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Differential Effects of Cholera Toxin Pre-treatment on *In Vitro* vs. *In Vivo* Immunocyte Responses (40111)

SUSAN F. LYONS AND HERMAN FRIEDMAN

Departments of Microbiology and Immunology, Albert Einstein Medical Center and Temple University School of Medicine, Philadelphia, Pennsylvania 19141

Cholera exoenterotoxin (CT) derived from vibrios has marked immunoregulatory properties when administered together with an antigen into an experimental animal or when added to lymphoid cells immunized *in vitro* (1–6). Although CT is known to stimulate adenylate cyclase activity and alter cyclic adenosine monophosphate (cAMP) levels in lymphoid as well as nonlymphoid tissues (7–9), the mechanism of immunologic modulation induced by the toxin is unknown. In the present study the *in vitro* hemolytic antibody plaque response to sheep red blood cells (SRBC) was found to be markedly enhanced when murine spleen cells were obtained from mice treated with CT on the same day of culture initiation or one day earlier, similar to the enhancement of the immune response in vivo when mice were given CT and RBCs at the same time (3, 5). Furthermore, similar to the effects observed *in vivo*, splenocytes from mice treated several days earlier with CT were markedly impaired in their in vitro immune responsiveness to the sheep RBCs (3). In contrast, CT induced only enhanced antibody responses when added to normal splenocytes *in vitro*, either on the day of culture initiation or several days earlier.

Methods and materials. Experimental animals. Adult male Balb/c mice 8–10 weeks of age and weighing 18 to 20 grams each, were obtained from Cumberland View Farms, Clinton, TNN and housed in groups of 6 to 8 in plastic mouse cages and fed Purina mouse pellets and water *ad libitum.*

Antigen. Sheep erythrocytes were obtained in Alsever's solution from Becton-Dickinson Company, Cockeysville, Maryland. The RBCs were washed with culture medium by centrifugation and resuspended to a 0.1% concentration containing approximately 2×10^7 SRBC per ml.

Cholera toxin. The toxin was obtained from Dr. H. Miller, National Institute of Allergy and Infectious Diseases, Bethesda, MD, and had been prepared by Dr. Richard Finkelstein, Microbiology Department, University of Texas, Southwestern Medical School, Dallas, TX. The lyophilized toxin was reconstituted with sterile water and diluted with phosphate buffered saline containing 0.1% human serum albumin to a concentration of 200 μg/ml. The toxin was stored at −70° in small aliquots and immediately before use diluted in pyrogen-free saline.

In Vitro culture. The Marbrook culture system for in vitro immunization of splenocytes was used essentially as described earlier (10, 11). In brief, 5×10^6 washed splenocytes from normal or toxin treated mice were cultured in 0.5 ml minimal essential medium (MEM) containing 10% fetal calf serum (Flow Laboratories, Rockville, MD) on a sterile dialysis membrane in the inner chamber of a Marbrook vessel (Bioresearch Glass Company, Vineland, NJ). Eleven to twelve milliliter MEM were placed in the outer reservoir chamber. Cholera toxin (0.1 μg) and erythrocytes (2×10^6 SRBC) were added to the inner chamber either on the day of culture initiation or, alternatively, toxin was added on day 0 and SRBC was added on day +2. Controls consisted of splenocytes incubated similarly but in the absence of either toxin and/or SRBC, or both. All cultures were prepared in duplicate or triplicate and incubated, without rocking, at 37° in a humidified atmosphere of 83% N_2, 10% CO_2 and 7% O_2 for 5–6 days. Viability was assessed by the standard trypan blue dye exclusion procedure and total cell number determined with a hemocytometer.

Determination of antibody forming cells. The localized hemolytic assay in agar gel as described initially by Jerne and associates (12) was used to enumerate 19S IgM plaque forming cells (PFCs) in the culture chambers. In brief, 0.1 ml of a spleen cell suspension

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was added to 2 ml Difco Noble agar containing 0.1 ml of a freshly washed 10% suspension of SRBC. The agar cell mixture was then quickly poured onto the surface of a previously prepared 60 mm diameter petri plate containing a base layer of agar. After 1 hr incubation at 37° the plates were treated with several ml of a 1:10 dilution of guinea pig complement and incubated further for 30–60 min at 37°. Zones of hemolysis appearing on the surface of the agar plate were considered due to high efficiency IgM PFCs. The numbers of such PFCs for 10^6 viable cells or per whole culture were determined and the average for two to three chambers in each group calculated.

Experimental results. Immunization of normal splenocytes in vitro with SRBC resulted in a marked antibody response as evident by the appearance of numerous PFCs. When spleen cells were obtained from mice pretreated with 1.0 μg cholera toxin at various times before sacrifice varying numbers of PFCs appeared for similar numbers of spleen cells plated (Table I). The number of PFCs for spleen cell suspensions from CT treated mice was directly related to the time of the toxin pretreatment of the mice prior to culture initiation. There was little difference in the total number or viability of spleen cells in the cultures prepared from mice pretreated with toxin as compared to control mice. However, spleen cells from mice given toxin shortly before sacrifice showed a marked enhancement of PFC formation (approximately twofold) as compared to control cultures. Mice given toxin 1 day before sacrifice showed a less consistent enhanced responsiveness to SRBC, but in general there were usually more antibody producing cells for these cells as compared to controls.

Mice given CT 2 days before sacrifice showed a suppression of their splenic PFC responsiveness, similar to the suppression for CT-pretreated mice challenged directly and then tested for splenic PFCs (Table I). Furthermore, mice given CT 3 days before sacrifice showed an even greater suppression of their PFC response, similar to earlier results of *in vivo* studies in which CT-pretreated mice showed marked impairment of antibody responsiveness (5). No significant difference was observed for the number of "background" PFCs in splenocyte cultures incubated without SRBC, regardless of whether the spleen cells were obtained from CT pretreated or normal mice (Table I). In addition, there was little difference in the rate of appearance of PFCs in cultures prepared from normal vs. CT-pretreated mice (Table II). Although the magnitude of the peak response differed, in all cases the peak numbers of PFCs occurred on day +5 after immunization.

To determine whether the toxin influenced the immune response directly or because of a possible indirect effect not associated with lymphoid cells *per se,* spleen cells from normal Balb/c mice were cultured in vitro with SRBC, either with or without CT. When 0.1 μg CT was added to normal cultures on the day of *in vitro* immunization, there was a

TABLE I. IN VITRO ANTIBODY RESPONSE OF SPLEEN CELLS FROM NORMAL AND CHOLERA TOXIN PRETREATED MICE.

	Antibody PFC response[b]				
Spleen cell source[a]	Per 10^6 spleen cells	Percent of control	Per culture	Percent of control	Background PFC response per culture
Normal (controls)	822 ± 21	—	1603 ± 68	—	72 ± 15
CT-treated[c] day 0	1525 ± 210	186	2850 ± 360	178	79 ± 32
−1	1209 ± 56	147	2290 ± 216	143	64 ± 19
−2	448 ± 136	54	860 ± 130	54	74 ± 30
−3	273 ± 76	33	563 ± 198	35	68 ± 27
−7	734 ± 42	89	1350 ± 210	83	78 ± 24
−10	829 ± 38	108	1560 ± 220	97	74 ± 36

[a] 5 × 10^6 spleen cells from indicated group cultured *in vitro.*

[b] Average number of PFCs (±SE) for 6–8 cultures per group 5 days after *in vitro* immunization with 2 × 10^6 sheep RBCs; number of nucleated cells per culture averaged 1.8 × 10^6 viable cells ± 0.25 × 10^6 for all cultures at time of assay.

[c] Groups of 5–10 donor mice injected iv with 1.0 μg CT on indicated day before culturing.

marked enhancement of the PFC response. This dose of CT had no effect on cell viability or number. Similar effects were noted with CT ranging from 0.01 to 0.5 μg. Higher doses caused cell death and lower doses had no effect on antibody formation. The enhancement of antibody formation was as great if not greater than that which occurred when splenocytes were obtained from mice treated with CT on the day of sacrifice or 1 day earlier (Table III). In order to determine whether pretreatment of spleen cells *in vitro* with toxin could depress the immune response as occurred with pretreated mice, normal mouse spleen cell cultures were treated with 0.01–0.5 μg toxin on day 0 and SRBC added 2 days later. PFCs were assayed 4 days after *in vitro* immunization (i.e., 6 days after culture initiation). As can be seen in Table III, a marked enhancement of the PFC response was evident for these cultures treated with 0.1 μg CT before immunization, similar to the enhancement occurring when the same dose of CT was added to cultures on the day of *in vitro* immunization. When the CT dose was varied from 0.01 to 0.5 μg similar effects occurred. Such enhancement contrasted markedly with the depressed PFC response of spleen cells obtained from mice treated 2–3 days earlier *in vivo* with toxin.

TABLE II. CYTOKINETICS OF APPEARANCE OF ANTIBODY FORMING CELLS TO SRBC BY SPLEEN CELLS FROM NORMAL MICE PRETREATED WITH CHOLERA TOXIN AFTER IMMUNIZATION IN VITRO.

Time in days after *in vitro* immunization[a]	PFC per culture[b]		
		CT-pretreated on day[c]	
	Normal	0	−3
0	11 ± 3	35 ± 5	10 ± 6
+2	89 ± 19	148 ± 42	38 ± 17
+3	310 ± 26	570 ± 68	86 ± 28
+4	810 ± 38	1715 ± 195	193 ± 18
+5	976 ± 48	1930 ± 240	224 ± 65
+8	478 ± 39	1130 ± 140	110 ± 27

[a] 5 × 10^6 spleen cells from normal or CT pretreated (1.0 μg) mice immunized *in vitro* with 2 × 10^6 SRBC and PFC responses determined on indicated days thereafter.

[b] Average number of PFC for 3–5 culture per group (±SE).

Coculture of 5 × 10^6 spleen cells from three day CT pretreated mice together with an equal number of spleen cells from normal mice did not affect the expected immune response. In a typical experiment 1705 ± 122 PFCs occurred per culture containing normal splenocytes alone and 372 ± 96 PFCs developed in cultures containing the same number of spleen cells from three day CT pretreated mice. When equal number of these cells were cocultured together the total number of PFCs was 2218 ± 1491, a number quite close to that expected from the sum of the expected number of PFCs in cultures of normal and CT pretreated splenocytes. Varying the ratio of spleen cells from normal vs CT-pretreated mice similarly resulted in the expected number of PFCs. Thus it did not appear likely that the spleen cell suspension from CT pretreated mice contained "suppressor" cells which could affect the expected antibody response of spleen cells from untreated mice.

Discussion. Cholera toxin is known to modulate immune responses, both *in vivo* and *in vitro* (1–6). Earlier studies in this laboratory showed that injection of toxin into Balb/c mice prior to immunization with sheep RBCs

TABLE III. IN VITRO ANTIBODY RESPONSES OF SPLEEN CELLS FROM NORMAL MICE TREATED WITH CHOLERA TOXIN.

Culture group	Day culture immunized with SRBC[a]	CT-treatment of culture[b]	Antibody PF Response[c]			
			Per 10^6 spleen cells	Percent of control	Per culture	Percent of control
A_1	Day 0	−	533 ± 109	—	1005 ± 170	—
A_2	0	+	3951 ± 477	741	7540 ± 622	750
B_1	Day +2	−	339 ± 37	—	528 ± 46	—
B_2	+2	+	1715 ± 379	506	2680 ± 498	508

[a] 5 × 10^6 normal mouse spleen cells cultured in vitro and immunized either on day of culture initiation or 2 days later with 2 × 10^6 SRBC.

[b] Indicated spleen cell cultures treated on day of culture initiation (day 0) with 0.1 μg CT.

[c] Average PFC (±SE) response for 5–8 cultures per group; group A_1 and A_2 tested 5 days after culture initiation and *in vitro* immunization while group B_1 and B_2 tested on day 6 after culture initiation (4 days after *in vitro* immunization).

resulted in markedly depressed antibody responses (3, 5). Toxin treatment at the time of immunization in vivo was found to enhance the immune response. These contrasting effects of CT according to the time of toxin administration relative to the day of challenge immunization was attributed to effects on adenylate cyclase, the enzyme which catalyses the conversion of adenosine diphosphate to cAMP. The increased antibody responsiveness in mice given CT simultaneously with antigen or one day earlier is thought to be related to increased nucleotide levels (5, 8). On the other hand, decreased antibody formation in mice given CT several days before antigen appeared to be related to lower levels of cAMP. CT also affects the nucleotide levels of mouse lymphoid cells treated in vitro. However, only increased levels occurred, without the decrease.

In the present study, the enhancement of PFC responsiveness by normal splenocytes treated in vitro with CT, may also be related to altered cyclase and cAMP levels. The failure of CT pretreatment *in vitro* to depress the antibody response of mouse splenocytes immunized with SRBC could be due to the failure of the toxin to influence certain target cells, other than lymphocytes, which may be important for *in vivo* induced immunosuppression. It is also possible that lymphocyte depletion induced by CT injection into mice may influence the *in vivo* but not *in vitro* response to SRBC. Earlier studies in several laboratories, including this one, have shown a temporary decrease in lymphocyte number within 2–5 days after CT injection. However, maximum depression was never greater than 30%, while the depression of antibody forming cell often reached 70–80%, indicating that lymphopenia per se could not account for the observed immunologic impairment *in vivo*. However, CT appeared to have a preferential depressive effect on T cell number, since T dependent areas in the spleen were often affected after CT injection. Other studies in this laboratory, using immunofluorescent procedures, indicated a moderate depletion of lymphoid cells rich in theta antigen on their surface (presumably T cells) as well as cells with surface Ig globulins, presumably B cells. Depletion of lymphocytes *in vivo* may not directly be responsible for immunosuppression. Even thymic lymphopenia may not be a direct effect of the toxin. For example, it has been reported that observed depletion of lymphoid cells after CT treatment can be prevented by prior adrenalectomy of mice (13). Exogenous and endogenous adrenal glucocorticosteroid hormones are known to induce lymphocytopenia (13, 14). Stimulation of adrenal corticosteroid synthesis and release of corticotropin (ACTH) are influenced by cAMP (14). Thus it is possible that CT injection into mice causes an increase in the level of circulating adrenal cortical hormones which, in turn, produces an effect on lymphoid cells.

A marked dysfunction of lymphoid cells, even without their obstruction, may occur in terms of immune responsiveness. Even when similar numbers of spleen cells from normal and CT-pretreated mice are cultured with SRBC, the splenocytes from the toxin treated mice were found not to respond in a normal manner to the antigen. On the other hand, treating cells entirely in vitro with CT caused only an immunoenhancement when toxin was added 2 days before antigen to simulate as closely as possible the *in vivo* experiments which resulted in the immunosuppression. No impairment of immune responsiveness was evident in such *in vitro* treated cultures, as occurred when CT was given to mice 2–3 days before culture. Thus it seems likely that CT affects the immune response mechanism in a negative manner only when given *in vivo* so target cells other than those directly involved in antibody formation are influenced.

Studies in progress are directed at determining the nature of such possible target cells. It does not appear likely that CT induces or increases the number of, or activity of "suppressor" cells, since cocultivation of graded numbers of spleen cells from 3-day CT pretreated mice with equal numbers of spleen cells from normal mice did not influence the antibody response to SRBC *in vitro* (unpublished observations). The enhanced antibody response of spleen cells from mice given CT the day of sacrifice or by spleen cells treated *in vitro* with CT on the day of *in vitro* immunization or 2 days earlier, did not appear due to stimulation of helper or nonspecific enhancing lymphoid cells, since cocultivation experiments with normal spleen cells *in vitro* did not result in PFC responses any greater than would be expected from the sum of the cell population used (unpublished observations).

Summary. Cholera toxin is known to affect antibody responsiveness, both *in vivo* and *in vitro*. In the present study spleen cells from mice pretreated with cholera toxin were markedly deficient in their ability to respond to sheep red blood cells by forming hemolytic antibodies in vitro, whereas splenocytes from mice treated with toxin on the day of immunization or 1 day earlier, showed marked enhancement of antibody responsiveness upon *in vitro* challenge immunization. Spleen cells from normal mice treated *in vitro* with cholera toxin at the same time as immunization also showed a marked enhancement of the immune response. Furthermore, normal spleen cell cultures first treated with cholera toxin *in vitro* and then immunized 2 days later with sheep erythrocytes showed an increased rather than decreased PFC response. These results indicate that depression of antibody formation observed *in vivo* or *in vitro* with spleen cells from cholera toxin pretreated animals may reflect an indirect effect, whereas increased responses induced by toxin exposure on the day of immunization, either *in vivo* or *in vitro,* may reflect a direct enhancing effect on immunocytes *per se.*

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