Interleukin 2–diphtheria toxin fusion protein can abolish cell-mediated immunity in vivo

(delayed-type hypersensitivity/interleukin 2 receptors/T lymphocytes)

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Communicated by Eugene Braunwald, January 25, 1988 (received for review November 25, 1987)

ABSTRACT De novo expression of the interleukin 2 receptor (IL-2R) is a critical and pivotal event in initiation of an immune response. Targeting the low-affinity IL-2-binding p55 subunit of the high-affinity IL-2R with the rat anti-mouse IgM monoclonal antibody M7/20 suppresses a variety of T-cell-mediated reactions, including transplant rejection, autoimmunity, and delayed-type hypersensitivity (DTH). A hybrid IL-2-toxin gene was constructed from the diphtheria toxin gene by replacing the DNA encoding the diphtheria toxin receptor-binding domain with DNA encoding the receptor-binding domain of IL-2, and the fusion protein encoded by the hybrid gene was expressed in Escherichia coli (Williams, D. P., Parker, K., Bach, P., Bishai, W., Borowski, M., Genbauffe, F., Strom, T. B. & Murphy, J. R. (1987) Protein Eng. 1, 493–498). We examined the action of the chimeric IL-2-toxin fusion protein on in vivo T-cell mediated response, DTH. The IL-2-toxin fusion protein was found to be a potent immunosuppressive agent. Treatment of mice with the IL-2-toxin blocks DTH and prevents expansion of IL-2R+ T cells. Indeed, IL-2-toxin treatment targets IL-2R+ T cells in vivo and is shown to selectively eliminate their appearance in draining lymph nodes. DTH suppression was observed even in mice possessing high titers of antibodies to diphtheria toxoid.

De novo expression of the interleukin 2 receptor (IL-2R) is a critical and pivotal event in initiation of an immune response (1, 2). The presence of high-affinity IL-2Rs on all recently activated T cells and their absence from the surface of most resting or memory T cells raises the hope that therapeutic targeting of high-affinity IL-2Rs may provide highly effective and selective immunosuppression. Indeed, targeting the low-affinity IL-2-binding 5.5-kilodalton subunit (p55) of the high-affinity IL-2R with the rat anti-mouse IgM monoclonal antibody (mAb) M7/20 effectively suppresses a variety of T-cell-mediated reactions, including transplant rejection, autoimmunity, and delayed-type hypersensitivity (DTH) (3–6). In these instances, it is dubious that the anti-IL-2 mAb treatment causes immunosuppression merely by blocking the IL-2R, since successful therapy requires complement activation and a monoclonal antibody that defines the IL-2-binding domain of the IL-2R (5). Because IL-2 binds to the IL-2R with much higher affinity than the anti-IL-2 mAbs and because coating of the IL-2R with antibody does not guarantee lysis of the target cells, we examined the effect of a chimeric IL-2-toxin fusion protein on an in vivo T-cell-mediated response, DTH. Through protein engineering and recombinant DNA methods, a fusion gene encoding IL-2-toxin was constructed from the diphtheria toxin gene by replacing DNA coding for the diphtheria toxin receptor-binding domain with DNA coding for IL-2 (7). Recombinant strains of Escherichia coli that carry the hybrid gene produce a 68,086-dalton fusion protein, IL-2-toxin, that retains the immunologic determinants of both its diphtheria toxin component and its IL-2 component. IL-2-toxin is selectively cytotoxic for both murine and human T cells bearing high-affinity surface IL-2 receptors, whereas cells that do not express the multimeric high-affinity receptor (composed of p55 and p75 subunits) are resistant to IL-2-toxin action (8). The action of the hybrid toxin is specifically blocked by a molar excess of recombinant IL-2 or monoclonal antibody to the p55 (Tac antigen) subunit of the high-affinity IL-2 receptor. In addition, intoxication of IL-2R+ target cells by IL-2-toxin is blocked by lysosomotropic agents that prevent acidification of endocytic vesicles (6). These results indicate that IL-2-toxin, like diphtheria toxin itself, must pass through an acidic cellular compartment in order to exert its ADP-ribosyltransferase action on elongation factor 2 in the target-cell cytosol. In the present study we used a murine model of DTH to demonstrate that injections of purified IL-2-toxin selectively eliminate IL-2R+ CD4+ and IL-2R+ CD8+ T cells from draining lymph nodes and thereby suppress DTH far more potently than anti-IL-2 mAb. Thus, targeting of IL-2R+ activated T cells is a selective, precise immunotherapy capable of preventing a T-cell-mediated immune response in vivo.

MATERIALS AND METHODS

Mice. BALB/c and DBA/2 [a strain genetically deficient for expression of the fifth component of complement (C5)] mice were obtained from The Jackson Laboratory and then maintained in the animal facilities at Brigham and Women’s Hospital. All mice studied were males 5–10 weeks old.

DTH. Mice were immunized, by bilateral subcutaneous injections of 0.1 ml in the dorsum, with 10 mM trinitrobenzene sulfonic acid (Tnbs; Nutritional Biochemicals) in 0.5 M NaCl/0.02 M phosphate (phosphate-buffered saline, PBS) at pH 7.2–7.4. Seven days after immunization, these mice were challenged with a 25-μl injection of the Tnbs solution into the right hind footpad. Twenty-four hours later, bilateral footpad thickness determinations were obtained by use of a micrometer. These measurements were made by a person ignorant of the immunization protocol. DTH units were defined as differences of 0.01 mm in thickness between the injected and the noninjected footpad. Values given in tables are means ± SEM. PBS was injected into the footpads of immunized mice in order to measure background footpad thickness values.

Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; mAb, monoclonal antibody; DTH, delayed-type hypersensitivity; Tnbs, 2,4,6-trinitrobenzene sulfonic acid.
Experimental Design. Mice were given daily intraperitoneal (i.p.) injections of IL-2-toxin or a control substance, from the time of immunization (day 0) through day 7 of antigen challenge (day 6). The experimental treatment group received the chimeric IL-2-toxin (i.e., the product of a fusion gene in which the IL-2 sequence replaces the sequence encoding the receptor-binding domain of diphtheria toxin), which was purified from periplasmic extracts of *E. coli*(pAB1508) as described (7). Control animals were treated with purified CRM45, a 45-kilodalton nontoxic mutant form of diphtheria toxin that lacks the receptor-binding domain (9). CRM45, therefore, is almost identical to the diphtheria toxin domains represented in the IL-2-toxin. Other mice were given daily i.p. injections of 5 μg of M7/20, a rat IgM mAb raised against the mouse IL-2R.

Phenotyping of Lymph Node Cells. Single-cell suspensions (10⁶ cells per sample) of draining paracortical lymph nodes of the challenged limb or spleen cells from i.p. IL-2-toxin-treated and control mice were prepared for two-color immunofluorescence analysis as follows. First, to allow determination of CD4⁺ or CD8⁺ cells, the samples were incubated with a saturating amount of either anti-L3T4 (GK1.5, American Type Culture Collection), a rat IgG2b mAb, or anti-Lyt-2 (53-6.72, American Type Culture Collection), a rat IgG2a, in 50 μl of PBS at pH 7.4 containing 20% heat-inactivated mouse serum (Cappel Laboratories, West Chester, PA) and 0.1% sodium azide (PBS/serum). The cells were washed and then incubated with fluorescein-labeled rabbit anti-rat IgG (Cappel Laboratories) in 50 μl of PBS/serum. Then, to allow determination of p55-IL-2R⁺ cells, biotinylated purified M7/20 mAb was incubated with the cell preparation (50 μl) in PBS/serum. This was followed by incubation with phycoerythrin-avidin (Becton Dickinson) in PBS/serum. All incubations were performed on ice for 30 min, and the cells were washed three times in cold PBS and kept at 4°C in the dark until analysis. The cells were analyzed with a FACS-1 cell analyzer (Becton Dickinson FACS Systems) using a Consort 30 computer program supplied by Becton Dickinson. Background staining was determined by incubating cells with fluorescein-conjugated rabbit anti-rat immunoglobulin followed by biotin-labeled sheep anti-rat immunoglobulin and phycoerythrin-avidin.

Diphtheria Toxoid Immunization. Mice were immunized by sequential intramuscular injections of 50 μg of diphtheria toxoid (Massachusetts State Laboratory, Jamaica Plain, MA) on days 0 and 21, resting for 3 weeks, and then used for DTH experiments. For daily treatment with IL-2-toxin, the chimeric protein was injected intravenously (i.v.). Anti-diphtheria toxin titers were determined by an *in vitro* neutralizing cytotoxicity assay (10).

RESULTS

IL-2-toxin treatment by i.p. injection of BALB/c mice reduced DTH-induced swelling of the footpad from 42.5 ± 2.0 units in untreated animals to 20.6 ± 1.3 units in the treated group (*P < 0.005*, Table 1). Treatment of BALB/c mice with the nontoxic diphtheria toxin-related control protein, CRM45, had much less effect on DTH. IL-2-toxin was more potent than the anti-IL-2R mAb M7/20 (*P < 0.005*). Although the anti-IL-2R mAb suppressed DTH (35.6 ± 0.8 units) as compared to DTH in untreated mice (51.6 ± 0.5 units), IL-2-toxin was considerably more effective (18.6 ± 0.9 units) at a dose of 5 μg/day (Table 1). IL-2-toxin also suppressed DTH in the complement-deficient mouse strain DBA/2, from 47.0 ± 4.1 units in mice given CRM45 to 20.0 ± 1.7 units in mice given IL-2-toxin (Table 1). By comparison, anti-IL-2R mAb is ineffective in DBA/2 mice (5). IL-2-toxin suppressed DTH at doses of 500 and 50 μg/day but was not effective at a dose of 5 ng/day (Table 2). Anti-IL-2R mAb was ineffective at doses ranging from 500 to 5 ng/day (data not shown).

To further characterize the action of IL-2-toxin in immunosuppression, the monodispersed lymph node cells of the limb ipsilateral to the antigen-challenged footpad were phenotyped with T-cell-subset and p55-IL-2R markers. IL-2-toxin (5 μg/day, i.p.) selectively targeted and eliminated IL-2R-bearing T cells in draining lymph nodes. As determined by dual-beam flow cytometric analysis the percentage of CD4⁺ p55-IL-2R⁺ cells was reduced from 14% in Tnbs-immunized, untreated mice to 5% in Tnbs-immunized IL-2-toxin-treated animals (Fig. 1). Similarly, the percentage of CD8⁺ p55-IL-2R⁺ cells was reduced from 18% to 3% (Fig. 2). IL-2-toxin was so effective that it reduced the number of p55-IL-2R⁺ T cells to values similar to the levels detected in nonimmunized mice (5% for CD4⁺ and 3% for CD8⁺ cells). By contrast, we did not detect an increase in IL-2R expression in spleen cells after Tnbs immunization, and the low percentage of IL-2R⁺ cells (1-2%) was not altered by IL-2-toxin treatment.

To examine the effect of route of IL-2-toxin administration, we performed a similar series of experiments using i.v. rather than i.p. injection. Administration of IL-2-toxin by the i.v. injection resulted in a 1000-fold increase in potency (compare Tables 2 and 3). In these experiments, administration of as little as 5 ng of IL-2-toxin per day resulted in a marked immunosuppression.

To assess the effect of circulating anti-diphtheria toxin antibodies on the *in vivo* action of IL-2-toxin, mice were hyperimmunized with diphtheria toxoid. Three weeks after the second injection with toxoid, animals were x immunized with Tnbs and, after a week of IL-2-toxin treatment, were challenged by footpad injection of Tnbs. IL-2-toxin treatment resulted in immunosuppression in diphtheria toxoid-immunized animals (Table 3). Indeed, the dose–response curves of the control and toxoid-immunized groups are quite similar despite the high levels of circulating neutralizing antibodies.

<table>
<thead>
<tr>
<th>Table 1. IL-2-toxin is a more potent suppressor of DTH than anti-IL-2R mAb and is not dependent on complement</th>
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<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>IL-2-toxin</td>
</tr>
<tr>
<td>CRM45</td>
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</tbody>
</table>

*IL-2-toxin, CRM45, and anti-IL-2R mAb M7/20 were injected i.p. (5 μg/day).
*Genetically deficient in expression of complement component C5.*
DISCUSSION

There is little doubt that the administration of some, but not all, pan-T-cell and anti-CD4 mAbs causes profound immunosuppression. However, these mAbs target all or most T cells, and this broad cellular targeting may not be ideal. Since only a small fraction of the total T-cell population is involved in an acute rejection episode or in nominal antigen activation, several investigators have examined the therapeutic effect of mAbs directed against the IL-2R (3–6, 11–14). The IL-2R is found primarily on the surface of activated, proliferating T cells and has been reported to be present on some recently activated B cells and macrophages (15–18). mAbs directed to the IL-2R selectively targets only those recently activated immune cells. Therefore, therapies that target IL-2R-bearing cells offer a selective and discrete approach to suppressing unwanted immune reactions.

We previously reported the genetic construction of a chimeric toxin (IL-2-toxin) in which the portion of the diphtheria toxin structural gene encoding the toxin's receptor-binding domain was replaced with a synthetic gene encoding the receptor-binding domain of the human T-cell growth factor IL-2 (7). Since the target-cell sensitivity for
Table 3. IL-2-toxin suppresses DTH in mice primunized with diphtheria toxoid

<table>
<thead>
<tr>
<th>IL-2-toxin, ng/day (i.v.)</th>
<th>Control*</th>
<th>Preimmunized</th>
<th>Anti-diphtheria toxin units†</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>1 ± 2.1</td>
<td>2 ± 2.6</td>
<td>3.2</td>
</tr>
<tr>
<td>50</td>
<td>4 ± 2.1</td>
<td>1 ± 2.1</td>
<td>3.2</td>
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<tr>
<td>5</td>
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<td>16 ± 2.2</td>
<td>3.2</td>
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<tr>
<td>0</td>
<td>45 ± 3.5</td>
<td>39 ± 6.5</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Mice were given daily i.v. injections of IL-2-toxin (n = 5 mice per group). All three IL-2-toxin doses resulted in significant suppression of DTH as compared to untreated mice (P < 0.001).

*Control mice received the vehicle (0.15 M NaCl/0.01 M Tris-HCl, pH 8.0) with 0.1% human serum albumin.
†Measured by in vitro neutralizing cytotoxicity assay (10).

diphtheria toxin is dependent upon the presence of the diphtheria toxin receptor, we reasoned that the replacement of the toxin’s receptor-binding domain with that of a cell-specific growth factor should result in the formation of a new toxin with a precisely defined target-cell specificity (7, 19). In the present study, we attempted to target the chimeric toxin to the IL-2R in vivo. We anticipated that IL-2-toxin would be a more effective agent than anti-IL-2R mAb-toxin conjugate for delivery of toxin to IL-2R* cells, since it has been shown that anti-Tac mAb, as well as all other tested mAbs that bind to the p55 subunit of the IL-2R, are not endocytosed (20). Because internalization is a required step in the intoxication process, immunotoxins assembled with these anti-IL-2 mAbs are unlikely to efficiently deliver a fragment of microbial or plant toxin to the cytosol of target cells (21). In contrast, IL-2 undergoes obligatory endocytosis after binding to the high-affinity IL-2R. Thus, a chimeric toxin in which IL-2 serves as the ligand component should be internalized and deliver its toxophore to the target cells. Since only high- or intermediate-affinity IL-2Rs mediate IL-2 internalization, an IL-2-directed toxin is likely to be a more selectively targeted therapeutic probe than anti-IL-2 (p55) receptor antibody.

We have now tested the capacity of IL-2–toxin in a murine model of T-cell dependent immunity, DTH. Previous work demonstrated that DTH is dependent upon a small subset of activated IL-2R* T cells, inasmuch as anti-IL-2R mAb treatment greatly depletes the immune response (4, 5). The M7/20 anti-IL-2R mAb used in the previous studies defines the IL-2–binding polypeptide p55 (22). This polypeptide is a subunit of the multicentric, high-affinity IL-2R; p55 expressed as a monomer is a low-affinity IL-2R. In vitro studies showed that IL-2–toxin is highly potent and intoxicates only those cells that possess the multicentric, high-affinity receptor (8). Hence, the present study directly tests the capacity of IL-2–toxin to mediate immunosuppressive effects in vivo and also assesses, indirectly, whether the T cells responsible for mediating the DTH response possess high-affinity multicentric IL-2Rs. Administration of IL-2–toxin resulted in a dose-dependent suppression of DTH. IL-2–toxin appears to be a more potent immunosuppressive agent than anti-IL-2R mAb. When given i.p., IL-2-toxin doses as low as 50 ng/day were effective, whereas anti-IL-2R mAb was ineffective at doses of 5–500 μg/day. When the chimeric toxin was given i.v., it was effective at doses as low as 5 ng/day, and doses of 50 and 500 ng/day essentially abolished DTH.

DTH was quantitated as footpad swelling 24 hr after antigenic challenge by injection of the haptens into the footpad of experimental animals. In animals receiving this antigenic challenge, but not in immunized control animals, a large proportion of CD4+ and CD8+ T cells in draining lymph nodes, but not in the spleen, express the IL-2R as determined by immunofluorescence using the M7/20 mAb. In contrast, Tnbs-immunized animals that were treated with IL-2-toxin did not possess a greatly expanded population of IL-2R* lymph node T cells. The most facile interpretation of these data is that antigen-reactive cells expressing the high-affinity IL-2R are eliminated during the course of IL-2–toxin treatment.

Insofar as immunization with diphtheria toxoid is an important public health measure, it was of interest that toxoid-immune mice bearing high titers of neutralizing anti-diphtheria toxin antibodies were as susceptible to the immunosuppressive effects of the IL-2–toxin as non-toxoid-immune mice. The failure of anti-diphtheria toxoid antibodies to neutralize IL-2–toxin in vivo is of particular interest. These results are consistent with the observations of Zucker and Murphy (23), who demonstrated that only those anti-diphtheria toxoid mAbs that prevented toxin from binding to its receptor were neutralizing. In the case of the IL-2–toxin fusion protein, the diphtheria toxin receptor-binding domain has been replaced with IL-2 sequence. Nonetheless, in a series of preliminary experiments measuring the antitoxin activity of both human and murine sera, it was observed that both IL-2–toxin action and diphtheria toxin action can be blocked in vitro (P.B., unpublished observations). Paradoxically, the murine antitoxin, which fails to neutralize the action of IL-2–toxin in the in vivo DTH model, is capable of neutralizing the offspring of IL-2–toxin in vitro.

Nonetheless, the studies reported here demonstrate that the IL-2–toxin fusion protein is a potent immunosuppressive agent. Treatment with IL-2–toxin blocks DTH and prevents expansion of the IL-2R* T-cell population in the mouse. The suppression of DTH was observed even in mice possessing high titers of anti-diphtheria toxoid antibodies. Indeed, IL-2–toxin treatment targets IL-2R* T cells in vivo and was shown to selectively eliminate their appearance in draining lymph nodes.

We wish to thank Corinne Kennedy for her secretarial assistance and Chu Chang for her technical expertise. This work was supported in part by Public Health Service Grants DK36149 (V.E.K.), AI21628 (J.R.M.), AI22882 (T.B.S.), DK33929 (T.B.S.), and CA41746 (J.R.M.).


