spleen (not shown). In preliminary studies, Ly-1$^+$ thymic B cells have been purified on the FACS and found to have little or no responsiveness either to LPS or to anti-μ and IL-4. Likewise, depletion with anti-Ly-1 and complement enhances the responsiveness of the thymic B cell preparation to these stimuli. Therefore Ly-1 B cells may not contribute to the functional assays that were used to help monitor our enrichment method (Table I).

Peripheral Ly-1$^+$ B cells are known to be involved in "spontaneous" secretion of IgM, including autoreactive antibody (10). These B cells appear early in development (11) and are long lived (12). A regulatory role has been suggested in allotypic (13) and idiotypic cell-cell interactions (14–16). This being the case, it is possible that the Ig V region of thymic Ly-1 B cells shapes the repertoire of T cell antigen receptors by providing epitopes that correspond to antigen in association with self-

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MHC. Alternatively, products of the Ly-1 B cell repertoire may function to eliminate or inactivate self-reactive T cells by providing internal images of self during T cell development. We are now studying the ontogeny of these thymic B cells to clarify their function in normal and autoimmune-prone mice.

Summary

A small number of B cells are found in the thymus of normal mice. A population of B lymphocytes could be enriched to >90% purity by isolating a low-density fraction on Percoll density gradients and then depleting T cells with a mixture of anti-Thy-1, CD4, and CD8 mAbs and complement. Enrichment was monitored by surface Ig staining and by functional studies (responsiveness to LPS, and to anti-μ plus IL-4). When the phenotype of these B cells was studied by flow cytometry, 60-80% had the phenotype Ly-1$^+$ (CD5), Ia$^+$, B220^{low} (CD45R), and Mac-1$^+$ (CD 11b). In contrast, splenic B cells lacked CD5 and CD11b and expressed higher levels of B220 and Ia antigens. These results indicate that most thymic B cells have the phenotype of the Ly-1 B cell subset, which was identified previously as a trace subpopulation in some peripheral tissues and is thought to play a role in autoantibody formation.

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