Brief Definitive Report

DETECTION AND FUNCTIONAL STUDIES OF p60-65 (TAC ANTIGEN) ON ACTIVATED HUMAN B CELLS

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The proliferation and differentiation of B cells have been shown to be under the influence of many factors. In particular, interleukin 1 (IL-1) and B cell–stimulatory factor (BSF) have been shown to play significant roles in the activation of B cells (1). Although interleukin 2 (IL-2 or T cell growth factor) is important in T cell proliferation, its role in B cell activation remains uncertain. Some investigators suggest that IL-2 might be directly involved in B cell activation (2, 3), while others (4, 5) indicate that its action on B cells is indirect, inducing T cells to secrete BSF and other helper factors.

Uchiyama et al. (6) produced a monoclonal antibody, anti-Tac. This antibody was shown to react with the IL-2 receptor (7). In the present study, we established a monoclonal antibody, AT-1, with reactivities similar to anti-Tac. With AT-1, activated normal human B cells were shown to express IL-2 receptors. These activated B cells proliferated in the presence of recombinant IL-2 (rIL-2).

Materials and Methods

Cell Preparation. Peripheral blood mononuclear cells were isolated from leukocyte concentrates from normal donors, and tonsillar single-cell preparations were made from tonsils removed from patients undergoing tonsillectomy. Separation of sheep erythrocyte (SRBC) rosette-forming cells and monocytes from B cells was done as described (8). The resulting non-T cells from peripheral blood contained 60–80% membrane Ig $(mIg)^+$ cells with <0.5% T cells. The resulting non-T cells from tonsils were 90–95% mIg^+ with <0.5% T cells. For simplicity, these non-T preparations will be referred to as B cells.

B Cell Activation. Affinity-purified $F(ab)'_2$ rabbit anti-IgM antibodies were prepared as described (8). 10–25 μg/ml of this preparation, which was used in the majority of the experiments, did not give substantial stimulation of resting B cells. rIL-2 was purchased from Genzyme, Boston, MA. Lymphokine preparations were either purchased from Electro-Nucleonics, Inc., Silver Springs, MD or prepared in our laboratory. In the latter case, pooled mononuclear cells were stimulated with 10 μg/ml PHA-P (phytohemagglutinin) (Difco Laboratories, Inc., Detroit, MI) and 1% pokeweed mitogen (PWM) (Gibco Laboratories, Grand Island, NY). After 48 h, the supernatant was subjected to $(NH_4)_2SO_4$ precipitation at 30–80% saturation. The precipitate was dissolved in 0.1 M phosphate buffer, pH 7.4. After dialysis against this buffer, the dissolved proteins were fractionated on a DE52 column with a salt gradient (0–0.3 M NaCl). Fractions containing B cell stimulatory activity were pooled. This pooled preparation contained IL-2 and B cell differentiation factor(s) (termed CM-BSF). For B cell activation, 10% CM-BSF was used in conjunction with anti-IgM antibodies. Other mitogens were used at 0.005% vol/vol, for formalin-treated *Staphylococcus aureus* (a gift from Dr. S. Pahwa, North Shore Hospital,

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Manhasett, NY), 20% vol/vol with B95-8 supernatant as a source of Epstein-Barr virus (EBV), and 1% vol/vol for PWM.

Monoclonal Antibody Production. BC_3F_1 females were immunized with PHA-activated T cells. Their spleen cells were fused with SP2/0 tumor cells. Hybridoma supernatants were screened for their binding activity and blocking activity of T cell proliferation. The desired hybridomas were cloned on soft agar. Details of these procedures have been described previously (9).

Immunofluorescence Studies. Activated B cells were analyzed for IL-2 receptor expression by two-color fluorescence microscopy. Activated cells were stained with AT-1 at 4°C for 30 min. After extensive washings, the cells were stained for 30 min, with a fluorescein isothiocyanate–conjugated goat anti-mouse Ig to identify the AT-1$^+$ cells and rhodamine-conjugated anti-human IgM to identify B cells. After further washings, slides were made for fluorescence microscopy. For certain experiments involving only one color, the cells were analyzed with a Coulter Epics V flow cytometer (Coulter Electronics, Hialeah, FL). Integrated fluorescence of the gated population was measured and 10,000 cells were analyzed.

Other Assays. Lymphocyte proliferation assays and reverse plaque assays were performed as described previously (10). For cell iodination and labeling with [^{35}S]methionine as well as immunoprecipitation and autoradiography, they were performed as described (11, 12).

Results

Identification of p60-65 (Tac Antigen) by Monoclonal Antibody AT-1. Two monoclonal antibodies against activated human T cells were found to precipitate a p60-65 molecule from activated T cells. One of these, AT-1, an IgG1 antibody, was studied in detail. It inhibited mitogen- and alloantigen-induced T cell proliferation as well as IL-2-dependent proliferation of T blasts. Immunoprecipitation revealed that AT-1 and anti-Tac, kindly provided by Dr. T. Waldmann and Dr. T. Uchiyama, precipitated similar p60-65 molecules on activated T cells (Fig. 1, lanes *1* and *2*). After clearance with anti-Tac, either AT-1 or anti-Tac did not precipitate any molecule from the lysate of activated T cells (Fig. 1, lanes *3* and *4*). The reciprocal experiment showed similar results. Thus, AT-1 and anti-Tac bound to an identical protein on activated T cells.

Immunofluorescence and Biosynthetic Studies of p60-65 Expression on Activated B Cells. To define the reactivity of AT-1, a variety of target cells were used in immunofluorescence studies. AT-1 readily stained B blasts generated by anti-IgM antibody activation and CM-BSF. The cell preparation was shown to be free of SRBC rosette-forming cells (T cells). To show that the p60-65 molecule was synthesized by the activated B cells, we incubated them for 16 h with [^{35}S]-methionine. A p60-65 molecule was precipitated by AT-1 from the cell lysate of these B blasts (Fig. 2, lane *4*). For comparison, the p60-65 molecule precipitated from PHA-activated T blasts is shown on lane *2* of Fig. 2.

The kinetics of p60-65 expression by B blasts stimulated by anti-IgM and CM-BSF were studied (Fig. 3). Resting B cells did not stain for IL-2 receptors (Fig. 3*A*). As early as 12 h after activation, a minor population of B cells stained by AT-1 (Fig. 3*B*). By 60 h, >50% of the B blasts were positive. Further incubation did not increase the positivity of the B blasts. At these time intervals, the activated B blasts were shown to be free of T cells and stained for membrane IgM.

The expression of p60-65 by B blasts was not dependent on the mode of activation. By two-color fluorochrome analysis, tonsillar B cells activated by three different mitogens were able to express IL-2 receptors (Table I). It is of interest

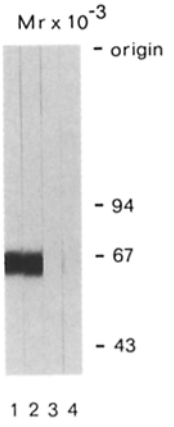

FIGURE 1

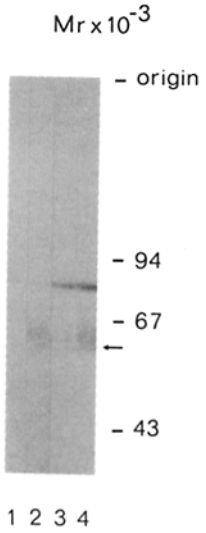

FIGURE 2

FIGURE 1. Immunoprecipitation of p60-65 by AT-1 and anti-Tac. I^{125}-labeled polypeptides precipitated by AT-1 (lane *1*) and by anti-Tac (lane *2*). No precipitated peptides were detected by either AT-1 (lane *3*) or anti-Tac (lane *4*) after the cell lysate was cleared with anti-Tac.
FIGURE 2. p60-65 molecules from activated T and B lymphocytes. 1×10^7 activated T lymphocytes and 2×10^7 activated B blasts were labeled with [^{35}S]methionine. Lysates were immunoprecipitated with control antibody and AT-1. No proteins were precipitated by the control antibody (lane *1* for T blasts and lane *3* for B blasts). p60-65 molecules were brought down by AT-1 from lysates of T blasts (lane *2*) and B blasts (lane *4*).

to note that the maximal expression was seen at day 5 of activation when formalinized staphylococci and EBV were used as activators. Peripheral blood B cells were also able to express IL-2 receptors when stimulated with anti-IgM and CM-BSF or with PWM in the presence of T cells.

Effect of rIL-2 on Activated B Cells. rIL-2 was assayed with PHA-stimulated T blasts. Maximum stimulation was obtained at 250–500 U/ml as defined by the manufacturer. At 250 U/ml, rIL-2 did not stimulate tonsillar B cells to proliferate (376 vs. 846 cpm for medium control). With anti-IgM at 10 μg/ml, rIL-2 showed minimal effect (706 cpm). With this dose of anti-IgM and 10% CM-BSF, significant proliferation was detected (11,117 cpm). Repeated experiments showed similar results, indicating rIL-2 had no effect on resting B cells.

To determine the effect of rIL-2 on activated B cells, tonsillar B cells were first activated with anti-IgM and CM-BSF for 3 d. The activated B cells were further treated with SRBC and Ficoll-Hypaque gradient sedimentation to deplete possible contaminating T cells. The remaining B blasts were cultured in the presence of rIL-2 and assayed for proliferation at various time intervals (Exp. 1, Table II). Significant proliferation was seen in the presence of rIL-2 at all time intervals. No SRBC rosette-forming cells were detected after rIL-2 induced proliferation in these cultures. Similar results were obtained in experiment 2. In general, rIL-2 induced less vigorous proliferation than CM-BSF.

The effects of rIL-2 on B cell differentiation to Ig-secreting cells or plaque-forming cells (PFC) were also investigated. The addition of 10% CM-BSF to

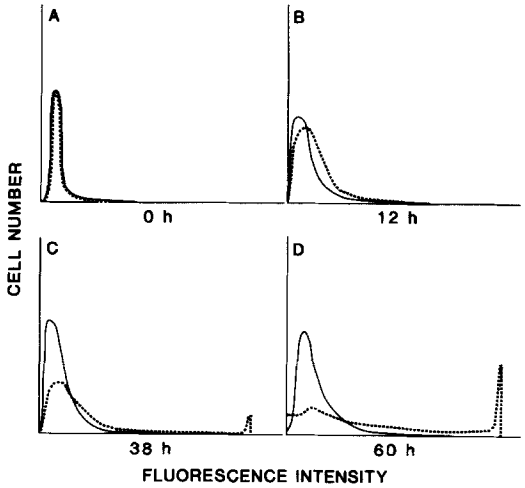

FIGURE 3. Cytofluorometric analysis of IL-2 receptor expression by human tonsillar B cells activated by anti-IgM and CM-BSF: (——) staining with control antibody, and (---) staining with antibody AT-1.

TABLE I

Expression of IL-2 Receptors on B Cells Activated by Various Mitogens

Cell source	Stimulation	Time of examination after activation	
		Day 3	Day 5
		%	
Tonsil B cells	Anti-IgM (25 μg/ml) + 10% CM-BSF	45.4 (35.4–50.1)	ND
	S. aureus	31.1 (21.8–48.5)	47.4 (35–59.7)
	EBV	6.9 (1.5–16.7)	42.8 (38.2–49.5)
Mononuclear cells	PWM	33.9 (17.1–46.5)	ND
PBL B cells	Anti-IgM (25 μg/ml) + CM-BSF	50.2 (39.8–57.2)	ND

ND, not done; PBL, peripheral blood leukocytes.

TABLE II

Effect of rIL-2 on Activated B Cells

Experiment	Time	Agents added	
		Medium	rIL-2 (500 U/ml)
	h	*cpm*	
1	24	8,988	18,329
	48	1,221	10,503
	60	1,332	8,561
		rIL-2 added	*cpm*
		U/ml	
2	24	0	1,752
		100	4,287
		200	6,702
		500	5,605
		10% CM-BSF	20,690

Tonsillar B cells were activated with rabbit anti-IgM (25 μg/ml) with 10% CM-BSF for 3 d. Cells were then washed three times and cell concentration adjusted to 10^6/ml. 10^5 cells/well were cultured with rIL-2 added at the indicated concentrations and cells harvested at the indicated time. No T cells were detectable at the beginning and the end of incubation period.

formalinized staphylococcus–stimulated B blasts induced 1,640 PFC/10^6, while 80 PFC/10^6 were detected in the control culture. Addition of rIL-2 to the activated B blasts did not increase PFC significantly (145 PFC/10^6 with 200 U rIL-2/ml). These results were confirmed in two additional experiments. Attempts to demonstrate synergy between a low dose of CM-BSF and rIL-2 gave varied results. In the best experiment, a twofold increase in PFC was seen when rIL-2 was added to a culture containing a suboptimal dose of CM-BSF.

Discussion

In the present investigation, monoclonal antibody AT-1 is shown to identify the putative IL-2 receptor (p60-65). The molecular weight was higher than that reported previously (7). The receptor of lower molecular weight was due to the expression of an aberrant receptor on HUT-102 cells (13). With this antibody, we have demonstrated the expression of IL-2 receptors by activated B cells by immunofluorescence and biosynthetic studies. Great care has been taken to ensure that our B blast preparations were devoid of T cells. The expression of IL-2 receptors by activated B cells is thus firmly established. Similar findings have been reported by others in a preliminary communication (14).

rIL-2 is shown in this study to induce B blast proliferation. It appears that three distinct interleukins (IL-1, IL-2, and BSF) play significant roles in B cell proliferation. They act at various times during B cell activation. BSF acts at 4–6 h, IL-1 at 16–20 h (1), and IL-2 at 2–3 d, after activation. The contributions of these factors to B cell activation in vivo remain to be determined.

The effect of IL-2 on the differentiation of B blasts is less clear. It can be concluded that IL-2 by itself induces little differentiation when added to activated blasts. Whether IL-2 can act synergistically with other helper factors on B cell differentiation needs to be resolved. This cannot be done unless various purified factors are available.

Relevant to this discussion is our recent finding in a child with severe combined immunodeficiency. The child had IgM^+ B cells. These B cells expressed IL-2 receptors upon activation by anti-IgM and B cell stimulation factors. However, the activated B cells failed to proliferate when IL-2 was added to the culture. These cells were also unable to differentiate in the presence of B cell differentiation factors. The failure to respond to IL-2 by IL-2 receptor–positive B blasts and the associated deficiency in this patient adds support to the hypothesis that IL-2 plays significant roles in normal B cell activation and differentiation.

Summary

A monoclonal antibody, AT-1, is shown to precipitate a p60-65 molecule identical to the Tac antigen. With AT-1, the expression of IL-2 receptors by normal activated human B cells from peripheral blood and tonsils is documented by biosynthetic and immunofluorescence studies. AT-1 precipitated a p60-65 protein from [^{35}S]methionine-labeled activated B cells, similar to that from activated T cells. The interleukin 2 (IL-2) receptor appeared shortly after activation with anti-IgM and B cell–stimulatory factor(s). Its expression reached its peak at 60–72 h with ~50% of the B blasts stained by AT-1. Other modes of activation of B cells, by T cell–independent, formalin-treated staphylococci and

Epstein-Barr virus, and by T cell–dependent pokeweed mitogen, also induced IL-2 receptor expression. The functional significance of this finding was investigated using recombinant IL-2 (rIL-2). While rIL-2 did not induce resting B cells to proliferate in the presence of anti-IgM, it induced activated B cells to proliferate in the absence of other factors. On the other hand, rIL-2 did not induce the differentiation of these activated B lymphocytes. These data suggest that IL-2 may play a significant role in B cell activation.

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