ACTIVATED B CELLS EXPRESS RECEPTORS FOR, AND PROLIFERATE IN RESPONSE TO, PURE INTERLEUKIN 2

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Interleukin 2 (IL-2)[1] was discovered through its function as a T cell growth factor (TCGF) (1, 2). Indeed, the availability of T cell lines for the quantitative measurement of TCGF activity has been crucial for the characterization of IL-2 (3, 4) and IL-2 receptors on activated T cells (5–7) in different species. Human IL-2, which acts on murine and human T cells (4, 8, 9), can now rapidly be purified to apparent homogeneity with monoclonal antibodies (8, 9) and its cDNA has been cloned (10, 11). Human IL-2 is a T cell–generated 15 kilodalton peptide hormone of 133 amino acids.

Although it was observed in several studies (3, 4, 12–14) that IL-2-rich T cell supernatants (SN) enhance the generation of plaque-forming cells (PFC) in certain B cell culture systems, it is widely believed that B cells do not directly respond to IL-2. Thus, a variety of other T cell–derived factors (but not IL-2) were reported to enhance the proliferation of B cells activated by either T-independent antigens (15), dextran (16), lipopolysaccharide (LPS) (17, 18), antiimmunoglobulin antibodies (anti-Ig) (16, 19–21), or other means (22). In one study (23), showing an effect of immunoaffinity-purified IL-2 on anti-Ig–induced B cell proliferation, the interpretation of the results was complicated because it was also observed that T cells participated in the B cell response. LPS-activated murine B cells were not found to bind radiolabeled IL-2 (5) but, in another study (7), such LPS blasts reacted weakly with an anti-IL-2 receptor monoclonal antibody.

However, there still exist controversies with regard to the activation signals that B cells require to respond to growth factors (24). A recent study (25) showed in a limit-dilution culture system for murine B cells that, in fact, either a cell contact–dependent T-B cell interaction or LPS was required in conjunction with anti-Ig for optimal induction of growth factor responsiveness. Whereas anti-Ig

[1] Abbreviations used in this paper: BCGF, B cell growth factor; Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; IL-1, interleukin 1; IL-2, interleukin 2; LPS, Escherichia coli lipopolysaccharide; MLC SN, secondary in vitro mixed leukocyte culture supernatant; PFC, plaque-forming cells; PHA, phytohemagglutinin; sIg, surface immunoglobulin; SN, culture supernatant; TCGF, T cell growth factor.

alone had no significant effect, B cells activated by LPS and anti-Ig together proliferated 10-fold better in response to T cell SN than B cells activated by LPS alone (25). Those results suggest that some previously reported B cell growth factors (BCGF) could have (directly or via other cells) participated in B cell activation but may be different from the essential growth factors acting on activated B cells.

In this study it was investigated whether IL-2 acts on B cell proliferation and whether activated B cells express IL-2 receptors. The results demonstrated (a) that either recombinant or immunoaffinity-purified human IL-2 stimulated the proliferation of LPS plus anti-Ig–activated purified murine B cells to the same extent as various T cell and spleen cell SN, (b) that very similar IL-2 concentrations were required for B vs. T cell proliferation, and (c) that LPS plus anti-Ig–activated B cells expressed IL-2 receptors.

Materials and Methods

Mitogens, Anti-Ig Antibodies. LPS (Westphal) from *Eschericha coli* 055:B5 was obtained from Difco Laboratories, Inc., Detroit, MI. Concanavalin A (Con A), phytohemagglutinin (PHA), and leukoagglutinin were obtained from Pharmacia, Inc., Uppsala, Sweden. Affinity-purified (on mouse Ig-Sepharose) rabbit anti-mouse Ig antibodies were prepared from hyperimmune polyvalent rabbit anti-mouse Ig antiserum and coupled to cyanogen bromide–activated Sepharose 4B (Pharmacia, Inc.) at 1 mg protein/ml of packed beads as described (25).

Culture Media, General Culture Methods. Cell cultures were performed in Dulbecco's modified Eagle's medium (DME) supplemented with additional amino acids and including 10 mM Hepes, penicillin-streptomycin, and 5×10^{-5} M 2-mercaptoethanol (26). Fetal calf serum (FCS) (lot 106183) was obtained from Seromed, Munich, Federal Republic of Germany. Cultures of 1 ml were performed in 24-well plates, cultures of 200 or 150 μl cultures in 96-well flat-bottomed plates (Costar, Data Packaging, Cambridge, MA).

Preparation and Immunohistochemical Analysis of B Cells. Surface Ig (sIg)-positive cells were obtained from C57BL/6 spleen cells by the panning method of Mage et al. (27). The efficiency of this method was checked by the quantitation of sIg^+ and $Thy-1^+$ cells on acetone-fixed cytocentrifuge smears using biotinylated horse anti-mouse Ig antibody (Vector Laboratories, Inc., Burlingame, CA) or biotinylated monoclonal anti-Thy-1 antibody (AT-15, provided by Dr. F. Fitch, University of Chicago) together with avidin-peroxidase conjugate (Vector Laboratories, Inc.) and hematoxylin counterstaining. 3,000 cells were scored. Freshly prepared B cells contained >97% sIg^+ cells and <0.3% $Thy-1^+$ cells. Control cells (Con A–stimulated thymocytes) were >98% $Thy-1^+$. In addition, it was found that B cells cultured during 4 d in the presence of LPS, anti-Ig, and EL-4 SN (see below) contained >99% Ig-positive cells and <0.2% $Thy-1^+$ cells.

Murine B Blast Assay for B Cell Growth Factor. To obtain activated B cells, 3×10^5 sIg^+ cells were cultured during 3 d in 1-ml cultures in the presence of 5 μg/ml of LPS, 10 μg/ml of Sepharose–anti-mouse Ig, and 10% FCS, followed by removal of anti-Ig beads from the culture suspension by sedimentation (10 min at 1 g), centrifugation of the cells, and resuspension in fresh DME. Assay cultures (150 μl, triplicates) were set up with 10^4 activated B cells, 10% FCS, and dilutions of tested supernatants. Thymidine incorporation was measured 3 d later (by standard procedure, using an automated cell harvester), after a 6-h pulse with 1 μCi of [³H]thymidine (Radiochemical Centre, Amersham, England) added in 50 μl of DME.

PFC Assay. PFC were measured using protein A (Pharmacia, Inc.)–coupled sheep erythrocytes (29) and a slide modification of the plaque assay (30) as described (26).

Analysis and Separation of Cells with the Fluorescence-activated Cell Sorter (FACS). A FACS II (Beckton, Dickinson & Co., Mountain View, CA) was used in conjunction with

monoclonal anti-Thy-1 and anti-Lyt-2 antibodies coupled to biotin, with fluoresceinated avidin as a second reagent as described (31, 32).

IL-2 Assay Using CTLL Cells. IL-2 biologic activity was determined by the IL-2-dependent stimulation of proliferation of the CTLL-2 murine cytotoxic T lymphocyte line (8). CTLL cells (4 × 10^3/culture of 200 μl) were cultured during 24 h, in the presence of DME–5% FCS and serial dilutions of tested SN. During the last 5 h, the cultures were pulsed with 1 μCi of [^{3}H]thymidine. The IL-2 concentration leading to 50% maximal thymidine incorporation was assigned a value of 1 U/ml. Thus a sample containing 100 U IL-2/ml would give 50% maximal stimulation at a dilution of 1:100 in the assay.

Immunoaffinity-purified Human IL-2. IL-2-containing SN were obtained from a subclone (6.8 b) of the IL-2 high-producer Jurkat 6.8 human T leukemia cell line in the absence of serum and the IL-2 purified on an immunosorbent column as described (8). Affi-gel 10 beads coupled with DMS-3 monoclonal anti-IL-2 antibodies were provided by Dr. K. A. Smith, Dartmouth, NH. The specific activity of the affinity-purified IL-2 was 1 U (see CTLL assay) per 0.3 ng protein. A single stained band of 15,000 mol wt was obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Recombinant human IL-2. The construction, cloning, and screening of a human IL-2 cDNA plasmid made from polyadenylated RNA isolated from PHA-stimulated human spleen cells and its expression in *E. coli* has been described (11). *E. coli*-derived recombinant IL-2 was purified to apparent homogeneity according to SDS-PAGE and freed of endotoxin (methods from Biogen SA, Geneva, Switzerland). The specific activity of the recombinant IL-2 was also 1 U/0.3 ng protein.

[^{3}H]IL-2-binding Assay. Biosynthetically radiolabeled IL-2 was prepared (5). Briefly, Jurkat cells were cultured in the presence of DME with 40 μM leucine and lysine (i.e., 5% of the levels in regular DME) and 10 mCi of both [^{3}H]leucine (160 Ci/mM; Amersham Corp., Arlington Heights, IL) and [^{3}H]lysine (85 Ci/mM; Amersham Corp.).[2] The radiolabeled IL-2 was immunoaffinity purified (8), tested for biological activity, and adjusted to 500 cpm/U IL-2 (25,000 cpm/pmol). The cell-binding assay was performed as described by Robb et al. (5). The LPS plus anti-Ig–activated B cells to be tested were obtained as follows. Cultures (250 μl) were set up with 3 × 10^4 C57BL/6 sIg$^+$ cells, 50 μg/ml LPS, 10 μg/ml Sepharose-anti-Ig, and 20% EL-4 SN (see below) in DME–10% FCS (using four complete 96-well plates). After 4 d, the cells were harvested and the anti-Ig beads removed, first by sedimentation (10 min at 1 g), and the remaining beads (~5%) by centrifugation (10 min at 850 g) of the cells (15 × 10^6 cells in 5 ml DME) on top of Ficoll (3 ml, density 1.077; Seromed) in 10-ml tubes. By microscopic counting it was found that all beads and also the dead cells sedimented to the bottom of the tubes whereas the activated B cells remained at the interface. The B cells had multiplied 10 times during the 4-d culture period and 75% of the viable cells were recovered after the sedimentation and centrifugation on Ficoll. LPS-activated B cells were obtained in the same way, i.e., the cultures also included 20% EL-4 SN. Con A–activated T cells were obtained by culturing C57BL/6 spleen cells during 4 d in the presence of 1 μg/ml Con A and 20% EL-4 SN in DME–5% FCS. Before the binding assay, all cells were washed three times in DME and incubated for 2 h at 37°C to promote the removal of cell-bound IL-2. Aliquots of 5 × 10^6 cells (in 200 μl DME) were incubated in the presence of [^{3}H]IL-2 for 20 min at 37°C, after which time 800 μl of cold DME was added. Free and cell-bound [^{3}H]IL-2 was determined as described (5).

Cell SN. Culture SN was obtained from various types of cells as described below, filtered (0.22 μm), and the IL-2 concentration measured in parallel with the BCGF activity. (a) PHA-stimulated human spleen cell SN (H-PHA SN) (144 U IL-2/ml): Mononuclear spleen cells (5 × 10^6/ml) were cultured during 36 h in the presence of 1% PHA and 2% human serum. The SN was concentrated two times by fractionated (NH$_4$)$_2$ SO$_4$ precipitation, i.e., the material soluble at 40% saturation was precipitated at 80% saturation followed by dialysis against RPMI (33). PHA SN was provided by Dr. L. Moretta,

Epalinges. (b) Con A–stimulated murine spleen cell SN (Con A SN) (540 U IL-2/ml): CBA spleen cells (10^6/ml) were cultured during 24 h in the presence of 4 μg/ml of Con A and 5% FCS. (c) Murine (BALB/c anti-DBA/2) secondary in vitro mixed leukocyte culture SN (MLC SN) (474 U IL-2/ml) was prepared as described (34). (d) EL-4 SN (600 U IL-2/ml): EL-4 thymoma cells (10^6/ml) were cultured during 40 h in the presence of 10 ng/ml of phorbol-12-myristate-13-acetate and 2% FCS (28). Different batches of SN were adjusted to 600 U IL-2/ml by 2–4-fold dilution in DME–2% FCS. (e) Murine cloned T helper cell SN (TH-HY 6 SN) (180 U IL-2/ml): H-Y-specific cloned T helper cells (26) (10^6/ml) were cultured during 24 h in the presence of 4 μg/ml of Con A and 5% FCS. (f) Murine T cell hybridoma SN (FS6 SN, 216 U IL-2/ml; AOFS SN, 366 U IL-2/ml): FS6-14.13 cells (10^6/ml) or AOFS21 cells (5 × 10^5/ml) (35) were cultured during 24 h in the presence of 2 μg/ml of Con A and 2% FCS. The FS6 SN was concentrated 10 times by fractionated $(NH_4)_2$ SO_4 precipitation as described above for PHA SN. The hybridomas, originally obtained from Dr. P. Marrack and Dr. J. Kappler, Denver, were provided by Dr. J. P. Corradin, Epalinges. (g) P388D$_1$ SN (no IL-2 activity): SN from ultraviolet light–stimulated P388D$_1$ cells (36) with potent IL-1 activity in a thymocyte proliferation assay was provided by Dr. J. Louis and Dr. R. Titus, Epalinges.

Results

Murine B Blast Assay for B Cell Growth Factor. In a recent report (25) by one of us it was shown that LPS and polyvalent rabbit anti-mouse Ig antibodies coupled to Sepharose beads (anti-Ig) together induced optimal growth factor responsiveness of murine B cells in a T cell–depleted polyclonal B cell system. Based on these results, a quantitative B blast assay for BCGF has now been developed. To obtain activated B cells, positively selected sIg$^+$ cells were cultured (3 × 10^5/ml) during 3 d in the presence of anti-Ig (10 μg/ml) and LPS, followed by one cell wash and depletion of anti-Ig beads by sedimentation. A concentration of 5 μg/ml of LPS was found to be optimal with regard to the subsequent detection of BCGF. On day 3, the B cells had multiplied by a mean 2.5 times and become >90% large cells according to FACS analysis. To further establish the optimal assay conditions, cultures (150 μl) were set up with different numbers of activated B cells suspended in fresh medium–10% FCS–10% EL-4 SN as a source of T cell factors (or control cultures without SN). 3 d later, thymidine incorporation was measured, after a 6-h pulse (see Fig. 1A). The highest stimulation index (cpm per test culture/cpm per control culture) was obtained in cultures with 10^4 activated B cells. This cell number was thus routinely used. It was also found in cultures with 10^4 B cells that the stimulation index was lower when thymidine incorporation was measured either on day 2 (because of a higher control response) or on day 4 (because of overgrowth of the cultures, data not shown). In confirmation of the immunohistochemical analysis of B cells (see Materials and Methods), T cells, i.e., Thy-1$^+$ cells, were not detectable by the FACS in either the starting or the LPS/anti-Ig–activated B cell populations (not shown) or in the cell population recovered after the 3-d cultures performed in the presence of EL-4 SN (see Fig. 1B).

Effects of IL-2 and Various T Cell SN in the B Blast Assay. Dose-response curves for the effects of IL-2 and different sources of T cell factors on thymidine incorporation in the B blast assay are shown in Fig. 2. Human IL-2, purified to apparent homogeneity according to SDS-PAGE analysis, was obtained either by immunoaffinity purification of IL-2 generated by Jurkat cells or by biochemical purification of recombinant IL-2 generated by *E. coli* (see Materials and Meth-
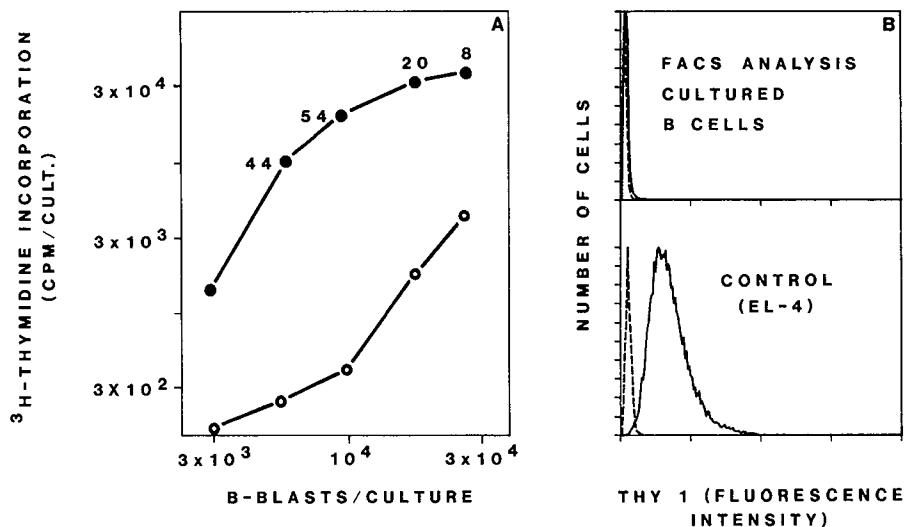
FIGURE 1. (A) Relationship between thymidine incorporation and the number of activated B cells added per culture in the murine B blast assay. Cultures (150 μl) with 10% EL-4 SN (●) or control cultures without SN (○) were set up with different numbers of sIg⁺ cells that had been precultured during 3 d in the presence of LPS (5 μg/ml) and Sepharose-coupled anti-Ig (10 μg/ml). Thymidine incorporation was measured 3 d later. The figure shows cpm per culture (y axis) vs. number of B cells per culture (x axis). In addition, stimulation indices (cpm per culture with SN/cpm per control culture) are indicated by numbers. (B) Absence of Thy-1-positive cells at the time of thymidine addition in the B blast assay. For the FACS analysis, the cultured B cells were harvested and either treated with biotinylated monoclonal anti-Thy-1 antibody and fluoresceinated avidin (—) or fluoresceinated avidin alone (---). EL-4 cells were used as the positive control. The fluorescence profiles were plotted by a computer using arbitrary linear scales for fluoresence intensity (x axis) and cell number (y axis).

ods). It was found that either affinity-purified or recombinant IL-2 supported B cell proliferation as well as (or better than) lectin-stimulated murine or human total spleen cell SN, murine MLC SN, or SN obtained from murine cloned T helper cells (TH-HY6) (25) and T cell hybridomas (FS6 and AOFS) (13, 14, 23, 35). TH-HY6 provide themselves, in conjunction with anti-Ig, all signals required by B cells for a PFC response (25). Moreover, as can be seen in Fig. 2, there was a striking correlation between the IL-2 activity of different T cell SN and their activity in supporting B cell proliferation. Only some batches of EL SN (e.g., batch 22) but not others (e.g., batch 25) led to a two- to threefold higher maximum proliferative response than IL-2. It is not known whether this variation was due to the presence of other growth factors (19) or phorbol ester in certain batches of EL-4 SN. Finally, the SN from the monocytic cell line P388D₁, which had a high IL-1 activity in a thymocyte proliferation assay (not shown) and was previously found (24) to strongly enhance T cell–dependent B cell activation, had absolutely no effect in the B blast assay when tested either alone or in conjunction with IL-2.

As shown in Fig. 3, the capacity of either recombinant IL-2, EL-4 SN, or murine MLC SN to support the generation of protein A–PFC in the B blast assay correlated with the capacity to support proliferation; i.e., the relationship between proliferation and differentiation into PFC was the same with different sources of T cell factors in this assay.
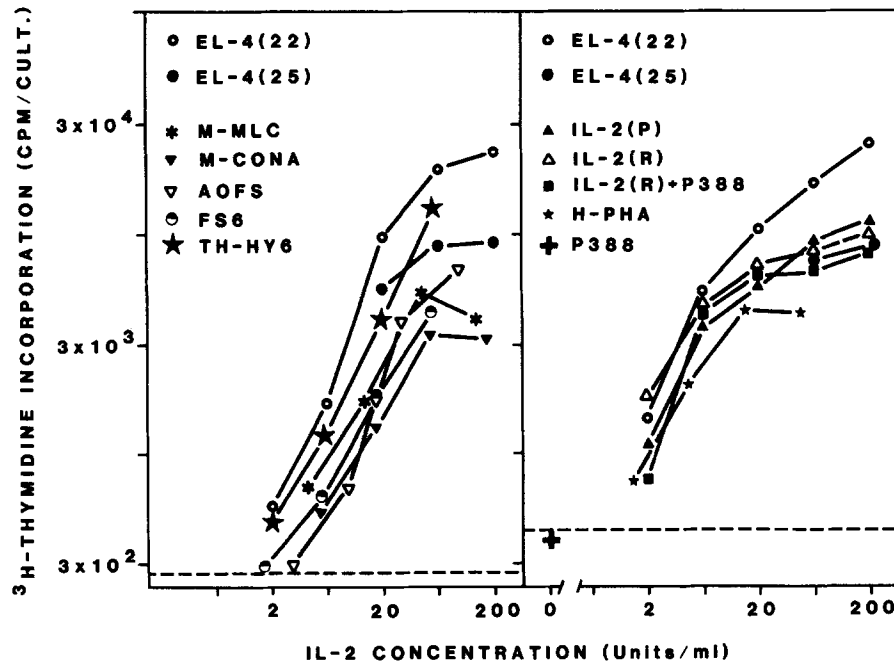
FIGURE 2. Correlation between IL-2 concentration and proliferation in the murine B blast assay: effect on thymidine incorporation of affinity-purified (P) or recombinant (R) human IL-2 or different cell supernatants: EL-4 SN (batch 22 and batch 25); murine MLC SN (M-MLC); Con A–stimulated murine spleen cell SN (M-CONA); murine T cell hybridoma SN (AOFS and FS6); murine cloned T helper cell SN (TH-HY6); PHA-stimulated human spleen cell SN (H-PHA); P388D₁ SN (P388). The SN concentration is expressed in units IL-2/ml, according to the activity in the CTLL assay. The highest SN concentration corresponded to 33% SN in the B blast assay, except for P388D₁ SN, which was tested at 3%. Two representative experiments are shown. The dotted lines indicate cpm per control culture (10^4 activated B cells).

Comparison of IL-2 Requirements for the Proliferation of T Cells vs. B Cells. 1 U of IL-2/ml was defined as the IL-2 concentration leading to 50% maximal thymidine incorporation in the 24-h assay using CTLL-2 cells. However, to compare the IL-2 requirements for the proliferation of normal, polyclonal T vs. B cell populations, a recently developed thymidine incorporation assay was used.[3] Cultures (200 μl) were set up with 2×10^3 FACS-selected Lyt-2⁺ (murine) splenic T cells, leukoagglutinin, and dilutions of recombinant human IL-2. As shown in Fig. 4, the concentration of IL-2 required in this system for 50% maximal thymidine incorporation on day 3 of culture (6 U/ml) was identical with that required in the B blast assay. Moreover, it appears that an increase from 80 to 160 U IL-2/ml still led to an increased proliferative response when the thymidine incorporation was measured on day 5. A similar requirement for a high IL-2 concentration was found for B cell proliferation in cultures with 10^4 B cells, LPS, and anti-Ig that lasted 5 d (data not shown).

Specific Binding of Radiolabeled IL-2 to LPS plus Anti-Ig–activated B Cells. Using

[3] Erard, F., P. Corthesy, M. Nabholz, J. W. Lowenthal, P. Zaech, G. Plaetinck, and H. R. MacDonald. Interleukin 2 is both necessary and sufficient for the growth and differentiation of lectin-stimulated cytolytic T lymphocyte precursors. Manuscript submitted for publication.
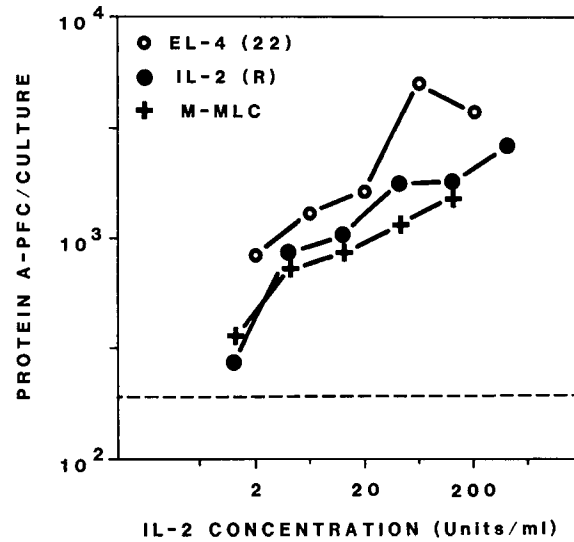
FIGURE 3.   Murine B blast assay: effects on protein A–PFC generation of recombinant (R) human IL-2, EL-4 SN (batch 22), and murine MLC SN (M-MLC). The dotted line indicates PFC per control culture.
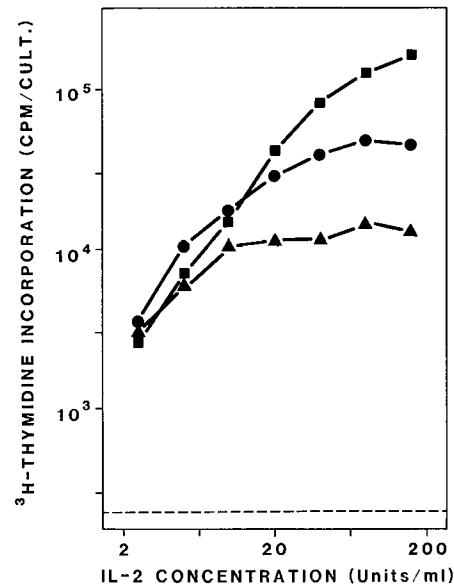

FIGURE 4.   Effect of recombinant IL-2 on T cell proliferation. Cultures (200 μl) were set up with 2×10^5 Lyt-2-positive (murine) splenic T cells (obtained by the FACS; 2% Lyt-2-negative cells), leukoagglutinin (0.5 μg/ml), and IL-2, at the concentrations indicated in the figure. Thymidine incorporation was measured on either day 3 (▲), 4 (●), or 5 (■) of culture, after a 5-h pulse with 1 μCi of [³H]thymidine. The dotted line indicates cpm/culture in the absence of IL-2 (day 5).

a radiolabeled IL-2 binding assay, Robb et al. (5) could detect IL-2 receptors on activated murine and human T cells quantitatively and measure their affinity.

TABLE I

[³H]IL-2 Binding to LPS plus Anti-Ig–activated B Cells vs. Con A–
activated T Cells

Cells	[³H]IL-2	[³H]IL-2 + cold IL-2	Inhibition
	cpm	*cpm*	*%*
Nonactivated B cells	45	ND	
LPS/anti-Ig–activated B cells	1,520	120	92
Con A–activated T cells	4,770	640	87

5×10^6 cells were incubated for 20 min at 37°C with radiolabeled IL-2 alone (25,000 cpm; see Materials and Methods) or together with a 50-fold excess of unlabeled IL-2; bound cpm were determined after subtraction of the machine background (55 cpm).

But these authors did not detect IL-2 receptors on LPS-activated murine B cells. In view of the results shown above, we performed this assay with B cells activated with LPS and anti-Ig for 4 d in the presence of EL-4 SN (LPS plus anti-Ig–activated B cells). Nonactivated B cells or B cells cultured during 4 d in the presence of LPS and EL-4 SN but not anti-Ig (LPS-activated B cells) were also tested. Since B cells cultured in the presence of anti-Ig and EL-4 SN alone died rapidly (24), such cells could not be analyzed.

By using immunoaffinity-purified [³H]IL-2 at a concentration that was saturating for T cells,[2] we found (see Table I) (*a*) that LPS plus anti-Ig–activated B cells bound [³H]IL-2 in a specific, cold-competable manner, (*b*) that the amount of [³H]IL-2 specifically bound by such activated B cells at equilibrium was, on a per cell basis, one-third of that bound by Con A–activated T cells, and (*c*) that nonactivated B cells (i.e., freshly prepared sIg⁺ cells) did not bind [³H]IL-2. Although anti-Ig beads were not detected by microscopic examination of LPS plus anti-Ig–activated B cells after Ficoll centrifugation, the possible influence of contaminating beads on the binding of [³H]IL-2 was also tested: 18 μl of packed beads (corresponding to a 50% removal from cultured B blasts) were found to have no effect on the binding of [³H]IL-2 to nonactivated B or T cells (data not shown).

Next, an IL-2-binding curve was obtained by incubating LPS plus anti-Ig–activated B cells in the presence of serial dilutions of [³H]IL-2 (Fig. 5*A*). The Scatchard plot established after correcting for nonspecific binding (Fig. 5*B*) indicated that thus-activated B cells expressed ~3,500 specific binding sites per cell with an apparent dissociation constant of 150 pM. In addition, it was confirmed that B cells activated by LPS alone did not specifically bind IL-2 (see Fig. 5*A*).

Discussion

This report shows that IL-2 is not only a TCGF but also a BCGF, that both activities occur at very similar concentrations of this lymphokine, and that appropriately activated B cells express IL-2 receptors. In particular, it demonstrates that two independently derived human IL-2 preparations, i.e., recombinant IL-2 and immunoaffinity-purified IL-2, of apparent homogeneity and high
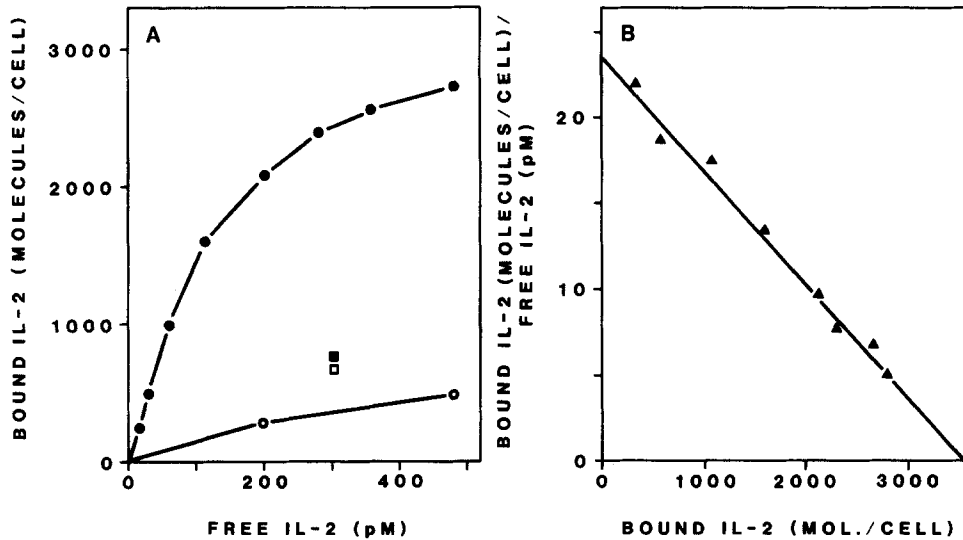
FIGURE 5. (A) [³H]IL-2-binding curve for LPS plus anti-Ig–activated B cells: total binding (●) obtained with serial dilutions of [³H]IL-2 and nonspecific binding (○) obtained in the presence of a 50-fold excess of unlabeled IL-2 are shown. A measurement of the total (■) and nonspecific (□) binding to B cells activated by LPS alone was included in the same experiment. (B) Scatchard plot of the data for LPS plus anti-Ig–activated B cells shown in A after correcting for nonspecific binding.

specific TCGF activity (1 U/per 0.3 ng protein, where 1 U/ml gives 50% maximal thymidine incorporation in the CTLL-2 assay), exhibited identical BCGF activity. It is, therefore, unlikely that contaminating substances caused the effects on B cells. It is also highly unlikely that the effect of IL-2 in the murine B blast assay using LPS plus anti-Ig–activated B cells was due to the IL-2-stimulated generation of other factors by contaminating T cells (37). It was previously shown (25) that LPS plus anti-Ig–dependent B cell activation itself requires neither T helper cells nor macrophages and, in the present study, T cell contamination, not detectable by the FACS, was <0.3% when checked by immunohistochemical analysis. Furthermore, a variety of sources of T cell factors, including total spleen cell SN, cloned T helper cell SN (25), and T hybridoma SN (13, 14, 23, 35), did not lead to stronger B cell proliferation than IL-2 alone. In fact, there appeared a striking correlation between the IL-2 TCGF activity and the BCGF activity of all tested supernatants. P388D₁ SN had no effect in this assay.

These results do not exclude the existence of BCGF distinct from IL-2. But, in providing more direct evidence than some previous studies (3, 4, 12–14, 23) for the functional role of IL-2 in B cell responses, the data raise questions regarding the conclusions reached by many other authors (15–22, 37), i.e., that IL-2 has no BCGF activity. Several aspects need to be discussed: (a) There exists no discrepancy with regard to the experimental data themselves in that the effects of IL-2 on LPS plus anti-Ig–activated murine B cells were previously not investigated. It is thus possible that some of the previously described factors participate (directly or via other cells) in B cell activation, whereas IL-2 would only act on appropriately activated B cells. Accordingly, it is conceivable that

different "activation factors" would be detected in different B cell activation systems (16). (*b*) In addition, different types of BCGF could theoretically act on different subsets of B cells (16). (*c*) Certain B cell lines may respond to other factors than activated normal B cells (20, 38). However, since most T helper cells are capable of generating IL-2 (39), the demonstrated functional role of IL-2 in B cell proliferation is certainly relevant in the physiologic B cell response.

In addition to the functional demonstration of the BCGF activity of IL-2, this study shows that LPS plus anti-Ig–activated B cells express IL-2 receptors; i.e., the receptor assay using radiolabeled IL-2 indicated a mean of 3,500 specific IL-2 binding sites per cell. It is known from limiting dilution analysis that in our system ~1 of 8 B cells become responsive to T cell–derived growth factors in the presence of LPS and anti-Ig (25). We assume that during 4 d of mass culture in the presence of EL-4 SN, these cells outgrow those that do not respond to IL-2, thereby generating a homogeneous population with regard to IL-2 receptor expression. However, this point needs further investigation, e.g., by FACS analysis of the proportion of B cells bearing IL-2 receptors, using anti-IL-2 receptor antibody. In any case, in agreement with previous observations by Robb et al. (5), B cells activated by LPS alone, like nonactivated B cells, did not significantly express IL-2 receptors. This also agrees with our previous finding (25) that anti-Ig is required for the induction of growth factor responsiveness of LPS-activated B cells. Whereas LPS plus anti-Ig–activated B cells expressed only two to three times fewer IL-2 receptors than we and others (5) detected on activated normal murine T cells, the receptor affinity was found to be five- (our unpublished data) to ninefold (5) lower than that of such T cells. This lower receptor affinity on B cells appears to contrast with the finding that B and T cell proliferation required similar IL-2 concentrations, but two points need to be discussed: (*a*) T and B cells may differ with regard to the level of IL-2 receptor occupancy required for cell cycle progression, and (*b*) the apparent receptor affinity measured with binding assays may be influenced by complex variables, including the cell activation stage (i.e., culture conditions), receptor modulation, and possible heterogeneity of the receptors themselves. Until these variables are known, the value of 150 pM should be considered a minimal estimate of the IL-2 receptor affinity on activated B cells.

A monoclonal antibody directed against the murine T cell IL-2 receptor was previously found by Malek et al. (7) to react, although weakly, with LPS-activated B cells. Our own studies in progress show that another monoclonal antibody against the murine T cell IL-2 receptor (obtained by Dr. M. Nabholz, Epalinges) inhibits B cell proliferation in an IL-2 dose–dependent way and that the radio-labeled antibody binds to LPS plus anti-Ig–activated B cells to almost the same extent as to Con A–activated normal T cells (unpublished data).

We are also currently investigating the effect of IL-2 on human B cell proliferation. But, in confirmation of previous studies (14, 24–26, 40–42) showing that murine or human B cell activation normally requires a direct T-B cell interaction (alone or in conjunction with T-dependent antigens or anti-Ig), and because LPS does not bypass this T helper signal in human B cell systems, we have thus far not obtained a T cell–free human BCGF assay. Nonetheless, in an assay using irradiated mutant EL-4 thymoma cells, which provide an activation

signal(s) to murine and human B cells, IL-2 optimally stimulated murine and human B cell proliferation (R. Zubler, C. Mingari, L. Moretta, and H. R. MacDonald, unpublished data). Thus, the previous observation that a monoclonal antibody reacting with the human T cell IL-2 receptor (6) did not react with human B cells (43) could have been related to a suboptimal B cell activation. In fact, it has been more recently (44) demonstrated that this antibody (anti-Tac) recognizes on certain human B tumor cells a molecule of the size of the IL-2 receptor (44).

Finally, we have observed (see Fig. 3) that IL-2 led to the generation of PFC in the murine B blast assay. However, the questions concerning the nature of the B cell differentiation signals in this assay and whether, in fact, differentiation signals are different from activation/proliferation signals in the B cell response (45), were not addressed in this study. The Ig class/subclass distribution of the PFC responses (46) remains to be studied in our systems.

Summary

In this study we investigated whether interleukin 2 (IL-2) acts on B cell proliferation and whether activated B cells express IL-2 receptors. First, the functional activity of immunoaffinity-purified or recombinant human IL-2 was studied in a B blast assay using positively selected murine surface Ig–positive cells that had been activated by lipopolysaccharide (LPS) plus anti-Ig antibodies (anti-Ig). In this assay, T cells were not detected by fluorescence-activated cell sorter analysis. It was found that both IL-2 preparations led to optimal B cell proliferation compared with supernatants obtained from murine or human spleen cells or murine cloned T helper cells. Second, we observed that the IL-2 requirement in this assay was about the same as in a proliferation assay using lectin-activated polyclonal murine Lyt-2-positive T cells. Third, analysis of the binding of radiolabeled imunoaffinity-purified IL-2 to B cells indicated that LPS plus anti-Ig–activated B cells expressed a mean of 3,500 IL-2 receptors per cell with an apparent dissociation constant of 150 pM. However, neither nonactivated B cells nor B cells activated by LPS alone exhibited significant specific IL-2 binding. The functional and the receptor data are consistent with the conclusion that IL-2 is a growth factor not only for T cells but also for B cells.

Note added in proof: Additional studies using very high [^{3}H]IL-2 concentrations indicate that activated B and T cells express both high and low affinity IL-2 receptors. The corresponding mean apparent dissociation constants (~30 pM and ~300 pM, respectively) do not differ significantly for B and T cells.

References

1. Morgan, D. A., F. W. Ruscetti, and R. C. Gallo. 1976. Selective in vitro growth of T-lymphocytes from normal human bone marrows. *Science (Wash. DC)*. 193:1007.

2. Gillis, S., and K. A. Smith. 1977. Long-term culture of tumor-specific cytotoxic T-cells. *Nature (Lond.)*. 268:154.

3. Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K. A. Smith. 1979. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. *J. Exp. Med.* 150:849.

4. Gillis, S., K. A. Smith, and J. Watson. 1980. Biochemical characterization of lymphocyte regulatory molecules. II. Purification of a class of rat and human lymphokines. *J. Immunol.* 24:1954.

5. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors. Quantitation, specificity, and biological relevance. *J. Exp. Med.* 154:1455.

6. Leonard, W. J., J. M. Depper, R. J. Robb, T. A. Waldmann, and W. C. Greene. 1983. Characterization of the human receptor for T-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 80:6957.

7. Malek, T. R., R. J. Robb, and E. M. Shevach. 1983. Identification and initial characterization of a rat monoclonal antibody reactive with the murine interleukin 2 receptor-ligand complex. *Proc. Natl. Acad. Sci. USA.* 80:5694.

8. Smith, K. A., M. F. Favata, and S. Oroszlan. 1983. Production and characterization of monoclonal antibodies to human interleukin 2: strategy and tactics. *J. Immunol.* 131:1808.

9. Robb, R. J., R. M. Kutny, and V. Chowdhry. 1983. Purification and partial sequence analysis of human T-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 80:5990.

10. Taniguchi, T., H. Matsui, T. Fujita, C. Takaoka, N. Kashima, R. Yoshimoto, and J. Hamuro. 1983. Structure and expression of a cloned cDNA for human interleukin 2. *Nature (Lond.)*. 302:305.

11. Devos, R., G. Plaetinck, H. Cheroutre, G. Simons, W. Degrave, J. Tavernier, E. Remaut, and W. Fiers. 1983. Molecular cloning of human interleukin 2 cDNA and its expression in *E. coli. Nucleic Acids Res.* 11:4307.

12. Swain, S. L., G. Dennert, J. F. Warner, and R. W. Dutton. 1981. Culture supernatants of a stimulated T-cell line have helper activity that acts synergistically with interleukin 2 in the response of B cells to antigen. *Proc. Natl. Acad. Sci. USA.* 78:2517.

13. Endres, R. O., E. Kushnir, J. W. Kappler, P. Marrack, and S. C. Kinsky. 1983. A requirement for nonspecific T cell factors in antibody responses to "T cell independent" antigens. *J. Immunol.* 130:781.

14. Roehm, N. W., P. Marrack, and J. W. Kappler. 1983. Helper signals in the plaque-forming cell response to protein-bound haptens. *J. Exp. Med.* 158:317.

15. Pike, B. L., D. L. Vaux, I. Clark-Lewis, J. W. Schrader, and G. J. V. Nossal. 1982. Proliferation and differentiation of single hapten-specific B lymphocytes is promoted by T-cell factor(s) distinct from T-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 79:6350.

16. Swain, S. L., M. Howard, J. Kappler, P. Marrack, J. Watson, R. Booth, G. D. Wetzel, and R. Dutton. 1983. Evidence for two distinct classes of murine B cell growth factors with activities in different functional assays. *J. Exp. Med.* 158:822.

17. Leanderson, T., E. Lundgren, E. Ruuth, H. Borg, H. Persson, and A. Coutinho. 1982. B-cell growth factor: distinction from T-cell growth factor and B-cell maturation factor. *Proc. Natl. Acad. Sci. USA.* 79:7455.

18. Lernhardt, W., C. Corbel, R. Wall, and F. Melchers. 1982. T-cell hybridomas which produce B lymphocyte replication factors only. *Nature (Lond.)*. 300:355.

19. Farrar, J. J., M. Howard, J. Fuller-Farrar, and W. E. Paul. 1983. Biochemical and

physicochemical characterization of mouse B cell growth factor: a lymphokine distinct from interleukin 2. *J. Immunol.* 131:1838.

20. Okada, M., N. Sakaguchi, N. Yoshimura, H. Hara, K. Shimizu, N. Yoshida, K. Yoshizaki, S. Kishimoto, Y. Yamamura, and T. Kishimoto. 1983. B cell growth factors and B cell differentiation factor from human T hybridomas. Two distinct kinds of B cell growth factor and their synergism in B cell proliferation. *J. Exp. Med.* 157:583.

21. Muraguchi, A., J. L. Butler, J. H. Kehrl, and A. S. Fauci. 1983. Differential sensitivity of human B cell subsets to activation signals delivered by anti-μ antibody and proliferative signals delivered by a monoclonal B cell growth factor. *J. Exp. Med.* 157:530.

22. Maizel, A., C. Sahasrabuddhe, S. Mehta, J. Morgan, L. Lachman, and R. Ford. 1982. Biochemical separation of a human B cell mitogenic factor. *Proc. Natl. Acad. Sci. USA.* 79:5998.

23. Parker, D. C. 1982. Separable helper factors support B cell proliferation and maturation to Ig secretion. *J. Immunol.* 129:469.

24. Zubler, R. H. 1984. Signal requirements for T-dependent and T-independent B cell activation. *Lymphokines.* 10:in press.

25. Zubler, R. H. 1984. Polyclonal B cell responses in the presence of defined filler cells: complementary effects of lipopolysaccharide and anti-Ig antibodies. *Eur. J. Immunol.* 14:357.

26. Zubler, R. H., and O. Kanagawa. 1982. Requirement for three signals in B cell responses. II. Analysis of antigen- and Ia-restricted T helper cell-B cell interaction. *J. Exp. Med.* 156:415.

27. Mage, M. G., L. L. McHugh, and T. L. Rothstein. 1977. Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture dishes coated with specifically purified anti-immunoglobulin. *J. Immunol. Methods.* 15:47.

28. Hilfiker, M. L., R. N. Moore, and J. J. Farrar. 1981. Biologic properties of chromatographically separated murine thymoma-derived interleukin 2 and colony-stimulating factor. *J. Immunol.* 127:1983.

29. Gronowicz, E., A. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* 6:588.

30. Pierce, C. W., B. M. Johnson, H. E. Gershon, and R. Asofsky. 1971. Immune responses in vitro. III. Development of primary M, G, and A plaque-forming cell responses in mouse spleen cell cultures stimulated with heterologous erythrocytes. *J. Exp. Med.* 134:395.

31. Heitzmann, H., and F. M. Richards. 1974. Use of avidin-biotin complex for specific staining of biological membranes in electron microscopy. *Proc. Natl. Acad. Sci. USA.* 71:3537.

32. MacDonald, H. R., R. K. Lees, B. Sordat, P. Zaech, J. Maryanski, and C. Bron. 1981. Age-associated increase in expression of the T cell surface markers Thy-1, Lyt-1, and Lyt-2 in congenitally athymic (nu/nu) mice: analysis by flow microfluorometry. *J. Immunol.* 126:865.

33. G. Pantaleo, L. Moretta, M. C. Mingari, and J.-C. Cerottini. 1983. Quantitative assessment of the pool size and subset distribution of cytolytic T lymphocytes within human resting or alloactivated peripheral blood T cell populations. *J. Exp. Med.* 158:571.

34. Ryser, J. E., J.-C. Cerottini, and K. T. Brunner. 1978. Generation of cytolytic T lymphocytes in vitro. IX. Induction of secondary CTL responses in primary long-term MLC by supernatants from secondary MLC. *J. Immunol.* 120:370.

35. Marrack, P., S. D. Graham, Jr., E. Kushnir, H. J. Leibson, N. Roehm, and J. W. Kappler. 1982. Nonspecific factors in B cell responses. *Immunol. Rev.* 63:33.

36. Kakiuchi, T., R. W. Chesnut, and H. M. Grey. 1983. B cells as antigen-presenting cells: the requirement for B cell activation. *J. Immunol.* 131:109.

37. Howard, M., L. Matis, T. R. Malek, E. Shevach, W. Kell, D. Cohen, K. Nakanishi, and W. E. Paul. 1983. Interleukin 2 induces antigen-reactive T cell lines to secrete BCGF-I. *J. Exp. Med.* 158:2024.

38. Maizel, A. L., J. W. Morgan, S. R. Mehta, N. M. Kouttab, J. M. Bator, and C. G. Sahasrabuddhe. 1983. Long-term growth of human B cells and their use in a microassay for B-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 80:5047.

39. Kelso, A., and H. R. MacDonald. 1982. Precursor frequency analysis of lymphokine-secreting alloreactive T lymphocytes. Dissociation of subsets producing interleukin 2, macrophage-activating factor, and granulocyte-macrophage colony-stimulating factor on the basis of Lyt-2 phenotype. *J. Exp. Med.* 156:1366.

40. Martinez-A., C., and A. Coutinho. 1981. B cell activation by helper cells is a two-step process. *Nature (Lond.).* 290:60.

41. Andersson, J., M. H. Schreier, and F. Melchers. 1980. T-cell-dependent B-cell stimulation is H-2 restricted and antigen dependent only at the resting B-cell level. *Proc. Natl. Acad. Sci. USA.* 77:1612.

42. Principato, M. A., G. S. Thompson, and S. M. Friedman. 1983. A cloned major histocompatibility complex–restricted trinitrophenyl-reactive human helper T cell line that activates B cell subsets via two distinct pathways. *J. Exp. Med.* 158:1444.

43. Uchiyama, T., S. Broder, and T. A. Waldmann. 1981. A monoclonal antibody (anti-Tac) reactive with activated and functionally mature human T cells. I. Production of anti-Tac monoclonal antibody and distribution of Tac(+) cells. *J. Immunol.* 126:1393.

44. Korsmeyer, S. J., W. C. Greene, J. Cossman, H. Su-Ming, J. P. Jensen, L. M. Neckers, S. L. Marshall, A. Bakhshi, J. M. Deeper, W. J. Leonard, E. S. Jaffe, and T. S. Waldmann. 1983. Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in hairy cell leukemia. *Proc. Natl. Acad. Sci. USA.* 80:4522.

45. Zubler, R. H., and A. L. Glasebrook. 1982. Requirement for three signals in "T-independent" (lipopolysaccharide-induced) as well as in T-dependent B cell responses. *J. Exp. Med.* 155:666.

46. Isakson, P., E. Pure, E. Vitetta, and P. Krammer. 1982. T-cell derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.