Human platelets are defective in processing of cholera toxin

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Cholera toxin is unable to elevate cyclic AMP levels in intact human platelets despite being very efficacious in this respect in other mammalian cells; in the presence of 0.5 mM-isobutylmethylxanthine, we found that 3–6 nM-cholera toxin over 3 h at 37°C elevated platelet cyclic AMP from 33 ± 13 to 39 ± 12 pmol/mg of protein (means $\pm$ S.D.; $n = 12$). We have investigated the basis for this lack of response. ^{125}I -labelled cholera toxin bound to platelets both saturably and with high affinity ($K_d \approx 60$ pM; $B_{\text{max}} \approx 50$ fmol/mg of protein). Incubation of platelets with the putative cholera toxin receptor monosialoganglioside G_{M1} enhanced ^{125}I -labelled cholera toxin binding at least 40-fold but facilitated only a minimal (≤ 3 -fold) elevation of platelet cyclic AMP levels. In contrast, dithiothreitol-activated cholera toxin markedly stimulated adenylate cyclase activity in platelet membranes. Platelet cytosol both enhanced stimulation of adenylate cyclase activity by activated cholera toxin (A_1 subunit) and supported stimulation by the A_1 – A_2 subunit of cholera toxin. Neither GTP nor NAD^+ , both necessary for response to cholera toxin, was lacking in intact platelets. However, we found that platelets were unable to cleave cholera toxin to the active A_1 subunit (as assessed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis). By contrast, murine S49 lymphoma cells were able to generate the A_1 subunit with a time course that closely resembled the kinetics of toxin-mediated cyclic AMP accumulation in these cells. Thus we conclude that human platelets are defective in their ability to process surface-bound cholera toxin. These results indicate that binding of cholera toxin to surface receptors is necessary, but not sufficient, for expression of the toxin effect and the generation of the A_1 subunit of the toxin may be rate-limiting for expression of cholera toxin response.

Cholera toxin, the causative agent of the disease cholera, is secreted by the bacterium *Vibrio cholerae* (Fishman, 1982*b*; van Heyningen, 1982). The toxin molecule (M_r 85000) is composed of three component polypeptides in the stoichiometry $1\text{A}_1:1\text{A}_2:5\text{B}$. By virtue of its B subunits, the toxin binds to cell-surface monosialoganglioside G_{M1} (II^3N -acetylneuraminylgangliosylceramide). After a 15–90 min lag, adenylate cyclase [ATP pyrophosphate-lyase (cyclizing); EC 4.6.1.1] activity is increased through ADP-ribosylation of the guanine nucleotide regulatory component of adenylate cyclase (Gill, 1977; Moss & Vaughan, 1979; Fishman, 1982*a*; Schleifer *et al.*, 1982; van

Heyningen, 1982). Cyclic AMP levels subsequently rise markedly in the affected cell (Gill, 1977; Moss & Vaughan, 1979; Fishman, 1982*a*; van Heyningen, 1982). Very little is known of the events that transpire between binding of the toxin to the cell surface and the ADP-ribosylation of the guanine nucleotide regulatory component of adenylate cyclase. However it is generally agreed that the toxin A_1 subunit must traverse the plasma membrane in order to activate the adenylate cyclase (Moss & Vaughan, 1979; Schleifer *et al.*, 1982).

Cholera toxin increases cyclic AMP levels in virtually all mammalian cells. However, we have found that intact human platelets are non-responsive to the toxin as judged by this criterion (Hughes & Insel, 1980). We reasoned that additional studies exploring the basis of this non-responsiveness might enlighten our understanding of the molecular basis of cholera toxin action. The present paper describes experiments designed to differentiate between the

Abbreviations used: G_{M1} , II^3N -acetylneuraminylgangliosylceramide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; IBMX, 3-isobutyl-1-methylxanthine; SDS, sodium dodecyl sulphate.

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numerous potential defects that might explain the non-responsiveness of intact platelets to cholera toxin. A preliminary report of some of these findings has appeared (Hughes *et al.*, 1982).

Materials and methods

FMN was a gift from the Sigma Chemical Co. The following chemicals and enzymes were purchased from Sigma: ATP, alcohol dehydrogenase (alcohol:NAD⁺ oxidoreductase; EC 1.1.1.1), cholera toxin A₁-A₁ subunits, creatine kinase (ATP:creatine *N*-phosphotransferase; EC 2.7.3.2), cyclic AMP, Hepes, luciferase (type V) from *Vibrio fischeri*, NAD⁺, NADH, phosphocreatine and semicarbazide hydrochloride. Cholera toxin was purchased from Schwarz-Mann, IBMX from Aldrich, [α -³²P]ATP (sp. radioactivity 77 Ci/mmol), cyclic [2,8-³H]AMP (38 Ci/mmol) and carrier-free Na¹²⁵I (>350 mCi/ml) from New England Nuclear. Bis-(2-ethylhexyl) sebacate and di-isobutyl phthalate were purchased from Eastman Kodak and G_{M1} was obtained from Supelco. Electrophoretic chemicals were purchased from Bio-Rad. All other chemicals were of analytical grade.

Isolation of human platelets and preparation of S49 lymphoma cells

Blood was drawn into one-tenth its volume of 3.8% (w/v) sodium citrate. After 20 min centrifugation at 300 *g*_{av} (20°C), the platelet-rich plasma was removed and centrifuged for 10 min at 500 *g*_{av}. The platelets were washed twice in buffer A (50 mM-Tris/HCl (pH 7.4)/85 mM-NaCl/8 mM-EDTA) and resuspended in the appropriate buffer. Murine S49 lymphoma cells (wild-type, clone 24.3.2) were grown and prepared for experiments as previously described (Darfler *et al.*, 1981).

Incubation of platelets with G_{M1}

Platelets were resuspended (0.1–0.5 mg of protein/ml) in Hank's balanced salt solution without the Ca²⁺ and Mg²⁺ salts. G_{M1} in chloroform was added to polypropylene tubes and the solvent was evaporated. A portion (20 ml) of platelet suspension was subsequently added and incubated at 37°C for 60 min. After incubation, 20 ml of buffer A was added and the platelets were washed three times by centrifugation at 4°C to remove free G_{M1}.

Cyclic AMP assay

Platelets were resuspended at a concentration of 0.2–0.3 mg of protein/ml in Hanks balanced salt solution (Hanks & Wallace, 1949) lacking Ca²⁺ and Mg²⁺ salts but containing 5 mM-sodium Hepes, pH 7.4, and 0.5 mM-IBMX. Platelet suspensions together with the indicated concentrations of cholera toxin were incubated in a 0.4 ml volume in triplicate

in polypropylene tubes for 3 h at 37°C. Trichloroacetic acid [0.1 ml; 40% (w/v)] was added to terminate the incubation. The tubes were centrifuged at 900 *g*_{av} for 10 min and the supernatant fractions were applied to columns containing 1 g of Dowex AG50 (X8) resin. The resin was washed twice with 1.2 ml of water to remove the trichloroacetic acid and cyclic AMP was eluted with a further 4 ml of water. Recovery of cyclic AMP (routinely 90% or better), determined by processing 0.5 ml of an identical solution containing approx. 30 000 c.p.m. of cyclic [³H]AMP through several columns, was used to correct the measured amount of cyclic AMP. The contents of each tube were freeze-dried and stored at 0°C. After reconstitution in 0.20 ml of 50 mM-sodium acetate buffer, pH 4.0, containing 0.2 mM-IBMX, a 0.030 ml portion from each tube was assayed for cyclic AMP using a competitive binding protein method.

Preparation of ¹²⁵I-labelled cholera toxin

¹²⁵I-labelled cholera toxin was prepared by using Chloramine-T as described by Cuatrecasas (1973). Typically, the toxin was iodinated to a specific radioactivity of 28.4 Ci/g (Darfler *et al.*, 1981). SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) of the ¹²⁵I-labelled cholera toxin with and without reduction by 20 mM-dithiothreitol showed that 80% of the ¹²⁵I label was on the A₁ subunit. The remaining 20% was on the B subunit.

Binding of ¹²⁵I-labelled cholera toxin to platelets

Platelets were resuspended in a modified Krebs-Ringer buffer [119 mM-NaCl/4.8 mM-KCl/3.6 mM-KH₂PO₄/2.5 mM-MgSO₄/16 mM-Na₂HPO₄/1 mg of bovine serum albumin/ml (pH 7.4)] at a concentration of approx. 1 mg of protein/ml. Binding of the ¹²⁵I-labelled cholera toxin to platelets was carried out by a modification of our previously-described procedure (Darfler *et al.*, 1981). 'Naive' platelets and platelets supplemented with G_{M1} (20–100 µg of platelet protein) were incubated with ¹²⁵I-labelled cholera toxin in triplicate at 25°C for 60 min in 1.5 ml polypropylene Microfuge tubes in final volumes of 0.2 ml or 1.0 ml, the latter being used for platelets pretreated with G_{M1} and representing a 5-fold dilution of platelets. Specific binding of cholera toxin was defined as the difference between the total amount of binding determined in triplicate and the non-specific binding determined in duplicate with a 100-fold greater concentration of unlabelled cholera toxin than ¹²⁵I-labelled cholera toxin. Bound toxin was separated by centrifugation for 2 min through 0.050 ml of oil [bis-(2-ethylhexyl) sebacate/di-isobutyl phthalate (4:35); density, 1.02 g/ml], contained in 0.4 ml or 1.5 ml polypropylene Microfuge tubes, in a Beckman Microfuge. The ends of the tubes were counted for ¹²⁵I radioactivity.

The rates of association and dissociation of ^{125}I -labelled cholera toxin were determined in a batchwise procedure for naive and G_{MI} -supplemented platelets. During incubation with 0.5 nM- ^{125}I -labelled cholera toxin either alone or in the presence of 50 nM unlabelled cholera toxin, triplicate 0.20 ml or 1.0 ml portions were withdrawn at timed intervals and specific binding was estimated. To measure the rate of dissociation, after a 45 min incubation a portion of the platelet suspension incubated with labelled toxin was transferred to another tube containing a 100-fold excess of unlabelled cholera toxin.

Preparation of a crude platelet membrane fraction

Platelets were prepared as described above and resuspended in a hypo-osmotic lysis buffer [5 mM-Tris/HCl (pH 7.5)/5 mM-EDTA] (approx. 1 mg of protein/ml). The platelets were freeze-thawed three times in liquid N_2 and the homogenate was washed twice at $3800g_{\text{av}}$ for 10 min at 4°C . The final pellet was resuspended in 5 mM-Tris/HCl, pH 7.5, at a protein concentration of 1–2 mg/ml. Adenylate cyclase was assayed in triplicate by a modification of our previous procedures (Insel *et al.*, 1978, 1982). Platelet membrane was added to a reaction mix comprising 50 mM-Tris/acetate, pH 7.6, 12 mM-magnesium acetate, 0.1 mg of bovine serum albumin/ml, 5 mM-phosphocreatine, 2 units of creatine kinase, 1 mM-dithiothreitol, 0.2 mM-EGTA, 2 mM- NAD^+ , 0.1 mM-GTP, 0.5 mM-cyclic [^3H]AMP (approx. 30000 c.p.m.), 0.5 mM- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (1×10^6 c.p.m.) and activated cholera toxin in a final volume of 0.050 ml. Cholera toxin was activated by incubation at a concentration of $500\mu\text{g/ml}$ with 20 mM-dithiothreitol for 10 min at 37°C . Adenylate cyclase reactions were conducted for 10–30 min at 30°C and were terminated by the addition of 0.10 ml of 'stop' solution [2% (w/v) SDS, 10 mM-ATP and 200 μM -cyclic AMP]. Stop solution was added to control tubes before addition of membranes. Cyclic AMP was purified by a two-column method (Insel *et al.*, 1978) and recovery of cyclic [^{32}P]AMP (generally $\geq 70\%$) was corrected by using the ^3H counts in each tube.

Assay of platelet NAD^+

Platelets were prepared as described for the cyclic AMP assay and incubated for 60 min at 37°C . Portions (0.10 ml) of platelet suspension were transferred to 1.5 ml Microfuge tubes containing 0.10 ml of 6 M-HClO₄. A series of NAD^+ standards (10 nM–10 μM) was identically treated. The tubes were incubated for 10 min at 37°C in order to destroy the platelets' endogenous NADH. After overnight incubation at 4°C the tubes were spun in a Beckman Microfuge for 60 s to remove precipitated protein. To 0.10 ml of each supernatant was added an equal

volume of 2 M-KHCO₃ solution. After evolution of CO₂ had ceased, the tubes were capped and incubated overnight at 4°C to allow complete precipitation of KClO₄. The tubes were spun in the Microfuge and a 0.10 ml portion of the supernatant was added to the following solution: 0.060 ml of 0.1 M-sodium pyrophosphate, pH 9.4, 0.020 ml of 6 M-ethanol, 0.010 ml of alcohol dehydrogenase (2 mg/ml) and 0.010 ml of 0.7 M-semicarbazide hydrochloride. After incubation for 30 min at 25°C (which allows complete conversion of NAD^+ into NADH) the tubes were placed on ice in the dark.

Platelet NAD^+ was assayed by conversion of NAD^+ into NADH and subsequent measurement of NADH using bacterial luciferase by a method described previously (Hughes, 1983). This assay was linear over the range 1–1000 pmol of NADH. Comparison of the standard curve for NAD^+ with that for untreated NADH demonstrated that the NAD^+ was quantitatively converted into NADH by the procedure. Furthermore, when NADH was carried through the procedure, it yielded less than 2% of the signal given by the same concentration of NAD^+ ; this was presumed to be due to NAD^+ contamination of the NADH, since the detection of NADH carried through the procedure was independent of the length of an acid incubation.

Generation of cholera toxin A_1 fragment

Platelets of S49 cells were incubated at 4°C for 60 min with 1 nM- ^{125}I -labelled cholera toxin in the modified Krebs–Ringer buffer used for cholera toxin-binding studies. The cells were then washed once at 4°C and resuspended in buffer at 37°C . After various times, 1 ml of cell suspension was spun through oil ($\rho = 1.02\text{ g/ml}$) and the pellet was resuspended in 0.2 ml of 10 mM-Tris/HCl (pH 7.4)/0.25 mM-MgCl₂/2 mM-phenylmethanesulphonyl fluoride. The S49 cell lysate was incubated with 0.1% (w/v) deoxyribonuclease II for 5 min at 20°C in order to degrade the large amount of cellular DNA. Similar treatment of platelet lysate produced no difference in the gel-electrophoretic pattern. The lysates were dissolved by adding SDS to 1% (w/v) and remaining particulate material was removed by centrifugation in a Beckman Microfuge for 2 min. One-sixth volume of 50% (w/v) sucrose and 0.01% (w/v) Bromophenol Blue was added to the samples, which were electrophoretically chromatographed on SDS/polyacrylamide gels (3% stack, 12.5% running gel) by the method of Laemmli (1970). After electrophoresis, the gels were sliced and counted for ^{125}I .

Protein determination

Protein was determined by the method of Peterson (1977), employing double-logarithmic transformation to yield a linear standard curve. Bovine

serum albumin, containing 1 mg of NaN_3/ml as a non-interfering bactericide, was employed for standards.

Results

Inability of cholera toxin to elevate cyclic AMP in intact human platelets

Incubation of washed human platelets from 10 different donors with 3–6 nM-cholera toxin for 3 h at 37°C in the presence of 0.5 mM-IBMX, a cyclic nucleotide phosphodiesterase inhibitor, increased cyclic AMP levels from 33 ± 13 to only 39 ± 12 (means $\pm$ S.D.; $n = 12$) pmol/mg of protein. This increase corresponded to a paired stimulation of 1.21 ± 0.20 -fold. Cyclic AMP levels did not increase with exposure to 6 nM toxin for 5 h, nor with 36 nM toxin for 3 h. The lack of response of human platelets to cholera toxin has been consistently recorded over a 2-year period using five different batches of cholera toxin. By contrast, other agents, including prostaglandins E_1 and I_2 and the diterpene forskolin, produced up to 30-fold increases in platelet cyclic AMP in parallel studies (Fig. 1). Our subsequent experiments were designed to explore the possible molecular basis for this non-responsiveness of intact platelets to cholera toxin.

Fig. 1. Response of cyclic AMP levels of intact platelets to various agents

Platelets were isolated as described and incubated at 37°C in the presence of 0.5 mM-IBMX. Cyclic AMP was measured after no further additions (basal; $n = 12$), after 3–6 nM cholera toxin for 3 h (CTx; $n = 12$), after 3 μM -prostaglandin E_1 for 1 min (PGE₁; $n = 3$), after 0.3 μM -prostaglandin I_2 for 1 min (PGI₂; $n = 6$) and after 10 μM -forskolin for 5 min (FORSK; $n = 2$). Results are means $\pm$ S.D. (indicated by the error bars).

Quantification of surface cholera toxin receptors and further enrichment of these receptors by incorporation of $\text{G}_{\text{M}1}$

To estimate the number of cholera toxin receptors on platelets, we prepared ^{125}I -labelled cholera toxin (Cuatrecasas, 1973; Darfler *et al.*, 1981). The iodinated toxin bound with high affinity ($K_d \approx 60 \text{ pM}$) to intact platelets ($B_{\text{max.}} \approx 50 \text{ fmol/mg}$ of protein) (Table 1, Fig. 2). This number of sites was far greater than the number we observed in parallel studies with S49 lymphoma cells (4 fmol/mg of protein) (Darfler *et al.*, 1981) and was comparable with measurements of the number of toxin sites on liver cells (Cuatrecasas, 1973; Gill, 1977; R. J. Hughes, unpublished work). We used wild-type S49 lymphoma cells for these studies on cholera toxin receptors and for subsequent experiments, because these cells show well-documented responses to cholera toxin (e.g., Darfler *et al.*, 1981, and references cited therein). When we measured toxin binding to a crude platelet membrane preparation, we found an increase in the number of toxin-binding sites expressed per mg of protein compared with those in intact platelets (Table 1), consistent with the notion that these sites are located on the platelet plasma membrane.

Although platelets possess a large number of cholera toxin receptors, there could be insufficient in number to support a response to the toxin. We therefore pre-incubated platelets with $\text{G}_{\text{M}1}$ [the putative receptor for cholera toxin (Cuatrecasas, 1973; Gill, 1977; Moss & Vaughan, 1979; Fishman, 1982a; van Heyningen, 1982)] to increase the number of toxin receptors. Incorporation of $\text{G}_{\text{M}1}$ markedly increased the concentration of binding sites, with little change in the affinity for ^{125}I -labelled

Table 1. Enhanced binding of ^{125}I -labelled cholera toxin by platelets supplemented with $\text{G}_{\text{M}1}$

Platelets were washed, incubated with or without $\text{G}_{\text{M}1}$ washed again and incubated in triplicate with six concentrations of ^{125}I -labelled cholera toxin ranging from 0.063 to 3 nM. Another set of tubes was run in duplicate using parallel conditions but also including a 100-fold excess of unlabelled toxin to determine non-specific binding. Scatchard analysis of the data gave values for $B_{\text{max.}}$ and K_d reported below, the results being expressed as means $\pm$ S.D. where several determinations were made.

$[\text{G}_{\text{M}1}] (\mu\text{M})$	$B_{\text{max.}}$ (fmol/mg of protein)	K_d (pM)
0	51 ± 27	64 ± 12 ($n = 3$)
0.025	870	190
0.075	1180	130
0.10	1250	32
Platelet membrane preparation	200 ± 120	240 ± 50 ($n = 3$)

Fig. 2. Concentration dependence of binding of ^{125}I -labelled cholera toxin to platelets

Naive platelets (a) and platelets supplemented with $0.1\text{ }\mu\text{M-GM}_1$ (b) were incubated with ^{125}I -labelled cholera toxin in the presence or absence of a 100-fold excess of unlabelled toxin. The specifically bound toxin (■) was defined as the difference between the total amount of binding (●) and the non-specifically bound toxin (▲) at each concentration of radioligand. Total binding was estimated in triplicate and non-specific binding in duplicate. The data are similar to those obtained in three experiments using different batches of cholera toxin.

cholera toxin (Table 1, Fig. 2). It appears that only a small portion of the added GM_1 was actually incorporated into the platelets. Nevertheless, at sufficiently high GM_1 concentrations (i.e. $1\text{ }\mu\text{M}$ and above), enough GM_1 could be incorporated such that measurement of ^{125}I -labelled cholera toxin was no longer feasible; these 'super-enriched' platelets bound up to 50% of the labelled toxin in the absence or presence of excess unlabelled toxin.

To characterize further the interaction of cholera toxin with its cell-surface receptor, we determined the rates of association and dissociation of ^{125}I -labelled cholera toxin in untreated and $0.1\text{ }\mu\text{M-GM}_1$ -supplemented platelets. Despite a 20-fold increase in the number of specific binding sites on GM_1 -supplemented platelets, the kinetic rate constants were comparable in untreated and GM_1 -supplemented platelets, yielding dissociation constants of 185 and 146 pM respectively (Fig. 3). The iodinated cholera toxin was judged to bind to a single class of sites on untreated and GM_1 -supplemented platelets, since Scatchard plots were linear and the dissociation of labelled toxin was monophasic (Fig. 3d).

Having established that GM_1 -enrichment increased the number of cholera toxin-binding sites, we examined the effect of GM_1 -enrichment on the ability of cholera toxin to stimulate platelet cyclic AMP levels (Fig. 4). Enrichment of platelets with GM_1 produced a slight increase in cyclic AMP levels in response to cholera toxin, although this response was never greater than 3-fold over basal, even when platelets were supplemented with sufficient GM_1 such that 'specific' cholera toxin binding could no longer be measured, as described above. Neither basal nor prostacyclin-stimulated cyclic AMP levels were altered after supplementation with GM_1 (results not shown). Furthermore, other cells having far lower numbers of cholera toxin receptors show prominent response to the toxin. For example, S49 lymphoma cells, which contain only 4 fmol of cholera toxin-binding sites/mg of protein (Darfler *et al.*, 1981), elicit a 10–30-fold response to cholera toxin. Thus, deficiency of toxin receptors is not able to account for the non-responsiveness of platelets to cholera toxin.

Stimulation of platelet adenylate cyclase by cholera toxin

To ascertain whether or not human platelet adenylate cyclase was defective in a manner that rendered it resistant to activation by cholera toxin, we measured adenylate cyclase activity in a crude membrane preparation. Dithiothreitol-activated cholera toxin stimulated adenylate cyclase activity more than 8-fold in the presence of GTP and NAD^+ (Fig. 5; see also Insel *et al.*, 1978, 1982). By contrast, treatment of intact platelets with $3.6\text{--}120\text{ nM}$ -cholera toxin resulted in only a 1.7-fold increase in adenylate cyclase activity subsequently measured in washed membranes from these cells, indicating that the high cyclic nucleotide phosphodiesterase activity of platelets (Hidaka & Asano, 1976) was not responsible for the failure of toxin to raise the intracellular cyclic AMP concentration. Rather, this result suggests that membranes of intact

Fig. 3. Kinetics of ^{125}I -labelled cholera toxin binding to platelets

Total (●) and non-specific (▲) binding of 0.5 nM - ^{125}I -labelled cholera toxin were followed as a function of time for naive (a) and $0.1\text{ }\mu\text{M}$ - G_{M1} -supplemented platelets (b). After 45 min, the rate of dissociation (■) of ^{125}I -labelled cholera toxin from its receptors was followed subsequent to the addition of unlabelled cholera toxin to a final concentration of 50 nM . The dissociation rate constant (k_2) was 0.017 min^{-1} for control (●) and 0.012 min^{-1} for G_{M1} -supplemented platelets (▲) respectively (d). The observed rate of association ($k_{obs.}$) for control (●) and G_{M1} -supplemented platelets (▲) was also calculated and the corrected association rate k_1 determined from the equation $k_{obs.} = \ln[B_{eq}/(B_{eq} - B_t)]/t = k_1[L] + k_2$ as $9.2 \times 10^7\text{ M}^{-1} \cdot \text{min}^{-1}$ and $8.2 \times 10^7\text{ M}^{-1} \cdot \text{min}^{-1}$ respectively (c) where L is the concentration of ^{125}I -labelled cholera toxin, B_t is the amount of toxin bound at

platelets have a decreased ability to utilize surface-bound toxin to activate adenylate cyclase.

Estimation of platelet NAD^+ and cytosolic factor

Having established that platelet adenylate cyclase was not itself refractory to cholera toxin-induced activation, we asked whether a paucity of NAD^+ in intact platelets could account for the lack of response of the intact platelets to the toxin. We found that intact platelets contained $1.10 \pm 0.06\text{ nmol}$ of NAD^+ /mg of protein (mean of three determinations, in each of which $n = 6$), a level comparable with that seen in other cells (Bergmeyer, 1974). We did not measure the GTP content of platelets because independent experiments on hormonal stimulation and inhibition of cyclic AMP synthesis indicated that the concentration of this nucleotide in intact platelets would be adequate to support the effect of cholera toxin (Jakobs & Schultz, 1979; Steer & Wood, 1979; Insel *et al.*, 1982; R. J. Hughes, unpublished work).

Fig. 4. Effect of cholera toxin on cyclic AMP levels of platelets pre-incubated with G_{M1}

Platelets were pre-incubated alone (▲) or with $0.01\text{ }\mu\text{M}$ - (▼), $0.025\text{ }\mu\text{M}$ - (●) or $0.05\text{ }\mu\text{M}$ - G_{M1} (■) and subsequently assayed for cyclic AMP accumulation in response to cholera toxin. Data from a representative experiment are shown. Accumulated data from five experiments show 36 nM -cholera toxin elevates cyclic AMP levels of platelets exposed to 0.01 , 0.025 and $0.05\text{ }\mu\text{M}$ - G_{M1} by 2.3 ± 0.5 , 2.9 ± 0.6 and 3.1 ± 1.2 -fold respectively.

time t , $B_{eq.}$ is that bound at equilibrium and B_0 that bound at time zero. Dissociation constants of 185 pM for naive and 146 pM for G_{M1} -supplemented platelets were calculated from k_2/k_1 .

Fig. 5. Activation of adenylate cyclase activity of human platelet membranes by cholera toxin

Platelet membranes were prepared as described in the text and incubated with dithiothreitol-activated cholera toxin under adenylate cyclase assay conditions for 10 min at 30°C. Results are expressed as the means of triplicate determinations.

A cytosolic protein factor has been described that is required for activation of adenylate cyclase by cholera toxin, particularly at low concentrations of toxin (Enemoto & Gill, 1979, 1980; Schleifer *et al.*, 1982). We assayed platelet cytosol for this activating ability by adding back cytosol to the adenylate cyclase assay mix (Fig. 6). We found that platelet cytosol was able to increase the activation of adenylate cyclase by the A_1 subunit of cholera toxin. Furthermore, the presence of platelet cytosol elicited stimulation of adenylate cyclase by the A_1 - A_2 subunits of cholera toxin. Thus, platelet cytosol must be capable of reducing the disulphide bond that links these two cholera toxin subunits. This action of cytosol from platelets was manifested in a dose-dependent manner and was mimicked by cytosol from S49 lymphoma cells (Fig. 6).

Generation of cholera toxin A_1 subunit

Since cholera toxin binds to platelets and can activate adenylate cyclase in membranes prepared from platelets, and intact platelets possess GTP, NAD^+ and the cytoplasmic factor that supports cholera toxin-activation of adenylate cyclase, we surmised that the A_1 subunit of cholera toxin must not be able to gain access to adenylate cyclase in these intact platelets. In order to test this hypothesis, we measured the generation of cholera toxin

Fig. 6. Effect of cytosol from platelets or S49 lymphoma cells on stimulation by cholera toxin of platelet membrane adenylate cyclase activity

Platelet membranes were prepared as described in the text and incubated with 50 nM-cholera toxin A_1 - A_2 ($\blacktriangle$ and $\triangle$), 50 mM-dithiothreitol-reduced A_1 - A_2 ($\blacksquare$ and $\square$) or under basal conditions ($\bullet$ and $\circ$). Cytosol, which was the supernatant of the initial centrifugation of these membrane preparations from platelets (filled symbols) or S49 cells (open symbols), was added to the adenylate cyclase assay medium that lacked dithiothreitol and the reaction was carried out for 30 min at 30°C. Platelet cytosol [due to its high cyclic nucleotide phosphodiesterase activity (Hidaka & Asano, 1976)], caused some degradation of cyclic AMP, resulting in a loss of up to 15% of cyclic $[^3H]$ AMP as compared with controls. The estimated amount of cyclic AMP generated in such tubes was corrected for this degradation.

A_1 subunit in intact platelets and S49 cells (Figs. 7 and 8). Platelets, in contrast with S49 cells, did not support a time-dependent increase in formation of the free A_1 subunit of cholera toxin. The time course for generation of the A_1 subunit by S49 cells closely resembles the kinetics of cyclic AMP accumulation that we and others find with these cells (Haga *et al.*, 1977; Darfler *et al.*, 1981; Insel & Koachman, 1982). The high-molecular-weight peaks, which are presumed to be oligomers of B subunits, are barely seen with S49 cells. The disappearance of these peaks in S49 cells, but not in platelets, after incubation at 37°C may indicate that cellular processing of cholera toxin involves cleavage of the B_5 pentamer to the B_1 monomer.

Discussion

Cholera toxin increases cyclic AMP levels in virtually all mammalian cells through the irreversible activation of adenylate cyclase (Gill, 1977;

Fig. 7. Generation of cholera toxin A_1 subunit by platelets and S49 lymphoma cells

Platelets (a) and S49 cells (b) were incubated with 1 nM ^{125}I -labelled cholera toxin (10^6 c.p.m./ml) at 4°C for 60 min. After one wash at 4°C , the pellets were resuspended in fresh buffer at 37°C for 10 (●) and 120 min (■). The cells were spun over oil and an extract was analysed for radioactivity as described in the Materials and methods section. The positions of the B_5 oligomer (M_r 57 000), A_1A_2 (M_r 27 000), A_1 subunit (M_r 22 000) and B_1 subunit (M_r 11 500) are indicated. The total radioactive counts on the gels reflects the toxin capacity of platelets and S49 lymphoma cells (Table 1, Darfler *et al.*, 1981). Similar results were obtained in three separate experiments. Both high-molecular-weight peaks observed with platelets appear to be oligomers of toxin B subunits, since they chromatograph as B monomers when the SDS-extract was boiled for 3 min before electrophoresis.

Moss & Vaughan, 1979; Fishman, 1982a; van Heyningen, 1982). The few exceptions to this rule include a cultured cell line, N.C.T.C. 2071, which is G_{M1} -deficient (Fishman *et al.*, 1980), human spermatozoa (Cheng & Boettcher, 1979), in which

Fig. 8. Time course of generation of cholera toxin A_1 subunit by platelets and S49 lymphoma cells

Platelets (●) and S49 cells (■) were incubated with 1 nM ^{125}I -labelled cholera toxin (10^6 c.p.m./ml) at 37°C for the times indicated. The cells were spun over oil and an extract was analysed for radioactivity. The generation of cholera toxin A_1 subunit is expressed as a percentage of the total radioactivity on the gel, which at 120 min was 20800 counts for the platelets and 1800 counts for S49 lymphoma cells. Similar results were obtained in two experiments. The value for platelets shown on the graph corresponds to $<0.02\%$.

the guanyl nucleotide regulatory component of adenylate cyclase may be defective (Stengel & Hanoune, 1981), and lastly, as we have shown here, human platelets.

Human platelets are unique among these unresponsive cells in both possessing a substantial number of cholera toxin receptors and a completely functional adenylate cyclase system (Jakobs & Schultz, 1979; Steer & Wood, 1979; Insel *et al.*, 1982). The cholera toxin receptors in platelets [as in other cells (Gill, 1977; Moss & Vaughan, 1979; Fishman & Atikan, 1980; Fishman, 1982a; van Heyningen, 1982)] appear to be composed of G_{M1} , since binding of ^{125}I -labelled cholera toxin to naive and G_{M1} -enriched platelets is identical except in the magnitude of toxin bound [G_{M1} constitutes such a small proportion of platelet membranes as to be virtually undetectable by other assay techniques (Snyder *et al.*, 1972)]. Nevertheless, binding of cholera toxin to G_{M1} appears to be necessary, but not sufficient, for subsequent activation of adenylate cyclase in intact human platelets.

Supplementation of platelets with G_{M1} , thereby raising the amount of cholera toxin bound to platelets, gives rise to some increase in cyclic AMP

in response to toxin. However, this response is minimal in comparison with that caused by other agents, such as prostacyclin, which causes a 17 ± 4 -fold increase in cyclic AMP. van Heyningen (1982) has proposed that cholera toxin may enter cells by a random process, the function of G_{M1} (cholera toxin receptor) being to increase the concentration of cholera toxin in the immediate vicinity of the cell and hence to increase the probability of toxin entry into the cell. We believe that the greatly increased cholera toxin-binding capacity of G_{M1} -supplemented platelets increases the probability of toxin entry and thereby leads to the slightly enhanced activation of adenylate cyclase activity in G_{M1} -supplemented compared with control platelets.

The events that transpire between binding of cholera toxin to the plasma membrane and activation of adenylate cyclase are unclear. Whereas some evidence indicates that lysosomal processing is obligatory (Houslay & Elliot, 1981), other work contradicts this (Gill *et al.*, 1981). Furthermore, although it is generally believed that the B subunits of cholera toxin do not enter the target cell, we have some evidence to suggest that cellular processing of cholera toxin involves cleavage of the B₅ pentamer to the B₁ monomer. Internalization of the toxin does appear to precede activation of adenylate cyclase (Fishman, 1982b), although it is unclear whether A₁-A₂, A₁ or some fragment of A₁ traverses the plasma membrane to affect the toxic action (van Heyningen, 1982). Some data addressing these possibilities are those made with phospholipid photoaffinity probes, indicating that the A₁ subunit of cholera toxin can only enter the plasma membrane after reduction of the disulphide bond that joins the A₁ and A₂ subunits (Tomasi & Montecucco, 1981) and additional findings with a glycolipid probe suggesting that only the A₁ subunit enters the plasma membrane (Wisnieski & Bramhall, 1981).

Our work with human platelets contributes new information regarding these issues. Since platelets possess both cholera toxin receptors and a functional adenylate cyclase system, the lesion that is responsible for the lack of response to cholera toxin must lie between binding of the toxin and activation of adenylate cyclase. In contrast with cholera toxin-responsive cells (Hagmann & Fishman, 1981; Figs. 7 and 8), intact platelets are unable to support a time-dependent generation of the A₁ subunit of cholera toxin. On the other hand, addition of platelet cytosol to an adenylate cyclase assay system comprising platelet membranes and the A₁-A₂ component of cholera toxin (in the absence of dithiothreitol) results in activation of adenylate cyclase (Fig. 6). This latter result indicates that platelet cytosol is capable of reducing the A₁-A₂

subunits of cholera toxin to free active A₁. Therefore, the most likely lesion in intact platelets is an inability to generate the A₁ subunit, thereby bringing this critical toxin component across the plasma membrane.

A close analogy to the platelet system appears to be the guinea-pig peritoneal macrophage, which, after treatment with cycloheximide, loses the ability to generate cyclic AMP in response to cholera toxin (Hagmann & Fishman, 1981). Such macrophages, which have decreased protein synthesis in the presence of cycloheximide, still possess toxin receptors and a functional adenylate cyclase system. Like human platelets, however, these macrophages have lost the ability to generate the A₁ cholera toxin subunit. This result led Hagmann & Fishman (1981) to propose that a protein with a short half-life may be required for generation of the A₁ subunit of cholera toxin and for toxin action.

We speculate that platelet membranes may lack a protein that is involved in translocation of critical toxin subunit(s) across the plasma membrane. Perhaps in most mammalian cells, receptor-mediated internalization brings the intact A₁-A₂ toxin fragment into a favourable reducing environment so that the A₁ subunit can be freed, thereby facilitating ADP ribosylation of the guanine nucleotide regulatory component of adenylate cyclase. If this model were true and since platelet cytosol can reduce A₁-A₂, the protein likely to be missing in platelets would be one involved in transmembrane transport (i.e., internalization) of cholera toxin. Alternatively, human platelets might lack activity of a membrane protein that is responsible for cleavage of cholera toxin within the plasma membrane. Further experiments are required to test these and other alternative hypotheses. Human platelets should prove useful in defining how mammalian cells process cholera toxin, since they are readily obtainable in a pure preparation and lack both cholera toxin response and generation of the A₁ subunit but retain toxin-mediated activation of adenylate cyclase activity in membrane preparations.

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