Brief Definitive Report

IDENTIFICATION, PURIFICATION, AND CHARACTERIZATION OF ANTIGEN-ACTIVATED AND ANTIGEN-SPECIFIC HUMAN B LYMPHOCYTES

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The activation of resting B lymphocytes and their transition to immunoglobulin (Ig)-secreting cells requires a complex series of regulatory signals. It has been demonstrated in animal as well as human models of B cell activation that small resting B cells can be triggered in a polyclonal manner by modulation of the surface membrane Ig (sIg) of the B cell by anti-Ig or other Ig-cross-linking substances such as *Staphylococcus aureus* Cowan strain I (SAC) (1, 2). These activated cells then increase in size (3) and become sensitive to proliferative signals from T cell-derived B cell growth factors (BCGF) (4). Subsequently, other T cell-derived differentiation factors can induce these activated and proliferating cells to terminally differentiate into Ig-secreting cells (2, 5–7). Precise study of antigen-specific human B cells has been hampered by the low precursor frequency of these cells in the peripheral blood B cell repertoire. In the present study, we have immunized normal subjects in vivo with a specific antigen, pneumococcal polysaccharide (PPS), and have noted a substantial but temporally restricted enrichment in the peripheral blood of activated PPS-specific B cells (8). This has allowed us to identify, purify, and characterize these activated antigen-binding B cells (ABC).

Materials and Methods

Immunizations. Normal volunteers received 0.5 cc of the 14 valent PPS vaccine (Pneumovax; Lederle Laboratories, Pearl River, NY).

Cell Separations. Buffy coats from 2 U of blood were obtained via standard blood bank procedures, and the mononuclear cell population was separated via Ficoll-Hypaque density centrifugation. T cells were depleted by rosetting with 2-aminoethylisothiorouronium bromide-treated sheep erythrocytes (9). The B cell- and monocyte-enriched population was depleted of monocytes by passage over a G-10 column (10). The resultant B cells were separated into ABC and non-ABC by rosetting with ox erythrocytes coated with PPS vaccine and by Ficoll-Hypaque density centrifugation. The ox erythrocytes were coated with PPS via the chromic chloride method. The Ficoll-Hypaque density centrifugation was repeated as needed to increase the purity to 60–70% rosette positive. Unless otherwise stated, all cells were obtained 7 or 8 d after immunization.

Monoclonal Antibodies. Mouse anti-human IgM, IgD, and IgG monoclonal antibodies were obtained from Bethesda Research Laboratories, Gaithersburg, MD and N. L. Cappel Laboratories, Cochranville, PA. The 4F2 monoclonal antibody was prepared as previously described (11).

Cell Size. Cell volume was quantitated with a Coulter channelizer (Coulter Electrics Inc., Hialeah, FL).

Cell Surface Isotype. 1×10^6 B cells were incubated with a 1:50 dilution of various monoclonal

antibodies or a control supernatant for 30 min at 4°C and then washed twice with phosphate-buffered saline (PBS). Subsequently, PPS-coated ox erythrocytes were added and the number of rosettes was scored after staining with 50 μg of acridine orange to produce nuclear fluorescence.

4F2 Immunofluorescent Staining. 1×10^6 cells were stained with 5 μg of purified 4F2 antibody, washed twice in PBS, and stained with a 1:50 dilution of an affinity-purified goat anti-mouse IgG. After two more washes, the cells were examined with the fluorescence-activated cell sorter (FACS) (B-D FACS Systems, Becton, Dickinson & Co., Sunnyvale, CA).

Cell Culture and Assays. 25,000 ABC were cultured in 200 μl of RPMI 1640 containing 10% fetal calf serum (Dutchland Laboratories, Inc., Denver, PA) supplemented with 20 μg/ml of gentamycin in round-bottomed microtiter plates (Linbro; Flow Laboratories, Inc., Rockville, MD). Some cultures contained $F(ab')_2$ fragment goat anti-human IgM antibody (50 μg/ml) purchased from N. L. Cappel Laboratories, SAC, prepared and used as previously described (2), 25% BCGF derived from the human T-T hybridoma $2B_{11}$ as previously described (12), or a combination of the above. Cultures were incubated in 100% humidity in 5% CO_2 at 37°C. Cultures were pulsed with 1 μCi of [^{3}H]thymidine over the last 16 h of a 3-d culture. Incorporation of [^{3}H]thymidine was measured by standard liquid scintillation counting techniques after harvesting by a Titertek cell harvestor (Flow Laboratories, Inc.). Plaque-forming cell (PFC) and ABC assays were performed as previously described (8).

Results and Discussion

Young adult normal volunteers were immunized with the PPS vaccine. 8 d after immunization, 5–20% of the circulating purified B cell pool were demonstrated to specifically bind to the immunizing antigen by forming rosettes with PPS-coated ox erythrocytes, and 0.5–2.0% of B cells spontaneously secreted anti-PPS antibody as detected in a PPS-specific hemolysis-in-gel PFC assay. Before immunization, there were no detectable spontaneous anti-PPS PFC and <1 ABC per 1,000 B lymphocytes. In addition, PPS in vitro could not stimulate human B cells to proliferate or differentiate either before or after in vivo immunization (8). Separation of the ABC from B cells that did not bind antigen was performed by density gradient centrifugation of the rosetted cells, and cell volume profiles were determined (Fig. 1). The ABC were shown to be large cells with an average cell volume of 320 μm^3. Morphologically, these cells have large nuclei with dark blue cytoplasms, and an occasional

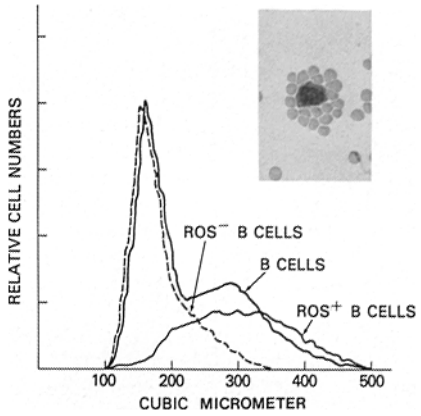

FIG. 1. Volume distributions of various lymphocyte cell populations and morphology of ABC. Purified B cells were separated into rosette-positive and rosette-negative cells, and the cell volume profiles were determined. The inset is a photomicrograph of a representative ABC.

cell was noted to be in mitosis (Fig. 1, inset). Thus, the size and morphology of these cells suggested that they had already been activated in vivo (13).

Next, the sIg isotypes of the ABC were examined by blocking the formation of rosettes with isotype-specific monoclonal antibodies (14). Two different monoclonal antibodies to IgD did not inhibit the formation of ABC. Of the expected number of ABC, 36 ± 15% were blocked by a monoclonal antibody to IgM, and 84 ± 5% were blocked by a monoclonal antibody to IgG. The addition of the IgM- and IgD-specific monoclonal antibodies resulted in inhibition similar to that found with the IgM monoclonal antibody alone. Thus, the majority of ABC were sIgG positive and sIgD negative. Furthermore, IgG was the predominant isotype spontaneously synthesized in vitro when these activated cells were placed in culture. Thus, the isotype expression of the ABC was also consistent with that of a preactivated cell (15).

We have described a mouse monoclonal antibody 4F2 which recognizes non-HLA, non-Ia cell surface determinants on human monocytes, but not on resting human B or T lymphocytes (11). However, the 4F2 antigen is expressed on lectin or alloactivated T lymphocytes as well as all dividing human T and B lymphoblastoid cell lines tested (11, 16). Thus, it has served as an excellent marker of activated human lymphocytes and can be used to distinguish a resting from an activated cell. Furthermore, 4F2 has been reported to recognize different molecular forms on human T and B lymphoblastoid cell lines (17).

The binding of 4F2 of ABC was demonstrated by immunofluorescently staining a

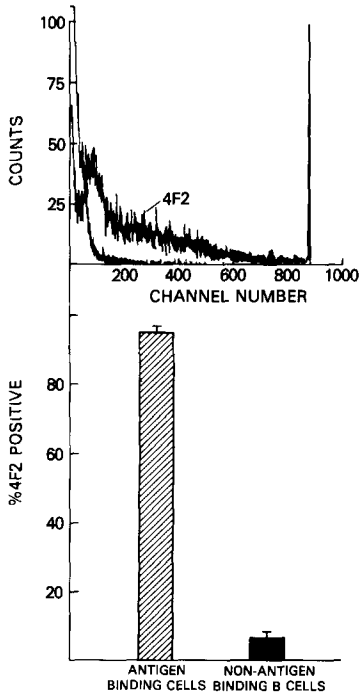

FIG. 2. Expression of an antigen recognized by the monoclonal antibody 4F2. In the upper portion of the figure, purified ABC were immunofluorescently stained with 4F2 and examined with the FACS. In the lower portion, B cells isolated from four individuals were similarly stained and then rosetted with PPS-coated ox erythrocytes and finally examined with a fluorescent microscope.

purified B cell population from a recently immunized subject followed by rosetting of the cells with PPS-coated ox erythrocytes (Fig. 2). 4F2-positive cells were demonstrated virtually exclusively in the ABC fraction with only a rare 4F2-positive cell found in the non-ABC fraction. Confirmation of the binding of 4F2 to ABC is shown in the FACS pattern (Fig. 2).

Given the fact that the ABC are activated, it is likely that they received an activation signal in vivo by modulation of their sIg with PPS antigen. In our recently described in vitro model of B cell activation (4), resting human B cells could be activated to proliferate in response to BCGF by prior stimulation with anti-μ or SAC (presumably by inducing the expression of BCGF receptors). In contrast, large activated cells could respond directly to BCGF, indicating that they already expressed receptors for the factor and could not be further activated by anti-μ (4).

In the present study, the ABC did not respond to signals capable of inducing proliferation of resting B cells, but they could respond directly to BCGF by increased proliferation. As shown in Fig. 3, when a monoclonal BCGF derived from a human T-T hybridoma ($2B_{11}$) (12) was added to cultures of ABC, high levels of tritiated thymidine incorporation were noted that were not further enhanced by the addition of anti-μ or SAC. As also noted in Fig. 3, the PPS-specific ABC manifested a relatively high background proliferation. This likely represented low levels of spontaneous proliferation of the ABC and was unrelated to the rosetting procedures required for isolation of the ABC (data not shown). Thus, the ABC, which are refractory to anti-μ and SAC but responsive to BCGF alone, differ from resting B cells, which respond poorly if at all to BCGF alone and show a substantial synergistic proliferative response to anti-μ plus BCGF.

These findings in an antigen-specific system can be integrated into the model of human B cell activation, proliferation, and differentiation. In this model, the initial signal is delivered in vivo by the immunizing antigen. Antigen-specific B cell activation occurs and is accompanied by cell enlargement and expression of the 4F2 activation

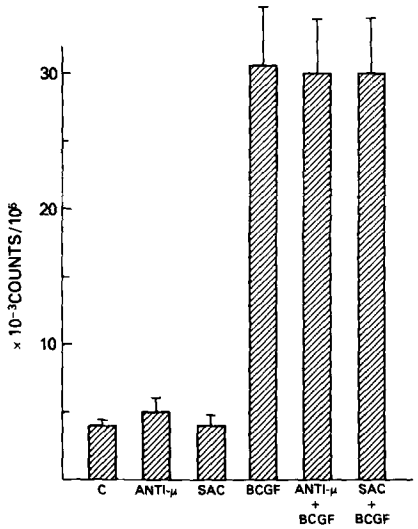

FIG. 3. Response to anti-μ, SAC, and BCGF. Induction of tritiated thymidine incorporation of ABC from two individuals.

antigen together with receptors for BCGF. At this point, the cell does not become further activated upon stimulation with anti-μ or specific antigen; however, it proliferates briskly to direct stimulation with BCGF. Further differentiation is manifested by continued proliferation and the synthesis and secretion of specific antibody.

Summary

Activated pneumococcal polysaccharide (PPS)-specific human B lymphocytes have been purified and examined in vitro. They are large cells that are refractory to further activation by antigen, anti-Ig, or *Staphylococcus aureus* Cowan strain I, but they respond directly by proliferation to B cell growth factors. In addition, they express the isotype pattern of activated cells and a surface marker of activated lymphocytes called 4F2. Finally, they contain a subset of cells that spontaneously secrete PPS-specific antibody.

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