FUNCTIONAL ANALYSES OF THYMIC CD5+ B CELLS
Responsiveness to Major Histocompatibility Complex Class II-restricted T Blasts but not to Lipopolysaccharide or Anti-IgM Plus Interleukin 4

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In a previous report, we demonstrated that a small number of B cells are present in the thymus of normal mice, and that the majority of thymic B cells show the phenotype observed for Ly-1+ (CD5+) B cells in other tissues (1). Thus, the majority of thymic B cells express surface CD5, IgM, B220 (CD45R), and Mac-1 (CD11b), and a lower amount of MHC class II relative to peripheral B cells. The functions of the CD5+ subset and the minor CD5− component in the thymus have not been determined, however, because of prior difficulties in isolating these B cells.

In this paper, we use a recent method for isolating thymic B cells to show that the CD5+ subset has low responsiveness to B cell stimulants, such as LPS or IL-4, but is induced to grow and make antibody after a direct interaction with MHC class II-reactive T blasts.

Materials and Methods

Mice. Female [BALB/c × DBA/2]F1 and C3H/HeN mice were purchased from CLEA Japan Inc. (Osaka, Japan) and maintained under specific pathogen-free conditions until use at 6–8 wk of age.

Antibodies. mAbs to Thy-1(F7D5) were purchased from Olac Ltd. (Bicester, UK), and mAbs to CD4 (GK 1.5), CD8 (HO-2.2 and 3.155), and CD5 (C3PO) were from the American Type Culture Collection (Rockville, MD). Unconjugated or FITC-coupled rabbit anti-mouse μ chain F[ab']2 fragment were obtained from Cappel Laboratories (Malvern, PA). Biotinilated mAbs against CD5 (53.7.3) and phycoerythrin (PE)-coupled avidin were from Becton Dickinson & Co. (Mountain View, CA).

Cell Preparation. Thymic B cells were prepared as described (1). Briefly, low density thymus cells floated with 65% Percoll were depleted of T cells by treating with a mixture of mAbs to Thy-1, CD4, and CD8 in the presence of low toxic rabbit complement. After washing with alkaline (pH ~7.4) RPMI 1640 medium to remove dead cells, the resulting population was used as thymic B cells. B cells from spleens or lymph nodes were prepared by the same procedure plus passage through a Sephadex G-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden).

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MHC class II-restricted T blasts were isolated as the released blast population produced from cell clusters of CD4+ T cells and allogeneic spleen dendritic cells as described (2).

Isolation of CD5+ and CD5- B Cells from Thymic B Cells. Thymic B cells were double stained with FITC-anti-μ and biotinated-anti-CD5 plus PE-avidine, and sorted into CD5+ and CD5- B cells using a FACStar. In some experiments, thymic and splenic B cells were treated with anti-CD5 (C3PO) mAb plus rabbit complement to remove CD5+ B cells.

Proliferative Assays. The proliferative assays were carried out as previously described (1). Assays for Polyclonal Ig Secretions. Cells (10^6/ml) were cultured with 10 μg/ml LPS for 1–5 d. The secretion of IgM and IgG was measured by a reverse plaque assay using class-specific rabbit anti-IgM and anti-IgG antibodies (ICN ImmunoBiochemicals, Lisle, IL).

Results

Low Responsiveness of Thymic B Cells to Standard B Cell Stimulation. The responses of thymic B cells and splenic B cells to LPS or IL-4 were compared. As shown in Fig. 1 A, the proliferative response of thymic B cells to LPS was significantly lower than that of splenic B cells at all time points assessed (day 2–5). This was also the case when the B cells were costimulated by anti-μ plus IL-4 (Fig. 1 B). Furthermore, IgM and IgG production of splenic B cells in response to LPS was much higher than those of thymic B cells (Fig. 2). Similar results were obtained in thymic B cells after passing through a Sephadex G-10 column (not shown). On the other hand, the addition of a graded dose of thymic B cells to splenic B cells did not inhibit the response to LPS (not shown), indicating that a low responsiveness of thymic B cells is not due to suppressor cells.

To clarify why thymic B cells have a lower responsiveness to B cell stimulants, we first examined the dose of LPS (10–50 μg/ml) or anti-μ (5–50 μg/ml) required for stimulation of thymic B cells and observed hyporesponsiveness to these at any dose (not shown). Then we eliminated CD5+ B cells by anti-CD5 mAb plus complement. Proliferation and Ig secretion of thymic CD5+ B cells were significantly higher than those of bulk thymic B cell populations (not shown). We then fractionated thymic B cells into CD5+ and CD5- B cells using a cell sorter after staining with FITC-anti-μ and biotinated anti-CD5 plus PE-avidine. More than 70% of thymic μ+ cells were CD5+ (Fig. 3). After sorting, each subset was cultured for 4 d with or without LPS. As shown in Table I, a very small number of IgM-producing cells, but not IgG-producing cells, were detected on day 0 (spontaneous Ig secretion) in CD5+ thymic B cells. After 4 d of stimulation with LPS, CD5+ thymic B cells again produced few IgM plaque-forming cells (PFCs) and no IgG PFCs. The CD5- thymic B cells did have spontaneous IgM/IgG-producing cells, in contrast, and also responded to LPS to produce many IgM and IgG PFCs.
Figure 2. Polyclonal Ig secretion of thymic B cells. Thymic (circles) or splenic (squares) B cells were cultured at 10^6 cells/ml/well in 24-well plates in the presence of 10 µg/ml LPS. The cells were harvested on the days indicated in the figure. IgM- (open symbols) and IgG- (closed symbols) producing cells were then determined by a reversed plaque assay using a class-specific anti-Ig antibody.

Figure 3. Double-staining profile of thymic B cells. Thymic B cells were stained with FITC-anti-μ F(ab')2 antibody, and biotinylated-anti-CD5 mAb plus PE-avidin. Dots, solid lines, and dashed lines represent 1, 10, and 100 cells, respectively.

Responses of Thymic B Cells with Class II-restricted T Blasts. We next investigated whether thymic B cells could respond better if stimulated by the direct interaction with T cells. Irradiated (25 Gy) alloreactive (Ia^d-specific) T blasts (5 × 10^5 or 10^5 cells) were added to 2 × 10^6 thymic B cells, and polyclonal Ig-secreting cells were assayed at 1–4 d. In fact, class II-restricted T blasts induced significant numbers of IgM, but not IgG, antibody-forming cells from thymic B cells, the response reaching a maximum at 3 d (Fig. 4 A). B cell proliferation was also observed (Fig. 4 B). The responses of thymic B cells stimulated by class II-restricted T blasts were much higher than those induced by LPS (Fig. 4). Both CD5^+ and CD5^- thymic B cell were proliferating in the presence of class II-restricted T blasts when assayed by flow cytometry, i.e., analyses of cell size and CD5/sIgM staining (not shown).

To establish that CD5^+ cells were responding to the T blasts, we did additional experiments in which CD5^+ cells were depleted after culture with T blasts. Elimination of CD5^+ cells just before the PFC assay caused a considerable reduction of the number of antibody-producing cells/culture, whereas treatment with anti-CD5 plus complement did not reduce the PFC in splenic B cell cultures (Table II). Also, the few antibody-producing cells that developed in thymic B cells upon stimulation with LPS were resistant to anti-CD5 mAb plus complement. We also found that
in contrast to splenic B cells, thymic B cells did not generate antibody-producing cells in the presence of antigen (SRBC) plus allogeneic MLR culture supernatant. These results indicate that direct interaction with T blasts is required for thymic B cells to proliferate and differentiate to antibody-producing cells.

Discussion

It has recently been shown that a small number of B cells are present in the thymus of normal mice (1) and humans (3). From our observations, it seems that the majority of these B cells are CD5+ (70-80% of sIgM-bearing cells in the thymus), whereas these cells are a minority in peripheral lymphoid organs. The function of CD5+ B cells present in the peritoneal cavity or spleen has been well characterized in autoimmune-prone mice, such as NZB and [NZB x NZW]F1 mice, or in normal BALB/c mice injected with LPS (4, 5). Here we have studied the function of thymic B cells in standard invitro assays of cell proliferation and differentiation. The results showed that thymic B cells did not respond well to stimulation with LPS or anti-μ plus rIL-4 (Figs. 1 and 2). The weak response that we observed could be ascribed to CD5+ B cells (Table II). This peculiar characteristic of CD5+ may only be in a population in the thymus, since CD5+ B cells in peritonium have been reported to respond to LPS stimulation (6).

![Table I: Polyclonal Ig Secretion of CD5+ and CD5- Thymic B Cells](https://example.com/table1)

| Cells             | Day 0  | Day 4  |
|-------------------|--------|--------|
| Bulk thymic B cells | 415 IgM 70 IgG | 2,750 IgM 650 IgG |
| CD5+ thymic B cells | 65 IgM 0 IgG | 75 IgM 0 IgG |
| CD5- thymic B cells | 640 IgM 55 IgG | 4,450 IgM 860 IgG |

Cells were assayed for polyclonal Ig secretion just after sorting on a FACS (see Fig. 3) or after sorting plus culture with 10 μg/ml LPS for 4 d at 10^6 cells/ml in 24-well plates.

![Figure 4: Stimulation of thymic B cells by direct interaction with MHC class II-restricted T blasts](https://example.com/figure4)

Thymic B cells were cultured at 2 x 10^6 in 24-well plates with CD4+ T blasts of 5 x 10^5 (O), or 10^5 (□) for polyclonal IgM secretion (A), or at 2 x 10^5 in 96-well plates with 3 x 10^4 (O), 10^4 (□), or 3 x 10^3 (▽) T blasts for proliferation (B). Thymic B cells cultured in the absence of T blasts (closed circles) or in the presence of LPS (open triangles, 10 and 25 μg/ml in A and B, respectively) were controls. CD4+ T blasts were Iaα specific and used for experiments after irradiation at 25 GY.
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TABLE II
Polyclonal Ig Secretion of CD5⁺ Thymic B Cells after Interaction with Ia-restricted CD4⁺ T Blasts

A number of IgM-secreting cells was assessed after 3-d culture of $2 \times 10^6$ B cells. The B cells were treated with anti-CD5 (C3PO) mAb and complement just before PFC assay. Irradiated Ia⁺-specific CD4⁺ T blast ($5 \times 10^5$), 10 μg/ml LPS, or 20% allogeneic MLR supernatant plus $2 \times 10^6$ SRBC were used for stimulation of the B cells. The viable cell recoveries after culture without stimulation or with T blasts, LPS, or allo-MLR sup + SRBC were 3 x $10^5$, 1.26 x $10^6$, and 1.24 x $10^6$ in thymic B cells, and 5.5 x $10^5$, 1.8 x $10^6$, and 1.6 x $10^6$ in splenic B cells, respectively.

| B cells from | Stimulation for B cells | Number of IgM-secreting cells/culture from treatment with: | Anti-CD5 |
|--------------|--------------------------|----------------------------------------------------------|----------|
|               |                          | None | C' alone | + C' |
| Thymus       | None                     | 35   | 30        | 10    |
|              | T blasts                 | 2,690| 2,490     | 750   |
|              | LPS                      | 670  | 645       | 590   |
|              | allo-MLR sup + SRBC      | 30   | 20        | 10    |
| Spleen       | None                     | 85   | 105       | 95    |
|              | T blasts                 | 8,900| 8,300     | 7,800 |
|              | LPS                      | 17,800| 19,600    | 17,600|
|              | allo-MLR sup + SRBC      | 2,700| 2,840     | 2,840 |

CD5⁺ B cells have been suggested to play an important role in maintaining IgM serum levels in normal mice (7). However, significant spontaneous secretion of IgM in the thymic B cells was not evident in our work (Fig. 2 and Table I), a result that is compatible with the work of Hayakawa et al. (4) using peritoneal CD5⁺ B cells. On the other hand, splenic or peritoneal CD5⁺ B cells have been reported to produce antibodies against native DNA or Bromeline-treated autologous erythrocytes when stimulated with LPS in vivo or even in vitro (5, 6, 8). This seems not to be the case in thymic B cells, since purified CD5⁺ thymic B cells cultured with LPS did not show any IgM production (Table I). In addition, no secretion of anti-ssDNA IgM antibody was detected (not shown). Possibly culture conditions such as density or time of culture explain the difference between thymic and splenic B cells. Alternatively, there is a qualitative difference in the responsiveness of splenic and thymic CD5⁺ B cells to LPS.

Interestingly, however, thymic B cells proliferated upon direct interaction with MHC class II-restricted CD4⁺ T blasts (Fig. 4). This response seems largely due to CD5⁺ B cells (Table II). In ongoing experiments, we find that thymic B cells stimulated with MHC class II-restricted T blasts are capable of producing rheumatoid factor (not shown). It has been shown that in patients with rheumatoid arthritis, CD5⁺ B cells produce rheumatoid factor (9, 10).

The actual function of CD5⁺ B cells in the thymus in situ is not clear. The findings shown here suggest that the role of thymic CD5⁺ B cells in situ may be to interact directly with developing thymic lymphoblasts, but not to respond to exogenous antigens binding to surface Ig. This possibility is now under investigation.
Summary

The function of thymic B cells in several standard in vitro assays was investigated. Thymic B cells, 75% of which were CD5+, showed a poor responsiveness to the mitogens LPS or anti-μ plus IL-4. Both proliferation and antibody formation were much lower in thymic than splenic B cell cultures. However, CD5- B cells purified using a cell sorter responded well to B cell stimulants, whereas purified CD5+ thymic B cells did not, indicating that CD5+ thymic B cells were unresponsive to B cell growth factor or LPS. Thymic B cells could be activated polyclonally by direct interaction with alloreactive T blasts, as manifested by DNA synthesis and antibody formation. These findings indicate that CD5+ thymic B cells may not be stimulated via sIg and IL-4, but require instead direct interaction with T blasts.

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References

1. Miyama-Inaba, M., S. Kuma, K. Inaba, H. Ogata, H. Iwai, R. Yasumizu, S. Muramatsu, and S. Ikehara. 1988. Unusual phenotype of B cells in the thymus of normal mice. J. Exp. Med. 168:811.
2. Inaba, K., and R. M. Steinman. 1984. Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. J. Exp. Med. 160:1717.
3. Issacson, P. G., A. J. Norton, and E. J. Davis. 1987. The human thymus contains a novel population of B lymphocytes. Lancet. ii:1488.
4. Hayakawa, K., R. R. Hardy, D. R. Parks, and L. A. Herzenberg. 1983. The "Ly-1 B" cell subpopulation in normal, immunodefective, and autoimmune mice. J. Exp. Med. 157:202.
5. Hayakawa, K., R. R. Hardy, M. Honda, L. A. Herzenberg, A. D. Steinberg, and L. A. Herzenberg. 1984. Functionally distinct lymphocytes that secrete IgM antibodies. Proc. Natl. Acad. Sci. USA. 81:2494.
6. Mercolino, T. J., L. W. Arnold, L. A. Hawkins, and G. Haughton. 1988. Normal mouse peritoneum contains a large population of Ly-1+ (CD5) B cells that recognize phosphatidyl choline. Relationship to cells that secrete hemolytic antibody specific for autologous erythrocytes. J. Exp. Med. 168:687.
7. Herzenberg, L. A., A. M. Stall, P. A. Lalor, C. Sidman, W. A. Moore, D. R. Parks, and L. A. Herzenberg. 1986. The Ly-1 B cell lineage. Immunol. Rev. 93:81.
8. Hardy, R. R., and K. Hayakawa. 1986. Development and physiology of Ly-1 B and its human homolog, Leu-1 B. Immunol. Rev. 93:53.
9. Casali, P., S. E. Burastero, M. Nakamura, G. Inghirami, and A. L. Notkins. 1987. Human lymphocytes making rheumatoid factor and antibody to ss-DNA belong to Leu-1+ B cells. Science (Wash. DC). 236:77.
10. Hardy, R. R., K. Hayakawa, M. Shimizu, K. Yamasaki, and T. Kishimoto. 1987. Rheumatoid factor secretion from human Leu-1 B cells. Science (Wash. DC). 236:81.