RECOMBINANT INTERLEUKIN 7, PRE-B CELL GROWTH FACTOR, HAS COSTIMULATORY ACTIVITY ON PURIFIED MATURE T CELLS

By PHILIP J. MORRISSEY, RAYMOND G. GOODWIN, RICHARD P. NORDAN, DIRK ANDERSON, KENNETH H. GRABSTEIN, DAVID COSMAN, JOHN SIMS, STEPHEN LUPTON, BRUCE ACRES, STEVEN G. REED, DIANE MOCHIZUKI, JUNE EISENMAN, PAUL J. CONLON, AND ANTHONY E. NAMEN

From the Immunex Corporation, Seattle, Washington 98101; the Laboratory of Genetics, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892; and The Seattle Biomedical Research Institute, Seattle, Washington 98109

The stimulation of resting T cells to an activated state is caused by signals received by the cells from their environment. To date, neither the number of signals required nor their exact nature are precisely known. Thus far, an understanding of the triggering events indicates that a necessary signal is delivered to the cell by the crosslinking of the antigen-specific receptor by its ligand (reviewed in reference 1). Also, it has become clear that at least one other signal is required for the T cell to proliferate and become functionally active. Previous evidence has indicated that the cytokine, IL-1, was able to provide this "second" signal to the T cell (2).

However, it has recently been demonstrated that another cytokine, designated IL-6, also has the ability to provide such a second signal to mitogen-stimulated T cells (3). This property of IL-6 was revealed in an assay system that used highly purified lymph node T cells. This cell population, devoid of any detectable APCs, does not proliferate in vitro in response to mitogen, mitogen plus IL-2, or mitogen plus IL-1 (4). However, when these T cells were cultured with mitogen plus IL-6 a strong proliferative response was observed (3). Thus, by using this unique assay system a novel pathway for T cell activation involving IL-6 was identified.

In this paper, we demonstrate the existence of another cytokine that has costimulatory activity with Con A when added to cultures of highly purified T cells. This 25-kD protein, designated IL-7, was first identified by its ability to support the growth of pre-B cells that were generated in Witte-Whitlock bone marrow cultures (5-7). The cDNA encoding biologically active murine IL-7 was isolated from a bone marrow stromal cell cDNA library and expressed in COS-7 cells (8). Surprisingly, when the rIL-7 was tested in the T cell activation assay, it had potent co-stimulatory activity. Highly purified T cells cultured with Con A plus IL-7 have significantly increased levels of IL-2 receptor expression, IL-2 production, and proliferation as has been reported for IL-6. Thus, IL-7, originally isolated for its potent effect on the growth of pre-B cells, has additional activating properties for mature, peripheral T cells.
Materials and Methods

**Mice.** BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Female mice used in these studies were between 14 and 18 wk of age.

**Assay for T Cell Activation.** The axillary, inguinal, cervical, and mesenteric lymph nodes were dissected and a single cell suspension was prepared by teasing the nodes apart and gently crushing the tissue fragments. After washing, the lymph node cells were either cultured directly, or further purification of the T cells was performed. Highly purified T cells were prepared according to the method of Garman and Raulet (4). The lymph node cells were passed over a nylon wool column, treated with anti-I-A\(^d\) mAb plus complement and passed over nylon wool a second time. These cells were >99% T cells with no contaminating macrophages or B cells detectable. These T cells were cultured in 96-well flat-bottomed microtiter plates (2 \(\times\) \(10^4\) purified T cells/well) or in 24-well plates at a density of 2 \(\times\) \(10^6\)/well. They were cultured in RPMI 1640 plus 5% FCS, sodium pyruvate, and nonessential amino acids. After 3 d of culture \([\text{H}]\)Tdr (0.5 \(\mu\)Ci/well, sp act 20 Ci/mM) was added for the last 6–8 h of culture. The cultures were harvested with an automated sample harvester and the incorporated \([\text{H}]\)Tdr determined by liquid scintillation spectrometry.

**Cytokines.** Murine rIL-7 was produced by transfection of COS-7 monkey kidney cells with the expression plasmid pDC201 containing the IL-7 cDNA insert 1046B as described (8). The supernatant had a biological activity of 6 \(\times\) \(10^4\) U/ml in the pre-B cell assay. rIL-7 was purified from the transfected COS-7 cell supernatants as previously described (5) and had a similar biological activity (4 \(\times\) \(10^6\) U/\(\mu\)g protein) as did natural material purified from the supernatant of the IxNA6 bone marrow stromal cell line. In addition, H2-N terminal amino acid sequence of the purified recombinant-derived IL-7 was identical to that from the purified natural material.

cDNA clones encoding human and murine IL-6 were isolated from libraries constructed from human peripheral blood monocytes and a murine T helper cell line (clone 7B9) mRNA. In each case the libraries were screened with oligonucleotide probes derived from the published gene sequences (9, 10). The cDNA clones were inserted into the expression vector pDC201 (8) and transfected into COS-7 cells to produce rIL-6. The COS-7 cell supernatants transfected with the human and murine IL-6 genes had biological activities of 7 \(\times\) \(10^5\) U/ml and 3 \(\times\) \(10^6\) U/ml respectively, in the T cell activating assay. In addition, COS-7 cell supernatants containing human IL-6 also induced Ig secretion by CESS cells (3 \(\times\) \(10^5\) U/ml of biological activity).

Malignant and human IL-2 and human IL-1\(\beta\) were produced and purified in house as previously described (11-13).

**Antibodies.** Ascites fluid from the MKD6 cell line was used as a source of monoclonal anti-I-A\(^d\) in the antibody plus complement treatment of the lymph node T cells (14). Culture supernatants of the 83-12-5 cell line was used as a source of anti-Lyt-2.2 mAb (courtesy of Dr. J. Bluestone, University of Chicago); supernatant from the GK1.5 cell line as a source of anti-L3T4 mAb (15) and supernatant from the 7D4 cell line as a source of anti-IL-2 receptor mAb (16). For immunofluorescent staining, FITC-conjugated anti-rat \(\kappa\) chain mAb (Becton-Dickinson, Mountain View, CA) was used as a second step. The anti-murine IL-2 mAb, S4B6, was a kind gift of Dr. T. Mosman (DNAX, Palo Alto, CA) (17) to S. G. Reed.

Rabbit antibody to murine IL-6 was produced in rabbits by a series of four intravenous injections of IL-6 encapsulated in liposomes. The IgG fraction of this antisera was purified by binding to protein A. The antibody was able to inhibit the IL-6-mediated proliferation of the IL-6-dependent plasmacytoma cell line T1165 (Nordan, R. P., submitted for publication).

**Immunofluorescent Staining and Flow Cytometry.** For antibody binding, \(10^6\) cells were incubated with saturating concentrations of antibody for 30 min at 4\(^\circ\)C. After each incubation the cells were washed with PBS containing 0.1% BSA and 0.1% NaN\(_3\). Samples were analyzed on an EPIC-C flow cytometer (Coulter Electronics, Hialeah, FL) using an argon laser that emitted visible light at 488 nm and 300 mW constant power. Data were collected on \(10^4\) viable cells. For single-color analysis, cells incubated with the FITC-conjugated second antibody alone were analyzed to determine background fluorescence levels.

**Preparation of Purified CD4\(^+\) or CD8\(^+\) T Cells.** CD4\(^+\) lymph node T cells were prepared by passing whole lymph node cells over nylon wool and treating the nylon nonadherent frac-
tions with anti-I-A\(^d\) mAb plus complement as usual. The cells were then treated with anti-Lyt-2.2 mAb plus complement and passed over a second nylon wool column. These cells were >98% CD4\(^+\) as assessed by immunofluorescent staining and flow cytometry analysis.

CD8\(^+\) cells were prepared by passing whole lymph node cells over nylon wool followed by treatment of the nonadherent fraction with anti-I-A\(^d\) mAb plus complement. These cells were treated with anti-L3T4 mAb (GK1.5) plus complement, then positively selected on anti-Lyt-2.2 coated plastic dishes (18). These cells were routinely >99% CD8\(^+\), as assessed by immunofluorescent staining and flow cytometry analysis.

**Results**

**IL-7 Induces Proliferation of Highly Purified T Cells Cultured with Con A.** It has been previously shown that highly purified T cells do not proliferate in response to Con A or Con A plus IL-2, but that an additional signal that is not IL-1 is required (4). Recently it has been demonstrated that this second signal, previously known as T-activating factor (TAF), could be provided by the lymphokine IL-6 (3). Thus, in this system, purified T cells cultured with Con A alone were not induced to proliferate whereas cells cultured with Con A plus IL-6 proliferated strongly. In our assessment of the biological activity of recombinant-derived IL-7 we have found that it will also induce the proliferation of highly purified T cells cultured with Con A. In Table I, a representative experiment showing the effect of both rIL-6 and rIL-7 on the proliferative response of highly purified BALB/c T cells is shown. In this experiment, the T cells responded minimally to Con A alone. This response was slightly increased by the addition of 50 ng/ml of murine rIL-2. This amount of IL-2 is saturating, as higher responses are not seen with greater amounts of IL-2 (data not shown). Also it can be seen that cells cultured with human rIL-6 in the absence of Con A did not respond, but in the presence of Con A, significant levels of proliferation were seen. Similarly, culturing the cells with murine rIL-7 alone did not cause proliferation but in the presence of Con A a strong proliferative response was observed.

It should be noted that culture supernatants from COS cells transfected with an irrelevant plasmid had no activity in this assay (data not shown). In Fig. 1, the activity of the purified murine rIL-7 in the T cell activation assay is shown. Here it can be seen that purified IL-7 has potent activating capability when added to purified T cells plus Con A. In this assay, the half-maximal stimulation occurred with 0.3 ng/ml of protein. Over a number of experiments, this value ranged from 0.07 to 0.4 ng/ml of IL-7. In the pre-B cell growth assay, the half-maximal stimulation occurred at 0.03 ng/ml (data not shown). Thus, these results demonstrate that recombinant-derived IL-7, a cytokine originally isolated because of its ability to support the growth of pre-B cells, also has strong co-stimulatory activity for mature T cells.

Both CD4\(^+\) and CD8\(^+\) Cells Respond to Con A Plus IL-7. Previously, there has been considerable evidence produced that the activation requirements for CD4\(^+\) and CD8\(^+\) T cells are fundamentally different (19, 20). For instance, it has been proposed that CD8\(^+\) cells required an additional signal in order to generate functional cytotoxic cells that is not required for the generation of functional helper cells (21). Therefore, it was of interest to determine the responsiveness of purified CD4\(^+\) and CD8\(^+\) T cells to Con A plus IL-7. CD8\(^+\) cells were purified from nylon nonadherent T cells by a combination of negative (anti-L3T4 plus complement treatment) and positive selection (adherence to anti-Lyt-2.2-coated plates). Cells prepared in this manner had no detectable CD4\(^+\) T cells. CD4\(^+\) cells were prepared from nylon nonadherent
lymph node cells by negative selection with anti-Lyt-2.2 mAb plus complement treatment. These cells were then passed over a second nylon wool column. This population of cells was routinely >98% CD4⁺ T cells. These cell populations were then cultured with Con A or Con A plus IL-6 or IL-7. The results of a representative experiment are shown in Table II. Here it can be seen that both the CD4⁺ and CD8⁺ T cells responded minimally to Con A alone, but the cells cultured with Con A and either IL-6 or IL-7 proliferated strongly. Analysis of the cell populations at the end of culture by immunofluorescent staining and flow cytometry showed that there was <2% contaminating cells. In addition, other cultures were subjected to two color-flow cytometry to determine the cell cycle status and proportion of each (CD4⁺ or CD8⁺) cell population. The results of this analysis (data not shown) confirmed the results obtained in the proliferative assay in that both CD4⁺ and CD8⁺ T cell subpopulations proliferated in response to Con A plus IL-7 and IL-6. Thus, both IL-6 and IL-7 have co-stimulatory activity for the CD4⁺ and CD8⁺ subpopulations of T cells.

**TABLE I**

Response of Purified T Cells to Con A Plus IL-7 or IL-6

| Cells cultured with: | Dilution of cytokine | [³H]TdR uptake: cpm (SEM) x 10⁻³ |
|----------------------|----------------------|-------------------------------|
| Con A                | 2 (1.2)              |                               |
| No additions         | 0.52 (.12)           |                               |
| Con A + IL-2         | 4.3 (0.9)            |                               |
| Con A + IL-7         | 1:10                 | 51.9 (9.3)                    |
|                      | 1:500                | 37.6 (3.2)                    |
|                      | 1:2,500              | 6.3 (1.5)                     |
| IL-7                 | 1:10                 | 3.6 (1.1)                     |
|                      | 1:500                | 18.3 (0.6)                    |
|                      | 1:2,500              | 5.0 (1.8)                     |
| IL-6                 | 1:10                 | 2.7 (1.4)                     |

Cells (2 x 10⁵/well) were cultured for 72 h with the indicated additions. The concentration of Con A was 2.5 µg/ml; the concentration of IL-2 was 50 ng/ml. IL-6 and IL-7 as COS cell supernatants were added at the dilutions indicated. The data are the arithmetic mean of quadruplicate cultures.

**FIGURE 1.** Co-stimulatory activity of purified murine rIL-7 for purified peripheral T cells. Dilutions of purified rIL-7 were added to cultures containing highly purified T cells plus Con A (2.5 µg/ml). [³H]Tdr uptake was assessed in the final 6 h of a 72-h culture. Response of the cells to Con A in the absence of IL-7 was 1,083 cpm. Response of cells alone was 486 cpm. The data are the average of triplicate values. The SEM was <5% of the mean and is omitted for convenience.
**TABLE II**

*Response of Purified L3T4+ or Lyt-2+ T Cells to Con A Plus IL-6 or IL-7*

| T cell subpopulation | Proliferative response to: |  |
|----------------------|-----------------------------|-----------------------------|
|                      | Con A  | Con A + IL-6 | Con A + IL-7 |
|                      |  cpm (SEM) x 10^-3          |  cpm (SEM) x 10^-3          |  cpm (SEM) x 10^-3          |
| CD4+                 |  3.2 (.56) | 31.8 (1.7) | 42.2 (3.0) |
| CD8+                 |  1.2 (.74) | 29.5 (2.1) | 36.1 (2.4) |

Purified CD4+ or CD8+ T cells (10⁵ cells/well) were cultured for 72 h. [³H]TdR was added for the last 6 h of culture. The data are the mean (SEM) of quadruplicate values. IL-6 and IL-7 were COS cell supernatants and were at a final concentration of 10% (vol/vol).

*IL-7 Induces IL-2 Receptors and IL-2 Production by Resting T Cells.* It has previously been demonstrated that cytokines with co-stimulatory activity for T cells (e.g., IL-1 and IL-6) act to induce the expression of IL-2 receptors and IL-2 production as well as proliferation (2, 3). We investigated whether IL-7 also induced these functional changes in the stimulated T cell population.

Highly purified T cells (2 x 10⁶/well) were cultured in 24 well plates with either no additives, Con A alone, or Con A plus IL-7. At 24 h, 0.1 ml was removed from the cultures and assayed for IL-2 activity by the induction of proliferation of CTLL cells in the presence of 11B11 mAb in order to neutralize any IL-4 activity (22). The results shown in Fig. 2 clearly demonstrate that incubation of the highly purified T cells with Con A plus IL-7 caused a substantial increase in the level of IL-2 production. At 48 h, the cells in these cultures were harvested and stained with the anti-IL-2 receptor antibody 7D4 plus fluoresceinated second step, and analyzed by flow microfluorimetry. As can be seen in Fig. 3, cells incubated with Con A alone expressed low levels of the IL-2 receptor (17% of the cells stained positively with a mean linear fluorescence of 15). Cells incubated with Con A plus IL-7 had high levels of IL-2 receptor expression (67% of the cells stained positively with a mean linear fluorescence of 15).
fluorescence of 83). Thus, the co-stimulatory activity of IL-7 appears to function by the induction of IL-2 receptor expression and IL-2 production. In our hands IL-6 also stimulates IL-2 production and IL-2 receptor expression (data not shown). Additionally, IL-7 was as potent as IL-6 in the induction of both of these functional changes.

**T Cell Proliferation Induced by IL-6 and IL-7 is Dependent on IL-2 Production.** T cells cultured with Con A plus IL-6 or IL-7 are induced to proliferate and to secrete IL-2. It was of interest to determine if IL-2 was necessary for proliferation or if the signals provided by Con A plus IL-6 or IL-7 were sufficient to drive proliferation. To approach this question, the anti-murine IL-2 mAb S4B6 was added to the cultures at their initiation to determine if the neutralization of the IL-2 produced would inhibit the proliferation. The results of such an experiment are shown in Table III. Here it can be seen that the addition of S4B6 to the cultures of T cells plus IL-6 or IL-7 totally inhibited the proliferative response. In addition, the response was restored by the addition of human IL-2 to the cultures. Since S4B6 does not bind or neutralize human IL-2, this result demonstrates that the inhibition of proliferation is not due to nonspecific effects but is due to the neutralization of IL-2 activity in the cultures by the mAb. Thus, these results show that the proliferative response

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**Figure 3.** Expression of IL-2-R by purified T cells cultured with Con A plus IL-7. Purified lymph node T cells were cultured for 48 h in 24-well plates (2 × 10⁶ cells/well in 2 ml) with 2.5 µg/ml Con A and 50 ng/ml purified rIL-7. The cells were harvested, stained, and analyzed as described in Materials and Methods.

**Table III**

| Purified T cells cultured with Con A plus: | [³H]Tdr uptake cpm (SEM) × 10⁻³ |
|------------------------------------------|-------------------------------|
| No additions                             | 4.6 (0.8)                     |
| IL-7                                     | 25.8 (1.1)                    |
| IL-7 + S4B6 (1:400)                      | 5.2 (0.2)                     |
| (1:1,000)                                |                               |
| IL-7 + S4B6 (1:400) + hu IL-2            | 64.5 (2.7)                    |
| IL-6                                     | 21.6 (3.1)                    |
| IL-6 + S4B6 (1:400)                      | 3.6 (1.2)                     |
| (1:1,000)                                | 3.5 (0.6)                     |
| IL-6 + S4B6 (1:400) + hu IL-2            | 57.5 (3.2)                    |

Purified T cells (2 × 10⁶/well) were cultured for 72 h with the indicated additives. IL-6 and IL-7 as COS cell supernatants were added at 10% (vol/vol) final dilutions. S4B6 mAb was ascites fluid. Human rIL-2 was used at a concentration of 25 ng/ml.
of highly purified T cells to Con A plus IL-6 or IL-7 is dependent on the production of IL-2.

The Co-stimulatory Activity of IL-7 Is Not Due to the Induction of IL-6. The action of IL-7 in the T cell activation assay could possibly be due to the induction of IL-6 production by the T cells. Thus, IL-7 would not act directly as a comitogen for the T cells, but as an inducer of IL-6 production. To address this possibility, a neutralizing anti-murine IL-6 antibody was added to cultures of highly purified T cells with Con A plus IL-6 or IL-7. If IL-7 acted to induce the production of IL-6 which was the co-mitogen, then the anti-IL-6 antibody should inhibit the IL-7-mediated response. The results of this experiment are shown in Table IV. Here it can be seen that the protein A-purified rabbit anti-murine IL-6 inhibited the response of the T cells stimulated with Con A plus IL-6 at all three doses of IL-6 used. In fact, at the highest concentration of antibody (1 μg/ml), the inhibition was >90%. In contrast, the response of the T cells to IL-7 was not significantly reduced by the addition of the antibody. It should be noted that the addition of an equivalent amount of purified rabbit anti-human IL-1 had no effect on the response of the T cells to the Con A plus IL-6 or IL-7. Thus, these results strongly suggest that action of IL-7 in the T cell activation assay is not via the induction of IL-6, but that IL-7 can act directly as a comitogen for T cells.

Discussion

The signals required to induce a proliferative response in T cells are complex. It is clear that a single stimulus is not sufficient, but that a series of signals must be encountered by the cell before activation occurs. In this report we have found that IL-7, a molecule discovered because of its potent activity on pre-B cells, also has costimulatory activity on purified T cells. This activity was revealed using the assay system of Raulet and Garman (4) in which T cells are highly purified by a

| Antibody concentration (μg/ml) | Proliferative Response to Con A Plus IL-6  | Proliferative Response to Con A Plus IL-7 |
|------------------------------|-------------------------------------------|------------------------------------------|
|                              | (1/1,000) | (1/5,000) | (1/25,000) | 25 ng/ml |
| 0                            | 119.7    | 97.5     | 86.3       | 133.3    |
| 0.1                          | 16.9     | 8.2      | 10.4       | 135.4    |
| 0.5                          | 45.9     | 15.1     | 12.9       | 138.6    |
| 0.25                         | 92.9     | 45.4     | 26.5       | 129.7    |
| 0.12                         | 94.8     | 67.7     | 38.6       | 132.5    |
| 0.06                         | 105.8    | 75.4     | 48.4       | 137.7    |
| 0.03                         | 110.3    | 83.8     | 68.8       | 133.5    |
| 0.015                        | 118.1    | 93.7     | 81.3       | 135.8    |

Highly purified T cells were cultured with Con A and the indicated amount of murine IL-6 or IL-7. The rabbit anti-mouse IL-6 was added in the indicated amounts at the initiation of culture. [3H]TdR uptake was measured on day 3. The response of the cells plus Con A only was 2,396 cpm.
combination of two passages through nylon wool and treatment with anti-Ia plus complement before culture. These manipulations result in a cell population that is highly enriched for T cells and functionally devoid of APCs. Cells prepared in this manner exhibit unusual requirements for triggering in vitro in that incubation with mitogen plus IL-2 does not result in significant cellular activation. Originally, Garman and Raulet (4) described a factor in the culture supernatant of Con A-stimulated murine spleen cells and certain T cell hybridomas that has co-stimulatory activity for these highly purified T cells. Recently, this activity has been attributed to IL-6 (3). Here we find that IL-7 also is active in this assay system.

In this report we have also provided evidence that IL-7 exerts its co-stimulatory effect by inducing the expression of IL-2 receptors and stimulating the production of IL-2. Thus, cultures of T cells with Con A plus IL-7 had significantly greater levels of IL-2 in the culture supernatant than cells incubated with Con A alone. Also T cells stimulated with Con A plus IL-7 had a greater percentage of cells expressing the IL-2 receptor and the mean level of receptor expression was also greater than that observed by the cells incubated with Con A alone. The ability of the anti-IL-2 mAb to inhibit the proliferation of the T cells incubated with Con A plus IL-7 further demonstrates that the production of IL-2 is necessary for the proliferative response. Thus, it appears that IL-7, as well as IL-6 (3), exerts co-stimulatory activity by inducing the production of IL-2 and the expression of IL-2 receptors.

It is possible that the activity of IL-7 in this assay system was to induce the production of IL-6 that then was the actual TAF (inducing IL-2 production and IL-2 receptor expression). However, the inability of the anti-IL-6 antibody to neutralize the IL-7-induced T cell proliferation strongly suggests that the IL-7, in of itself, has T cell activating properties.

It is not clear if the IL-6 and IL-7 induce the same or different subpopulations of T cells. Cell cycle analysis of the T cells stimulated with Con A plus IL-6 and IL-7 over many experiments has revealed that from 21 to 43% of the T cells were induced to cycle (data not shown). Cultures of T cells stimulated with Con A plus IL-6 and IL-7 have shown the effects of both cytokines to be synergistic and not additive, suggesting that at least some T cells respond to both factors. We are currently using limiting dilution analysis of T cells responding to Con A plus IL-6 and/or IL-7 to approach this question.

It is also apparent that these molecules are functionally pleiotropic. For instance, IL-6 also has potent activity on B cells, causing the induction of Ig synthesis and supporting the growth of B cell hybridomas and plasmacytoma (9, 23). IL-7 was also originally cloned because of its potent growth factor activity on pre-B cells (8). Thus both these factors have significant growth and differentiative capabilities for B cells. Of additional interest is the observation that IL-6 augmented the proliferation of PHA-activated human thymocytes (24), thereby suggesting that this cytokine may be involved in T cell differentiation. We have found that IL-7 also has comitogenic activity for thymocytes (Conlon, P. J., submitted for publication). Indeed IL-7 has co-stimulatory activity for the least mature CD4+CD8+ thymocyte population in both adult and fetal thymus. Thus, this cytokine can augment the proliferative response of immature as well as mature T cells. The range of activities associated with IL-7 indicates that it may play an important role in the production and function of B cells and T cells.
Summary

The activation of highly purified murine peripheral T cells in vitro by Con A is dependent on a co-stimulatory signal that is not IL-1 or IL-2. Previous evidence has demonstrated that the recently defined lymphokine IL-6 could provide costimulatory activity for purified T cells cultured with Con A. In this report we demonstrate that IL-7 also has potent co-stimulatory activity for purified murine T cells, as well as its previously described ability to support the growth of pre-B cells in Witte-Whitlock cultures. When rIL-7 was added to cultures of purified T cells together with Con A, it induced the expression of IL-2 receptors, IL-2 production, and consequently proliferation. In addition, IL-7 exhibited the same magnitude of activity in this assay as IL-6. Also, anti-IL-6 antibody which inhibited the IL-6-induced response had no effect on the IL-7 response. Thus, IL-7 does not act by inducing IL-6. These results demonstrate that IL-7, a potent growth stimulus for pre-B cells, also has a role in T cell activation.

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