Journal of Supramolecular Structure 12:273–291 (1979)
Tumor Cell Surfaces and Malignancy 605–623

Isolation of Cholera Toxin Receptors From a Mouse Fibroblast and Lymphoid Cell Line by Immune Precipitation

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Cholera toxin receptors have been isolated from both a mouse fibroblast (Balbc/3T3) and mouse lymphoid cell line labeled by the galactose oxidase borotritiide technique. Tritiated receptor-toxin complexes solubilized in NP40 were isolated by addition of toxin antibody followed by a protein A-containing strain of Staphylococcus aureus. In both cell types by far the major species of toxin receptor isolated was ganglioside in nature, although galactoproteins were also present in the immune complexes. Whether the galactoproteins form part of a toxin-receptor complex or are artifacts of the isolation procedure is presently unclear.

The relative specificity of cholera toxin for a carbohydrate sequence in a glycolipid suggests that the toxin might prove a useful tool in establishing the function and organization of glycolipids in membranes. For example, interaction of cholera toxin with the mouse lymphoid cell line was shown to result in patching and capping of bound toxin, raising the possibility that the glycolipid receptor interacts indirectly with cytoskeletal elements. Cholera toxin might also be used to select for mutant fibroblasts lacking the toxin receptor and therefore having an altered glycolipid profile. Such mutants might prove useful in establishing the relationship (if any) between modified glycolipid pattern and other aspects of the transformed phenotype. Attempts to isolate mutants, based on the expectation that growth of cells containing the toxin receptor would be inhibited by the increase in cAMP levels normally induced by cholera toxin, proved unsuccessful. Cholera toxin failed to inhibit significantly the growth of either Balbc or Swiss 3T3 mouse fibroblasts although it markedly elevated cAMP levels.

Key words: cholera toxin – receptors, cell growth, glycolipids – transformation, organization in membranes, glycolipids as cell surface receptors

Abbreviations. Ganglioside structure and nomenclature: GM_3, Cer-Glc-Gal-AcNeu; GM_2, Cer-Glc-Gal(AcNeu)-GalNac; GM_1, Cer-Glc-Gal(AcNeu)-GalNac-Gal; GD_{1a}, Cer-Glc-Gal(AcNeu)-GalNac-Gal(AcNeu); GD_{1b}, Cer-Glc-Gal(AcNeu)$_2$-GalNac-Gal; GT, Cer-Glc-Gal(AcNeu)$_2$-GalNac-Gal(AcNeu). Cer, ceramide; Glc, glucose; Gal, galactose; GalNac, N-acetylgalactosamine; AcNeu, N-acetylneuraminic acid. Glycolipids referred to in this paper are glycosphingolipids. PBS, phosphate buffered saline; PMSF, phenylmethylsulphonylfluoride; SDS, sodium dodecylsulphate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; $PGF_{2\alpha}$, Prostaglandin $F_{2\alpha}$.

Received April 23, 1979; accepted August 29, 1979.

0091-7419/79/1202-0273$03.50

One of the many changes associated with the transformed phenotype is loss of the ability to synthesize the more complex cell surface glycolipids. While the observation is well documented and there are few if any exceptions, the significance of the change has remained obscure (for a review, see Critchley and Vicker [1]). Recent interest has centered around the idea that glycolipids act as cell surface receptors. For example, glycolipids have been implicated as important determinants of cellular interaction [1–5] and as receptors for the glycoprotein hormones and certain bacterial toxins (for reviews see Refs. 1 and 6–8). That glycolipids act as receptors for the glycoprotein hormones appears somewhat surprising in that glycoprotein receptors for thyrotropin and human chorionic gonadotropin have previously been isolated [9, 10]. However, the primary determinant of receptor activity is probably a specific carbohydrate sequence, which may therefore appear on both glycolipids and glycoproteins. Perhaps the best evidence relating to a possible receptor role for glycolipids stems from studies on the interaction of cholera toxin with target cells: a) Ganglioside GM_1 is the best inhibitor of toxin binding to cells [11]; b) cells lacking GM_1 are unresponsive to toxin, and such cells become toxin-responsive following insertion of exogenous GM_1 into their membranes [12, 13]; c) GM_1 but not other glycolipids causes a change in the fluorescence spectrum of the toxin [14, 15]; d) saturating levels of toxin protect the terminal galactose residue of GM_1 from galactose oxidase [14, 16]. However, while the evidence is substantial it is largely indirect, and in light of the apparent dual nature of the glycoprotein hormone receptors, it remains important to establish by a more direct approach the nature of the cholera toxin receptor.

If it were shown that cholera toxin is specific for a carbohydrate sequence in glycolipid, the toxin might prove a useful tool to probe the function and organization of glycolipids in membranes. For example, interaction of cholera toxin with lymphocytes is known to result in capping of the bound toxin (and presumably the toxin receptor) in a manner which is inhibited by azide, colchicine, and cytochalasin [17–19]. If one assumes the glycolipid nature of the toxin receptor, then one of a number of possible explanations for the phenomenon is that the glycolipid receptor is associated with a transmembrane protein which in turn interacts with the cytoskeletal system. In addition the toxin could be used to derive mutant cell lines lacking the toxin receptor and therefore defective in their ability to synthesize complex glycolipids. Such cells would then be monitored for the expression of any parameters of the transformed phenotype which might arise as a consequence of loss of complex glycolipids. This approach would offer a useful alternative to the so-called "add-back" experiments where one attempts to look for reversion of transformed cell characteristics following incorporation of glycolipids into the transformed cell membrane [20, 21]. While such an approach has been extremely important in establishing the significance of loss of a high-molecular-weight cell surface protein (fibronectin) on transformation [22, 23], it has not been particularly helpful in the case of glycolipids.

In this paper we report experiments in which we have isolated the cholera toxin receptor from mouse lymphoid and fibroblast cell lines by immune precipitation. In both cases the *major* species of toxin receptor would appear to be glycolipid in nature.

MATERIALS AND METHODS

Cell Culture

The mouse lymphoid cell line (AT5) was obtained from Dr. M. Boss of the Imperial Cancer Research Fund Laboratories (ICRF), London. It was derived from a tumor formed in an inguinal lymph node following injection of Abelson virus into a Balbc mouse. The

cell line was grown in suspension in Dulbecco's modified Eagle's medium containing 10% heat-inactivated calf serum and maintained at densities below 1×10^6/ml. Balbc/3T3 cells (clone A31) were also obtained from ICRF.

Scheme for Isolation of the Cholera Toxin Receptor

Terminal galactose residues are an important determinant of cholera toxin receptor activity [11]. We therefore initially chose to label surface components by the galactose oxidase borotritiide technique (see Fig. 1). Labeled cells were then exposed to an excess of cholera toxin; the mouse lymphoid and fibroblast cell lines used in the study bound approximately 1×10^6 molecules of toxin per cell, which is equivalent to 0.16 μg of toxin bound per 10^6 cells. The amount of toxin added to labeled cells was therefore based on this figure. The apparent dissociation constant for cholera toxin is of the order of 10^{-10} M [24] and although some toxin can be removed by washing, about 50% remains apparently irreversibly bound. Toxin-receptor complexes were then solubilized in 1% NP40 and incubated with toxin antibody, and the immune complexes were adsorbed to a protein A-containing strain of Staphylococcus aureus [25] (Fig. 2). Pilot experiments with a [^{3}H]GM_1

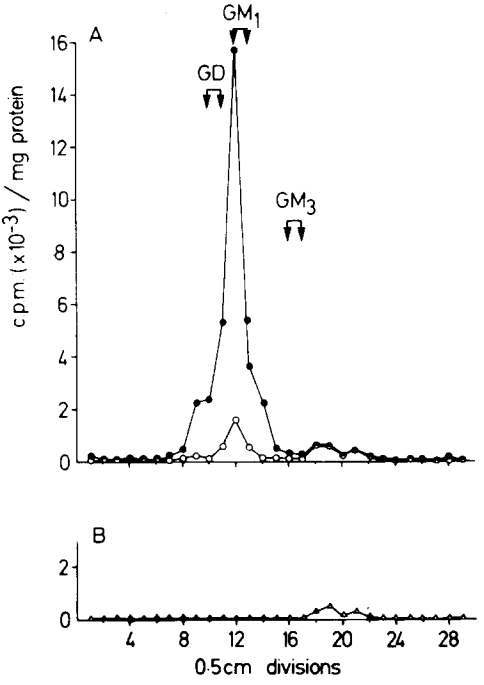


Fig. 1. Surface labeling of the gangliosides of a mouse lymphoid cell line by the galactose oxidase borotritiide technique. Cells (5×10^7) were incubated with galactose oxidase (2 units/ml) with and without neuraminidase (25 units/ml) in 5 ml of Hanks' balanced salt solution (HBSS) at 37°C for 3 h in a 5% CO_2 atmosphere. Cell viability was not significantly impaired under these conditions as monitored by dye exclusion. Following two cycles of washing in HBSS, cells were incubated with 1 mCi of NaB^3H_4 in 1 ml of PBS, pH 7.8 (stock 10 mCi/ml in 0.01 M NaOH stored at -70°C) for 15 min at 20°C. Cells were washed three times with PBS/1 mM PMSF, pH 7.4, and gangliosides were extracted and separated by thin-layer chromatography on silica gel G precoated plates using the solvent chloroform:methanol: water (60:35:8). Following visualization of glycolipid standards with iodine vapor, the plate was marked in 0.5-cm divisions and the silica gel was transferred to vials for scintillation counting. A: Galactose oxidase alone (o——o); with neuraminidase (•——•). B: No enzyme.

standard, cholera toxin, and toxin antibody showed that 1% NP40 did not reduce the recovery of GM_1 in the bacterial pellets. This suggests that NP40 does not inhibit binding of GM_1 to toxin or toxin antibody to toxin. In similar experiments, 1% deoxycholate reduced the recovery of GM_1 by about 50%. Bacterial pellets were then extracted for analysis of adsorbed lipid or protein. Lipids were extracted and partitioned against water [26] to give organic-phase lipids (primarily cholesterol, phospholipids, and neutral glycolipids) and aqueous-phase lipids (primarily gangliosides). The aqueous-phase material was saponified with 200 μl of 0.1 M NaOH in methanol for 4 h at 20°C prior to desalting and separation of the gangliosides by thin-layer chromatography. Organic-phase lipids were separated by two-dimensional thin-layer chromatography [27]. Glycoproteins were extracted by boiling with 2% SDS in electrophoresis sample buffer and the sample was reduced with dithiothreitol prior to SDS-PAGE [28].

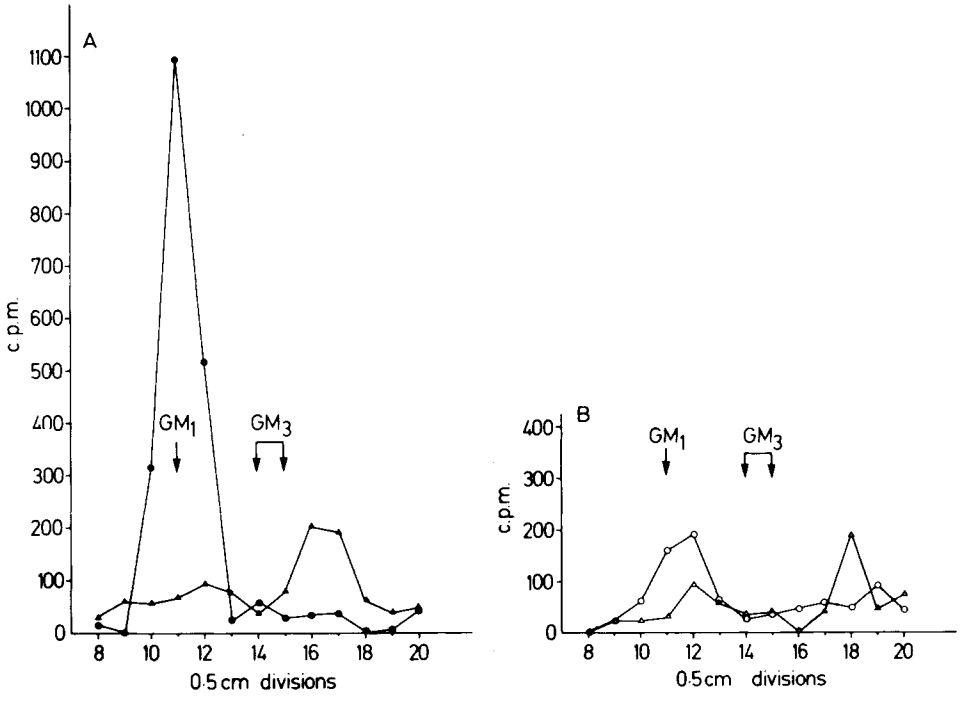


Fig. 2. Isolation of the cell surface ganglioside receptors for cholera toxin from galactose oxidase borotritiide-labeled cells. Galactose oxidase borotritiide-labeled cells (2×10^8) of the mouse lymphoid cell line were incubated with and without 50 μg of cholera toxin in 1 ml of PBS/0.05% bovine serum albumin/1 mM PMSF for 30 min at 20°C. The cells were washed twice with PBS/PMSF, extracted with 8 ml of 1% NP40 in 0.15 M NaCl, 5mM EDTA, 50 mM Tris (NET) buffer, pH 8/PMSF (0°C, 1 h), and centrifuged at 100,000g for 1 h. Aliquots (1 ml) of the supernatant were incubated with 40 μl of toxin antibody or preimmune serum for 1 h at 4°C, 400 μl of a 10% suspension of a protein A-containing strain of Staphylococcus aureus (in 0.5% NP40 NET, pH 8, 0.25% gelatin, or bovine serum albumin) was added [25], and incubation continued for a further 30 min. The bacterial pellet (1,000g, 10 min, 4°C) was washed three times with the same buffer, and gangliosides were extracted and separated as described in Figure 1. A: Plus toxin; ●——●, plus toxin antibody; ▲——▲, plus preimmune serum. B: Minus toxin; ○——○, plus toxin antibody; △——△, plus preimmune serum.

Antibodies to Cholera Toxin

Rabbit antibodies were prepared by injection of 30 μg of cholera toxin in 1 ml of complete Freund's adjuvant into two subcutaneous and four intramuscular sites. The procedure was repeated 3 weeks later and after a further 10 days the rabbit was bled. Preimmune serum was obtained from the same animal.

Materials

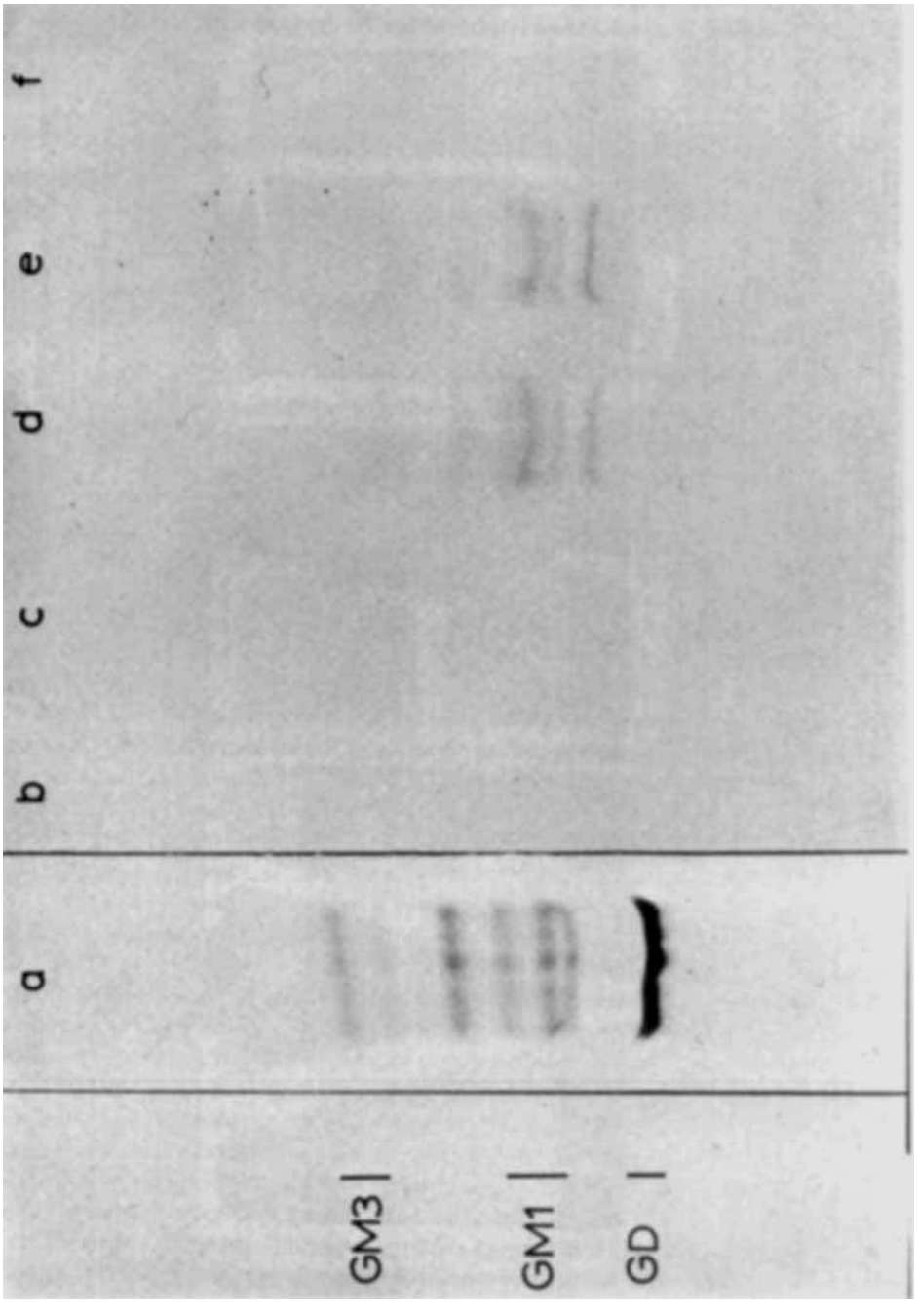
NaB^3H_4 (7 Ci/mmole), [1-^{14}C] palmitate (50 mCi/mmole), and carrier-free $^{125}I^-$ were obtained from the Radiochemical Centre. Cholera toxin was purchased from Schwarz Mann, galactose oxidase from Worthington Biochemicals, neuraminidase from Behringwerke, lactoperoxidase from Calbiochem, and ganglioside standards from Supelco.

RESULTS

Isolation of the Cholera Toxin Receptor From a Mouse Lymphoid Cell Line

Galactose oxidase borotritiide labeling profiles of cell surface gangliosides of a mouse lymphoid cell line are shown in Figure 1a. The main peak of radioactivity had a similar mobility on thin-layer chromatograms to a GM_1 standard. Other gangliosides known to be synthesized by these cells from metabolic labeling studies (Fig. 3a) were poorly labeled, probably because they lacked terminal galactose or N-acetylgalactosamine residues. The extent of the labeling could be increased approximately tenfold by preincubation of the cells with neuraminidase, which presumably converted some di- and trisialogangliosides to GM_1. There was no labeling of gangliosides in the absence of galactose oxidase (Fig. 1b). Cells labeled by this procedure were exposed to cholera toxin and lysed in 1% NP40, and a soluble fraction was obtained by centrifugation at 100,000g for 1 h. Aliquots of the supernatant were incubated with toxin-antibody and the toxin-receptor-antibody (immune) complexes adsorbed to a protein A-containing strain of Staphylococcus aureus. Separation of labeled gangliosides which bound to the bacterial pellets by thin-layer chromatography showed a peak of radioactivity with similar mobility to GM_1 (Fig. 2a). Elution of the peak followed by rechromatography in a different solvent (chloroform–methanol–2.5 M ammonium hydroxide containing 20 mg/100ml $CaCl_2 \cdot 2H_2O$, 60:35:8) again showed a single major peak of radioactivity coincident with a GM_1 standard. The ganglioside was not found adsorbed to the bacterial pellet if cholera toxin or toxin antibody was omitted during the isolation procedure (compare Fig. 2a and b).

Because galactose oxidase borotritiide labeling of cellular lipid is restricted mainly to a component(s) with similar chromatographic properties to GM_1, these experiments do not resolve the issue of whether cholera toxin is specific for GM_1. We have attempted to answer the question by applying the method of immunoprecipitation described to cells metabolically labeled with [^{14}C] palmitate. Separation of the gangliosides from cells labeled in this manner shows a complex profile including molecules with mobilities similar to gangliosides GM_3, GM_2, GM_1, and GD (Fig. 3a). No one ganglioside species predominates. Analysis of [^{14}C] palmitate-labeled gangliosides in the immune complexes adsorbed to protein A showed a considerably less complex pattern, suggesting that the toxin was interacting specifically with certain cell surface gangliosides (Fig. 3d, e). However, although gangliosides with mobilities similar to GM_1 predominated, the profile suggested considerable heterogeneity. Again virtually no ganglioside was found adsorbed to the protein A if cholera toxin or toxin antibody was omitted during the isolation procedure (Fig. 3b, c, f).



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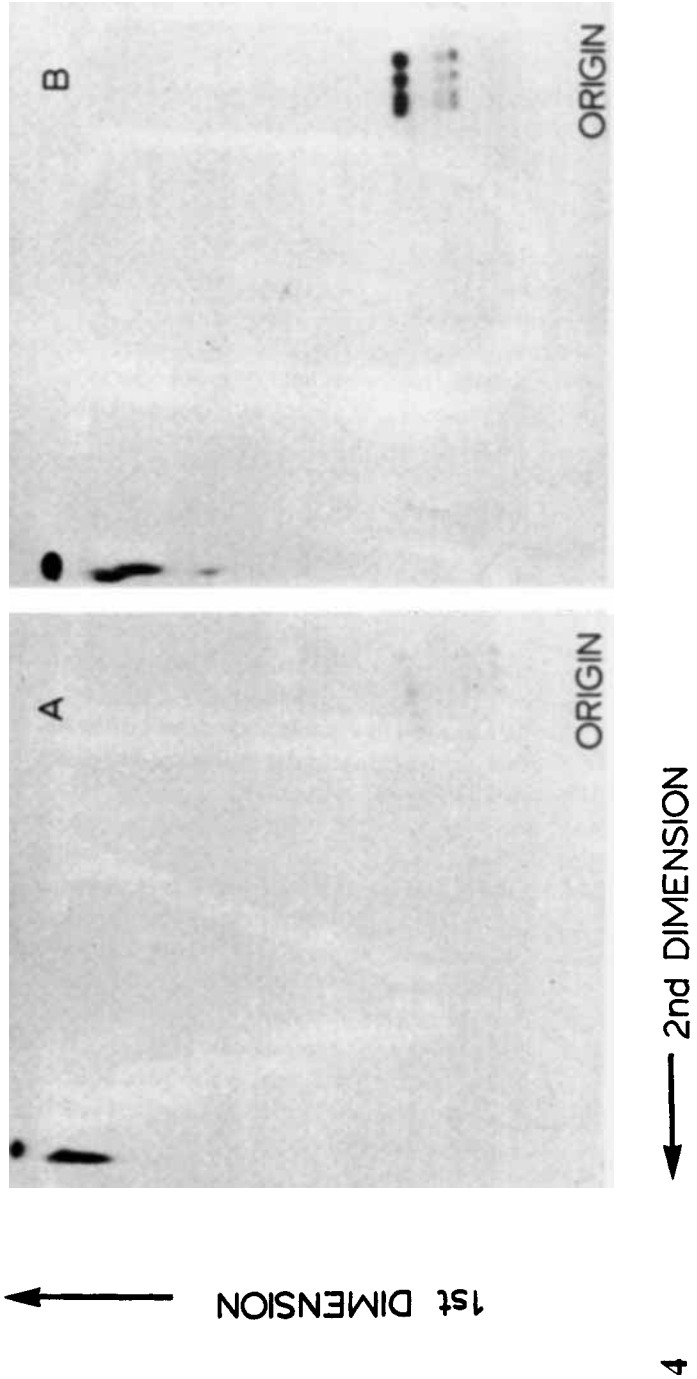
Such experiments strongly suggest that gangliosides, predominantly those with the mobility of GM_1, are at least part of the cholera toxin receptor at the surface of this lymphoid cell line.

During the course of experiments with [^{14}C] palmitate-labeled cells it became clear that other lipids apart from gangliosides were adsorbed to the protein A. Two-dimensional thin-layer chromatography showed that neutral lipids, phosphatidyl choline, and sphingomyelin were the major components (Fig. 4). These lipids were bound to protein A in the absence of cholera toxin or toxin antibody, although levels generally increased when toxin and antibody were included in the isolation procedure. The extent of the increase was variable, however (by a factor of 1.0–4).

A similar experimental approach was used to examine whether any cell surface glycoproteins bind cholera toxin. Galactose oxidase borotritiide labeling profiles of the cell surface glycoproteins separated by SDS-PAGE are shown in Figure 5. As for labeling of gangliosides, labeling of glycoproteins was markedly enhanced by neuraminidase (Fig. 5a, b). Labeling in the absence of enzyme was largely restricted to molecules traveling with the marker dye, much of which probably represents lipid (Fig. 5c). The increased labeling in this region in neuraminidase-treated cells is in accord with increased labeling of gangliosides similar to GM_1 (Fig. 1a). Similar analysis of the toxin-receptor-antibody complexes adsorbed to protein A showed that the major species of toxin receptor traveled with the marker dye and is therefore most likely lipid (Fig. 6). The only lipids found in the immune complexes which are specifically labeled by the galactose oxidase borotritiide technique are gangliosides similar to GM_1. This result therefore suggests that such glycolipids are quantitatively the most important species of toxin receptor. Interestingly, a peak of radioactivity corresponding to a glycoprotein in the molecular weight range 80,000–90,000 has also consistently been found in the immune complexes. The glycoprotein was not adsorbed to protein A if cholera toxin or antibody was omitted during the isolation procedure. Figure 7 shows a more complete analysis of a similar experiment using slab gels and fluorography. Analysis of the immune complexes again shows that quantitatively the major species of toxin receptor travels with the marker dye, and is therefore presumably lipid. Comparison of immune complexes isolated from cells exposed to galactose oxidase with or without neuraminidase shows that recovery of this species is dramatically increased in cells exposed to neuraminidase (Fig. 7f, g). The observation is in agreement with the marked increase in labeling of molecules with similar chromatographic properties to GM_1 following exposure of cells to neuraminidase, and again points to gangliosides as the main species of toxin receptor. As before, a glycoprotein of approximately 80,000–90,000 MW was also found as a minor component of the labeled material present in the immune complexes. This is most clearly seen in cells exposed to neuraminidase (Fig. 7f), although a glycoprotein with a slightly greater apparent molecular weight is also present in the immune complexes isolated from cells treated with galactose oxidase alone (not visible in Fig. 7). This glycoprotein corresponds to one of the major galactose oxidase borotritiide-labeled glycoproteins on this lymphoid cell line. It is indeed the major galactoprotein present in the

Fig. 3. Isolation of cell surface ganglioside receptors for cholera toxin from [^{14}C] palmitate-labeled cells. The lipids of the mouse lymphoid cell line were labeled by growing the cells in medium containing 1 μCi/ml [^{14}C] palmitate [27]. The cells were then exposed to cholera toxin and the toxin receptor complexes isolated as described in Figure 2. a, labeling profile of total cell gangliosides; b,c, cells not incubated with cholera toxin; b, plus toxin antibody; c, plus preimmune serum; d–f, cells incubated with toxin; d,e, plus toxin antibody; f, plus preimmune serum.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NP40 supernatant, although it is very much less well labeled by lactoperoxidase catalyzed iodination (Fig. 7i), suggesting that it is not quantitatively a major cell surface protein. Analysis of immune complexes isolated from iodinated cells has not as yet revealed the presence of any other cell surface glycoproteins.

Interaction of low concentrations of cholera toxin with the lymphoid cell line leads to patching and some capping of the toxin (Fig. 8a). Such redistribution was not observed if high concentrations of toxin were used (Fig. 8b), a finding that agrees with previous work [17].

Isolation of Cholera Toxin Receptor From Balbc/3T3 Cells

Using methods similar to those described in this paper, we have previously shown that a ganglioside with mobility similar to GM_1 is also at least part of the cholera toxin receptor on mouse fibroblasts [8]. We have recently separated the components present in immune complexes prepared from neuraminidase galactose oxidase-labeled fibroblasts by SDS-PAGE. The major labeled component traveled with the marker dye coincident with a [^{3}H] GM_1 standard, as expected (Fig. 9). Labeling of this component was dependent on galactose oxidase, and the major glycolipid in mouse fibroblasts with terminal galactose has a similar mobility to GM_1 [8]. There is thus little doubt that quantitatively the most important species of toxin receptor on mouse fibroblasts is also a ganglioside. However, as was the case with the lymphoid cell line, a glycoprotein was also present in the immune complexes obtained from mouse fibroblasts. The glycoprotein was not adsorbed to protein A in the absence of antibody.

Examination of One Possible Method of Selection of Mutant Balbc/3T3 Cells Lacking Cholera Toxin Receptors

Our attempts at using cholera toxin to select for mutants of Balbc or Swiss 3T3 cells defective in complex glycolipid biosynthesis have progressed more slowly than expected, owing to unforeseen difficulties with the mutant selection procedure. Cholera toxin has been shown to inhibit mitogen-stimulated DNA synthesis in lymphocytes [17, 32], and serum-stimulated DNA synthesis in a mouse kidney epithelial cell line AL/N [33] and human fibroblasts [34], in accordance with the idea that cAMP is involved in regulation of cell division [35, 36]. One mutant selection procedure tested was therefore based on the assumption that only Balbc/3T3 cells defective in toxin receptor, and therefore complex glycolipid biosynthesis, would be able to grow in the presence of cholera toxin. We were surprised to find that cholera toxin (1 pg–10 μg/ml) failed to markedly inhibit serum-stimulated uridine uptake (data not shown) or DNA synthesis and cell growth in Balbc or Swiss 3T3 cells, two processes previously reported to be inhibited by cAMP [35–37] (Fig. 10). This result may be explained by the observation that cAMP levels elevated (8-fold to

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Fig. 4. Characterization of neutral lipids and phospholipids bound to protein A from NP40 extracts of [^{14}C] palmitate-labeled mouse lymphoid cells. A: Cells not exposed to cholera toxin; NP40 extract incubated with toxin antibody. B: Cells exposed to toxin; NP40 extract incubated with toxin antibody. Lipids were separated by two-dimensional chromatography on silica gel G plates and detected by autoradiography as previously described [27]. Neutral lipids travel at the solvent front in both systems; phosphatidyl choline and sphingomyelin stay at the origin in the second dimension. In this experiment toxin antibody led to a fourfold increase in the amount of neutral lipid and phospholipid adsorbed to the bacterial pellet.

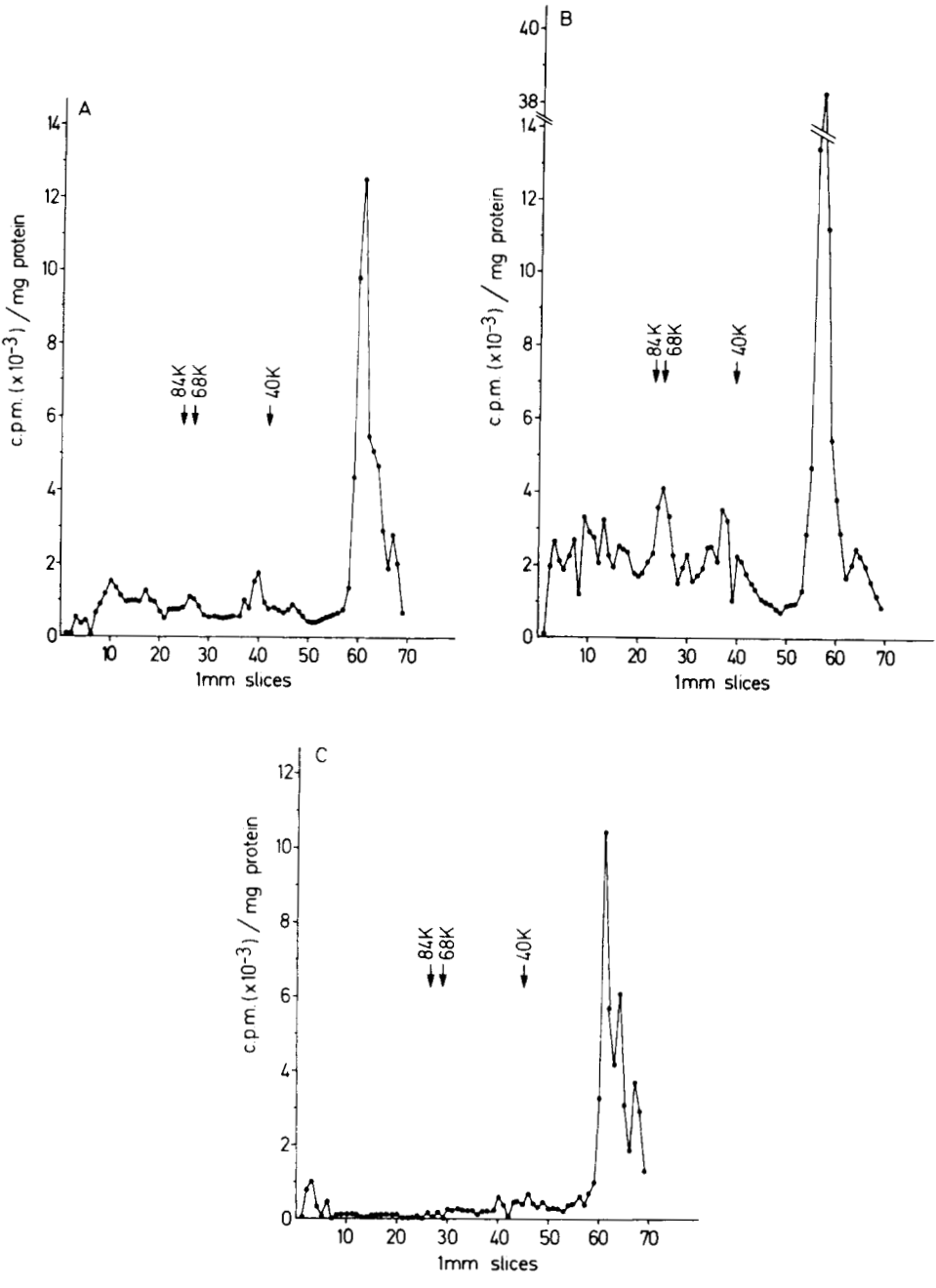

Fig. 5. Surface labeling of the glycoproteins of a mouse lymphoid cell line by the galactose oxidase borotritiide technique. Cells were dissolved by boiling in 2% SDS in electrophoresis sample buffer, aliquots were taken for protein, and the sample was reduced with 0.1 M dithiothreitol. Labeled proteins were separated by SDS-PAGE (7.5% disks) and radioactivity was detected as previously described [29]. A: Cells incubated with galactose oxidase alone; B: neuraminidase and galactose oxidase; C: No enzyme.

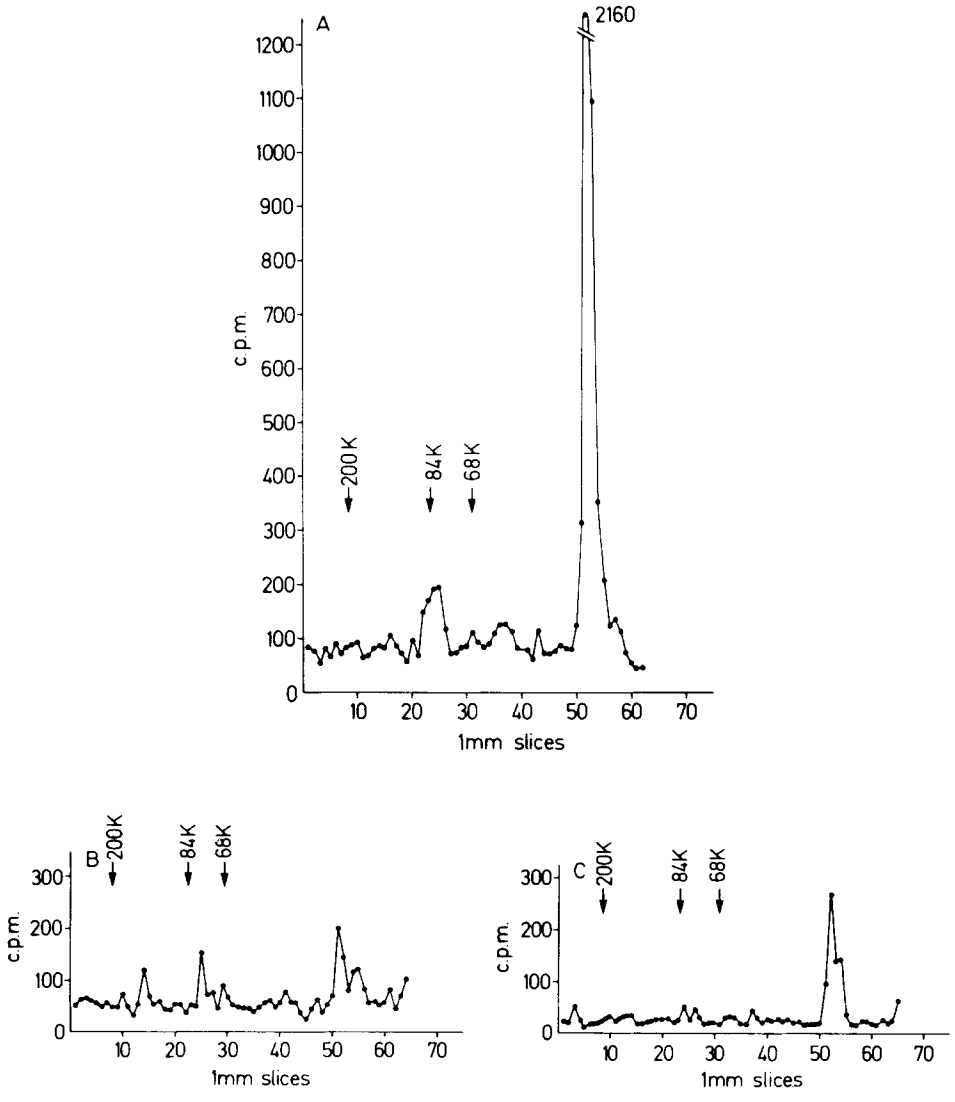

Fig. 6. Analysis of the galactose oxidase borotritiide-labeled glycoproteins which bind to protein A during the isolation of the cholera toxin receptor from a mouse lymphoid cell line. Methodology as described in Figure 2 except that components bound to protein A were solubolized by boiling of the bacterial pellet with 200 μl of 2% SDS in electrophoresis sample buffer plus 0.1 M dithiothreitol. Separation of the extracted material was carried out by SDS-PAGE (7.5% disks) [28, 29]. A: Cells exposed to cholera toxin; NP40 extracts were incubated with toxin antibody; B: as in (A) except preimmune serum was used; C: cells not exposed to cholera toxin; NP40 extracts were incubated with toxin antibody. Identical volumes of the NP40 extracts from equivalent numbers of cells were used during immune adsorption of samples A, B, and C.

30-fold increase) by exposure of cells to cholera toxin in serum-free medium were markedly reduced on addition of serum, although cAMP levels remained substantially elevated over those in quiescent cells (Fig. 11). Inclusion of a phosphodiesterase inhibitor partially prevented the reduction in cAMP levels produced by serum, and markedly elevated levels were maintained for at least 32 h after the addition of complete growth medium. However, although cholera toxin potentiated the inhibition of DNA synthesis produced by low concentrations of a phosphodiesterase inhibitor, the effect was sufficiently incomplete to make this simple approach to mutant selection inapplicable.

DISCUSSION

Our present data strongly suggest that gangliosides with similar chromatographic properties to GM_1 are quantitatively the major species of cholera toxin receptor in both mouse lymphoid and fibroblast cell lines. While GM_1 is the best inhibitor of cholera toxin binding yet reported [11], there is no guarantee that the cell surface receptor will be identical to GM_1. For example, while GD1b is the best commercially available inhibitor of thyrotropin binding to thyroid plasma membranes [14], a more complex ganglioside has recently been isolated from thyroid tissue, which is a far more potent inhibitor of hormone binding [38]. Indeed the apparent heterogeneous nature of the gangliosides in immune complexes isolated from [^{14}C] palmitate-labeled cells might suggest that a number of species of cell surface ganglioside act as the toxin receptor. Such an interpretation is complicated by the fact that gangliosides such as GM_1 tend to resolve into several bands on thin-layer chromatography, probably because of heterogeneity in the sphingosine and fatty acid moieties.

The significance of galactoproteins in the immune complexes obtained from both mouse lymphoid and fibroblast cell lines is somewhat uncertain. Based on an analogy with the ABO blood group system [39], there is no reason to suppose that similar carbohydrate sequences which specify toxin receptor activity might not exist in both glycolipid and glycoproteins. The structure of cholera toxin also has certain similarities to that of the glycoprotein hormones [14, 40], which themselves apparently utilize both glycolipid and glycoprotein receptors [7, 14]. In addition, low levels of a number of cell surface galactoproteins from mouse fibroblasts which cross-react with anti-GM_1 antibodies have recently been tentatively identified [41]. It is therefore perhaps not surprising that a cell surface glycoprotein was present in the immune precipitates. However, there remains some doubt about the validity of this interpretation. First, phospholipid and neutral lipids were adsorbed to the bacterial pellet. A low level of contamination of the bacterial pellet with NP40-extracted [^{14}C]-palmitate-labeled lipid may not be surprising in that they might in some way be retained by the bacterial membrane. However, there was some increase in the level of neutral lipid and phospholipid adsorbed to the bacterial pellets under conditions where specific adsorption of the toxin receptor was expected, although the extent of the increase was variable. One possible conclusion from this result is that mixed micelles of ganglioside, phospholipid, and some protein remain in the supernatant even after centrifugation of the NP40 extract at 100,000g for 1 h. Such micelles containing other components apart from the true toxin receptor may also be adsorbed to the bacterial protein A under these conditions, although the major cell surface proteins labeled by lactoperoxidase-catalyzed iodination were not found in the immune complexes. Alternatively it may be of genuine interest that other lipids apart from gangliosides and a galactoprotein are specifically adsorbed to the bacterial pellet. It is conceivable that a ganglioside-phospholipid-galactoprotein complex exists in

the membrane [7], and mild detergents such as NP40 fail to disrupt such complexes. We are presently attempting to use other detergents with different characteristics to resolve this question. A further concern is that the glycoprotein is an artifact of the galactose oxidase procedure. The enzyme creates an aldehyde group on the six carbon of terminal galactose and N-acetylgalactosamine residues. It is therefore possible that the aldehyde created on the terminal galactose of GM_1 formed a Schiff's base with an amino group on a protein in close proximity to GM_1, and the linkage was stabilized and labeled by borotritiide reduction. Under such circumstances one could generate an artifact, ie, a GM_1-containing glycoprotein. However, as the terminal galactose of GM_1 would appear to be an important determinant of its toxin-binding activity, it would seem unlikely that such a protein artifact would bind toxin.

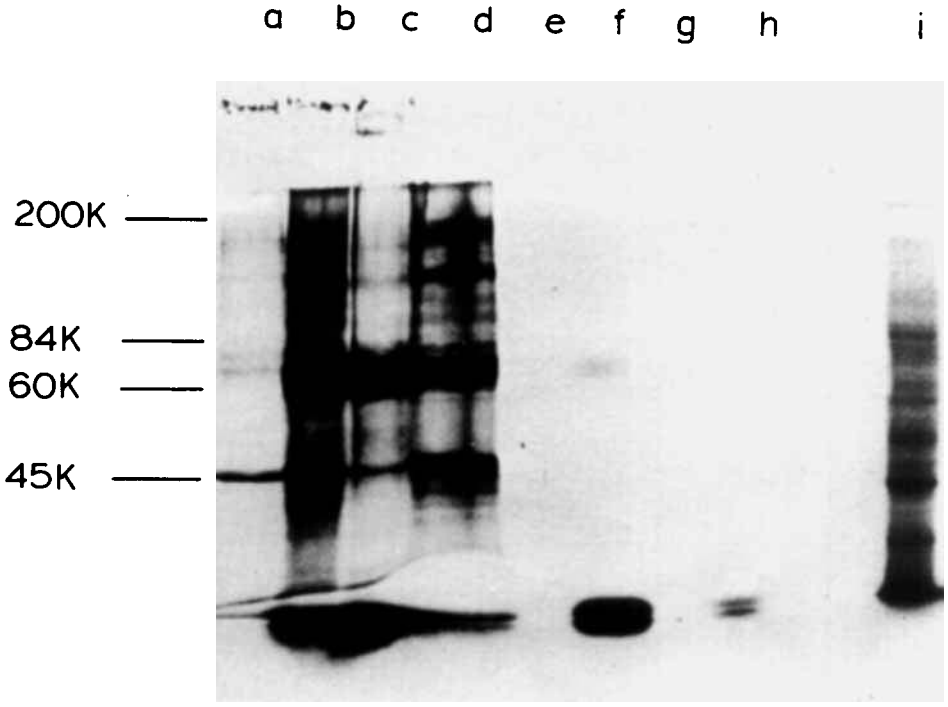

Fig. 7. Fluorographic detection of mouse lymphoid cell surface proteins labeled by the galactose oxidase technique. Methodology as previously described in Figures 2 and 6, except that proteins were separated by SDS-PAGE (7.5% slabs) and labeled proteins detected by fluorography [29, 30]. a, Cells incubated with galactose alone. b, As in (a) plus neuraminidase. c, NP40 extract of cells labeled as in (b). d, NP40 pellet of cells labeled as in (b). e,f, Analysis of glycoproteins from NP40 extracts of cells labeled as in (b) and exposed to cholera toxin, which bound to protein A; e, NP40 extract incubated with preimmune serum; f, Toxin antibody; g,h, As in e,f, except that cells were labeled with galactose oxidase alone; g, NP40 extract incubated with preimmune serum; h, toxin antibody. i, Cells labeled by lactoperoxidase-catalyzed iodination [31]. Tracks (a/b) contained equivalent amounts of protein (140 μg). Track (c) contained half the protein equivalent of track (d), ie, 100 μg and 200 μg, respectively. The amount of NP40 extract used in the immune precipitation procedure was equivalent on a cell basis (e–h).

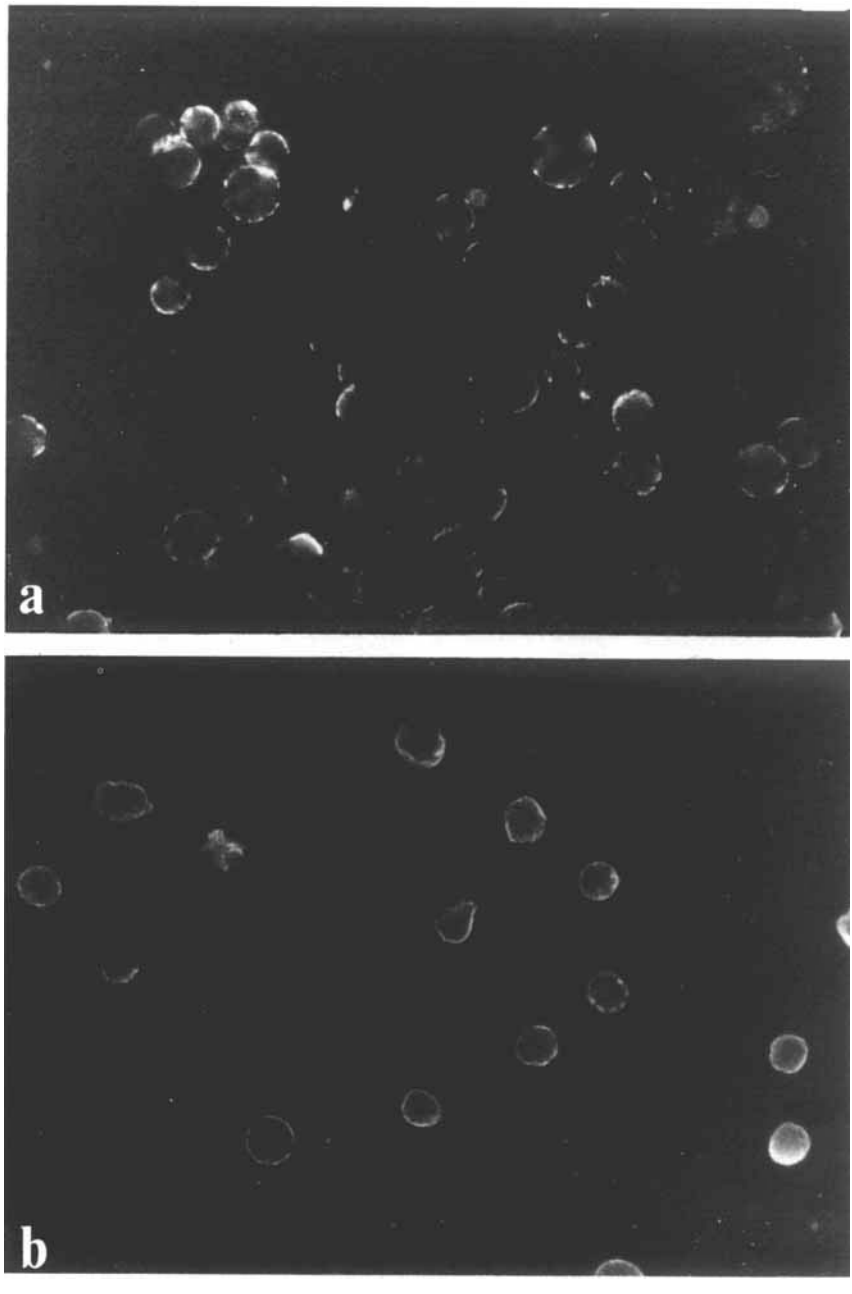

Fig. 8. Detection of cholera toxin bound to the surface of a mouse lymphoid cell line by indirect immunofluorescence. Cells (2×10^6) were incubated with 1 μg/ml (a) or 10 μg/ml cholera toxin (b) in PBS at 0°C for 15 min. Unbound toxin was removed by washing, and the cells were incubated with rabbit anti-cholera toxin (1:10) for 30 min at 20°C. The cells were washed twice and incubated with FITC-labeled goat anti-rabbit antibodies (1:10) for 30 min at 37°C. Excess FITC-labeled antibody was removed by washing, and the cells were suspended in 50% glycerol and viewed in a Zeiss microscope equipped with epifluorescence optics. Photographs were taken on Kodak plus X with exposure times of up to 2 min (× 187).

While it therefore remains somewhat unclear whether we have isolated a genuine glycoprotein toxin receptor, there is little doubt that quantitatively the major species of toxin receptor is ganglioside in nature in both cell lines tested. One is therefore faced with attempting to explain how interaction of cholera toxin with a mouse lymphoid cell line leads to capping. Previous data from other laboratories on capping of toxin in peripheral lymphocytes suggests that the microfilament and microtubule systems are involved [17–19]. As glycolipids are unlikely to interact directly with the cytoskeleton, it is possible that the interaction is mediated by association between the ganglioside receptor and a transmembrane protein which itself is not a toxin receptor. Using the myosin affinity technique, we are presently attempting to see whether gangliosides can be cross-linked to specific proteins in membranes, and whether they indirectly interact with the cytoskeleton [42]. Alternative explanations for capping of toxin ganglioside complexes based on membrane flow [43] should not be ignored, although we have noted that toxin does not cap in

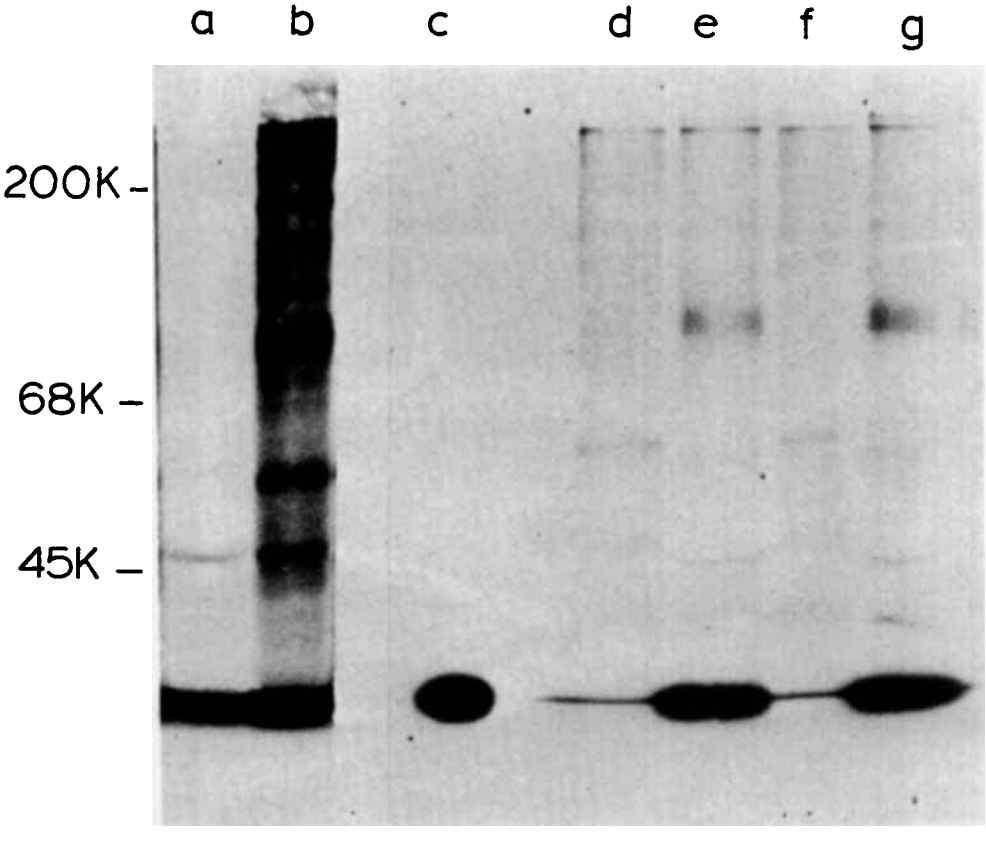

Fig. 9. Fluorographic detection of Balbc/3T3 cell surface proteins labeled by the neuraminidase galactose oxidase borotritiide technique. Cells were labeled in monolayer [29] and labeled proteins were separated and detected as outlined in Figure 7. a, Borotritiide-labeling profile without galactose oxidase; b, labeling profile following incubation with neuraminidase and galactose oxidase; c, [^{3}H]GM_1 standard; d–g, Cells labeled as in (b) were exposed to cholera toxin (25 μg in 1 ml PBS, 0.1% BSA), unbound toxin was removed by washing, and the cells were extracted with 4 ml of 1% NP40 in PBS/PMSF for 1 h, 0°C. Toxin-receptor complexes were isolated as described in Figure 2; d,f, NP40 extracts incubated with preimmune serum; e,g, NP40 extracts incubated with toxin antibody.

all cell types tested. For example, a myeloma cell line (MOPC 21) that lacks toxin receptor binds the toxin after incubation of the cells with GM_1. The interaction leads to patching but not capping, as detected by indirect immunofluorescence. Similarly, toxin bound to Balbc/3T3 fibroblasts does not patch or cap under the conditions we have tested so far.

Our results with mouse fibroblasts also suggest that gangliosides represent the predominant species of cholera toxin receptor. It should therefore be feasible to use the toxin to select for mutants defective specifically in glycolipid biosynthesis as outlined in the introduction. The specificity of the toxin offers a major advantage over those approaches previously used to isolate mutants with altered cell surface carbohydrate profiles such as lectin resistance [44, 45] or reduced cell adhesion [46]. The failure of cholera toxin to inhibit serum-stimulated DNA synthesis in Balbc and Swiss 3T3 cells may be related to the rapid reduction in cAMP levels produced when toxin-treated cells are exposed to serum. Assuming that adenyl cyclase is irreversibly activated by cholera toxin [47], then either cAMP was rapidly lost into the medium or it was degraded by an intracellular phosphodiesterase. Although elevated levels of cAMP can lead to a slow increase in phosphodiesterase activity

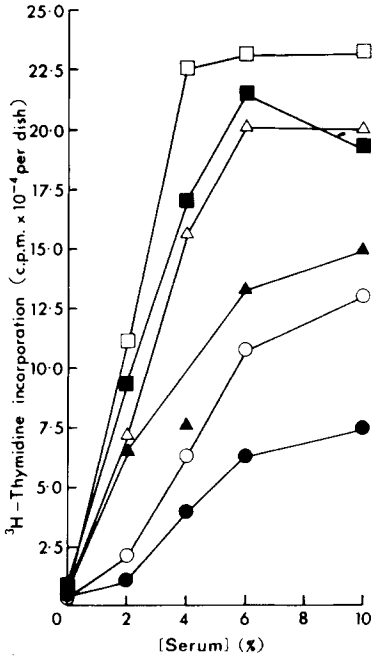

Fig. 10. Effect of cholera toxin and/or a phosphodiesterase inhibitor on serum-stimulated DNA synthesis in Balbc/3T3 cells. Quiescent cells (35-mm dishes) were preincubated for 3 h with serum-free medium alone (□——□) or medium containing 100 ng/ml cholera toxin (■——■), 100 μM isobutylmethylxanthine (△——△), 100 ng/ml cholera toxin and 100 μM isobutylmethylxanthine (▲——▲), 500 μM isobutylmethylxanthine (○——○), or 100 ng/ml cholera toxin and 500 μM isobutylmethylxanthine (●——●). Varying concentrations of serum were then added and the cells were pulsed 20 h later for 1 h with 0.4 μCi/ml [methyl-^{3}H] thymidine (28 Ci/mmole). Cells were extracted with 10% trichloracetic acid and ethanol, then solubilized in 0.2 M NaOH. Aliquots were taken for scintillation counting. Each point represents the average incorporation into two separate dishes. Similar results were obtained by autoradiography.

[48, 49], Pledger et al [50–52] have shown that serum rapidly activates a cellular phosphodiesterase in BHK21 cells by an unknown mechanism. However, while a phosphodiesterase inhibitor potentiated the action of cholera toxin in raising cAMP levels in Balbc/3T3 cells, it did not prevent a significant drop in cAMP levels in response to serum. That this reduction in cAMP levels produced by serum was probably not the key element in the lack of growth inhibition by cholera toxin is suggested by our recent experiments with $PGF_{2\alpha}$, fibroblast growth factor, and insulin. Combinations of these three agents are mitogenic for quiescent Swiss 3T3 cells (eg, percentage nuclei labeled by [^{3}H] thymidine 28 h after addition of $PGF_{2\alpha}$ (200 ng/ml) plus insulin (50 ng/ml) to quiescent cells was 33%) even in the presence of cholera toxin (1 ng–1 μg/ml), yet they failed to produce a rapid reduction in cAMP levels elevated by cholera toxin. Whatever the basis of the serum effect,

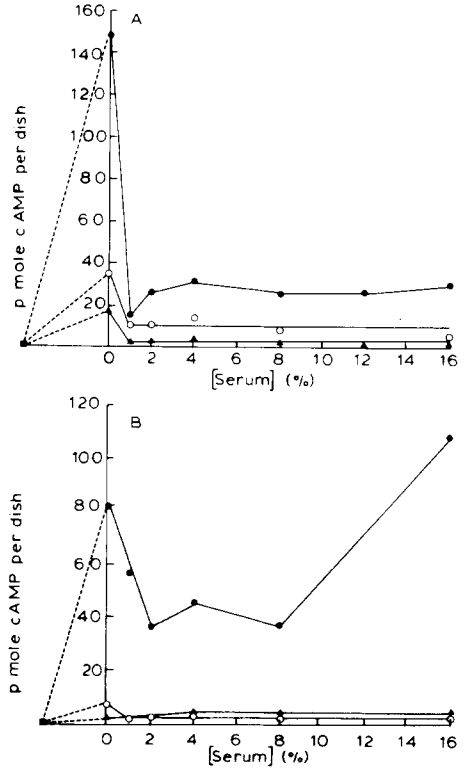

Fig. 11. Effect of serum on the cholera toxin-induced increase in cAMP levels in Balbc/3T3 cells. Quiescent Balbc/3T3 cells (35-mm dishes) were incubated with either cholera toxin or isobutylmethylxanthine, or both, for 3 h in serum-free medium. Twenty minutes after the addition of a variety of concentrations of serum the cells were processed for cAMP determination. A: 1 ng/ml cholera toxin (▲——▲); 10 ng/ml cholera toxin (○——○); 100 ng/ml cholera toxin (●——●). B: 10 ng/ml cholera toxin (○——○); 1 mM isobutylmethylxanthine (▲——▲); 10 ng/ml toxin plus 1 mM isobutylmethylxanthine (●——●). The concentration of cAMP in quiescent untreated cells was approximately 1–2 pmoles/30-mm dish (10–20 pmoles/mg protein). Cyclic AMP was assayed using the system supplied by the Radiochemical Centre. Growth medium was rapidly removed from cell monolayers, 50% acetic acid was added, and the extract was centrifuged prior to freeze-drying. The extract was reconstituted in the Tris/EDTA buffer provided prior to assay. Treatment of samples prepared in this way with cyclic nucleotide phosphodiesterase (Sigma) showed that the material assayed was cAMP.

levels of cAMP in toxin-treated cells (with or without a phosphodiesterase inhibitor) remained substantially elevated for several days after addition of complete growth medium, compared with those in quiescent cells. However, cholera toxin failed to inhibit significantly the growth of either Balbc or Swiss 3T3 cells, a process previously reported to be inhibited by cAMP [35, 36]. Interestingly, cholera toxin and cAMP have recently been reported to be mitogenic for rat Schwann cells [53]. Alternative ways of using cholera toxin as a method of selection for cells deficient in complex glycolipid biosynthesis are presently under study.

ACKNOWLEDGMENTS

The authors would like to thank Drs. M. Boss, M. F. Greaves, and D. Cicco of the Imperial Cancer Research Fund, London, for supplying the mouse lymphoid cell line and cholera toxin antibodies; and Dr. R. O. Thompson of the Wellcome Laboratories, Beckenham, London, for the cholera toxin used in the early stages of this work. The work was supported by a Medical Research Council project grant.

REFERENCES

1. Critchley DR, Vicker M: In Poste G, Nicolson GL (eds): "Cell Surface Reviews." Amsterdam: Elsevier/North-Holland, 1977, vol 3, p 307.
2. Marchase RB: J Cell Biol 75:237, 1977.
3. Obata K, Oide M, Handa S: Nature 266:369, 1977.
4. Lingwood CA, Ng A, Hakomori S: Proc Natl Acad Sci USA 75:6049, 1978.
5. Hakomori S: Biochim Biophys Acta 417:53, 1975.
6. Fishman PH, Brady RO: Science 194:906, 1976.
7. Kohn L: In Cuatrecasas P, Greaves MF (eds): "Receptors and Recognition." London: Chapman Hall, 1978, Series A, vol 5, p 135.
8. Critchley DR, Ansell S, Dilks S: Biochem Soc Trans 7:314, 1979.
9. Tate RL, Holmes JM, Kohn LD, Winand RJ: J Biol Chem 250:6527, 1975.
10. Dufau MI, Ryan DW, Baukal AJ, Catt KJ: J Biol Chem 250:4822, 1975.
11. Cuatrecasas P: Biochemistry 12:3547, 1973.
12. Moss J, Fishman PH, Manganiello VC, Vaughan M, Brady RO: Proc Natl Acad Sci USA 73:1034, 1976.
13. Fishman PH, Moss J, Vaughan M: J Biol Chem 251:4490, 1976.
14. Mullin BR, Fishman PH, Lee G, Aloj SM, Ledley FO, Winand RJ, Kohn LD, Brady RO: Proc Natl Acad Sci USA 73:842, 1976.
15. Fishman PH, Moss J, Osborne JC: Biochemistry 17:711, 1978.
16. Moss J, Manganiello VC, Fishman PH: Biochemistry 16:1876, 1977.
17. Revesz T, Greaves MF: Nature 257:103, 1975.
18. Craig S, Cuatrecasas P: Proc Natl Acad Sci USA 72:3844, 1975.
19. Sedlacek HH, Stark J, Seiler FR, Zeigler W, Weigandt H: FEBS Lett 61:272, 1976.
20. Keenan TW, Schmidt E, Franke WW, Weigandt H: Exp Cell Res 92:259, 1975.
21. Laine RA, Hakomori S: Biochem Biophys Res Commun 54:1039, 1973.
22. Hynes RO, Ali IU, Destree AT, Mautner VE, Perkins ME, Senger DR, Wagner DD, Smith KK: Ann NY Acad Sci 312:317, 1978.
23. Yamada KM, Olden K, Pastan I: Ann NY Acad Sci 312:256, 1978.
24. Cuatrecasas P: Biochemistry 12:3567, 1973.
25. Kessler SW: J Immunol 115:1617, 1975.
26. Fishman PH, Moss J, Manganiello VC: Biochemistry 16:1871, 1977.
27. Critchley DR, Macpherson I: Biochim Biophys Acta 296:145, 1973.
28. Laemmli UK: Nature 222:680, 1970.
29. Critchley DR: Cell 3:121, 1974.
30. Laskey RA, Mills AD: Eur J Biochem 56:335, 1975.

31. Hynes RO: Proc Natl Acad Sci USA 70:3170, 1973.
32. Holmgren J, Lindholm L, Lonnroth I: J Exp Med 139:801, 1974.
33. Hollenberg MD, Fishman PH, Bennett V, Cuatrecasas P: Proc Natl Acad Sci USA 71:4224, 1974.
34. Hollenberg MD, Cuatrecasas P: Proc Natl Acad Sci USA 70:2964, 1973.
35. Pastan I, Johnson GS, Anderson WB: Ann Rev Biochem 44:411, 1975.
36. Willingham MC: Int Rev Cytol 44:329, 1976.
37. Rozengurt E, Jimenez de Asua L: Proc Natl Acad Sci USA 70:3609, 1973.
38. Mullin BR, Pacuska T, Lee G, Kohn LD, Brady RO, Fishman PH: Science 199:77, 1977.
39. Watkins WM: Science 152:172, 1966.
40. Kurosky A, Markel DE, Fitch WM: Science 195:299, 1977.
41. Tonegawa Y, Hakomori S: Biochem Biophys Res Commun 76:9, 1977.
42. Koch GLE, Smith MJ: Nature 273:274, 1978.
43. Bretscher MS: Nature 260:21, 1976.
44. Meager A, Ungkitchanukit A, Nairn R, Hughes RC: Nature 257:137, 1975.
45. Stanley P, Narasimhan S, Siminovitch L, Schacter H: Proc Natl Acad Sci USA 72:3323, 1975.
46. Pouyssegur J, Willingham M, Pastan I: Proc Natl Acad Sci USA 74:243, 1977.
47. Gill DM: Adv Cyclic Nucleotide Res 8:85, 1977.
48. Manganiello V, Vaughan M: Proc Natl Acad Sci USA 69:269, 1971.
49. D'Armiento M, Johnson GS, Pastan I: Proc Natl Acad Sci USA 69:459, 1972.
50. Pledger WJ, Thompson WJ, Strada ST: J Cyclic Nucleotide Res 1:251, 1975.
51. Pledger WJ, Thompson WJ, Strada ST: Nature 256:729, 1975.
52. Pledger WJ, Thompson WJ, Strada ST: Biochem Biophys Res Commun 70:58, 1976.
53. Raff MC, Hornby-Smith A, Brookes JP: Nature 273:672, 1978.