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The Differential Effect of Cholera Toxin on the Lymphocyte Stimulation Induced by Various Mitogens

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Summary. BALB/c spleen cells (5×10^6) were cultured in 1 ml of serum-free RPMI 1640 medium for 3 days in order to examine the effect of cholera enterotoxin (CN) and its spontaneously formed toxoid (CD) on lymphocyte stimulation. Stimulation was assessed after addition of [^{3}H]thymidine for the last 16 hours of culture. One microgram of CN per culture markedly reduced the baseline of [^{3}H]thymidine incorporation and the stimulation due to phytohaemagglutinin (PHA), concanavalin A (con A) and bacterial lipopolysaccharide (LPS). One microgram of CD diminished the base-line to half, abolished the response to PHA, reduced the response to con A and had very little effect on the LPS-induced stimulation. One-tenth the amount (0·1 μg) of both CN and CD affected only the PHA reaction. A secondary response to haemocyanin *in vitro* was not decreased by this lower dose. The effects of 1 μg of CN on the LPS response could be reduced by pretreatment of the cells with CD, whereas the PHA reaction remained markedly diminished. Dibutyryl-cAMP added to culture tubes had a similar effect to 1 μg of CN, affecting the PHA response much more than the response to LPS. Spleen cells of mice immunized with CD gave a significant proliferative response to both 1 μg of CD and CN. The results are interpreted as indicating a strong inhibitory effect of CN mediated by accumulation of intracellular cAMP. CD-immunized cells contain specific receptors for both CD and CN which probably compete with the sites responsible for adenylate cyclase stimulation by CN.

INTRODUCTION

Recently, the enterotoxin of *Vibrio cholerae* has been purified and some of its effects upon cells have been more clearly defined (Finkelstein and LoSpalluto, 1969). This enterotoxin called choleragen (CN) is a protein with a molecular weight of 84,000 and is made up of approximately six subunits (LoSpalluto and Finkelstein, 1972). In the intestinal mucosa and in other cells, this toxin appears to stimulate the enzyme adenylate cyclase, which in turn causes production of an excessive amount of cyclic adenosine 3′,5′-monophosphate (Kimberg, Field, Johnson, Henderson and Gershon, 1971; Pierce, Greenough and Carpenter, 1971). Such a stimulation of adenylate cyclase often results in functional

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alterations in many cells, including lymphocytes, where it inhibits elaboration and action of macrophage migration inhibition factor (MIF) (Pick, 1972; Koopman, Gillis and David, 1973), lymphotoxin production (Lies and Peter, 1973) and the stimulation of DNA brought about by a plant lectin phytohaemagglutinin (PHA) (Smith, Steiner, Newberry and Parker, 1971). In addition to the cholera enterotoxin there is a spontaneously formed toxoid, choleragenoid (CD), which has a molecular weight of 58,000 (LoSpalluto and Finkelstein, 1972). It appears to be antigenetically similar to CN and inhibits the effects of CN on gut cells, presumably by competitive binding (Peterson, LoSpalluto and Finkelstein, 1972). In the present paper the results of experiments investigating the influence of both CN and CD on the [^{3}H]thymidine incorporation of mouse spleen cells stimulated in culture by non-specific agents and specific antigen are reported. These results indicate that CN in high dose inhibits the lymphocyte stimulation in general and is much more efficient at this than CD. The same high dosage of CN does not inhibit CD-sensitized lymphocytes but stimulates them. At a lower dosage, both CD and CN interfere with the reaction to PHA only, sparing the effects of other stimulants.

MATERIALS AND METHODS

Animals

Four to 6-week-old male BALB/c/M mice were obtained from the Institut für biologisch-medizinische Forschung AG (Füllinsdorf, Switzerland), and were maintained under standard laboratory conditions. Some mice were immunized by one intraperitoneal injection of 0·1 mg of Keyhole limpet haemocyanin (KLH) (Calbiochem) adsorbed on bentonite (Gallily and Garvey, 1968) and were used 4–8 weeks afterwards. Other mice were injected similarly with 4 μg of choleragenoid (CD) on bentonite at 2 weeks intervals.

Cell suspensions

Spleen cell suspensions were prepared as described previously (Vischer, 1972a) in Eagle's minimum essential medium (MEM) (Microbiological Associates) and washed twice before use. In some experiments, cell suspensions (20×10^6/ml) were incubated at 37° for 30 minutes with 1 μg of CD per millilitre. The cells were then washed twice with MEM. Control cells were treated identically, omitting the CD.

Cultures

5×10^6 spleen cell aliquots were cultured in 1 ml of serum-free RPMI 1640 medium (Microbiological Associates) with additional HEPES buffer (Calbiochem) for 3 days as described previously (Vischer, 1972b). Phytohaemagglutinin P (PHA) (Difco), concanavalin A (con A) (Calbiochem), lipopolysaccharide *S. typhi* 0901 (LPS) (Difco) or KLH were added to some cultures in 5-μl volumes, to give optimal concentration as determined from a response curve. In certain experiments, dibutyryl cyclic adenosine 3′,5′-monophosphate (cAMP) (Sigma Chemical Company) was added to the culture tubes to give a final concentration of 10^{-3} M or 10^{-5} M.

Choleragen (CN) and choleragenoid (CD) were kindly provided by Dr R. A. Finkelstein (University of Texas Southwestern Medical School, Dallas) and had been prepared as previously described (Finkelstein and LoSpalluto, 1970; Finkelstein, Fujita and LoSpalluto, 1971). They were appropriately diluted in MEM and added in 5-μl volumes to cultures. Sixteen hours prior to harvesting the cells, 0·5 μCi of [^{3}H]thymidine (The

TABLE 1

EFFECT OF CHOLERAGEN (CN) AND CHOLERAGENOID (CD) ON THE LYMPHOCYTE STIMULATION INDUCED BY PHA, LPS AND CON A

	Ct/minute	Ct/minute with 1 μg of CN	Inhibitory index*	Ct/minute with 1 μg of CD	Inhibitory index*	Ct/minute with 0·1 μg of CN	Inhibitory index*	Ct/minute with 0·1 μg of CD	Inhibitory index*
No stimulant	9909	1562	0·16	5426	0·55	10,112	1·02	9269	0·94
LPS	53,548 (5·40)	11,759 (7·53)	0·22	58,403 (10·76)	1·09	51,470 (5·08)	0·96	57,077 (6·16)	1·07
PHA	122,905 (12·40)	4985 (3·19)	0·04	5448 (1·00)	0·04	9325 (0·92)	0·08	12,623 (1·36)	0·10
Con A	311,527 (31·44)	81,285 (52·04)	0·26	124,380 (22·92)	0·40	162,307 (16·05)	0·52	223,075 (24·07)	0·72

5×10^6 BALB/c spleen cells in 1 ml of RPMI 1640, cultured for 3 days. 0·5 μCi of [^{3}H]thymidine was added for the last 16 hours of culture, and radioactivity was measured by liquid scintillation counting. Ct/minute are the mean of triplicate tubes. The numbers in parentheses = stimulation index = (ct/minute of stimulated cultures)/(ct/minute of unstimulated cultures).

* Inhibitory index = (ct/minute of cultures with CN or CD)/(ct/minute of the control cultures).

Radiochemical Center, Amersham, Bucks) (specific activity 5 Ci/mmol) were added to the tubes and the cultures precipitated with 10 per cent cold trichloracetic acid. The precipitate was then washed and prepared for liquid scintillation counting as described previously (Vischer, 1972a). The results are expressed as the mean net ct/minute of triplicate culture tubes. The technique gave replicate values consistently within 10–15 per cent. In some experiments, the stimulation index was calculated. This is the ratio of the ^3H counts in stimulated cultures to the ^3H counts in unstimulated control cultures. The inhibitory index is the ratio of the ^3H counts in cultures with CN or CD divided by ^3H counts in control cultures without CN or CD. For statistical analysis, Student's *t*-test was used.

RESULTS

In Table 1, the effect of both CN and CD on the stimulation induced by LPS, PHA and con A is shown. One microgram of CN had a definite inhibitory action on unstimulated and on all stimulated cultures. The same amount of CD reduced the base-line proliferation to half, diminished the response to con A, abolished the response to PHA, but had no definite effect on the stimulation with LPS. A similar picture of sensitivity of the response to the different mitogens emerged when only a tenth of the dose of both CN and CD was used. The reaction to PHA was the most sensitive to suppression by 1 μg of CD. Table 2 summarize all these results giving the mean inhibitory indices from several experiments.

TABLE 2

EFFECT OF CHOLERAGEN (CN) AND CHOLERAGENOID (CD) ON THE [^{3}H]THYMIDINE INCORPORATION IN SPLEEN CELL CULTURES*

	Inhibitory index†			
	CN (1 μg)	CD (1 μg)	CN (0·1 μg)	CD (0·1 μg)
Unstimulated cultures (n = 8)	0·103 ± 0·022	0·514 ± 0·055‡	0·801 ± 0·090	0·848 ± 0·031
Cultures stimulated by LPS (n = 5)	0·162 ± 0·090¶	0·720 ± 0·106§¶	0·857 ± 0·057**	0·947 ± 0·072**
Cultures stimulated by PHA (n = 5)	0·020 ± 0·004	0·068 ± 0·014§	0·122 ± 0·046	0·188 ± 0·067

* 5×10^6 BALB/c spleen cells were cultured for 3 days.
† (Ct/minute of cultures with CN or CD added)/(untreated control cultures) ± standard error of the mean.
‡ $P < 0·01$ against CN.
§ $P < 0·001$ against CN.
¶ $P < 0·01$ against PHA.
** $P < 0·001$ against PHA.

The secondary proliferation *in vitro* in response to a soluble antigen (KLH) was not reduced by 0·1 μg of either CD or CN; the results of two of this series of experiments are reported in Table 3. The mean inhibitory index for these experiments was 1·16 ± 0·157 (s.e.) for CN and 1·08 ± 0·191 for CD, which is not significantly different from the results obtained with LPS.

Pretreatment of the cells for 30 minutes before the beginning of the cultures with CN or CD had an effect very similar to that obtained when these substances were present for the

TABLE 3

EFFECT OF CHOLERAGEN (CN) AND OF CHOLERAGENOID (CD) ON THE SECONDARY PROLIFERATIVE RESPONSE OF KLH SENSITIZED SPLEEN CELLS TO ANTIGEN *in vitro*

	Ct/minute	Ct/minute with CN (0·1 μg)	Inhibitory index*	Ct/minute with CD (0·1 μg)	Inhibitory index*
Experiment 1					
No antigen	3452	1891	0·55	1561	0·45
KLH (5 μg)	8610	12,104	1·41	11,416	1·33
Experiment 2					
No antigen	5321	2306	0·43	2555	0·48
KLH (5 μg)	11,306	13,535	1·20	13,524	1·20

5×10^6 Spleen cells were cultured for 4 days in 1 ml of RPMI enriched with 5 per cent rabbit serum.

* Inhibitory index = (ct/minute of cultures with CN or CD)/(ct/minute of control cultures).

whole culture period. In addition, as can be seen in Table 4, pretreatment of the spleen cells with CD protected them from some of the effects of 1 μg of CN; the base-line incorporation was undiminished by CN, the response to LPS was only slightly affected, but the PHA response remained profoundly decreased by CN.

TABLE 4

INFLUENCE OF PRETREATMENT OF SPLEEN CELLS WITH CHOLERAGENOID (CD) ON THE EFFECT OF CHOLERAGEN (CN) DURING CULTURES

	Ct/minute untreated cells			Ct/minute cells pretreated with CD		
Additions for stimulation	No toxin added	CN added (1 μg)	Inhibitory index*	No toxin added	CN added (1 μg)	Inhibitory index*
None	5219	399	0·08	3714	5151	1·39
LPS	42,966	1631	0·04	44,765	24,378	0·54
PHA	95,588	753	0·01	51,462	4071	0·08

Cells (20×10^6/ml) incubated with 0·25 μg of CD per millilitre for 30 minutes and washed twice with MEM. Untreated cells were handled correspondingly. The cells were subsequently cultured and stimulated in the presence of 1 μg of CN as indicated.

* Inhibitory index = (ct/minute of cultures with CN)/(ct/minute of control cultures).

That 1 μg of CN does not have a toxic effect on lymphoid cells can be inferred from two observations: CN does not, at least for 24 hr, change cell morphology and decrease viability as judged by examination of the suspensions by phase contrast microscopy. Secondly, as seen in Table 5, which shows the results of one of four similar experiments, spleen cells from CD-immunized mice were perfectly capable of expressing a secondary response to 1 μg of CD or CN, showing marked increase in [^{3}H]thymidine incorporation. CN or CD added to such sensitized cells inhibited the PHA response much less than would be expected from the experiments reported in Tables 1 and 2.

Since CN appears to exert its effect on cells in general by stimulation of adenylate cyclase, dibutyryl cAMP was added to certain culture tubes in some experiments. As can be seen in Table 6, this cAMP derivative mimics the effect of CN and affects the response to PHA much more than the response to LPS.

Table 5

Stimulation of choleragenoid (CD) sensitized spleen cells by choleragen (CN) and choleragenoid *in vitro*

Additions	Ct/minute	
	No stimulant	PHA
	5728	59,042
CD (1 μg)	17,114	21,637
CN (1 μg)	12,348	16,598
CD (0·25 μg)	11,344	39,458
CN (0·25 μg)	8616	11,614

5×10^6 Spleen cells from CD-sensitized mice cultured in 1 ml of RPMI 1640 medium for 3 days.

Table 6

Effect of choleragen (CN) and of dibutyryl-cAMP on the stimulation induced by PHA and LPS

Additions for stimulation	Ct/minute		Inhibitory index†	Ct/minute with cAMP (10^{-3} M)*	Inhibitory index†	Ct/minute with cAMP (10^{-5} M)*	Inhibitory index†
	Controls	CN (1 μg)					
None	6608	4317	0·65	10,153	1·54	17,288	2·62
PHA	33,198	5455	0·16	3156	0·10	10,057	0·30
LPS	41,822	10,102	0·24	25,636	0·61	43,795	1·05

5×10^6 spleen cells were cultured for 3 days.

* Dibutyryl cAMP was added to give a final concentration of 10^{-3} M or 10^{-5} M.

† Inhibitory index = (ct/minute of cultures with CN or cAMP)/(ct/minute of control cultures).

DISCUSSION

Cholera toxin exerts its effect on gut and other cells through a delayed stimulation of adenylate cyclase, thus inducing an increase in intracellular cyclic AMP (Lichtenstein, Henney, Bourne and Greenough, 1973). Similar effects have been observed on other cell types such as cytotoxic lymphocytes (Sultzer and Craig, 1973). Metabolic functions such as the production of migration inhibition factor (Pick, 1972), its action (Koopman *et al.*, 1973), cytotoxin production (Lies and Peter, 1973), IgE-mediated release of histamine by leucocytes (Lichtenstein *et al.*, 1973) and macromolecular synthesis by spleen cells induced by PHA, LPS and con A have thus been inhibited (Sultzer and Craig, 1973; Holmgren, Lindholm and Lonnorth, 1974). Cholera toxin has even been proposed as a tool for testing the dependency of a metabolic process on cAMP (Bourne, Lehrer, Lichtenstein, Weissman and Zurier, 1973). Stimulation of lymphocytes is also regulated by cAMP (Whitfield, Rixon, MacManus and Balk, 1973); low levels seem to slightly stimulate DNA synthesis and mitosis, whereas excessive amounts inhibit these processes (Quagliata, Lawrence and Phillips-Quagliata, 1973). It seems very likely that in the present experiments, CN exerts its effect on lymphocytes, as it does on other cells, through an accumulation of cAMP. As a pharmacological effect, it is dose-dependent. The same

dose, in micrograms, of the toxoid CD, corresponding to about a third more molecules, has a much smaller effect on the parameters tested, thus confirming the absence of a marked biological activity of CD (Peterson, LoSpalluto and Finkelstein, 1972).

At the lower dose, both CN and CD (0·1 μg) have a very similar action, reducing marginally only the base-line incorporation, the response to LPS and con A, and not altering the specific stimulation induced by KLH. The PHA response, however, remained inhibited. The same occurred in the experiments where some of the effects of the higher dose of CN were partially inhibited by pretreatment of the cells with CD; whereas the action of CN on non-stimulated or LPS-treated cells was diminished, the inhibition of the PHA response by CN persisted.

Competition for the cell surface receptors between PHA and both CN and CD could be an explanation for this high sensitivity of the PHA response. Indeed, both CN and CD attach themselves very quickly to the lymphocyte surface, as shown by Holmgren *et al.* (1974). However, extensive attempts to inhibit the PHA-induced agglutination of murine erythrocytes and thymocytes by CN or CD gave negative results (Vischer, unpublished results). Similarly, Holmgren *et al.* (1974) could not show interference of binding between another mitogen, con A and CN or CD. The experiments where dibutyryl-cAMP was added to the cultures point towards another mechanism for this difference among lymphocyte stimulants in sensitivity to CN: the response to PHA seems to be much more sensitive to the effect of cAMP than the response to LPS. No explanation for the variable susceptibility of lymphocyte stimulants to cAMP is available at the moment. It might indicate that the various stimulants act on cells in different ways. It is also possible that the susceptibility of cells to cAMP might depend on the time course of the stimulation. Preliminary experiments carried out in our laboratory demonstrate that the PHA-induced protein synthesis begins within a few hours, whereas that induced by LPS requires longer times. Another possibility would be that various lymphocyte populations are reacting differently to an increase of cAMP.

That the presence of 1 μg of CN does not itself kill or inactivate the spleen cells in a non-specific way is indicated by the intact morphology of the spleen cells in these experiments and, in addition, by the experiments with cells from mice immunized with toxoid. Antigenic cross-reactivity between CD and CN is well known. Spleen cells obtained from immunized animals were stimulated *in vitro* by 1 μg of either CD or CN. An increase in [^{3}H]thymidine incorporation was observed instead of the profound decrease usually seen with 1 μg of CN. This could be interpreted as indicating that the avidity of the specific, membrane-incorporated antibodies is greater than that of the normal surface sites for adenylate cyclase stimulation. The cell-surface antibody-bound CN would thus be unavailable to stimulate this enzyme. An alternative explanation could be that antibody released into the culture by some of the spleen cells from immunized animals could bind CN or CD, thus inactivating it and stimulating the cells by the immune complexes (Harris, 1968). We would consider this unlikely since the spleen cells were washed before culture and both CN and CD adhere to the cells immediately after contact, before appreciable amounts of antibody can be released.

Purified cholera toxin thus suppresses lymphocyte stimulation *in vitro*, probably by a mechanism involving accumulation of intracellular cAMP. The response to PHA is much more sensitive to both cholera toxin and added dibutyryl cAMP than the response to LPS. The suppression of the lymphocyte stimulation by CN can be blocked by either pretreatment of the cells with the natural toxoid, probably by direct competition with CN, or it

can be blocked by previous immunization of the lymphocyte donors. In this instance the competition for CN would be between the natural sites of CN and CD for stimulation of adenylate cyclase and the membrane-bound, specific antibody molecules.

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