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Monoclonal antibody to amphomycin. A tool to study the topography of dolichol monophosphate in the membrane *

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ABSTRACT

Understanding the topographical orientation of dolichol monophosphate (Dol-P) in the membrane of the endoplasmic reticulum (ER) is of utmost importance for studying the regulation of asparagine-linked protein glycosylation in eukaryotic cells. This was practically impossible due to the nonavailability of a suitable probe. Recent studies on the specific interaction between a lipopeptide, amphomycin, and Dol-P, provided an insight to develop a monospecific antibody to amphomycin which could recognize the amphomycin–Dol-P complex in order to detect Dol-P immunocytochemically in the ER membrane. We report herein the successful production of a monoclonal antibody to amphomycin. The antibody belongs to the IgG + IgM subclasses and is specific for amphomycin when analyzed by the enzyme-linked immunoassay and immunoblot procedures. The antibody recognizes with equal potency both the native amphomycin and also mild acid-hydrolyzed amphomycin from which *N*-terminal fatty acylated aspartic acid has been removed. Preincubation of amphomycin with the antibody partially reduced the inhibitory action of amphomycin on dolichol phosphate mannosyltransferase (EC 2.4.1.83). Furthermore, exposure of capillary endothelial cells to amphomycin, followed by the monoclonal antibody to amphomycin, followed sequentially by staining with FITC-conjugated goat anti-mouse IgG and examination under a fluorescent microscope gives intense fluorescence at the perinuclear region of the cell with a structure reminiscent of the ER.

INTRODUCTION

It is now well recognized that glycosylation of asparagine residue(s) present in a sequence Asn-X-Ser/Thr is initiated by a cotranslational transfer of a fourteen-sugar long oligosaccharide chain ($Glc_3Man_9GlcNAc_2$) from its lipid carrier in eukaryotic cells[1,2]. The assembly of $Glc_3Man_9GlcNAc_2$-PP-dolichol is a multistep

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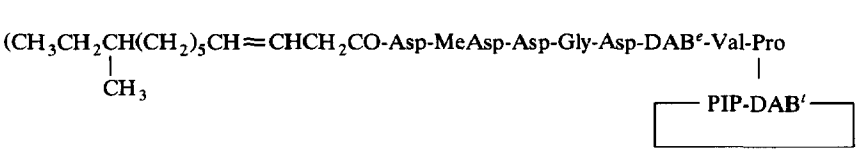


Fig. 1. Structure of amphomycin. DAB^e, D-*erythro*-α,β-diaminobutyric acid; DAB^l, L-*threo*-α,β-diaminobutyric acid; and PIP, D-pipecolic acid.

process carried out by a multienzyme complex in endoplasmic reticulum (ER) through a sequential transfer of *N*-acetylglucosamine, mannose, and glucose either from their nucleoside diphosphate sugar donors or from their dolichol monophosphate sugar derivatives[3,4]. Studies on the topography of the assembly reaction have claimed that the biosynthesis of dolichol derivatives occurs on both sides of the ER membrane[5,6]. For example, $Man_5GlcNAc_2$-PP-dolichol has been detected on the cytoplasmic side of the ER membrane[7], while the subsequent dolichol intermediates, with additional mannose and glucose residues, face the luminal side[7,8]. In addition, evidence has been presented that $GlcNAc_2$-PP-dolichol is synthesized on the cytoplasmic side of the ER as well[9,10].

Less understood in this sequence of events, however, is the orientation of the dolichol monophosphate (Dol-P) in the ER membrane—a phenomenon that needs to be studied. Amphomycin, a lipopeptide antibiotic[11–13] whose structure is given in Fig. 1 has been found to inhibit Man-P-Dol, Glc-P-Dol, and GlcNAc-PP-Dol formation in eukaryotes as well as in plants[14–16]. Detailed mechanistic studies also provide strong evidence that the primary target of amphomycin inhibition of monosaccharide–lipid synthesis is Dol-P where both the polyprenyl monophosphate and the lipopeptide are engaged in a complex formation in the presence of Ca^{2+} (refs. 17 and 18). This finding, therefore, suggests that perhaps targeting the Dol-P–amphomycin complex with a monospecific anti-amphomycin antibody may provide an excellent tool to delineate the topography of Dol-P in the ER membrane.

In the present investigation we have explored this possibility by developing a monoclonal antibody to native amphomycin. The antibody recognizes native amphomycin, as well as the peptide from which an *N*-terminal fatty acylated aspartic acid residue has been removed. Preincubation of amphomycin with the monoclonal antibody partially reversed the inhibitory effect of amphomycin on Man-P-Dol synthase activity. Furthermore, immunocytochemical analysis with cultured cells documented a distinct staining pattern for amphomycin on an area where the endoplasmic reticulum is normally located.

MATERIALS AND METHODS

Dolichol monophosphate, peroxidase-conjugated goat anti-mouse IgG, bovine serum albumin (BSA, crystallized), hydrogen peroxide, dimethyl sulfoxide (Me_2SO) and Triton X-100 were from Sigma Chemical Co. Phosphatidic acid, phosphatidyl-

choline, phosphatidylinositol and phosphatidylserine were from Serdary Research Laboratories, Canada. Bio-Gel A 1.5 m (200–400 mesh), Tween-20, and Enhanced Colloidal Gold Total Protein Detection Kit were from Bio-Rad Laboratories. Adenosine 5′-monophosphate as well as FITC-conjugated goat anti-mouse IgG were from Boehringer Mannheim. GDP-[U-^{14}C]mannose (307 mCi/mmol) was supplied by Amersham Corp. Amphomycin (calcium salt) was a gift from Bristol Laboratories and Dr. M. Bodanszky, Case Western Reserve University. All other chemicals and reagents were of analytical reagent grade. For all purposes, the lipopeptide was dissolved in 10% Me_2SO.

Production of monoclonal antibodies.—The amphomycin was diluted at 250 μg/mL in phosphate buffered saline (PBS), pH 7.2, and emulsified with complete Freund's adjuvant (1:1, v/v). Two Balb/c mice were injected intraperitoneally with 200 μL of the antigen preparation. Thirty days later the mice received a second injection of 50 μg of antigen in 100 μL of PBS. Three days after the booster, the mice were bled from the retroorbital plexus and sacrificed by cervical dislocation. Mononuclear splenic cells were obtained by centrifugation on a Ficoll-Hypaque gradient. These cells were mixed with murine myeloma cells (SP2/O Ag14; 10:3) in the presence of 50% polyethylene glycol 1500 (PEG). After 2 min, the PEG was diluted with RPMI-1640 medium and the cells were centrifuged at 1200 rpm for 4 min. The cells were suspended in HAT medium (Dulbecco's modified Eagle medium containing 1×10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, 1.6×10^{-9} M thymidine and 10% fetal calf serum) and dispersed into 96-well plates. The cells were fed with the HAT medium (50% vol) on day 5 after fusion. When the growth of hybrids was detected microscopically the supernatant culture fluid was removed and assayed for production of immunoglobulin and specific antibody by enzyme-linked immunoassay (ELISA). Cells from the antibody-positive wells were cloned by limiting the dilution. Positive subclones were injected into mice to produce ascites.

Production of ascitic fluid.—Three 6–8-weeks-old Balb/c mice were primed intraperitoneally with 0.5 mL of pristane, and 5–8 days later they were injected with the selected positive subclone $4c_9$ (10^6 viable cells in 0.5 mL of complete Dulbecco's Eagle modified medium). After 10–14 days, mice were tapped for ascites fluid. The fluid was centrifuged to remove red blood cells and debris, and analyzed for the production of antibodies by ELISA.

Assessing the production of antibodies.—When the growth of hybrids was macroscopically detected (10–14 days later), the culture fluid was removed and assessed for the production of antibodies by ELISA[19]. In brief, 200 μL of the culture supernatants were dispensed in 96-well microtiter plates (Costar) and stored overnight at 4°. The plates were washed and incubated with 100 μL of BSA for 45 min at room temperature. After incubating for 2 h with goat anti-mouse IgG conjugated with peroxidase (1:500), the color was developed with freshly prepared substrate (40 mg orthophenylenediamine in 24.3 mL of 0.1 M citric acid, 25.7 mL of 0.2 M sodium phosphate dibasic, and 50 mL of distilled water containing 40 μL

of 30% hydrogen peroxide), and the plates were read at 490 nm after 20 min. Positive, negative, and conjugated controls were included. Positive controls were serum samples from the immune mice whose spleens were used for hybridization. Mean absorbance readings greater than 0.2 A above the mean absorbance readings of the negative controls were considered as positive. Amphomycin-specific antibodies were detected following the above procedure by sensitizing the plates with amphomycin antigen diluted in PBS at 20 μg/mL and then exposing the wells to the hybrid culture supernatants.

Assay for the transfer of [^{14}C]mannose from GDP-[^{14}C]mannose into endogenous acceptors.—ER membranes prepared from rat parotid acinar cells[20] were used as enzyme. Protein concentration was determined by Bradford's procedure[21] using BSA as standard.

Intracelluar localization of amphomycin.—Capillary endothelial cells from the vascular bed of bovine adrenal medulla[22] were subcultured on glass cover slips (18-mm diameter) for three days under standard conditions. The cells were then washed with PBS (pH 7.4) and fixed for 60 s in ice-cold MeOH. The cells were washed twice with PBS and exposed to amphomycin (5 μg/mL) for 5 min at room temperature. After washing three times with PBS the cells were exposed to mouse monoclonal antibody to amphomycin which had been diluted 1:10 in PBS for 30 min. The cells were washed three times and reincubated with FITC-conjugated goat anti-mouse IgG (1:80 dilution) for 30 min. After washing three times with PBS, the cover slips were mounted and examined in an Olympus BH-2 fluorescent microscope. Appropriate controls for amphomycin, conjugated IgG and normal mouse serum were processed identically.

Measurement of radioactivity.—All radioactive samples were counted in Ready Protein (Beckman) scintillation cocktail.

RESULTS

Hybrid selection and characterization of the clones.—The hybridoma method followed here would allow only the hydrid cells (hybrid between the spleen and myeloma cells) to proliferate. Therefore, successful fusion would yield antibody producing clones only. The results summarized in Table I identified two positive clones, 4c and $4c_9$. Isotyping of the antibody produced by these clones indicated that they belong to the immunoglobulin subclasses IgG + IgM. Furthermore, the results also suggested that clone $4c_9$ is not only more potent in immunoglobulin production, but it is also more specific for the antibody to amphomycin as compared to the 4c clone. Consequently, clone $4c_9$ was used in the rest of the study.

Enrichment of the anti-amphomycin antibody.—Large amounts of antibody could be obtained by growing the hybridoma cells and their progeny in bottles. But the most convenient way to generate an antibody with a high titer is by injecting the hydrid cells into Balb/c mice and collecting the ascitic fluid. When the cells

TABLE I

ELISA reactivity of hybrid supernatants [a]

Clone	Absorbance at 490 nm	
	Production of immunoglobulin (IgG + IgM)	Production of specific antibody
4c	0.29	0.12
$4c_9$	0.97	0.39
Immune serum	2.13	2.04
PBS	0.00	0.00

[a] After detecting the growth of the hybrids, the culture fluid was assayed for the production of antibodies. The plates were coated in triplicate with 200-μL of samples overnight at 4°. The plates were washed with PBS–0.5% Tween-20 (PBST), and 100 μL of BSA was then added in each well, and kept for 45 min at room temperature. The plates were washed again with PBST, and 100 μL peroxidase-conjugated goat anti-mouse IgG was added. After 2 h, the wells were washed five times with PBST and incubated with freshly prepared orthophenylenediamine substrate and hydrogen peroxide. Absorbance was measured after 20 min at 490 nm. For detecting amphomycin-specific antibodies, the plates were coated with amphomycin.

positive for anti-amphomycin antibody were injected into mice and analyzed for the immunoglobulin levels in the ascitic fluid, all of them gave very high absorbance reading (> 2) even at a 1:3,000 dilution (Table II), strongly suggesting that the production of antibody with a high titer had been achieved.

The specificity of the immunoglobulin present in the ascitic fluid was tested against amphomycin by ELISA. The plates were coated with 1 or 5 μg of amphomycin per well and the assay was performed according to the procedure above but with ascitic fluid as the source of antibody. The results shown in Figs. 2a and 2b indicated that amphomycin-specific antibody was present in the ascitic fluid

TABLE II

Determination of immunoglobulin in ascitic fluid [a]

Mouse	Ascitic fluid dilution	Absorbance at 490 nm
1	1:500	1.91
	1:1500	> 2.00
	1:3000	1.97
2	1:500	1.91
	1:1500	> 2.00
	1:3000	1.85
3	1:500	> 2
	1:1500	> 2
	1:3000	> 2

[a] The 96-well microtiter plates were coated in triplicate with 2 μg of amphomycin overnight at 4°. The plates were washed with PBST and incubated with the ascitic fluid for 45 min at room temperature. The plates were then washed three times with PBST, and 100 μL of peroxidase-conjugated goat anti-mouse IgG was added and processed as described in Table I.

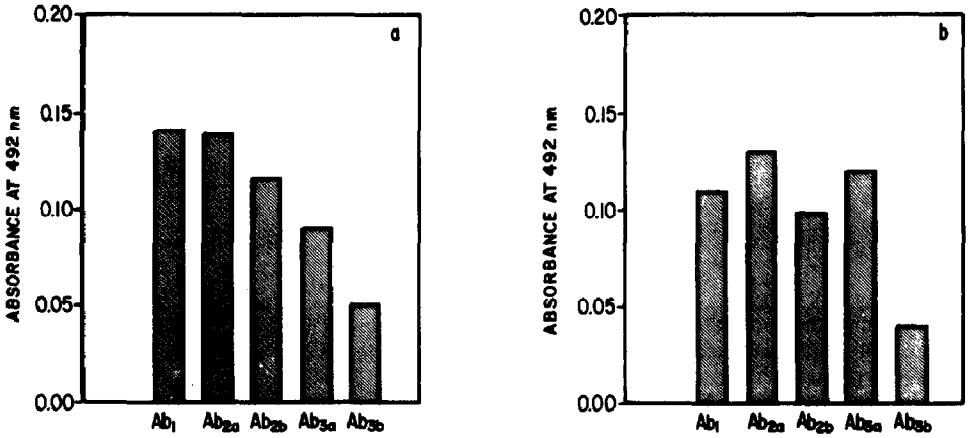


Fig. 2. Enzyme-linked immunoassay of amphomycin. The 96 well microtiter plates were coated with either 1 μg or 5 μg of amphomycin diluted in PBS, pH 7.4 (total vol 50 μL) overnight. The plates were washed with PBS, pH 7.4, containing 0.5% Tween-20 (PBST) and incubated for 2 h at room temperature with ascitic fluid (diluted 1:500 in PBS) obtained from five mice. At the end of the incubation, the plates were washed again with PBST and incubated for 45 min at room temperature with peroxidase-conjugated goat anti-mouse IgG (diluted 1:500 in PBS containing 1% BSA) and followed as described in the Materials and Methods section. The absorbance was taken after 40 min in an automated ELISA plate reader at 490 nm. (a) 1 μg of amphomycin; (b) 5 μg of amphomycin. AB_1, collection of ascitic fluid from mouse 1 at day 5; AB_{2a}, collection of ascitic fluid from mouse 2 at day 5; AB_{2b}, collection of ascitic fluid from mouse 2 at day 9; AB_{3a}, collection of ascitic fluid from mouse 3 after day 5 and AB_{3b}, collection of ascitic fluid from mouse 3 at day 9.

collected from the mice. These results also indicated that the amount of antibody present in the ascitic fluid was relatively higher in samples collected 5 days after the immunization as compared to that collected after 9 days.

The specificity of the anti-amphomycin antibody was also tested by dot-blot analysis. In this experiment, 0.05–2.00 μg of amphomycin was spotted on nitrocellulose strips which were then treated with the enriched monoclonal antibody at a 1:100 dilution, followed by color development with alkaline phosphatase-conjugated goat anti-mouse IgG. The results in Fig. 3 indicate that the antibody reacted positively with the amphomycin. A slight reaction was observed in the BSA control dot. It was concentrated only at the point of application and a reaction pattern very different from that of amphomycin was observed.

Evidence for detecting native and delipidated structures by the anti-amphomycin antibody.—Amphomycin is an undecapeptide containing either 3-isododecenoic or 3-anteisotridecenoic acid, attached to the *N*-terminal aspartic acid residue by an amide linkage (Fig. 1). It has been shown earlier that removal of the fatty acylated aspartic acid residue at the *N*-terminus completely abolished its biological activity[14]. In order to understand the role of the fatty acylated aspartic acid residue on the development of antibody production and antigen recognition, the ELISA test was performed with the native as well as mild acid-hydrolyzed (0.25 acetic acid, 100°, 2

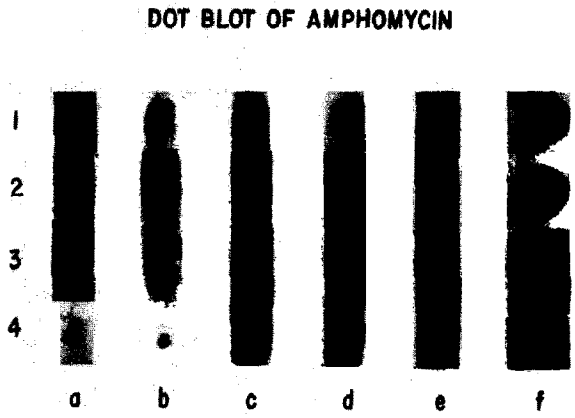


Fig. 3. Immunoblot of Amphomycin. Amphomycin (0.05 μg to 2.0 μg) was spotted on nitrocellulose strips, and the strips were washed for 20 min with Tris-buffered saline, pH 7.5 (TBS) containing 5% dry milk. After 1 min in water, the strips were incubated with a mouse monoclonal antibody to amphomycin (1:100 dilution) for 3 h at room temperature. The strips were then washed three times with TBS, pH 7.5, and reincubated for 3 h with alkaline phosphatase-conjugated goat anti-mouse IgG (1:50 dilution). After washing three times with TBS, pH 7.5, containing 0.05% Tween-20 and twice with TBS, pH 7.5, the color was developed with the appropriate substrate (1 mL of 30 mg/mL of *p*-nitrobluetetrazolium chloride + 1 mL of 15 mg/mL of 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt + 98 mL of 0.1 M $NaHCO_3$ buffer, pH 9.8, containing 1 mM $MgCl_2$). 1, 0.05 μg amphomycin; 2, 1.00 μg amphomycin; 3, 2.00 μg amphomycin; 4, 5.00 μg BSA. a, Ascitic fluid from mouse 1 collected at day 5; b, ascitic fluid from mouse 2 collected at day 5; c, ascitic fluid from mouse 2 collected at day 9; d, ascitic fluid from mouse 3 collected at day 5; e, ascitic fluid from mouse 3 collected at day 9; f, gold staining for protein.

h) amphomycin. The results, shown in Fig. 4, indicate that the monoclonal antibody to amphomycin recognizes both native and mild acid-hydrolyzed amphomycin.

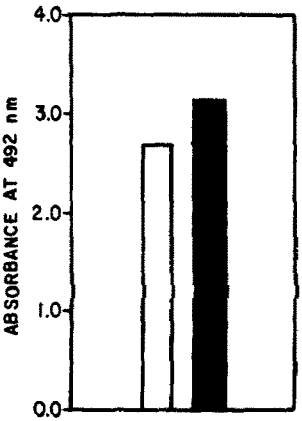


Fig. 4. Enzyme-linked immunoassay of native and acid-hydrolyzed amphomycin. Amphomycin was hydrolyzed in 0.25 M acetic acid for 2 h at 100°. ELISA was performed with the native and acid-hydrolyzed amphomycin as described in Fig. 2. □, Native amphomycin; ■, acid-hydrolyzed amphomycin.

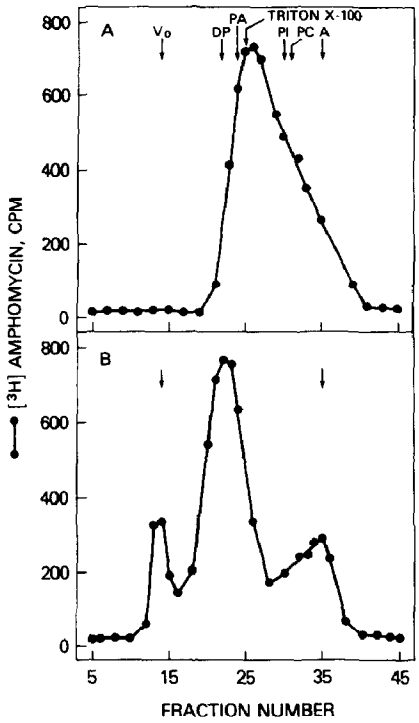


Fig. 5. Selective affinity of [^{3}H]amphomycin for dolichol monophosphate. Amphomycin was radiolabelled according to the procedure earlier described[18]. A, [^{3}H]Amphomycin (10000 cpm) was reacted with 50 μg of phosphatidylserine in 20 mM Tris-HCl buffer, pH 7.0 containing 10 mM $CaCl_2$ and 0.2% Triton X-100 at room temperature. The mixture was then applied to a Bio-Gel A-1.5 m column (0.64 × 25 cm). The column was washed and equilibrated with the same buffer; 0.5 mL-fractions were collected and counted. B, 160 nmols of dolichol monophosphate was first mixed with 160 nmols of phosphatidylserine and then reacted with [^{3}H]amphomycin (10000 cpm). The rest of the procedure was as described for A. V_0, Blue dextran 2000; A, [^{3}H]amphomycin; DP, Dol-P + [^{3}H]amphomycin; PI, phosphatidylinositol + [^{3}H]amphomycin; PC, phosphatidylcholine + [^{3}H]amphomycin; PA, phosphatidic acid + [^{3}H]amphomycin; Triton, [^{3}]Triton X-100.

Ability of the anti-amphomycin antibody to block the inhibitory action of amphomycin on dolichol phosphate mannosyltransferase activity.—Earlier studies indicated that amphomycin inhibited dolichol phosphate mannosyltransferase (EC 2.4.1.83) activity in eukaryotic ER membranes by interacting with dolichol monophosphate[14,18]. Since, the ER membranes not only contain dolichol monophosphate but also other phospholipids, the question was raised whether amphomycin could form complexes with other phospholipids in the same manner as it does with Dol-P, and what happens when both are present at the same time? In order to answer these questions, first a variety of phospholipids were reacted with [^{3}H]amphomycin and then monitored by use of Bio-Gel A 1.5 m column chromatography. The column profiles (Fig. 5a) for [^{3}H]amphomycin in the presence of phosphatidic acid, phosphatidylserine, phosphatidylinositol, and phosphatidylcholine indicate that these phospholipids seem to have interacted with am-

TABLE III

Dolichol phosphate mannosyltransferase activity in the endoplasmic reticulum (ER) membranes before and after treating amphomycin with mouse monoclonal ($4c_9$) antibody [a]

Treatment	Man-P-Dol (pmol/mg protein/5 min)
No addition	5.99 ± 0.14
+ Amphomycin (1.5 μg)	0.60 ± 0.03
+ Amphomycin (1.5 μg) + Antibody (1:666 dilution)	1.98 ± 0.14

[a] ER membranes from the rat parotid gland was incubated with 2.5 μM GDP-U-[^{14}C]mannose (specific activity 310 cpm/pmol), 5 μM $CaCl_2$, 2 mM 5'-AMP and 0.05% Me_2SO at 37° for 5 min in a total volume of 100 μL in the presence or absence of amphomycin. The effect of anti-amphomycin was tested by preincubating amphomycin (20 μg) with 2 μL of ascitic fluid on ice for 30 min, and then an aliquot of 1.5 μL was added to the enzyme assay. Man-P-Dol was extracted and counted. The results are mean ± SEM from two experiments.

phomycin to varying degrees and eluted out at positions different from the dolichol monophosphate–amphomycin complex. However, when an equimolar mixture of phosphatidylserine and Dol-P was reacted with [^{3}H]amphomycin, the radioactivity eluted out in the area where the dolichol monophosphate–amphomycin complex normally elutes and not in the area of the phosphatidylserine–amphomycin complex. The appearance of a short radioactive peak at the V_0 was due to the formation of larger Dol-P vesicles during sonication. Thus, the lipopeptide has selective affinity for Dol-P. If this is true, then preincubation of amphomycin with a specific antibody would prevent its interaction with Dol-P. This was examined by studying the reversibility of amphomycin-mediated inhibition of dolichol phosphate mannosyltransferase activity. It has been observed that amphomycin alone at 1.5 μg would inhibit the enzymic activity by 89% but a partial recovery of the enzyme activity was obtained after pretreating amphomycin (1.5 μg) with the monoclonal antibody (Table III).

Immunochemical localization of amphomycin.—After being convinced that the mouse monoclonal antibody is indeed active against amphomycin, we wanted to localize amphomycin immunocytochemically following its insertion into the cell. The hypothesis was that, if Dol-P on the ER membrane is oriented towards the cytoplasmic side, then once the cells are exposed to amphomycin, the lipopeptide would quickly get inside and form complexes with Dol-P. Since, the monoclonal antibody recognizes the acid-hydrolyzed amphomycin; therefore, the antibody would have no difficulty in identifying amphomycin even though part of the molecule would be engaged in complex formation with Dol-P in the ER membrane. Fluorescent-tagged antibody would then easily monitor this interaction.

We used an established capillary endothelial cell line which has the capability for protein *N*-glycosylation[23] for this study, and the results are shown in Fig. 6. In Figs. 6a and 6b the cells were stained with FITC-conjugated goat anti-mouse IgG. The antibodies used were AB_1 for Fig. 6a and AB_{2a} for Fig. 6b (see Fig. 2). In Fig.

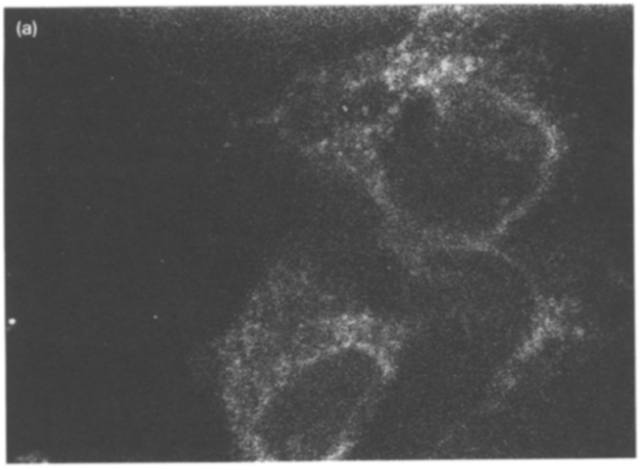
6c the cells were exposed to normal mouse serum. The immunofluorescence in Figs. 6a and 6b clearly indicates that the fluorescence stain is distributed in the perinuclear region of the cell, in an area where the endoplasmic reticulum is normally located, whereas in the sample treated with normal mouse serum, the fluorescence was localized in the nucleus (Fig. 6c).

DISCUSSION

The topographical orientation of Dol-P in the bilayer of the endoplasmic reticulum had to be resolved before investigation into the regulation of the cascade pathway of protein *N*-glycosylation. This is important especially when current evidence predicts the utilization of a common Dol-P pool for the pathway[24], asymmetric distribution of Dol-P in the lipid bilayer[5,6], and also with a possibility that $Man_5GlcNAc_2$-PP-Dol could flip from the cytoplasmic side to the luminal side. It was however, noted earlier that the hydrocarbon chain of dolichol in its extended form is longer than the width of a typical biological membrane[1] and the $t_{1/2}$ for the "flip-flop" of polyprenyl phosphates in artificial membranes is greater than 5 h[25]. Therefore, it was expected that with the development of a monoclonal antibody against Dol-P-specific lipopeptide, amphomycin, many of the above ambiguities would be clarified. The difficulty that is generally encountered in developing antibodies to small molecules due to their poor antigenic responses was not observed in the present case; hence, the construction of an adduct with BSA[26] was not required. It is also worth mentioning here that this is the first report about the production of a monoclonal antibody against amphomycin.

Different levels of anti-amphomycin immunoglobulin in ascitic fluid in each mouse is most likely due to the varying immunologic capability of each animal. Recognition of mild acid-hydrolyzed amphomycin by the antibody to the same degree as the native amphomycin indicated that the peptide portion of the lipopeptide is immunogenic and has contributed to the antibody production. The antibody, when tested for its ability to block the amphomycin-mediated inhibition of dolichol phosphate mannosyltransferase activity in the endoplasmic reticulum from rat parotid acinar cells, produced only a partial reversal of the inhibition. This could be explained by the fact that the antibody binds to the peptide part of the lipopeptide, keeping its Dol-P binding site intact. This is extremely important for detecting the Dol-P–amphomycin complex, and is supported by the observation above that anti-amphomycin antibody binds to the mild acid-hydrolyzed am-

Fig. 6. Immunocytochemical localization of Dol-P in capillary endothelial cells. The capillary endothelial cells were cultured (as described in the Materials and Methods section) in minimal essential medium with Earle's salt containing 10% fetal bovine serum (heat-inactivated), penicillin (50 units/mL) and streptomycin (50 μg/mL) at 37° in a CO_2 incubator and processed for immunological detection of inserted amphomycin by fluorescence microscopy: a, antibody AB_1; b, antibody AB_{2a}; c, normal mouse serum. The nomenclature AB_1 and AB_{2a} are adopted from Fig. 2. Magnification, ×980.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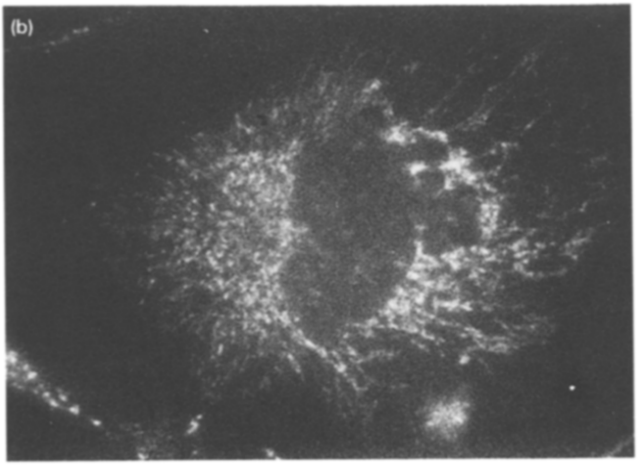
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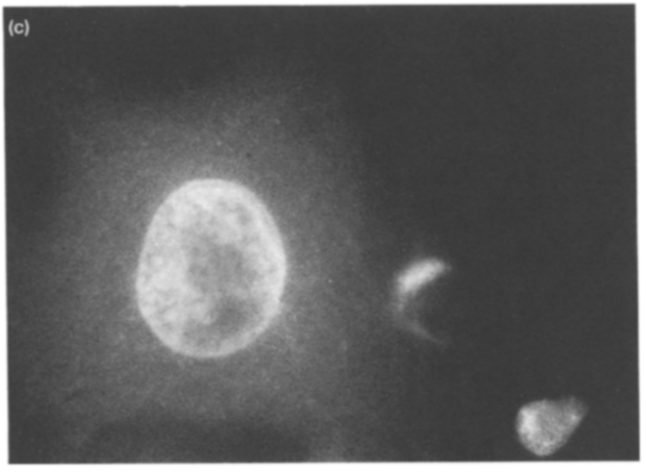
(c)

phomycin with equal potency. Hence, the monoclonal antibody to amphomycin thus generated will provide an excellent tool for immunocytochemical localization of Dol-P in the ER membrane. Experiments described in Fig. 6 clearly indicate that FITC-conjugated goat anti-mouse IgG reacted positively with amphomycin bound to the cell structures which apparently correspond to the ER membrane. All other areas, including the plasma membrane, remained insensitive. Furthermore, neither amphomycin alone, nor FITC-conjugated goat anti-mouse IgG alone, gave positive reactions. This led to the conclusion that anti-amphomycin antibody is certainly a powerful tool to localize Dol-P in the ER membrane. Due to the inherent limitation of light microscopy, the exact orientation of Dol-P in the ER membrane could not be defined. Electron microscopy is currently being used to elucidate more precisely the orientation of Dol-P in the ER membrane.

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