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### Research Article

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# 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  $\mu$ g 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  $\rightarrow$  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 cholera toxin.

## 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 cytotoxicity in vitro are those agents which stimulate lymphocyte cyclic 3',5'-adenosine monophosphate (cAMP)<sup>1</sup> accumulation. The overall

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<sup>1</sup>Abbreviations used in this paper: cAMP, cyclic 3',5'-adenosine monophosphate; MEM, Eagle's medium.

efficacy of these agents in inhibiting cytotoxicity 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 cytotoxicity in long-term (> 4 h) cultures (5). The prostaglandins E<sub>1</sub> and E<sub>2</sub> on the other hand, raised lymphocyte cAMP levels for periods in excess of 4 h and strongly inhibited in vitro cytotoxicity 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 cytotoxicity without affecting cell viability (8, 9). These findings suggested to us that cholera toxin might effectively suppress lymphocyte-mediated cytotoxicity in vivo and thus afford a pharmacologic means of control of an efferent arm of the immune response. Such studies are the subject of this communication.

## METHODS

*Lymphocyte-mediated cytotoxicity.* 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 cytotoxic 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 10<sup>7</sup> cells given intraperitoneally (i.p.). For cytotoxic assays, 5  $\times$  10<sup>6</sup> washed cells in 1 ml Tris buffer were incubated for 30 min with 0.2 ml of <sup>51</sup>Cr as sodium chromate (Amersham/Searle Corp., Arlington Heights, Ill.; sp act between 100 and 200  $\mu$ Ci/ $\mu$ g Cr). The cells were then washed three times in MEM containing 10% heat-inacti-

vated fetal calf serum, counted, and routinely diluted to  $10^6$  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% CO<sub>2</sub>, 90% air. Routinely,  $10^6$  <sup>51</sup>Cr target cells and  $10^7$  lymphocytes were used. When fewer lymphocytes from immune animals were assayed, the total lymphocyte concentration was adjusted to  $10^7$ /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 <sup>51</sup>Cr content measured in a  $\gamma$ -spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). The percentage of the <sup>51</sup>Cr 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 <sup>51</sup>Cr 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 decomplexed (56°C, 40 min) before use. The maximal serum dilution causing agglutination was recorded.

**cAMP measurements.** The method of Brown, Albano, Ekins, and Sgherzi 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, 8).

**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 has previously been 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 mouse 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 anti-cholera toxin antibody. The inhibition of histamine release by cholera toxin has been significantly reversed by 1:10<sup>4</sup> dilutions of dog antitoxin antiserum (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  $10^7$  DBA/2 mastocytoma cells (P815) i.p. Cholera enterotoxin (1  $\mu$ g) 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 alloantigenic stimulation, and the cytolytic activity of these populations towards <sup>51</sup>Cr-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 toxin 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  $\mu$ g 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  $\mu$ g 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  $\mu$ g) 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.<sup>2</sup>

Although cholera toxin has been established as an immunogen (15), 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

<sup>2</sup> Northrop, R. S. Personal communication to the authors.

skin, and were given 1  $\mu\text{g}$  cholera toxin 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 titers 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- $\mu\text{g}$  doses of cholera toxin 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  $\mu\text{g}$ ) doses of cholera toxin 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 cholera enterotoxin in vitro was mediated via stimulation of the adenylate cyclase system (8). To study whether there was a correlation in vivo between these immunosuppressive effects and cellular cAMP levels, the following experiment was devised. Adult C57BL/6 mice were given  $10^7$  DBA/2 mastocytoma cells i.p. One-half of these mice (30 animals) were given 1  $\mu\text{g}$  cholera enterotoxin i.p. At daily intervals thereafter spleens were removed from both normal animals and those receiving cholera toxin, 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 cholera enterotoxin and subsequently were assayed for cAMP content. As can be seen (Fig. 2), cAMP levels in vitro and in vivo were elevated for prolonged periods by cholera enterotoxin treatment. These levels fell to base-line 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 cholera 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

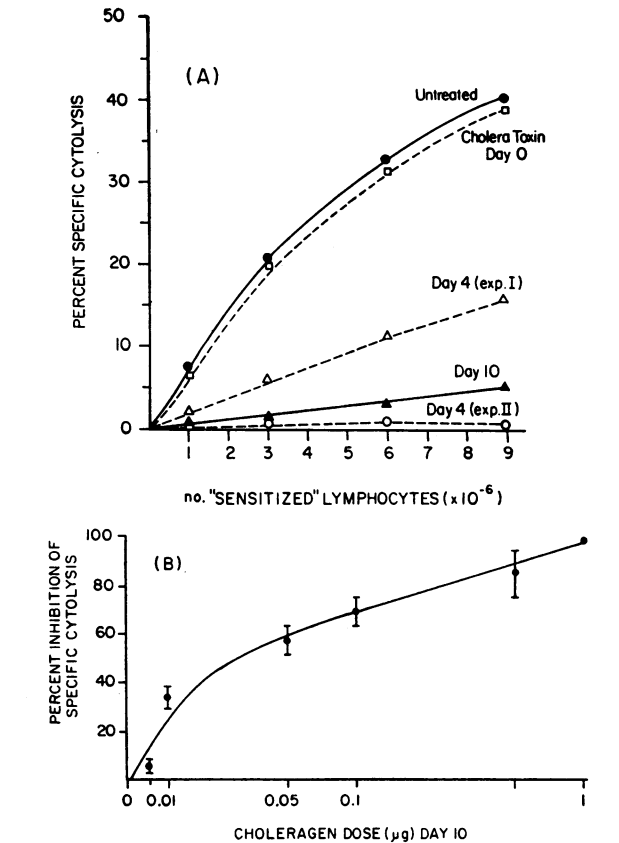


FIGURE 1 Effect of in vivo administration of cholera enterotoxin on the cell-mediated immune response to allogeneic cell suspension. The lytic activity of spleen cell suspensions from adult C57BL/6 mice 11 days after antigenic challenge with  $10^7$  DBA/2 mastocytoma cells i.p. is shown. (A) Animals received either antigen (untreated) or a dose (1  $\mu\text{g}$ ) of cholera toxin. Cholera toxin 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  $10^6$   $^{51}\text{Cr}$  mastocytoma cells. Specific cytolysis was calculated as described in the text. Values are means of triplicate assays. (B) Animals received various doses of cholera toxin i.p. on day 10. Spleen cell suspensions (pools of at least four similarly treated animals) were assayed in vitro 24 h later.  $10^7$  spleen cells were incubated for 4 h with  $10^6$   $^{51}\text{Cr}$  mastocytoma cells and specific cytolysis evaluated. This value was expressed as a percentage of the cytolysis observed with unsuppressed animals and recorded as the percent inhibition of specific cytolysis, relative to the unsuppressed controls. The values shown are means of triplicate assays  $\pm$  SEM.

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 cholera toxin administration.

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

TABLE I  
Immune Responses in C57BL/6 Mice to DBA/2 Allografts: Effect of Cholera Toxin

Antigen	Cholera toxin days after allografting	Agglutination titer*	Cytolytic activity‡		
			Spleen	Draining nodes	Graft rejection§
DBA/2	1 µg (i.p.)				day
	None	8,32,32,32,64	12.7	22.0	8-9
Skin	0 (day of graft)	2,4,4,8	<2	<2	8-10
	2	<2, <2,4,8	<2	not tested	8
Graft	4	<2,2,2,4	<2	4.0	7-8

\* Highest serum dilution causing DBA/2 mastocytoma agglutination. Titer of individual sera in each group, 11 days after grafting.

‡ Percent specific cytolysis of  $10^5$  DBA/2 mastocytoma cells in 4 h at 37°C by  $5.0 \times 10^6$  lymphoid cells. Pooled cell suspensions (four animals) were used, values given are means of triplicate assays. Replicate assays varied less than  $\pm 5\%$  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.

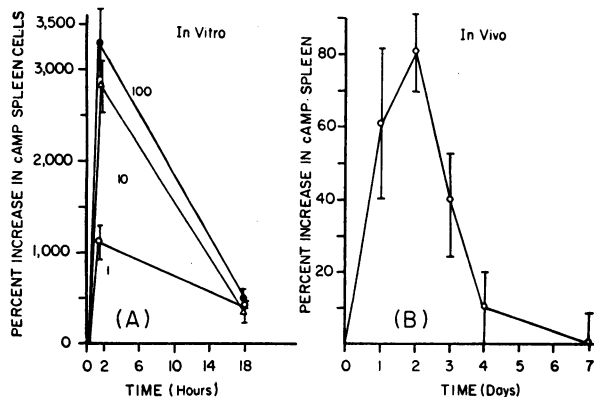


FIGURE 2 Effect of in vivo and in vitro cholera enterotoxin stimulation on splenic cAMP levels. (A) Single cell suspensions ( $10^7$  leukocytes/ml) of C57BL/6 spleens in Eagle's medium containing 10% inactivated fetal calf serum were treated with varying doses of cholera toxin, ranging from 1 ng/ml (lower curve) to 100 ng/ml (upper curve). Control cell suspensions contained no cholera toxin. 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  $\pm$  SEM. Spleen cells with no cholera toxin contained  $0.15 \pm 0.04$  pmol/ $10^7$  leukocytes at time 0,  $0.17 \pm 0.1$  pmol/ $10^7$  cells after 90 min in culture and  $0.16 \pm 0.12$  pmol/ $10^7$  cells after 18 h culture. (B) Adult C57BL/6 mice were immunized i.p. with  $10^7$  DBA/2 mastocytoma cells. Half of these animals additionally received 1 µg cholera 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  $\pm$  SEM of that in the control group. The administration of allograft itself did not cause a rise in cAMP levels. The cAMP content of spleens at time zero was  $593 \pm 56$  pmol/g wet weight.

tive lymphoid cells have differentiated (11), would seem to be a direct analogy of events previously observed in vitro and related to the elevated cAMP levels in the effector cell population (5, 8). Suppression by administration of cholera toxin on day 4, however, may be due to inhibition of antigen-induced clonal proliferation, for several investigations have recently shown that mitogen induced lymphocyte transformation is inhibited by agents that elevate cAMP levels (16, 17). Alternatively, or additionally, it may be that increased cAMP levels 4 and 5 days after antigenic stimulation, a time of rapid cellular proliferation, causes lysis of newly differentiated cells, a phenomenon recently described with cultured lymphoma cells by Daniel, Litwack, and Tompkins (18).

We suggest that the decrease in serum antibody titers observed in cholera toxin-treated animals could be due to diminished capacity of plasma cells to secrete antibody. Evidence that this can occur has recently been provided by the observation that adenylate cyclase stimulation of plasma cells in vitro leads to a fall in splenic plaque-forming cell numbers against sheep erythrocytes.<sup>3</sup> The relatively long half-life of mouse immunoglobulin (19) would be compatible with the observation that treatment of animals with cholera toxin on day 10 had no effect on serum antibody titers measured 24 h later.

Although cholera toxin treatment markedly suppressed the development of antibody formation and cytolytically active lymphocytes against skin allografts, interference with the time of onset of skin graft rejection was only rarely observed. One assumes that this relates to the small number of effector cells required for skin rejection;

<sup>3</sup> Melmon, K., J. Weinstein, and H. R. Bourne. 1973. Personal communication.

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 I). 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.

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