Brief Definitive Report

DEVELOPMENT OF Ly-1$^+$ B CELLS IN IMMUNODEFICIENT CBA/N MICE

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Developmental analyses of B cell differentiation are consistent with the existence of at least two distinct lineages. Thus, adult bone marrow solely regenerates the commonest (Ly-1$^-$) lineage, while Ly-1$^+$ B cells reconstitute the Ly-1$^+$ B cell lineage (1). CBA/N mice carrying the X-linked immunodeficiency gene (*xid*) show a defective differentiation of B lymphocytes (2). They lack all Ly-1$^+$ B cells as well as the normally predominant subpopulation within the Ly-1$^-$ lineage (3). Functional studies (4) indicated that Ly-1$^+$ B cells are responsible for the production of most of the autoantibodies, while those B cells in the Ly-1$^-$ lineage participate in the conventional responses to "foreign" antigens. These observations may account for both the protection conferred against autoimmune disease, when the *xid* genetic defect is bred into lupus-prone strains, and the CBA/N mice unresponsiveness to many bacterial antigens (5, 6).

Administration of the immunosuppressant cyclosporine A (CsA) at the time of *autologous* bone marrow reconstitution results in systemic autoimmunity in CBA/N mice. Besides, these mice show a severe diminution, if not absence, of bone marrow pre-B cells, but increased amounts of activated B cells and autoantibodies (7). We have now studied the possibility that Ly-1$^+$ B cell precursors may exist in CBA/N mice and report here experiments indicating that this is indeed the case.

Materials and Methods

Animals. CBA/N mice were kindly provided by Dr. Tjio from the National Institutes of Health, Bethesda, MD. They were maintained in conventional animal care facilities or in sterile hoods, and when submitted to experimental conditions at 6 to 12 wk of age, were given oral antibiotics.

Autoimmune Disease Induction and Cell Transfers. Adult CBA/N mice received irradiation (800 cGy) after shielding of a leg (7). They were injected intraperitoneally with CsA (kindly provided by Dr. J. F. Borel, Sandoz, Basel, Switzerland) daily at 15 mg/kg/mouse,

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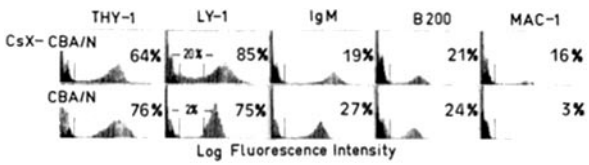

FIGURE 1. Phenotypic analysis of spleen cells from transferred CBA/N. Spleen cells from CBA/N mice were obtained 2 wk after the transfer of 5×10^6 spleen cells from either CsX-CBA/N (*top*) or CBA/N (*bottom*) mice. Cells were stained with irrelevant antibody, anti-Thy-1, FITC–anti-Ly-1, FITC–anti-IgM, anti-B220, and anti-Mac-1. Uncoupled antibodies were revealed by FITC-labeled MARK-1 antibody. Fluorescence distribution curves for the indicated antigens were superimposed on background staining profiles. Results represent the percentage of cells labeled above the threshold for positive staining indicated by the vertical bars.

continuously from the date of irradiation. Control mice were treated with CsA alone (Cs-mice) or irradiated and saline injected (X-mice), as described before (7). Here, spleens from irradiated and CsA-treated mice (thereafter referred to as CsX-CBA/N), as well as controls, were used as a source of donor cells 4–8 wk after irradiation. Recipients were unmanipulated adult CBA/N mice who were injected intravenously with 5×10^6 total spleen cells from each group of donor mice.

Antibodies. FITC- and biotin–anti-Ly-1 mAbs antibodies as well as phycoerithrin (PE)-labeled avidin were purchased from Becton Dickinson & Co., Mountain View, GA. J1j (anti-Thy-1), RA3-2C2/1 (anti-B220), and M1/70 (anti-Mac-1) are available from the American Type Culture Collection, Rockville, Bethesda, MD. Biotin–goat anti–mouse μ chain, FITC-conjugated sheep anti–mouse μ-chain and FITC-conjugated MARK-1 antibodies (anti-rat κ-chain) were the kind gift of Drs. F. Pitcheda (German Cancer Institute, Heidelberg, Federal Republic of Germany), L. Forni (Basel Institute for Immunology, Basel, Switzerland), and P. Pereira (Institut Pasteur, Paris, France), respectively.

Cell Staining and Flow-cytometry Analysis. Bone marrow, lymph nodes, and spleen cells from transferred mice were examined for surface expression of differentiation antigens after immunofluorescence staining with the indicated antibodies, as described elsewhere (7). All the procedure was performed at 4°C in PBS with 0.1% sodium azide. The frequencies of Ly-1$^+$ B cells were obtained from two-color stainings of cells with fluoresceinated anti-IgM and biotinated anti-Ly-1 antibodies plus PE. Isotype-matched antibodies of irrelevant specificity and either the fluoresceinated second-step reagent or PE were used to ensure specificity and standardize the background settings (3, 7). A similar procedure was used to define Mac-1$^+$ B cells. Immunofluorescence of 5×10^4 live cells was analyzed with an Epics-C flow cytometer, and the frequencies were estimated using the consort computer statistical package V2.2A (Coulter Electronics Inc., Hialeah, FL).

Results

Previous studies (8) used CBA/N mice as a unique "test tube" in which selective reconstitution of the defective B-cell populations by congenic, non-*xid* bearing, CBA/Ca haematopoietic precursors was demonstrated. If precursors for lymphoid lineage(s) absent in CBA/N mice were distinctively developed in the spleen of the irradiated and CsA treated CBA/N (CsX-CBA/N) mice suffering systemic autoimmunity (7), they would expand in the permissive microenvironment of unmanipulated syngeneic *xid* recipients after adoptive transfer. Such transferred mice were sequentially analyzed for the expression of markers present on the lymphocyte and myelomonocytic differentiation lineages. These studies showed that 2–3 wk after the spleen cell transfers, the numbers of Ly-1$^+$ cells in the spleen of these recipient mice, but not in control groups, greatly exceeded the numbers of Thy-1$^+$ cells (Fig. 1). In agreement, two-color stainings demonstrated a population defined as IgM$^+$ Ly-1^{dull}, accounting for most (>95%) B cells present in spleen of the transferred mice (Fig. 2).

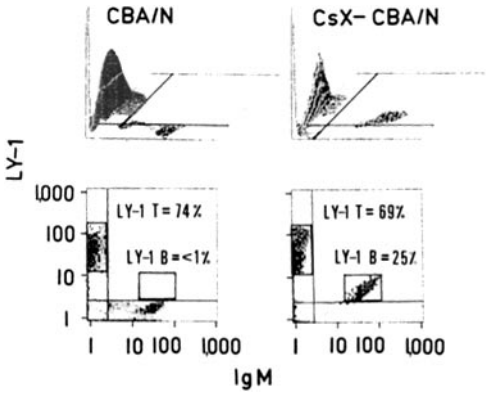

FIGURE 2. Selective expansion of Ly-1^{dull} B cells in syngeneic recipients after transfer of CsX-CBA/N mice spleen cells. Spleen cells from CBA/N mice transferred with CBA/N (*left*) or CsX-CBA/N (*right*) spleen cells were costained with FITC–anti-IgM (*green*) and anti-Ly-1 + PE-avidin (*red*). Three-dimensional diagrams represent the logarithm of green and red fluorescence along the *x*- and *y*-axis, respectively, and the number of cells along the *z*-axis. For integration and statistical study, data were displayed as two-color dot plots (*bottom*). Thresholds for positive staining are depicted by continuous lines. Rectangular gates were the boundaries used to define Ly-1^{bright} IgM$^-$ (T cells) and Ly-1^{dull} IgM^{bright} (B cells) live cells. Note B cells in recipients of CBA/N cells (22%) are IgM$^+$ but fall into Ly-1$^-$ lineage.

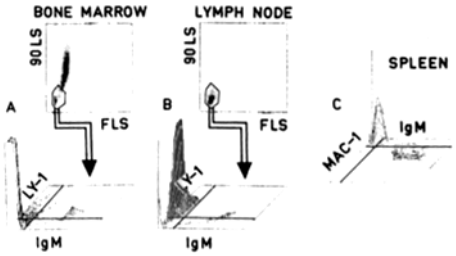

FIGURE 3. Ly-1^{dull} IgM^{bright} Mac-1$^+$ lymphocytes are the predominant B cell lineage present in the transferred mice. Bone marrow, lymph node, and spleen cells of syngeneic recipients of CsX-CBA/N spleen were costained with either FITC–anti-IgM and biotin–anti-Ly-1 + PE-avidin (*A* and *B*), or Mac-1 + FITC–MARK-1 and biotin–anti-IgM + PE-avidin (*C*). Two-parameter diagrams depict size (FLS) × wide angle (90°) distribution, and the gate set for defining lymphoid-like populations in bone marrow and lymph nodes. Analyses of two-color immunofluorescence (see Fig. 2) showed that >90% B cells in bone marrow and lymph nodes are Ly-1^{dull}, and that spleen Ly-1$^+$ B cells coexpress Mac-1 antigen. Recipients of CBA/N mice cells lack Ly-1$^+$ B cell lineage (reference 1; not shown).

Bone marrow precursors for the lymphocytes and myelomonocytes can be discriminated by means of their light scatter properties, and thus can be conveniently gated in forward light scatter (FLS) × 90° light scatter (90° LS), two-parameter, displays for an independent analysis (7). As shown in Fig. 3*A*, the studies revealed a marked depletion of cells of the lymphoid lineage in the bone marrow of transferred mice, as opposed to control groups of sham-transferred mice, revealing a striking similarity to the findings in donor mice. In agreement, pre-B cells defined as sIg$^-$ B220$^+$ precursors were again virtually absent (reference 7, data not shown). In fact, the multiparameter analyses of the residual population demonstrate that the few B cells present mostly belong to the Ly-1$^+$ lineage. Furthermore, B cells, rare (<3%) in lymph nodes from these mice, are again Ly-1$^+$ B cells (Fig. 3*B*).

The numbers of Mac-1$^+$ cells in the spleen of the recipient mice were markedly augmented when compared with the control groups (Fig. 1). Interestingly, spleen B cells from the recipient, besides being Ly-1$^+$ sIg$^+$, also coexpress the Mac-1 antigen (Fig. 3*C*). Although Mac-1 (CD11b) is currently defined as a differentiation antigen specifically expressed in myelomonocytic lineage (9), its presence in some Ly-1$^+$ pre-B and B cell lines has been reported (1).

Discussion

The most straightforward interpretation of our results would be that Ly-1$^+$ B cells, abnormally present in CsX-CBA/N mice, selectively expand in the recipient

CBA/N microenvironment. This would imply that the lack of Ly-1$^+$ B cells in immunodeficient CBA/N mice is not the consequence of a primary defect in their precursors. Rather, our findings suggest a failure, in *xid* strains, of the regulatory mechanisms allowing the initial expansion of Ly-1$^+$ B cells in neonates, and their maintenance in adult peritoneum and spleen (1). Alternatively, the existence of a suppressive mechanism, released by Cs treatment, specifically blocking Ly-1$^+$ B cell development in *xid* mice may be proposed. However, the mechanism would be expected to operate in the unmanipulated recipient CBA/N. Here, only 5×10^6 spleen cells were inoculated in a syngeneic system, making this explanation unlikely. Furthermore, recent experiments (Marcos, M. A. R., C. Martinez-A, M. L. Gaspar, et al., unpublished observations) show that transfer of thymocytes from CsX-CBA/N mice are also able to promote selective development of Ly-1$^+$ B cells in the syngeneic recipients.

Interestingly, the existence of hypercycling, T cell–mediated mechanisms in the expansion of idiotypically defined B cell subpopulations has been proposed (10). We have shown that the influence of B cells on T cell repertoires is exerted mostly in the neonatal period (11), when Ly-1$^+$ B cells are a major subpopulation (1). We therefore suggest that distinctive T cells expanded in CsX-CBA/N mice (7) would select Ly-1$^+$ B cell precursors from the differentiating pool, which in turn would then be engaged in mutual selection. In agreement with this, we have recently observed a bias of primordial T cell repertoires for interactions with B cells in the Ly-1$^+$ lineage (Marcos, M. A. R., A. de la Hera, P. Pereira, M. L. Toribio, A. Coutinho, and C. Martinez-A, manuscript submitted for publication) which may contribute to the preferential expansion of this subset.

Primary immunodeficiency defects in CBA/N mice have formerly been placed at the B cell level. Novel formulations of B cell development pathways should take into consideration present evidence for interplay among T and B lymphocyte subpopulations within the two lineages of differentiation (10–12). Besides, the differentiation of common B cell precursors in CBA/N mice shows a strong T cell dependence (13–15). Thus, in addition to the defects of the major subpopulation I spontaneously caused by *xid* genes (3), the alterations in intrathymic development present in CsX-CBA/N and transferred mice (7) may contribute to the paucity of B cells observed in the major (Ly-1$^-$) lineage. The expansion of formerly absent Ly-1$^+$ B cells with depletion of common B cells are not the unique phenomena occurring in CBA/N recipients. They display a systemic autoimmune disease indistinguishable from that previously reported in the donors (reference 7, Marcos, M. A. R., M. L. Gaspar, C. Marquez, et al., manuscript in preparation). Similarly, inadequate expansion of Leu-1$^+$ (CD5) B cells has recently been implicated in the pathogeny of certain human diseases (1). It suggests that direct interactions among lymphocytes in distinct lineages might play an essential role in the physiology and pathology of the immune system.

Summary

Spleen cells from CBA/N mice developing a systemic autoimmune disease after daily injection of CsA during an autologous bone marrow reconstitution were transferred into unmanipulated syngeneic recipients. Adoptive transfer allowed the development of Ly-1$^+$ B cells, which shared Mac-1 differentiation

antigen expression with the myelomonocytic lineage. Interestingly, expansion of formerly absent Ly-1$^+$ B cells was paralleled by a severe reduction in common, Ly-1$^-$, B cell development in the recipient. We conclude that precursors for Ly-1$^+$ B lineage do exist in CBA/N mice.

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