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Immunology

Autocrine growth inhibition of a cloned line of helper T cells

(interleukin 1/interleukin 2/immunoregulator)

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ABSTRACT **The growth of T lymphocytes is dependent on the T-cell growth factor interleukin 2 (IL-2), which causes T cells bearing high-affinity receptors for IL-2 to proliferate. Most cloned helper-T-cell lines can be shown to both produce and respond to IL-2; thus, growth of such cells is by an autocrine mechanism. We report that the failure of the cloned murine T-cell line D10.G4.1 to respond to its own IL-2 results from the secretion, by the same cells, of a potent inhibitor of the IL-2-driven T-cell proliferative response. This inhibition can be overcome by increasing the number of IL-2 receptors expressed by the target cell. In the cloned T-cell line producing the inhibitory substance, this increase in IL-2 receptors is driven by the monokine interleukin-1. We propose that this inhibitor of IL-2 responses may play a role in preventing "bystander" activation of T cells by IL-2 released *in vivo* and could be a potent pharmacologic agent.**

The growth of many cell types has been shown to depend upon growth factors (1). In the case of T lymphocytes, the major stimulus to growth is the T-cell-derived growth factor interleukin 2 (IL-2) (2). This protein interacts with specific high-affinity receptors expressed on activated T cells, causing them to proliferate (3). The gene encoding IL-2 has been cloned (4), and these findings have been confirmed using recombinant-derived IL-2 (rIL-2).

Given this apparently general rule regarding the central role of IL-2 in T-cell growth, it was puzzling that a cloned helper-T-cell line, D10.G4.1 (D10), that produces large amounts of IL-2 upon crosslinking of its specific receptor for antigen by a monoclonal anti-receptor antibody, does not proliferate in response to this antibody (5, 6). This finding was more puzzling in light of the observation that this cloned T-cell line will proliferate in response to exogenous IL-2 (5).

In analyzing the growth requirements of the D10 cloned T-cell line, we have discovered that it produces not only IL-2 but also an inhibitor of its own response and of the response of many cloned T-cell lines to IL-2. Thus, this cloned T-cell line demonstrates not only autocrine growth but also autocrine growth inhibition. In this report, we describe the biological properties and preliminary chemical characterization of this growth-inhibitory substance. Further, we propose a biological role for such growth-inhibitory substances in increasing the specificity of T-cell growth in responses to antigen *in vivo*.

MATERIALS AND METHODS

Animals. BALB/cByJ and AKR/J mice were obtained from The Jackson Laboratory.

Cloned T-Cell Lines. The AKR/J cloned T-cell line D10.G4.1 (D10) has been described extensively (5–9). It is specific for the hen egg white protein conalbumin in the context of I-A^k and also responds to the monoclonal anti-receptor antibody 3D3 (see below). The BALB/cByJ cloned T-cell lines 5.5 and 5.9.24 have been described in detail elsewhere (10–12); they are specific for hen ovalbumin and I-E^d or I-A^d, respectively. These cloned T-cell lines were maintained by feeding with syngeneic mitomycin C-treated spleen cells and specific antigen (100 μg/ml) every 1–3 weeks in Click's EHAA medium supplemented with 10% fetal bovine serum and 5% IL-2-containing supernatant of Con A-treated rat spleen cells (13). Between feedings with cells and antigen, the cells were fed at intervals with the same medium.

Culture Supernatants. *Interleukin 1.* Murine IL-1 was prepared as described (6) by stimulating the murine macrophage cell line P388.D1 with bacterial lipopolysaccharide (1 μg/ml) for 24 hr.

Interleukin 2. IL-2 was produced by stimulating the T-cell hybrid AOFS, kindly donated by P. Marrack and J. Kappler (National Jewish Hospital, Denver, CO), with Con A (2.5 μg/ml) for 24 hr. Methyl α-D-mannoside (20 mg/ml) was added to these supernatants to inactivate the Con A.

Recombinant-derived human IL-2. Human rIL-2 was a kind gift of R. Robb (E.I. du Pont de Nemours, Glenolden Laboratories, Glenolden, PA). It was produced in *Escherichia coli* from cloned Jurkat IL-2 cDNA and was purified by high-performance liquid chromatography.

D10 supernatants. D10 cells (10^5 per ml) were stimulated overnight in Click's EHAA medium containing proteins for serum-free culture (HB101) at 1% (vol/vol) from Hana Biologics (Berkeley, CA), either with antibody 3D3 (10 ng/ml) or with Con A (2.5 μg/ml). Con A-containing supernatants were neutralized with methyl α-D-mannoside (20 mg/ml), and all assays using such supernatants contained methyl α-D-mannoside (5 mg/ml).

Bioassays. *Proliferation assays.* Cells plus supernatants were mixed in 200 μl volumes of Click's EHAA/10% fetal bovine serum in flat-bottom multiwell trays. After culture for 48 hr at 37°C in a 5% CO_2 atmosphere, 1 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine was added to each culture. Sixteen hours later, cells were harvested with a PHD harvester (Cambridge Technologies, Cambridge, MA), and incorporated ^{3}H in triplicate cultures was determined. Standard deviations of mean cpm incorporated were <20% and have been omitted.

IL-1 assay. IL-1 was assayed as previously described by comitogenesis of D10 cells and 3D3 (7, 14). Such supernatants caused no growth of D10 in the absence of 3D3 and were without activity in the IL-2 assay.

IL-2 assay. IL-2 was assayed by measuring the incorporation of [^{3}H]thymidine by the IL-2-dependent T-cell line HT-2. After 36 hr of culture, cells (10^4) were incubated for 16 hr with 1 μCi of [^{3}H]thymidine.

Inhibitor assay. This is described in detail in the *Results*.

Monoclonal Antibody. The monoclonal anti-receptor antibody 3D3 was produced and assayed as described (5). All

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Abbreviations: IL-1, interleukin 1; IL-2, interleukin 2; rIL-2, recombinant IL-2.

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batches tested thus far maximally stimulate cloned line D10 at protein concentrations of about 1 ng/ml. Batches are produced as ascites fluid, partially purified by precipitation with saturated ammonium sulfate, dialyzed, sterile-filtered, and stored at −70°C in aliquots.

RESULTS

D10 Cells Stimulated by Anti-Receptor Antibody Produce Both IL-2 and an Inhibitor of D10 Proliferation. When D10 cells are stimulated with the monoclonal anti-receptor antibody 3D3, they produce large amounts of IL-2, as detected by bioassay of supernatants on HT-2 cells (6). However, D10 cells fail to proliferate under these conditions unless IL-1 is also added to the cultures (Table 1). In order to explore further the growth characteristics of the D10 line, supernatant from D10 cells was compared to another source of IL-2, supernatant from the T-cell hybrid AOFS. The D10 supernatant contains 4-fold more IL-2 than a supernatant derived from AOFS (Fig. 1*A*). As shown previously (5), AOFS supernatants also stimulate proliferation by D10 cells in the absence of 3D3 (Fig. 1*B*). These findings indicate that D10 both produces IL-2 and expresses functional receptors for IL-2. Why, then, does D10 not proliferate when it produces its own IL-2? A preliminary answer emerges when one examines the effects of a supernatant from D10 stimulated with 3D3 on the growth of D10 cells. In the absence of additional 3D3, this supernatant at high concentrations does not significantly stimulate growth of D10 cells; however, as the supernatants are diluted, D10 cells show increasing proliferative responses (Fig. 1*B*). These titration curves strongly suggest that D10 cells, stimulated by crosslinking of their receptors by the monoclonal antibody 3D3, secrete an inhibitor of their own response to IL-2, to which HT-2 cells are relatively resistant.

IL-1 added together with 3D3 leads to intense proliferative responses by D10 cells, as reported previously (6). Thus, if D10 makes an inhibitor of its own response to IL-2, IL-1 in some way blocks this inhibitory pathway. Previous studies have shown that the combination of IL-1 and 3D3 causes a 10-fold increase in IL-2 receptors on D10 cells (6).

D10 Supernatants Inhibit the Response of Other Cloned T-Cell Lines to IL-2. The cloned T-cell line D10 has a number of unusual properties and thus might be unique in its susceptibility to inhibition by D10 supernatants. Therefore, we tested a number of cloned T-cell lines for their ability to proliferate in the presence of rIL-2, D10 supernatant, or a mixture of rIL-2 and D10 supernatant (Fig. 2). The BALB/c cloned T-cell lines 5.5 and 5.9.24 respond well to rIL-2 but do not proliferate in response to D10 supernatants that, as judged by their ability to stimulate the proliferation of HT-2 cells, contain significantly more IL-2. Further, these D10 supernatants can inhibit the response of 5.5 or 5.9.24 cells to rIL-2; in the case of 5.5 cells, this can occur at very low (<1:2000) levels of crude 24-hr culture supernatant.

These experiments show that D10 supernatants can inhibit the response of several cloned T-cell lines not only to D10-derived IL-2 but also to rIL-2. As the monoclonal antibody 3D3 does not react specifically with clones 5.5 or 5.9.24, and as analogous results are obtained whether the supernatant is obtained by stimulation with Con A or 3D3 (Fig. 3), the inhibitory substance is not 3D3. Finally, as clones D10 and 5.5 differ in antigen specificity and major histocompatibility complex (MHC) restriction and derive from mice differing at MHC, immunoglobulin heavy chain, and many other loci, this inhibitor is not demonstrably genetically restricted in its action on its target cell and is thus distinct from most known suppressor factors. For simplicity, we shall refer to the inhibitory substance(s) in these supernatants as inhibitor.

Table 1. Cloned T-cell line D10.G4.1 requires two signals to proliferate: IL-1 and crosslinking of the antigen–Ia receptor

Stimulus	[^{3}H]Thymidine incorporation,* cpm
None	131
3D3†	777
IL-1‡	275
3D3 + IL-1	32,542

*[^{3}H]Thymidine incorporated by 2 × 10^4 D10.G4.1 cells on day 3 after stimulation; means of triplicate cultures. Standard deviations, <20% of the mean, are omitted.
†Monoclonal anti-D10.G4.1 receptor antibody (20 ng/ml).
‡Supernatant (3% final concentration) of lipopolysaccharide activated P388.D1 cells.

IL-1 Does Not Block Either Inhibitor Secretion or Inhibitor Action. It seems highly likely that the failure of D10 cells to respond to their own endogenously produced IL-2 is the result of a dominance of inhibitor over IL-2, as is seen clearly in Figs. 1 and 2. However, as reported previously and repeated in Table 1, addition of IL-1 to D10 cells can overcome the effects of the inhibitor and cause growth of D10 cells stimulated by 3D3. This effect could result from several possible actions of IL-1. To test for some of these, we stimulated D10 cells with 3D3, with 3D3 and IL-1, or with nothing. These supernatants were then assayed for their ability to stimulate growth of HT-2 cells, 5.9.24 cells, or 5.9.24 cells stimulated with rIL-2. As seen in Fig. 3*A*, IL-1 did not significantly increase the amount of IL-2 secreted by D10 cells stimulated with 3D3, nor did it affect secretion of inhibitor, as 5.9.24 cells did not grow in response to either 3D3-induced supernatant. Further, the response of 5.9.24 cells to rIL-2 is equally inhibited by both supernatants. Thus, IL-1 does not overcome the effects of inhibitor by stimulating the production of more IL-2 or by suppressing inhibitor secretion. In the absence of 3D3, neither IL-2 nor inhibitor is secreted (data not shown).

IL-1 also does not inhibit the action of inhibitor directly. When D10 supernatant induced by 3D3 alone is mixed with IL-1 and added to 5.9.24 cells, growth is not induced, nor is the inhibition of the response of 5.9.24 cells to rIL-2 affected

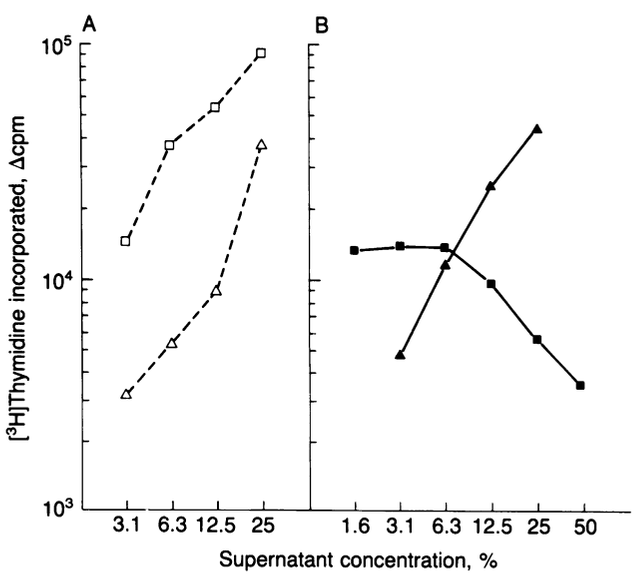

FIG. 1. Culture supernatant of D10 cells induced by 3D3 contains high levels of IL-2 but inhibits growth of D10 cells. Various amounts of D10 (□, ■) or AOFS (△, ▲) supernatant were added to 10^4 HT-2 (*A*) or 2 × 10^4 D10 cells (*B*).

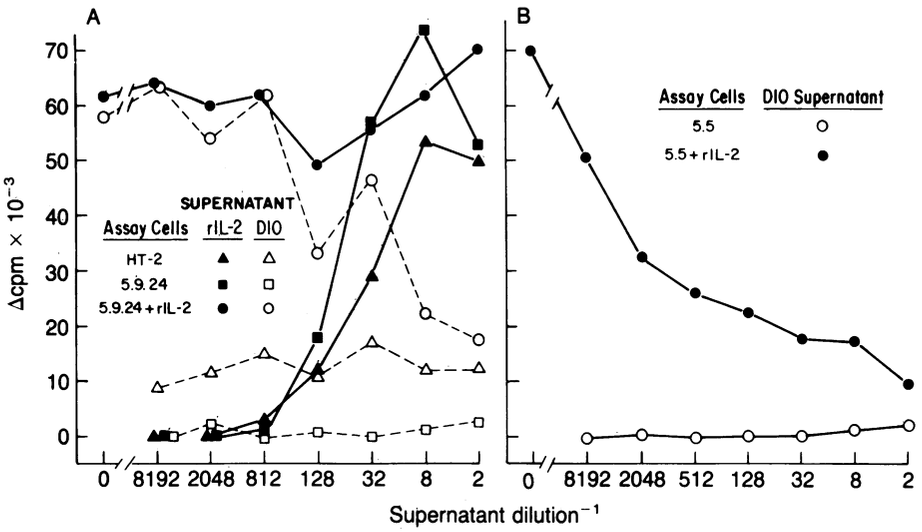

FIG. 2. D10 supernatant inhibits the response of cloned T-cell lines to rIL-2. (*A*) D10 supernatant induced by Con A stimulation causes growth of HT-2 cells (△) to a titer greater than that of rIL-2 at 4 units/ml (▲) but does not stimulate growth of 5.9.24 cells (□), which respond well to rIL-2 at 0.5 unit/ml (■). rIL-2 is not inhibitory to growth of 5.9.24 cells (●), whereas D10 supernatant is (○). (*B*) Clone 5.5 T cells do not grow in response to the same D10 supernatant as in *A* (○), but their response to rIL-2 (0.5 unit/ml) is well inhibited by the same D10 supernatant (●).

by addition of IL-1. Thus, IL-1 appears to prevent neither inhibitor synthesis nor its action on a variety of target cells.

Inhibitor Is a Nondialyzable, Heat- and Acid-Stable Molecule of $M_r \approx 12{,}000$. To further characterize the inhibitory molecule contained in D10 supernatant, we subjected the supernatant to treatment with acid, heat, or dialysis. The inhibitory activity in culture supernatants is stable at 4°C for >6 months and at 56°C for 30 min. It is stable in 0.01 M HCl for at least 30 min and is thus distinct from an acid-labile inhibitor previously described in crude, IL-2-containing supernatants (15). It does not pass through dialysis tubing with a nominal molecular weight cutoff of 6000. When samples are run on Sephadex G-50 Superfine and assayed for IL-2 on HT-2 cells, several peaks of activity are found, the smallest of which correlates with M_r 15,000, as determined by a separate run of molecular weight markers. As shown in Fig. 4, the inhibitor appears to be slightly smaller than this species of IL-2. Further, in partially separating the inhibitor from IL-2, the degree of inhibition has been increased to >95%, which is better than is seen with crude, IL-2-containing supernatants (see Figs. 2 and 3). Thus, we estimate the molecular weight of the inhibitory substance to be about 12,000.

DISCUSSION

The cloned T-cell line D10.G4.1 has several unique characteristics, among which are (*i*) the ability to respond to soluble

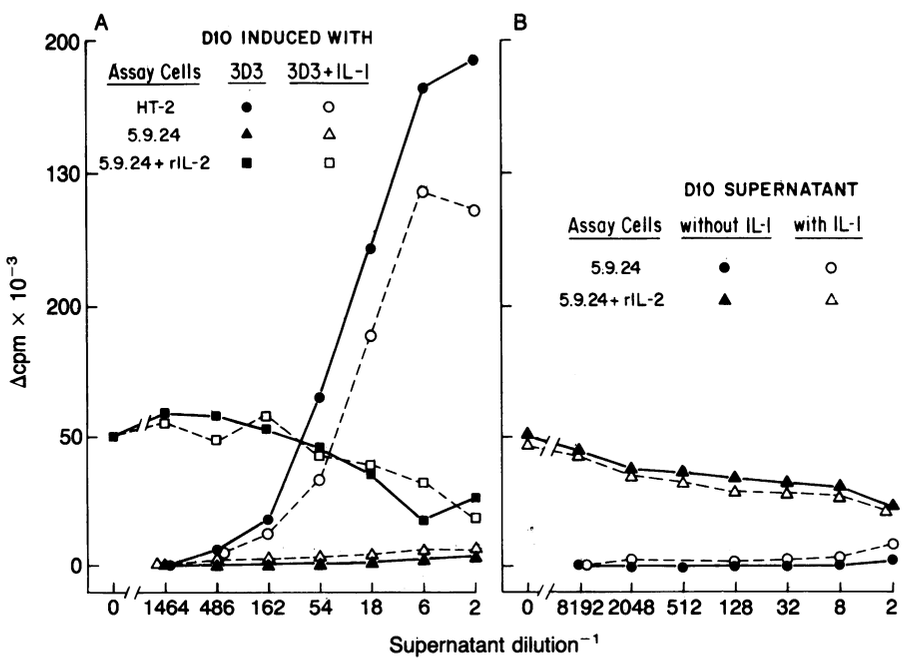

FIG. 3. IL-1 prevents neither production nor effect of inhibitor. (*A*) D10 cells were activated with 3D3 (●, ▲, ■) or with 3D3 plus IL-1 (○, △, □). Supernatants were assayed on HT-2 (●, ○) or 5.9.24 cells alone (▲, △) or with rIL-2 at 0.5 unit/ml (■, □). (*B*) The same supernatant induced with 3D3 and used in *A* was mixed with a maximally stimulating concentration of IL-1 (○, △) (5% P388.D1 supernatant) or tested directly (●, ▲) on 5.9.24 cells (●, ○) or 5.9.24 cells plus rIL-2 at 0.5 unit/ml (▲, △).

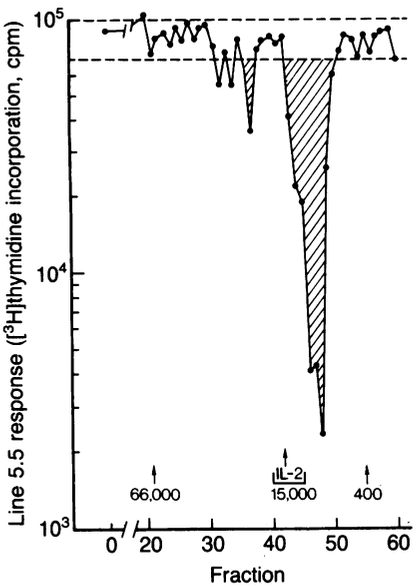

FIG. 4. Size-separation of IL-2 and inhibitor on Sephadex G-50 Superfine. D10 supernatant was size-fractionated on a Sephadex G-50 Superfine column (1.5 × 30 cm) by elution with 5 mM sodium phosphate buffer (pH 6.0) containing polyethylene glycol 6000 (30 μg/ml). Fractions were assayed, at 20% final concentration, for IL-2 activity on HT cells (data not shown) or for inhibitor activity on line 5.5 cells with rIL-2 (0.5 unit/ml). Positions of molecular weight markers and the major IL-2 peak are indicated by arrows. Inhibitory activity emerges at $M_r \approx 12{,}000$ as a single peak (hatched area). Broken horizontal lines denote range of control responses of assay cells (line 5.5) to rIL-2.

monoclonal anti-receptor antibody by secretion of IL-2 and (*ii*) the requirement for IL-1 for optimal growth. This IL-1-dependence is the basis for a highly sensitive IL-1 assay that has been useful in characterizing IL-1 (7, 14). It has been proposed (6, 7) that the IL-1-induced increase in IL-2 receptors, detected by a monoclonal anti-IL-2 receptor antibody, was the reason for this sensitivity to and requirement for IL-1 in D10 growth. However, the finding that D10 would proliferate strongly in response to exogenous IL-2, including rIL-2, at levels lower than those contained in supernatants of D10 stimulated with 3D3, suggested that D10 cells possess biologically active IL-2 receptors in the absence of either IL-1 or 3D3 and, thus, that the failure of D10 to respond to the IL-2 it produced must have a more complex explanation. In the present experiments, we found that D10 indeed secretes not only its own growth factor but also an inhibitor of that growth factor, to which it is sensitive and to which the IL-2-indicator line HT-2 is relatively resistant.

The present studies provide evidence that this inhibitor is distinct from lymphotoxin (16), interferon γ (17), and inhibitor of DNA synthesis (18), all of which can block T-cell proliferation. This distinction is based in part on the biochemical data and in part on the fact that D10 and 5.9.24 differ markedly in their susceptibility to lymphotoxin and interferon γ yet are identical in their susceptibility to the inhibitor in D10 supernatants (Figs. 1 and 2; unpublished data). Further, D10 secretes neither lymphotoxin nor interferon γ in detectable amounts (19). The "inhibitor of DNA synthesis" has been well-characterized and blocks thymidine uptake in all cell lines tested, and its action is not reversible by either IL-1 or IL-2 (18, 20). A similar conclusion has been reached by Honda *et al.* (21), who have recently observed a similar inhibitory molecule in supernatants from Con A-stimulated cells.

We have tested several cloned helper-T-cell lines for production of the inhibitor in response to Con A as well as for susceptibility to it, and all both produce and respond to the inhibitor (P.J.C. and C.A.J., unpublished results). We have chosen 5.5 cells to be our standard assay population because they are highly sensitive, respond well to rIL-2, and are highly resistant to lymphotoxin and interferon γ, which could be confused with the inhibitor (22).

The biological significance of an autocrine growth inhibitor is not clear, although it seems logical that if cells can produce factors that stimulate growth they should also make molecules that inhibit growth. In the specific case of helper T cells, however, another function for this inhibitor can be envisaged. This is based on the hypothesis that the resistance of HT-2 cells to the effects of the inhibitor is due to the very high number of high-affinity IL-2 receptors found on such cells; HT-2 has 10 times as many high-affinity IL-2 receptors as do resting D10 cells (R. Robb and J.B.H., unpublished observations). Further, while the production of IL-2 makes it difficult to measure IL-2 receptors on stimulated D10 cells, a rapid, 10-fold increase in surface molecules reacting with anti-IL-2 receptor antibody has been detected on D10 cells stimulated in the presence, but not the absence, of IL-1 (6). Thus, we propose that the biological role of an inhibitor of IL-2 responses is to prevent the "bystander" activation of T cells bearing low numbers of high-affinity IL-2 receptors during immune responses *in vivo*. As a D10 cell requires two signals (namely, receptor crosslinking and IL-1) to express IL-2 receptors (6) in numbers sufficient to respond to its own IL-2 in the presence of its own inhibitor, so a T cell undergoing stimulation on the surface of a macrophage or other antigen-presenting cell would have its receptors crosslinked by antigen and also receive IL-1 as either a membrane signal (23) or a secreted product. Such cells would secrete both IL-2 and an inhibitor of IL-2 responses that acts preferentially on T cells with low numbers of high-affinity IL-2 receptors. In this way, a nonspecific growth factor becomes more highly specific for cells actually undergoing antigenic stimulation *in vivo*. Whether these speculations prove true or not, an inhibitor of IL-2 responses has interesting potential as a pharmacologic agent. IL-2 plays a central role in immune responses, and a specific inhibitor of IL-2 responses could be used to control unwanted or excessive immune responses, as in autoimmunity, allergy, or graft rejection.

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