Up-regulation of interleukin 4/B-cell stimulatory factor 1 receptor expression

JUNICHI OHARA* AND WILLIAM E. PAUL

Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892

Contributed by William E. Paul, August 8, 1988

ABSTRACT The expression of interleukin 4 (IL-4) receptors on resting T and B lymphocytes was enhanced 4- to 8-fold by IL-4 stimulation of these cells. Other agents such as lipopolysaccharide and anti-IgM for B cells and concanavalin A for T cells also caused increased IL-4 receptor expression, although to a somewhat smaller degree than IL-4. Using a newly developed flow cytometric analysis based on the binding of biotinylated IL-4 and phycoerythrin-streptavidin, it was observed that receptor up-regulation in a T-cell population treated with IL-4 was a feature of the majority of the T cells. Analysis of IL-4 by cross-linkage of 125I-labeled IL-4 to IL-4 receptor with disuccinimidyl suberate indicated that the IL-4-IL-4 receptor complex was the same size in the resting and up-regulated cells, implying that the same receptor species found in resting cells was up-regulated in response to IL-4.

Recent studies have shown that lymphocyte proliferative and differentiation responses to antigenic stimulation are controlled by endogenous hormone-like substances designated lymphokines. Among these, interleukin 4 (IL-4)/B-cell stimulatory factor 1 is particularly notable in its range of biologic activities. IL-4 has been purified to homogeneity (2, 3) and cDNA clones for IL-4 (4–6) have been obtained. An anti-IL-4 monoclonal antibody that inhibits its biologic activities has also been generated (7). Recently, high-affinity IL-4 receptors have been demonstrated on mouse and human cells of various lineages by using 125I- or 35S-labeled IL-4 (8–13). High-affinity receptors are expressed on normal resting B cells and T cells.

We have reported that stimulation of B cells with lipopolysaccharide (LPS) or with anti-IgM and of T cells with concanavalin A (Con A) increased the receptor expression (8). In this study, we demonstrate that resting B cells and T cells increase receptor number upon stimulation with IL-4 alone.

MATERIALS AND METHODS

Animals, Culture Medium, and Cell Preparation. BALB/c, C3H/HeN, and DBA/2 female mice, 8–12 weeks old, were obtained from the Frederick Cancer Research Facility (Frederick, MD). Cells were cultured in RPMI 1640 medium (Biofluids, Rockville, MD) supplemented with 10% fetal bovine serum (Biofluids), L-glutamine (2 mM), and gentamicin (50 μg/ml). Purified B cells were prepared from spleen (14), and purified T cells were prepared from mesenteric lymph nodes as described (15).

Cytokines. Purified IL-4 was prepared as described from supernatant (Sn) fluids of cells of the thymoma line EL-4 that had been activated with phorbol 12-myristate 13-acetate (PMA) (3) and from the culture Sn of SF9 cells infected with a recombinant IL-4 baculovirus (C. Watson, J.O., and W.E.P., unpublished data). Recombinant mouse IL-4 from a yeast expression system was kindly provided by Immunex (Seattle). Biological units of chemically purified IL-4 and of recombinant IL-4 were determined by both an anti-IgM costimulation assay, using purified B cells, and a T-cell proliferation assay, using the IL-4-responsive CT.4R cell line as described elsewhere (J. Hu-Li, J.O., C. Watson, and W.E.P., unpublished data). One unit is equal to 3–5 pg (2) of purified IL-4. Recombinant human interleukin 2 (IL-2) was kindly provided by Cetus (Emeryville, CA).

Chemicals. PMA and avidin-Sepharose 4B were purchased from Sigma. A23187 was from Calbiochem. LPS and Con A were obtained from Difco. Goat anti-IgM antibody was prepared and purified in our laboratory. The monoclonal rat anti-mouse IgM antibody Bet-2 (16) was used as a 1:10 dilution of a 10-fold concentrated Sn of hybridoma cells. Disuccinimidyl suberate and N-hydroxysuccinimide d-biotin were from Pierce. Streptavidin–phycoerythrin (PE) conjugate was obtained from Becton Dickinson.

125I-Labeled IL-4 (125I-IL-4) Binding Assay. Purified IL-4 was 125I-labeled as described (8). Equilibrium constants and receptor numbers were determined by Scatchard analysis. In experiments in which only receptor number was determined, cells were incubated with a single concentration (124 pM) of 125I-IL-4 sufficient to bind to ~80% of receptors. More than 100-fold excess of nonradioactive IL-4 was added to some reaction mixtures as an inhibitor. Specifically bound IL-4 was determined from the difference between bound radioactivity in the absence or presence of inhibitor.

In vitro. Purified baculovirus-derived recombinant IL-4 (350 μg) and N-hydroxysuccinimide d-biotin (500 μg) reacted in 0.5 ml of sodium bicarbonate (0.1 M; pH 7.8) for 2 h at room temperature. The protein was extensively dialyzed against phosphate-buffered saline (PBS). The biological activity of biotinylated recombinant IL-4 was measured by the [3H]thymidine uptake assay using CT.4R cells. All of the biological activity of the biotinylated IL-4 preparation was removed by passage over an avidin-Sepharose 4B column, implying that all biologically active IL-4 had been biotinylated.

Flow Cytometric Analysis of IL-4 Receptors. Cells (5 × 10⁶) were incubated with medium only or with 2.4 × 10⁶ units of recombinant IL-4 in 50–100 μl (2.5 × 10⁻⁵ M) of staining buffer (PBS with 0.1% azide and 5% fetal calf serum) for 30 min at 4°C. Without washing, biotinylated IL-4 in 15–30 μl was added to a final concentration of 6.5 × 10⁻¹⁰ M and incubation continued for an additional 30 min at 4°C. The cells were then washed twice and exposed to a streptavidin–PE conjugate (5 μl; Becton Dickinson) in a total vol of 100 μl of 30 min at 4°C. The stained cells were thrice washed and then analyzed by FACSCAN (Becton Dickinson).

Cross-Linkage of 125I-IL-4 to Cell-Surface Molecules. 125I-IL-4 (5.8 × 10⁻⁸ M) was cross-linked to cell-surface receptors with 3 mM disuccinimidyl suberate as described (8) and

Abbreviations: IL, interleukin; 125I-IL-4, 125I-labeled IL-4; MFI, mean fluorescence intensity; LPS, lipopolysaccharide; Sn, supernatant; PE, phycoerythrin; PMA, phorbol 12-myristate 13-acetate. *To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
the cross-linked complex was analyzed by electrophoresis on 20% polyacrylamide gels in 0.1% NaDodSO₄.

RESULTS

Up-Regulation of IL-4 Receptor Expression. Resting B and T lymphocytes express relatively small numbers of IL-4 receptors (Table 1). Stimulation of resting B cells with LPS or with anti-IgM antibodies increased receptor number 4- to 5-fold; stimulation of resting T cells with Con A also caused an increase in numbers of IL-4 receptors (8, 9). These same cell populations showed somewhat greater increases in numbers of IL-4 receptors in response to IL-4 itself. Both purified T-cell-derived IL-4 and recombinant IL-4 caused increases in numbers of IL-4 receptors on B and T cells (Table 1) and anti-IL-4 monoclonal antibodies inhibited this increased receptor expression (data not shown). By contrast, recombinant human IL-2 did not affect expression of IL-4 receptors.

By itself, PMA caused a modest up-regulation in T-cell IL-4 receptors while the calcium ionophore A23187 had no effect. Treatment of T cells with IL-4 and PMA for 1 or 2 days did not reproducibly increase receptor number above that induced by IL-4 alone.

Further support for an increase in receptor number in lymphocytes cultured with IL-4 comes from experiments in which [125I]-IL-4 was cross-linked to cell-surface receptors for IL-4. Spleen cells were cultured for 24 hr with no IL-4 or with 2 or 10 units of recombinant IL-4 per ml. [125I]-IL-4 was cross-linked to cell-surface receptors with disuccinimidyl suberate; NaDodSO₄/PAGE revealed the presence of a complex with an Mr of ~80,000 in each case. Stimulation with IL-4 caused a striking increase in the intensity of the Mr, 80,000 band when compared to unstimulated cells (Fig. 1).

Higher molecular weight bands were also observed to increase in intensity in lysates of cells treated with IL-4. Increased receptor number in T cells could be detected within 6-12 hr of initiation of culture with IL-4 and plateaued between 18 and 24 hr (Fig. 2B). B cells showed similar time dependency (data not shown). Maximal up-regulation was observed with 0.5 and 5 units/ml; B and T lymphocytes had similar dose-response curves, although T cells generally demonstrated a greater degree of induction (Fig. 2A).

These results indicate that both B and T lymphocyte preparations showed increased IL-4 receptor expression as a result of stimulation with IL-4 alone. However, since the measurements are based on the binding of [125I]-IL-4 to whole cell populations, they do not distinguish between up-regulated expression by a small fraction of the cells or by the majority of the cells in the population under examination. To study this, we developed a ligand binding assay suitable for flow cytometric measurements that enabled us to examine the proportion of the cells that showed heightened expression of IL-4 receptors.

For this purpose, we prepared biotinylated IL-4 that retained biological activity. Cells that had bound biotinylated IL-4 were enumerated and the degree of IL-4 binding was determined by the use of PE-conjugated streptavidin and measurement of fluorescence on individual cells with a Becton Dickinson FACSscan. Specific binding of biotinylated IL-4 was determined by the addition of a very large molar excess of purified nonbiotinylated IL-4 before the addition of biotinylated IL-4. The degree of fluorescence [mean fluorescence intensity (MFI)] observed under these conditions was assumed to represent "nonspecific" binding and was subtracted from MFI obtained without such preincubation. The difference in the MFIs (AMFI) represents the binding of IL-4 to its receptor.

To optimize the technique, we used a cell line (CT.4R) with a large number of IL-4 receptors (~15,000 per cell) and added various amounts of biotinylated IL-4 to cells that had been preincubated with no IL-4 or with excess nonbiotinylated IL-4. Table 2 shows that specific (i.e., IL-4 inhabitable) binding occurred with all concentrations of biotinylated IL-4 used. For future work, we chose a concentration of biotinylated IL-4 (6.5 × 10⁻¹⁰ M) that gave specific binding 60-75% of maximal but that had a relatively low nonspecific binding. The inverse of the equilibrium constant of the interaction of IL-4 and the IL-4 receptor (~0.5 × 10⁻¹⁰) is...
privately lower than the concentration of biotinylated IL-4 used, and most cell lines and normal cell populations have many fewer receptors than CT.4R cells, suggesting that, in most circumstances, we will detect the majority of IL-4 receptors with this method.

To show that we can distinguish cells bearing various numbers of IL-4 receptors by flow cytometric analysis, we determined receptor numbers on two T-cell sublines derived from CTLL cells. Scatchard analysis indicated that one line, CT.EV, had 2400 receptors per cell, while the other, CT.4R, had 15,600 receptors per cell. Using the binding of biotinylated IL-4 and detection with PE-streptavidin, CT.EV cells had a ΔMFI of 2.3 fluorescence units, while the ΔMFI for

---

Table 2. Binding of biotinylated IL-4 to CT.4R T-cell line

<table>
<thead>
<tr>
<th>Biotinylated IL-4, M</th>
<th>Preincubation</th>
<th>Modal fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Preincubation</td>
<td>Medium IL-4 Δ</td>
</tr>
<tr>
<td>0</td>
<td>0.05</td>
<td>4.27</td>
</tr>
<tr>
<td>2.2 × 10⁻¹⁰</td>
<td>0.05</td>
<td>4.27</td>
</tr>
<tr>
<td>6.5 × 10⁻¹⁰</td>
<td>0.05</td>
<td>5.93</td>
</tr>
<tr>
<td>15.9 × 10⁻¹⁰</td>
<td>0.05</td>
<td>6.82</td>
</tr>
<tr>
<td>58.5 × 10⁻¹⁰</td>
<td>0.05</td>
<td>7.93</td>
</tr>
</tbody>
</table>

CT.4R cells (5 × 10⁴) were incubated with medium or purified baculovirus-derived recombinant IL-4 (6 × 10⁻⁸ M) for 20 min at 4°C. Biotinylated IL-4, at the final concentration indicated, was added and incubation continued for an additional 30 min. Cells were washed twice and PE-streptavidin (5 μl) was added in a total vol of 100 μl for 30 min. The cells were washed again and analyzed on a FACSCAN by logarithmic amplification. Both median fluorescence intensity and mode of fluorescence intensity are reported. Δ, Fluorescence intensity in cells preincubated in medium minus fluorescence intensity in cells preincubated in excess IL-4.

CT.4R cells was 11.4 fluorescence units (Fig. 3). The ratio of receptor number (6.5) and of ΔMFI (5.0) was sufficiently similar to indicate that the fluorescence binding approach could give useful information about the relative number of receptors on different cell populations and on the distribution of receptors in a cell population.

We used this method to determine whether IL-4 up-regulation of IL-4 receptor expression on normal T cells was a property of the majority of the T cells or of small subpopulations. Resting lymph node T cells were cultured with medium or recombinant IL-4 (100 units/ml) for 22 hr and the specific binding of biotinylated IL-4 was measured. T cells cultured in medium only had barely detectable IL-4-inhibitable binding (ΔMFI = 0.8) (Fig. 4), while the T-cell population cultured with IL-4 expressed a ΔMFI of 4.1. This verifies by another technique that IL-4 enhances the expression of the IL-4 receptor on resting T cells. In addition, the shape of the fluorescence histograms indicates that the great majority of the cells cultured in IL-4 express more IL-4 receptors than do cells cultured in medium. Since IL-4 does not cause proliferation of resting T cells and since the viability of cells cultured in IL-4 for 24 hr is excellent (16), we conclude that the IL-4-induced up-regulation of IL-4 receptors is a process that occurs in virtually all lymph node T cells.

DISCUSSION

In this study, we have confirmed the previous findings of IL-4 receptor up-regulation by mitogens reported by our group as well as others (8, 9) and demonstrated that IL-4 is a potent inducer of IL-4 receptors on both B and T lymphocytes. The up-regulated IL-4 receptors on stimulated lymphocytes appeared to be identical to those expressed on unstimulated lymphocytes in terms of the size of the molecular complex formed by cross-linking 125I-IL-4 to the IL-4 receptor on cell surfaces. Although we have not determined the mechanism of receptor up-regulation, the fact that 6 hr is required before a substantial increase in receptor number is observed suggests that new receptor synthesis rather than redistribution of receptors is necessary. Furthermore, we observed that cycloheximide blocked receptor up-regulation (I.O., unpublished observation), further supporting the idea that new receptor synthesis is required for IL-4-induced increase in receptor number.

The up-regulation of IL-4 receptors expressed on IL-4-stimulated peripheral small dense T cells was confirmed by a newly developed flow cytometric approach with biotinylated
immunology: ohara

fluorescence intensities (10^5 units/ml) CT.4R and CT.EV cells. IL-4 great the technique IL-4-mediated T-cell clones flow cytometric detection. Indeed, populations. Consequently, increase IL-2. Thus, to restiing T cells. The addition of lymph mesenteric populations. Although both the IL-4 receptor and the p55 chain of the IL-2 receptor increase in expression as a result of exposure to their ligands, the control of these receptors is not identical. Thus, IL-2 failed to cause increased IL-4 receptor expression on resting T cells in contrast to the striking effect that IL-4 exerts on IL-4 receptor expression in these cells. IL-4 by itself fails to cause a detectable increase in p55 expression on resting T cells, although in combination with PMA, IL-4 does cause some up-regulation of p55 (18). Furthermore, the relative degree of up-regulation of the two receptors appears different. IL-4 receptors are detectable on resting T cells and increase by ~5-fold in response to IL-4. By contrast, p55 expression is usually undetectable on resting T cells and stimulated cells show increases of great magnitude (19). Of course, it may be more relevant to compare regulation of IL-4 receptor expression to that of the p70 chain of the IL-2 receptor. p70 appears to mediate the signaling function of the receptor, but little is known about the regulation of the level of its expression; by contrast, p55 by itself appears not to cause cellular activation in response to IL-2.

Recently, a T-cell line (CT.4R) that can grow in IL-4 alone or in IL-2 alone was derived from a subline of the IL-2-dependent T-cell line CTLL (J. Hu-Li, J.O., C. Watson, and W.E.P., unpublished data). CT.4R cells express small amounts of the p55 chain of the IL-2 receptor when grown in IL-4 but rapidly up-regulate their level of expression of p55 when IL-2 is added and rapidly diminish their p55 expression when IL-2 is removed. On the other hand, CT.4R cells grown in IL-4 expressed >6-fold more IL-4 receptors on their cell surface (15,600 receptors per cell) than cells of the parental

---

**Fig. 3.** IL-4 receptor expression on CT.4R cells and CT.EV cells. CT.4R cells grown in IL-4 (500 units/ml) and CT.EV cells grown in IL-2 (10 units/ml) were harvested after a 3-day cultivation and subjected to a flow cytometric analysis of IL-4 receptors. Cells (5 × 10^6 cells in 50–100 µl) were incubated with biotinylated IL-4 (6.5 × 10^9 M) in the presence or absence of purified nonbiotinylated IL-4 (6 × 10^-5 M). The cells were washed, stained with PE-streptavidin-PE (Becton Dickinson), and analyzed on a FACSCAN. Ten thousand cells were analyzed. Mean fluorescent intensities are shown in parentheses. Receptor number was independently measured by Scatchard analysis of ^125I-IL-4 binding to CT.4R and CT.EV cells.

---

**Fig. 4.** Up-regulation of IL-4 receptor expression on IL-4-stimulated small resting T cells. Small resting T cells purified from BALB/c mesenteric lymph nodes were cultivated for 22 hr in the presence or absence of recombinant IL-4 (100 units/ml) at a cell density of 5 × 10^5 cells per ml. Flow cytometric analysis of IL-4 receptors expressed on T cells was conducted as described in Materials and Methods and in the legend of Fig. 3. Specific binding of biotinylated IL-4 to IL-4 receptors was shown by the addition of purified nonbiotinylated IL-4 before the addition of biotinylated IL-4.
Immunology: Ohara and Paul

CTLL line grown in IL-2 (CT.EV; 2400 receptors per cell). CT.4R cells are much more sensitive to IL-4 than are CT.EV cells; indeed, the latter cells cannot be propagated in IL-4 alone. These results suggest the existence of distinct regulatory mechanisms of growth factor receptor expression by their specific ligands. Furthermore, the fact that the same cell line displays independent ligand-mediated regulation of two growth factor receptors suggests that the signaling mechanisms used by these receptors are not identical.

The authors express great appreciation and thanks for the expert technical participation of Mrs. Cynthia Watson and Mr. Charles Hoes in the performance of these experiments, and Ms. Shirley Starnes for her excellent editorial assistance.
