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Role of the A Subunit of Pertussis Toxin in Alteration of Chinese Hamster Ovary Cell Morphology

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The mechanism by which pertussis toxin induces morphological changes in Chinese hamster ovary cells was studied to determine whether the resulting clustered growth pattern is due to toxin-catalyzed ADP-ribosylation of a cellular substrate. While pertussis toxin was extremely potent in inducing morphological changes in Chinese hamster ovary cells, preparations of isolated A subunit or B oligomer exhibited greatly reduced activity. The clustered growth response of these cells correlated with ADP-ribosylation of a 41-kilodalton cellular substrate for the toxin in that the toxin concentration and time of exposure to the toxin required for ADP-ribosylation were the same as those needed for alterations in cellular morphology. Moreover, pertussis toxin modified by either chemical or photolytic methods exhibited similar decreases in the ability to ADP-ribosylate the cellular substrate and alter cell morphology. These results suggest that clustering of Chinese hamster ovary cells is due to toxin-catalyzed ADP-ribosylation of a 41-kilodalton substrate. Therefore, alteration in Chinese hamster ovary cell morphology can be used as a measure of toxin activity. This assay should prove to be a useful tool in the development and evaluation of new pertussis vaccines.

Pertussis toxin, an exotoxin produced by *Bordetella pertussis*, comprises two components, an enzymatically active A subunit and a B oligomer which is responsible for binding of the toxin to eucaryotic cell surfaces (24). The B oligomer, composed of five subunits ranging in molecular weights from 23,000 to 9,300, exhibits certain activities (25). For example, the B oligomer agglutinates erythrocytes and stimulates mitosis of lymphocytes (15, 25). The A subunit, a single polypeptide chain having a molecular weight of 28,000, catalyzes the ADP-ribosylation of a family of GTP-binding regulatory proteins found in eucaryotic cells (4, 6, 7). In the absence of a protein substrate, the A subunit will catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide (11). The ADP-ribosyltransferase activity of the toxin is believed to be responsible for a number of biological effects observed both in vivo and in vitro (15). For example, ADP-ribosylation of the GTP-binding protein termed G_i can result in interference with the ability of the cell to respond to hormones which inhibit cyclic AMP production or mobilize calcium (1, 8, 18).

Previously, it has been shown that injection of mice with inactivated pertussis toxin or with antibodies against pertussis toxin will protect against subsequent challenge with *B. pertussis* (12, 13, 16, 20). Inactivated pertussis toxin, therefore, is being used as a component of new acellular pertussis vaccines under development (21). A simple, sensitive assay which can detect residual ADP-ribosyltransferase activity is needed for proper testing of these vaccines. Such an assay also would be useful for examining sera for the presence of toxin-neutralizing antibodies. Many of the assays which are currently used to measure the ADP-ribosyltransferase activity of the toxin are cumbersome as well as expensive and therefore are not considered useful for routine assay purposes. A possible exception is an assay first described by Hewlett et al. (5) in which exposure of Chinese hamster ovary (CHO) cells to pertussis toxin was shown to result in a clustered growth pattern. This assay is simple to perform and exquisitely sensitive (10 pg of toxin can be readily detected). However, the mechanism by which the clustered growth pattern is mediated remains unknown. Previously, it has been shown that pertussis toxin will ADP-ribosylate a 41-kilodalton protein in CHO cells (10). If the CHO cell assay is to be useful for detecting small amounts of active toxin, the characteristic morphological alteration should be a reflection of ADP-ribosylation of this substrate within the CHO cells.

We studied the mechanism by which pertussis toxin induces the morphological changes of CHO cells. In this report, we describe our findings which demonstrate that the A subunit of the toxin is required for changes in the morphology of these cells and that the clustered growth pattern correlates with ADP-ribosylation of a cellular protein having a molecular weight of 41,000.

MATERIALS AND METHODS

CHO cell assay. CHO cells (ATCC CCL 61) were prepared as previously described (5). CHO cells (10^4) were added to each well of a 96-well microtiter plate containing 200 μl of medium (Ham F-12 containing 10% fetal calf serum) per well, or culture dishes (60 cm^2) containing 20 ml of medium per dish were seeded with 10^6 CHO cells. Toxin or modified toxin was added to give final concentrations which differed by approximately a factor of 3 as indicated in the figure legends. After incubation for 24 to 72 h in a CO_2 incubator at 37°C, the cells were examined under a microscope to determine the extent of clustering. The clustered morphology usually appeared 16 to 24 h after exposure of the cells to toxin. Changes in morphology of both unclustered and clustered cells did not occur after this time.

Preparation of CHO cell membranes. The medium was removed from the culture plates, and the attached cells were washed once with 5 ml of phosphate-buffered saline. A 0.8-ml volume of 25 mM Tris hydrochloride (pH 7.5) containing 0.3 M sucrose was added, and the cells were removed from the plates with a rubber policeman. After centrifugation of the cell suspension at 500 × *g* for 5 min, the packed cells

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were suspended in 20 volumes of 10 mM Tris hydrochloride (pH 7.5) containing 5 mM $MgCl_2$ and homogenized in a tissue grinder (25 strokes). The homogenate was centrifuged (500 × *g* for 5 min), and the pellet containing the membranes was frozen.

ADP-ribosylation of proteins. ADP-ribosylation of CHO cell membrane fractions (300 μg of protein) was performed by incubation of membrane fractions with pertussis toxin and [*adenylate*-^{32}P]NAD (10 to 50 Ci/mmol; New England Nuclear Corp., Boston, Mass.) as described previously (2). Samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9) followed by autoradiography.

Mitogenic action of pertussis toxin and B oligomer. Spleen cells prepared from male BALB/c mice were suspended (10^6 cells per ml) in 200 μl of RPMI 1640 culture medium containing 10% fetal calf serum, 2 mM glutamine, and 50 μg of gentamicin per ml with or without holotoxin or B oligomer and incubated for 48 h in a CO_2 incubator at 37°C. After 24 h, 0.2 μCi of [^{3}H]thymidine (18.2 Ci/mmol; New England Nuclear) per 2 × 10^5 cells was added in a volume of 25 μl, and the incorporation of radioactivity into the cells during the subsequent incubation was measured. Assays were performed in triplicate.

Hemagglutination activity. Goose erythrocytes were washed twice and suspended in phosphate-buffered saline to make a 0.7% (vol/vol) solution. Toxin was serially diluted (twofold) with phosphate-buffered saline in a multiwell plate to give various concentrations in a total volume of 50 μl. An equal volume of the erythrocyte solution was added. After 1 h at room temperature, the extent of agglutination of erythrocytes was determined by visual inspection.

NAD glycohydrolase activity. Pertussis toxin or modified toxin (2 μg) was assayed in a volume of 50 μl of 50 mM potassium phosphate buffer, pH 7.5, containing 19 μM [*carbonyl*-^{14}C]NAD (56 mCi/mmol; Amersham Corp., Arlington Heights, Ill.), 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate) (Calbiochem-Behring, La Jolla, Calif.), 1 mM dithiothreitol, 1 mg of ovalbumin per ml, and 0.1 M NaCl for 3 h at 30°C. The reaction was stopped by using the method described by Carroll et al. in which [^{14}C]nicotinamide is isolated (3). Assays were performed in triplicate.

Preparation of pertussis toxin and its subunits. Pertussis toxin was purified from culture supernatants of *B. pertussis* 114 as previously described (22). The A subunit was dissociated from the B oligomer by incubation in 10 mM sodium phosphate buffer, pH 7, containing 3 M urea, 1% CHAPS, and 100 μM ATP (buffer A) for 15 min. The solution containing 1.0 mg of protein was applied to a column (0.38 cm^2 by 2 cm) of carboxymethyl Sepharose CL-6B (Pharmacia, Uppsala, Sweden) which had been equilibrated with buffer A. The A subunit did not bind to the column and was eluted with an additional 0.8 ml of buffer A. The column was then washed with 6 volumes of buffer A. The B oligomer was eluted from the column with 1.0 ml of 0.2 M potassium phosphate buffer, pH 7.5, containing 2 M urea.

The extent of contamination of the A subunit preparation with B oligomer was measured by testing for hemagglutination activity. The A subunit preparation exhibited hemagglutination activity at a concentration of 10 μg/ml, whereas the B oligomer preparation agglutinated erythrocytes at a concentration of 0.044 μg/ml, suggesting that the A subunit preparation contained 0.44% B oligomer by weight. The extent of contamination of the B oligomer preparation with the A subunit was determined by measuring ADP-ribosyltransferase activity. A 3-μg amount of the B oligomer exhibited ADP-ribosyltransferase activity equivalent to that exhibited by 50 ng of the holotoxin (of which 12 ng is A subunit). Thus, the B oligomer preparation contains approximately 0.4% A subunit by weight.

TABLE 1. Induction of CHO cell clustering by pertussis toxin and isolated components

Addition	Concn[a] (ng/ml)
Pertussis toxin	0.1
A subunit	30.0
B oligomer	10.0
A subunit + B oligomer[b]	0.1

[a] Minimum concentration required for maximal cluster response.
[b] Toxin components were mixed in equimolar amounts.

Modification of pertussis toxin with glutaraldehyde. A 1% solution of glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) was added to pertussis toxin (65 μg) in 50 mM potasssium phosphate buffer, pH 7.5, containing 0.3 M urea to give a final glutaraldehyde concentration of 0.04% and a final volume of 750 μl. After incubation at room temperature for 2 h, the reaction was stopped by the addition of lysine to give a final concentration of 19 mM.

Modification of pertussis toxin with *N*-ethylmaleimide. Pertussis toxin (0.33 mg/ml) was incubated in 50 mM potassium phosphate, pH 7.5, containing 2 mM dithiothreitol, 3 M urea, 0.1 mM ATP, and 1% CHAPS for 3 h at room temperature to dissociate the A subunit from the B oligomer. A solution of *N*-ethylmaleimide (Sigma Chemical Co., St. Louis, Mo.) in H_2O was then added so that the final concentration of *N*-ethylmaleimide was 6 mM (a total of 1.5 μmol), and the protein concentration was 0.25 mg/ml. After incubation at room temperature for 1 h, the reaction was stopped by the addition of 1.5 μmol of dithiothreitol. Preliminary experiments indicated that after modification with *N*-ethylmaleimide, the A subunit did not readily reassociate with the B oligomer.

Photolysis of pertussis toxin. Pertussis toxin (0.2 mg/ml) in 50 mM sodium phosphate, pH 7.0, containing 1% CHAPS was placed in 75-μl droplets on strips of Parafilm and irradiated for 10 min at a distance of 5 cm with an 8-W germicidal lamp (G8-TS; Sylvania, Danvers, Mass.).

RESULTS

Effect of pertussis toxin components on clustering of CHO cells. A comparison of the effects of pertussis holotoxin, isolated A and B subunits, and reconstituted toxin on CHO cell morphology is shown in Table 1. Pertussis toxin induced a maximal clustering of CHO cells at concentrations as low as 0.1 ng/ml. In contrast, the isolated A subunit and B oligomer were much less effective. These components altered CHO cell morphology at 30 or 10 ng/ml, respectively. If, however, the A subunit was added to the B oligomer in equimolar quantities, the ability of the mixture to induce morphological changes was regained, with clustering of CHO cells occurring at 0.1 ng of protein per ml.

The B oligomer preparation used in these studies possesses the biological activities normally associated with the binding component of the toxin. The B oligomer was as effective on a molar basis as the holotoxin in agglutinating goose erythrocytes. The minimum concentrations of pertussis toxin and B oligomer required for hemagglutination were 0.67 and 0.49 pmol/ml, respectively. In addition, the isolated

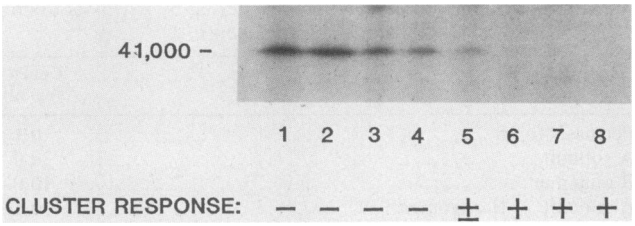

FIG. 1. Effect of pertussis toxin concentration on ADP-ribosylation of 41-kilodalton protein and induction of morphological response of CHO cells. CHO cells were incubated without (lane 1) or with pertussis toxin at the following concentrations (nanograms per milliliter): 0.003 (lane 2), 0.01 (lane 3), 0.03 (lane 4), 0.1 (lane 5), 0.3 (lane 6), 1.0 (lane 7), and 3.0 (lane 8). After 48 h, cells were examined to determine whether clustering had occurred. Cells were then harvested, and the membrane fraction was prepared. Samples of this fraction (300 μg) were incubated with [^{32}P]NAD and pertussis toxin and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. An autoradiogram of a portion of the gel is shown. Symbols: −, no clustering; ±, some cells unaffected and others clustered; +, maximal clustering of cells.

B oligomer exhibited the ability to stimulate lymphocyte mitosis, although it was only 40% as effective as the holotoxin on a molar basis. The concentrations required for maximal mitogenic activity of pertussis toxin and B oligomer were 21 and 56 pmol/ml, respectively. In the absence of mitogen, [^{3}H]thymidine was incorporated to the extent of 250 cpm/2 × 10^5 cells. Incorporation in the presence of pertussis toxin and B oligomer was 9,000 and 10,700 cpm/2 × 10^5 cells, respectively.

CHO cell clustering occurs in parallel with ADP-ribosylation of a 41-kilodalton protein. The altered morphology of CHO cells was evident after 24 h of exposure of cells to the toxin. After that time, the extent of ADP-ribosylation of a 41-kilodalton toxin substrate could be measured by harvesting those CHO cells, preparing a membrane fraction, and incubating this fraction with [^{32}P]NAD and pertussis toxin. Any substrate which had not been ADP-ribosylated by the toxin within the cell is free to be modified in vitro and thus becomes radiolabeled. In contrast, any protein ADP-ribosylated within the CHO cell can no longer serve as a toxin substrate and will not incorporate radiolabel.

Incubation of CHO cells with ≥0.3-ng/ml concentrations of toxin resulted in greater than 90% in vivo ADP-ribosylation of the 41-kilodalton substrate as indicated by densitometric scans of the autoradiogram (Fig. 1). The addition of less toxin resulted in incomplete ADP-ribosylation of this protein within the cell. The minimum concentration of toxin needed in this experiment for maximal clustering of these cells was determined to be 0.3 ng/ml (Fig. 1), the same concentration as that needed for ADP-ribosylation of the cellular substrate. It should be noted that the concentration of toxin required for maximal clustering of cells (0.3 ng/ml) in this experiment differed by 1 dilution from that observed at other times (Table 1). This amount of variation from experiment to experiment was typical. For this reason, when the extent of clustering of CHO cells was to be directly compared with the extent of ADP-ribosylation of the cellular substrate, cells were first incubated with toxin, and after about 48 h alterations in morphology were recorded. The same cells were then immediately harvested, and the extent of ADP-ribosylation which had occurred during incubation with the toxin was measured.

The minimum time required for exposure of CHO cells to pertussis toxin in order for the cells to exhibit the clustered morphology was examined, and the results are shown in Fig. 2. A 20-min exposure to pertussis toxin was sufficient for alterations in morphology to occur. The same exposure time was required for eventual complete ADP-ribosylation of the 41-kilodalton substrate (Fig. 2).

Chemical and photolytic modifications of pertussis toxin. Various modifications of the toxin will result in perturbation of its enzymatic activity, reflecting alterations in the A subunit, and of its hemagglutination activity, indicating changes in the B oligomer. The NAD glycohydrolase activity of toxin was significantly decreased when the protein was modified with glutaraldehyde or *N*-ethylmaleimide (Table 2). The hemagglutination activity of toxin modified by exposure to glutaraldehyde or UV light was decreased. Thus, the modified toxins were altered in the A subunit, B oligomer, or both. It should be noted that while alterations in NAD glycohydrolase activity or hemagglutination activity reflect certain changes in the toxin components, other changes such as association state of the subunits or the ability of the A subunit to enter the eucaryotic cell may not be detected by these assays.

The modified toxins were tested for their ability to ADP-ribosylate the 41-kilodalton CHO cell protein as well as to promote the clustered response of these cells. All of the modified toxins exhibited a decreased ability to both ADP-ribosylate and induce clustering of CHO cells compared with the native toxin (Table 2). While the potencies of these modified toxins were reduced, ADP-ribosylation of the 41-kilodalton substrate always correlated well with the clustering of CHO cells.

DISCUSSION

Previous studies indicated that pertussis toxin-induced morphological changes of CHO cells were not due to alterations in adenylate cyclase since clustering occurred in the absence of hormones, and basal cyclic AMP levels were unaffected by the toxin (5). Moreover, the morphological change induced by pertussis toxin was strikingly different from that caused by cholera toxin, which alters cell morphology by increasing cyclic AMP levels (5). Recently, activities of pertussis toxin not involving adenylate cyclase have been identified (14, 17, 23, 26). Thus, in spite of the absence of changes in cyclic AMP levels, toxin-catalyzed ADP-ribosylation could result in altered CHO cell morphology.

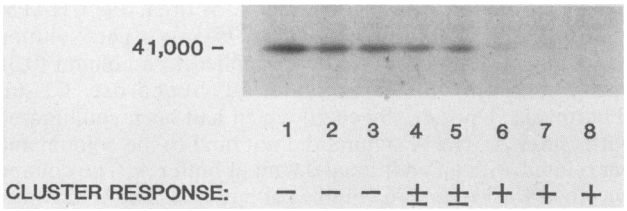

FIG. 2. Effect of time of incubation of CHO cells with pertussis toxin on ADP-ribosylation of 41-kilodalton substrate and induction of clustering response. Cells were incubated without (lane 1) or with pertussis toxin (1 ng/ml) for 30 s (lane 2), 1 min (lane 3), 3 min (lane 4), 10 min (lane 5), 20 min (lane 6), 60 min (lane 7), or 42 h (lane 8). At the indicated times medium containing toxin was removed, cells were washed twice, and new medium was added. After incubation of the cells for a total of 42 h, cells were examined to determine the extent of clustering. Cells were then harvested, and membranes were prepared and ADP-ribosylated as described in the text. An autoradiogram of a portion of the resulting sodium dodecyl sulfate-polyacrylamide gel is shown. Symbols are as in the legend to Fig. 1.

TABLE 2. Activities of pertussis toxin and modified toxins

Addition	% Activity of unmodified toxin			
	NAD glycohydrolase	Hemagglutination	CHO cell clustering	ADP-ribosylation of 41-kilodalton protein
Pertussis toxin	100[a]	100[b]	100[c, d]	100[d, e]
Glutaraldehyde-treated toxin	14	3	0.3	0.3
UV-treated toxin	84	13	0.8	0.8
N-Ethylmaleimide-treated toxin	<5	100	0.05	0.05

[a] 100% = 304 pmol of NAD hydrolyzed per min per mg.
[b] 100% = 0.078-μg/ml minimum protein concentration required for hemagglutination.
[c] 100% = 0.3-ng/ml minimum protein concentration required for positive CHO cell response.
[d] CHO cells were incubated with toxin or modified toxins (each added to give various concentrations differing by approximately a factor of 3) as described in the text. After the extent of clustering was determined, the extent of ADP-ribosylation of the 41-kilodalton substrate within those cells was measured.
[e] 100% = 0.3-ng/ml minimum protein concentration required for complete ADP-ribosylation of the 41-kilodalton substrate.

Pertussis toxin catalyzes the ADP-ribosylation of a 41-kilodalton protein of CHO cells (10). If the clustering phenomenon is due to ADP-ribosylation of this protein, neither the isolated A nor the isolated B component of the toxin should induce the altered morphology since the enzymatically active A subunit does not enter the cell and ADP-ribosylate its substrate in the absence of the B oligomer (24). Sato and co-workers (19) have shown that the A subunit is ineffective in clustering CHO cells. Moreover, their studies demonstrated that isolated subunits of the B oligomer, including the S_5 subunit as well as a mixture of the S_2, S_3, and S_4 subunits, did not induce an altered cell morphology. In those studies, no information was obtained concerning the ability of the intact B oligomer to cluster CHO cells. Results from our study indicate that while the toxin was shown to be effective in inducing clustering of CHO cells, neither the A nor the B component seems to have a substantial effect on CHO cell morphology. The small amount of clustering activity exhibited by the subunit preparations is likely due to contamination of each of the isolated components with minute quantities of the other, probably resulting in the formation of a small amount of holotoxin. The extent of contamination of the subunit preparations which was measured (see Materials and Methods) is sufficient to account for the observed clustering activity of the A and B preparations at concentrations of 30 and 10 ng/ml, respectively. Thus, clustering activity does not appear to be intrinsic to the B oligomer since this component has little effect on CHO cell morphology, yet other biological activities attributed to the B oligomer, such as hemagglutination activity and mitogenicity, are readily manifested in the absence of the A subunit. These data are therefore consistent with the hypothesis that clustering of CHO cells is caused by ADP-ribosylation of a 41-kilodalton protein.

Clustering of CHO cells parallels ADP-ribosylation of the 41-kilodalton substrate. The concentration of pertussis toxin needed for induction of altered cell morphology was found to be identical to that required for complete ADP-ribosylation of the 41-kilodalton substrate (Fig. 1). This concentration of toxin was 2 to 4 orders of magnitude lower than concentrations of the toxin required for mitogenesis or hemagglutination (25, 27), activities attributed to the action of the B oligomer and not ADP-ribosylation.

Exposure of CHO cells to the toxin for periods as brief as 20 min resulted in the ADP-ribosylation of the 41-kilodalton protein as well as alterations in cell morphology. Shorter incubation periods were inadequate for both ADP-ribosylation and CHO cell clustering. The 20-min period of exposure to the toxin required for these effects may reflect the length of time needed for binding of the toxin to the cell surface. Subsequent events leading to the ADP-ribosylation of the 41-kilodalton substrate and CHO cell clustering would then occur during the next 16 to 24 h.

The studies reported here demonstrate that chemical and photolytic modifications of pertussis toxin can alter one or more of the components of the molecule, resulting in decreased ability of the toxin to ADP-ribosylate cellular substrates or induce clustering of CHO cells. If CHO cell clustering is due to ADP-ribosylation of a cellular component, the clustering effect of the toxin should decrease in parallel with its ability to ADP-ribosylate cellular substrates irrespective of the method used for modification of toxin. When pertussis toxin was modified by reaction with glutaraldehyde or *N*-ethylmaleimide or by exposure to UV light, changes in the A subunit, B oligomer, or both were detected. While each type of modification greatly reduced the ability of the toxin to induce clustering of CHO cells, ADP-ribosylation of the 41-kilodalton substrate always correlated with the ability of the modified toxin to alter CHO cell morphology.

These results indicate that CHO cell clustering appears to be due to ADP-ribosylation of a 41-kilodalton protein. Events occurring after ADP-ribosylation of the protein which may lead to altered cell morphology remain to be elucidated. Nonetheless, these data indicate that the CHO cell clustering assay can be used for detection of active toxin and should prove to be a valuable tool for use in the development of new pertussis vaccines.

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