In Vivo Suppression of the Immune Response to Alloantigen by Cholera Enterotoxin

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**ABSTRACT** The immune response of C57BL/6 mice to allogeneic (DBA/2) mastocytoma cell suspensions was profoundly suppressed by intraperitoneal administration of 1 μg cholera enterotoxin 4 days after antigenic stimulation. The immune response assayed 11 days after antigen showed decreased cytolytically active thymus-derived (T) lymphocytes and markedly depressed serum-agglutinating titers. A comparable suppression of the immune response to skin allografts (DBA/2 → C57BL/6) was also effected by cholera toxin administration, although there was no prolongation of allograft survival.

The mechanism of the immune suppression is apparently related to the known adenylate cyclase stimulatory activities of choleragen.

**INTRODUCTION**

The cytolytic activity that “sensitized” lymphocytes exhibit towards alloantigen-bearing target cells in vitro is widely considered to be of importance in vivo, particularly in allograft rejection (1, 2). Recent evidence strongly suggests that the effector cells in such cytolytic phenomena are predominantly, if not exclusively, thymus-derived (T) lymphocytes (3, 4). Our previous studies (5-8) have indicated that among reversible inhibitors of T-cell mediated cytolyis in vitro are those agents which stimulate lymphocyte cyclic 3',5'-adenosine monophosphate (cAMP)

1 accumulation. The overall efficacy of these agents in inhibiting cytolyis appears related to the length of time over which they cause elevation of cAMP (5). Thus, isoproterenol was found to augment lymphocyte cAMP levels only temporarily (< 1 h) and was an inefficient inhibitor of cytolyis in long-term (> 4 h) cultures (5). The prostaglandins E1 and E2 on the other hand, raised lymphocyte cAMP levels for periods in excess of 4 h and strongly inhibited in vitro cytolyis for similar periods (5, 7). We have recently had occasion to study the effects of another adenylate cyclase-stimulating agent, cholera enterotoxin (8). This agent was found to cause a slow but extremely protracted (> 24 h) elevation of lymphocyte cAMP levels in vitro, and at concentrations of 1 ng/ml, effectively suppressed lymphocyte-mediated cytolyis without affecting cell viability (8, 9). These findings suggested to us that cholera toxin might effectively suppress lymphocyte-mediated cytolyis in vivo and thus afford a pharmacologic means of control of an effecent arm of the immune response. Such studies are the subject of this communication.

**METHODS**

**Lymphocyte-mediated cytolyis.** The procedure employed has previously been described in detail (10, 11). A murine mastocytoma cell line (P815 of the DBA/2 strain) was used both as immunizing alloantigen and as a target cell in cytolytic assays. The mastocytoma was serially passaged in ascitic fluid through adult DBA/2 female mice. For immunization, the cells were aspirated into Eagle’s medium (MEM) (Microbiological Associates, Inc., Bethesda, Md.) containing 200 IU heparin/ml, washed twice in Tris buffer (10), and 10⁶ cells given intraperitoneally (i.p.). For cytolytic assays, 5 × 10⁶ washed cells in 1 ml Tris buffer were incubated for 30 min with 0.2 ml of ²⁶Cr as sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.; sp act between 100 and 200 μCi/μg Cr). The cells were then washed three times in MEM containing 10% heat-inacti-
vated fetal calf serum, counted, and routinely diluted to 10⁶ cells/ml in the same medium.

Spleen cells obtained 10 days after immunization and target cells were incubated together in a reaction volume of 1 ml MEM containing 10% heat-inactivated fetal calf serum. These cell mixtures were incubated for 6 h at 37°C in an atmosphere of 10% CO₂, 90% air. Routinely, 10⁶ ⁵¹Cr target cells and 10⁷ lymphocytes were used. When fewer lymphocytes from immune animals were assayed, the total lymphocyte concentration was adjusted to 10⁷/ml by the addition of normal syngeneic C57BL/6 spleen cells.

At the end of incubation the cells were spun down (1,000 rpm for 5 min), an aliquot of cell-free supernate taken, and its ⁵¹Cr content measured in a γ-spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). The percentage of the ⁵¹Cr which was released into the supernate on incubation was equated with the percentage of target cell lysis (10, 11). The percentage of specific cytolysis was evaluated by subtracting from the lysis obtained with immune C57BL/6 lymphocytes that amount of ⁵¹Cr released in the presence of the same number of normal C57BL/6 spleen cells (11).

Agglutinating antibody. Sera obtained from C57BL/6 mice, at various times after sensitization, were assayed for agglutinating antibody activity against DBA/2 alloantigens by the method of Stimpfling (12). The DBA/2 mastocytoma cell was employed as the target cell and polyvinylpyrrolidone was used as an agglutination-augmenting agent. All sera were decompounded (56°C, 40 min) before use. The maximal serum dilution causing agglutination was recorded.

cAMP measurements. The method of Brown, Albano, Ekins, and Sgerzzi was used (13), employing a modified extraction procedure (8). The application of this assay to lymphoid cell suspensions has previously been described in detail (5, 6).

Skin allografts. Young adult male C57BL/6 mice were anesthetized with 1 mg Nembutal i.p. A 1.0 × 0.5 cm piece of full thickness dorsal skin was excised and a similar-sized DBA/2 female skin sutured in place. Toenails were clipped. The grafted mice were housed individually with open grafts and provided with ad lib. mouse chow and water. The grafts were inspected daily.

Cholera enterotoxin. Purified cholera enterotoxin was generously supplied by Dr. Richard A. Finkelstein, Dallas, Tex., and was previously described in detail (14).

Assay for antibody production against cholera enterotoxin. The method used has previously been described in detail (8). Human peripheral leukocytes from donors sensitive to ragweed were incubated with 2.5 ng/ml of cholera enterotoxin and with various dilutions of serum, obtained on different occasions after cholera toxin immunization. After 60 min incubation, antigen E was added and histamine release from the leukocytes measured (8). Cholera toxin markedly inhibited histamine release in this system, an inhibition that was specifically reversed by anticholera toxin antibody. The inhibition of histamine release by cholera toxin has been significantly reversed by 1:10 dilutions of dog antitoxin antisera (8). None of the mouse sera employed, even at final dilutions of 1/10, reversed the cholera toxin effect.

RESULTS

Adult C57BL/6 mice were given 10⁶ DBA/2 mastocytoma cells (P815) i.p. Cholera enterotoxin (1 µg) was administered i.p. on the day of antigenic challenge, or on days 4, 7, or 10 thereafter. Pooled spleen-cell suspensions were made from each group (six mice/group) 11 days after allantigenic stimulation, and the cytolytic activity of these populations towards ⁵¹Cr-labeled DBA/2 mastocytoma cells was assessed (10, 11). Typical results are shown in Fig. 1 A. Cholera enterotoxin administration at the time of immunization with DBA/2 mastocytoma cells had no effect on the subsequent development of cytolytically active cells in the spleen.

When cholera was given 4 days after antigen, however, a marked suppression of cytolytic activity was observed, ranging between 70 and 100% suppression in four experiments. Cholera toxin administration on days 7, 8, or 9 after antigen challenge invariably resulted in a complete inhibition of cytolytic activity as assessed in vitro on day 11 (results not shown).

Varying the i.p. dose of cholera enterotoxin given on day 10 demonstrated that a partial (30–40%) suppression of cytolytic activity, measured in vitro 24 h later, could be accomplished with as little as 0.01 µg of toxin (Fig. 1B).

The agglutination of DBA/2 mastocytoma cells by sera obtained from each of the groups represented in Fig. 1A was also assessed (12). Animals given cholera toxin at the time of mastocytoma cells exhibited serum titers (geometric mean: 243; range, six animals 108–324) indistinguishable from the untreated groups (geometric mean: 187; range, 108–324). Animals receiving cholera toxin 4 days after antigen showed markedly depressed titers of serum antibody on day 11 (mean 15: range 9–27). Animals which did not receive cholera toxin until day 10 exhibited normal antibody titers on day 11 (mean 187: range 108–324).

Administration of cholera toxin i.p. in quantities up to 1 µg had no demonstrable effect either on lymphoid cell numbers (spleen or axillary nodes) or on cell viability as assessed by vital dye exclusion tests. When larger doses (4 µg) of cholera toxin were employed, however, evidence of cellular depletion, particularly in the splenic lymphoid cell pool, was observed; a finding compatible with the observation of Northrop.

Although cholera toxin has been established as an immunogen (13), none of the regimes which we employed led to detectable antibody formation, despite the fact that the antibody assay system used (reversal of the toxin-induced inhibition of histamine release) was capable of detecting antitoxin antibody in nanogram amounts (8, 14).

As cholera enterotoxin caused a profound suppression of the immune response to allogeneic cell suspensions, similar studies were made employing skin allografts as antigen. Adult C57BL/6 mice were grafted with DBA/2

*Northrop, R. S. Personal communication to the authors.
skin, and were given 1 µg choleragen i.p. on the day of grafting or on days 1, 2, or 4 thereafter. The animals were all exsanguinated on day 11. Spleens, and in some cases, lymph nodes draining the grafted site, were collected and pooled cell suspensions prepared from at least four similarly treated animals. As previously, the cytolytic activity and the serum agglutination titer were measured against DBA/2 mastocytoma cells. The results are shown in Table I. Marked immunosuppression of both serum antibody titers and cell-mediated immune responses was observed in treated animals, including, in some cases, the total absence of cytolytically active lymphocytes in both spleens and draining lymph nodes. Graft prolongation, however, was only rarely observed. The summation of experiments involving approximately 150 allografts is shown in Table I. In further experiments, not shown here, daily 1-µg doses of choleragen have been given. The suppression of lymphocyte-mediated cytotoxicity and of antibody formation was readily and reproducibly observed. But graft rejection was usually unaffected. Likewise, large (5-10 µg) doses of choleragen given i.p. on the day of grafting have failed to cause significant prolongation of skin allograft survival.

Our previous studies have indicated that the suppression of cytolytic activity by choleragen enterotoxin in vitro was mediated via stimulation of the adenylate cyclase system (8). To study whether there was a correlation between these immunosuppressive effects and cellular cAMP levels, the following experiment was devised. Adult C57BL/6 mice were given 10⁷ DBA/2 mastocytoma cells i.p. One-half of these mice (30 animals) were given 1 µg choleragen enterotoxin i.p. At daily intervals thereafter spleens were removed from both normal animals and those receiving choleragen, and the cAMP content of both groups individually measured (8, 13). Additionally, normal C57BL/6 spleen-cell suspensions were cultured for varying periods of time in vitro in the presence of choleragen enterotoxin and subsequently assayed for cAMP content. As can be seen (Fig. 2), cAMP levels in vitro and in vivo were elevated for prolonged periods by choleragen enterotoxin treatment. These levels fell to baseline values, however, by 30-36 h in vitro and by 72 h in vivo. The data presented here thus demonstrates immune suppression which follows, by a considerable period, transitory elevation of cAMP levels.

**DISCUSSION**

These studies demonstrate that choleragen enterotoxin administered in vivo can cause a profound suppression of the immune response both to allogeneic cell suspensions and to skin allografts. The mechanism of such suppression is unclear. Our working hypothesis based on previous experiments (5, 8) has been that increased cAMP levels caused by adenylate cyclase stimulation inhibits the synthesis and/or release of soluble mediators required for the lytic activity of T lymphocytes. The present in vivo studies show, however, a suppression of cytolytically active effector cells at a time considerably later than the observed increased cAMP levels after choleragen enterotoxin administration.

The suppression of cytolytic activity by choleragen enterotoxin treatment on day 10, a time when many cytolytically ac-

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**Figure 1** Effect of in vivo administration of choleragen enterotoxin on the cell-mediated immune response to allogeneic cell suspension. The lytic activity of spleen cell suspensions from adult C57BL/6 mice 11 days after antigenic challenge with 10⁷ DBA/2 mastocytoma cells i.p. is shown. (A) Animals received either antigen (untreated) or a dose (1 µg) of choleragen. Choleragen was given on the day of antigenic challenge (day 0), on day 4 or on day 10. The assay employed a 4 h incubation of various numbers of immune spleen cells with 10⁶ ³⁵Cr mastocytoma cells. Specific cytalysis was calculated as described in the text. Values are means of triplicate assays. (B) Animals received various doses of choleragen i.p. on day 10. Spleen cell suspensions (pools of at least four similarly treated animals) were assayed in vitro 24 h later. 10⁶ spleen cells were incubated for 4 h with 10⁶ ³⁵Cr mastocytoma cells and specific cytalysis evaluated. This value was expressed as a percentage of the cytalysis observed with unsuppressed animals and recorded as the percent inhibition of specific cytalysis, relative to the unsuppressed controls. The values shown are means of triplicate assays ±SEM.
TABLE I
Immune Responses in C57BL/6 Mice to DBA/2 Allografts: Effect of Cholera Toxin

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Cholera toxin days after allografting</th>
<th>Agglutination titer*</th>
<th>Cytolytic activity‡</th>
<th>Graft rejection§</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/2</td>
<td>1 µg (i.p.)</td>
<td>8,32,32,32,64</td>
<td>12.7</td>
<td>22.0</td>
</tr>
<tr>
<td>Skin</td>
<td>None</td>
<td>2,4,4,8</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Graft</td>
<td>(day of graft) 2</td>
<td>&lt;2, &lt;2, &lt;2, &lt;2, &lt;2</td>
<td>&lt;2</td>
<td>not tested</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&lt;2, &lt;2, &lt;2, &lt;2, &lt;2</td>
<td>&lt;2</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* Highest serum dilution causing DBA/2 mastocytoma agglutination. Titer of individual sera in each group, 11 days after grafting.
‡ Percent specific cytolyssis of 10⁷ DBA/2 mastocytoma cells in 4 h at 37°C by 5.0 × 10⁶ lymphoid cells. Pooled cell suspensions (four animals) were used, values given are means of triplicate assays. Replicate assays varied less than ±5% of value shown.
§ Range of time for skin allograft rejection. The values given represent at least 20 animals for each suppressive regime employed. The normal values represent >50 grafts.

![Figure 2](http://www.jci.org)

**Figure 2** Effect of in vivo and in vitro cholera enterotoxin stimulation on splenic cAMP levels. (A) Single cell suspensions (10⁷ leukocytes/ml) of C57BL/6 spleens in Eagle's medium containing 10% inactivated fetal calf serum were treated with varying doses of cholera toxin, ranging from 1 ng/ml (lower curve) to 100 ng/ml (upper curve). Control cell suspensions contained no choleragen. cAMP levels were assayed after various times in culture and expressed as a percentage of control values. The values shown are means of triplicate assays ±SEM. Spleen cells with no choleragen contained 0.15±0.04 pmol/10⁷ leukocytes at time 0, 0.17±0.01 pmol/10⁷ cells after 90 min in culture and 0.16±0.02 pmol/10⁷ cells after 18 h culture. (B) Adult C57BL/6 mice were immunized i.p. with 10⁷ DBA/2 mastocytoma cells. Half of these animals additionally received 1 µg choleragen enterotoxin i.p. At time zero, and at daily intervals thereafter, the animals from each group were sacrificed and cAMP levels of individual spleen samples measured. The mean cAMP content of the cholera toxin-treated animals was recorded as a percentage ±SEM of that in the control group. The administration of alloantigen itself did not cause a rise in cAMP levels. The cAMP content of spleens at time zero was 593±56 pmol/g wet weight.

a number presumably beyond the limits of detection of the cytolytic assay system employed. Although unlikely, it is of course conceivable that the cells responsible for in vitro cytolytic activity play no major role in allograft rejection. Alternatively, it may be that the toxin stimulates a process necessary for allograft rejection and that this compensates for the relative disappearance of cytolytically active cells.

It is of interest to note that cholera toxin administration on day 0 had no effect on the immune response to allogeneic cell suspensions (Fig. 1A), but markedly suppressed the response to skin allograft (Table 1). This finding may be related to the nature of the antigenic stimulus and the timing of the host's proliferative response.

In any event, it appears that significant immunosuppression in vivo can be induced by an agent that causes increased levels of cAMP. The optimum dose and time of administration of cholera toxin and its possible synergy with other immunosuppressive regimens needs further evaluation, as does the mechanism by which transient increases in cAMP levels can apparently obviate an immune response 7-10 days later. Nonetheless, the present results suggest that such agents afford a novel means of immunosuppression, and imply a favorable prognosis for the pharmacologic control of the immune response.

ACKNOWLEDGMENTS

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REFERENCES