CAPPING OF EXOGENOUS FORSSMAN GLYCOLIPID ON CELLS

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ABSTRACT

When motile cells are incubated with Forssman glycolipid, the antigen is incorporated into the cells' plasma membranes. If cross-linked by antibody, the patched glycolipids cap. This process is sensitive to those drugs that are known to inhibit capping of protein antigens. The results support a flow mechanism for capping.

KEY WORDS Forssman glycolipid · antigen capping · membrane flow

The mechanism whereby surface antigens, after cross-linking by appropriate antibodies, form a cap at one region of the cell (11) is unknown. Various schemes put forward to explain the phenomenon can be subdivided into two main classes. One set of models suggests that when antigens are cross-linked to form patches, they are directly or indirectly recognized by cytoskeletal elements within the cell cytoplasm, and that these elements move the patch to a pole of the cell where they form a cap. This view is supported by many reports (see the review by de Petris [5]), which provide indirect evidence for it. The alternative set of models suggests that the process of capping reflects a polar movement of the cell's surface membrane (1) or selected components of it (3). There is little published evidence in favor of these flow mechanisms.

It is difficult to conceive of any direct experiment to distinguish these quite different models. However, one scheme requires recognition of the patch from within the cell; the other does not. If an antigen on the surface of a cell, which is known not to be in contact with the cytoplasm, were found to cap when cross-linked by antibody, then this could be construed as evidence for a flow scheme. The ideal antigen to use in this case would be a lipid, because by its nature it extends only half-way through the bilayer and does not extend to the cytoplasm.

Here we show that various cells, which lack Forssman antigen (FA),[1] will bind a monoclonal anti-Forssman antibody after the cells have been incubated in Forssman glycolipid. This leads to a uniform cover of antibody on the cell surface. When cross-linked, these glycolipids cap.

MATERIALS AND METHODS

The monoclonal antibody M1/22.25, which recognizes a FA determinant (10), was used to assay FA during its purification from sheep erythrocytes by its ability to inhibit M1/22.25 cytotoxicity for sheep erythrocytes. The glycolipid was purified by the procedure of Fraser and Mallette (8) from 164 g of an acetone powder of sheep erythrocytes, the final stages of purification being thin-layer chromatography in chloroform:methanol:water (65:25:4; R_f = 0.23) and *n*-propanol:water (70:30; R_f = 0.68). In both these systems, and in three additional ones (chloroform:methanol:water [60:45:10; R_f = 0.8]; chloroform:methanol:2.5 N NH_4OH [130:90:18; R_f = 0.45]; and *n*-propanol:6 N NH_4OH [2:1; R_f = 0.5], the material contained a single component as seen by primuline staining, which comigrated with the biological activity.

[1] *Abbreviations used in this paper:* CS, calf serum; DNP, 2,4 dinitrophenol; FA, Forssman antigen; M1/22.25, cell line producing monoclonal anti FA antibodies; R.anti-Rat Ig-Fl or -Ft, rabbit anti-rat immunoglobulin antibodies conjugated to fluorescein or ferritin; PBS, phosphate-buffered saline.

The yield of FA glycolipid (9.1 mg) is considerably higher than reported (12); this is probably due to a different breed of sheep being used.

Insertion of FA glycolipid into various cells (10) which are FA negative was carried out by incubating them at $\sim 10^7$ cells/ml in RPMI-1640 containing 10% calf serum (CS) and FA at 1 mg/ml (unless otherwise stated) for 2 h at 37°C. The cells were washed three times in phosphate-buffered saline (PBS) containing 10% CS.

In some cases, cells were fixed before labeling with antibodies. This was done in 4% glutaraldehyde in PBS for 30 min at 23°C. The cells were then washed in PBS, held in 0.1 M L-lysine in PBS for 30 min at 23°C, and then washed in PBS.

For fluorescence microscopy, cells were indirectly labeled by incubation in excess M1/22.25 antibodies in PBS with or without 0.2% sodium azide for 30 min at 23°C. These monoclonal antibodies had been partially purified from tissue culture medium by precipitation with 45% ammonium sulphate. After a single wash, the cells were incubated for 30 min in Miles-Yeda rabbit anti-rat immunoglobulin antibodies conjugated to fluorescein (R.anti-Rat Ig-Fl) diluted 1:5 in PBS containing 10% CS and with, or without, 0.2% sodium azide at the temperature specified. Washed cell suspensions were mounted on slides and examined in a Zeiss photomicroscope modified for epifluorescence microscopy (Carl Zeiss, Inc., New York). At least 200 cells were counted where figures are given. Photographs were taken on Kodak Tri-X film.

For immunoelectron microscopy, cells were labeled first with excess M1/22.25 antibodies as described above, followed by 10 mg/ml R.anti-Rat Ig-ferritin (Ft) (7). The labeled cells were fixed and prepared for electron microscopy (7).

RESULTS

The monoclonal antibody M1/22.25 has been shown to recognize a FA determinant (10). We have used these antibodies to assay this antigen during its purification from sheep red blood cells. The M1/22.25 reactive molecule, a glycolipid, behaves during this isolation like FA (8). It is a pure preparation, because its ability to inhibit M1/22.25 cytotoxicity for sheep erythrocytes comigrates with a single glycolipid in five thin-layer chromatography systems.

Mouse thymocytes, which are FA negative (10), were used to test the incorporation of exogenously added FA into their membranes. The thymocytes were incubated in various concentrations of FA, and then labeled by successive incubation in M1/22.25 antibodies in azide at 23°C, followed by R.anti-Rat Ig-Fl at 23°C in azide. The cells were examined for fluorescent staining. As seen in Fig. 1, positive cells can be detected following incubation in as little as 10 μg/ml FA. The FA glycolipid

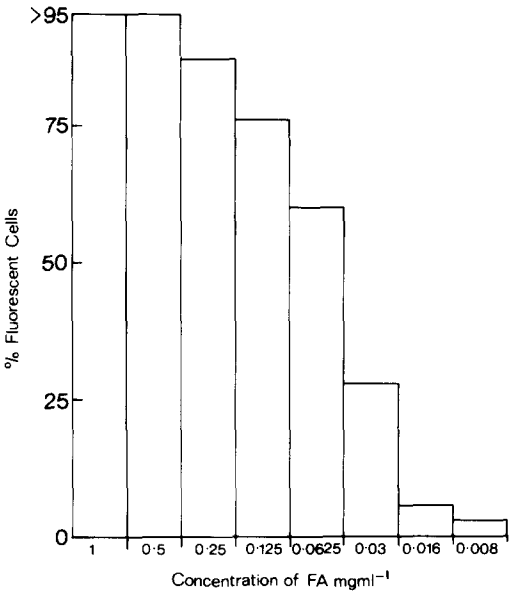

FIGURE 1 Insertion of FA glycolipid into mouse thymocytes.

can also be inserted into the membranes of a variety of other cell types which do not normally express this antigen, including lymphomas and fibroblasts (Table I). Not all cell types incorporate the same amount of the glycolipid, as judged by the relative intensities of their fluorescence. For example, the Abelson lymphoma cells are only weakly fluorescent whereas the other cell types exhibit much stronger fluorescence. Following the initial incubation with Forssman glycolipid, thymus, N115, P815, and BW cells were washed and incubated for a further 18 h in RPMI-1640 containing 10% CS. These cells were then labeled with M1/22.25 antibodies and R.anti-Rat Ig-Fl; the majority of cells retain the added FA which is thus stably bound to these cells. When the FA-incubated cells are labeled with directly conjugated fluorescent M1/22.25 antibodies, they show a smooth, nonpatchy membrane fluorescence (data not shown). A similar distribution is obtained if, after the FA incubation, the cells are prefixed with glutaraldehyde and indirectly labeled (Fig. 2 *a* and *b*). When the FA-positive cells are labeled at 4°C in the presence of azide by indirect immunofluorescence, the antigen is observed to have a patchy distribution over the whole cell (Fig. 2 *c* and *d*).

The smooth distribution of the inserted FA as visualized by direct immunofluorescence or indirectly following fixation suggests that the antigen is not simply sticking to the surface of the cells. To investigate this further, P815 cells with inserted

TABLE I

Insertion of FA Glycolipid into Various Cells

Cell line	Strain	Cell type	Fluorescent cells +FA*	Fluorescent cells −FA‡	Capped cells
			%		%
EL4	C57BL/6	Thymoma	>95	0	>95
B10A.A2.P5	B10A	Abelson lymphoma	>95	0	40
BW 5147	AKR	Thymoma	>95	0	>95
P815	DBA/2	Mastocytoma	>95	0	27
N115	A/J	Neuroblastoma	>95	0	17
PY-3T3	Swiss	Polyoma transformed fibroblasts	>95	0	14

Cells were incubated (*) in FA or (‡) in its absence, and then indirectly labeled at 4°C and in azide. An aliquot of the labeled cells was washed in PBS containing 10% CS and incubated for a further 30 min at 37°C, and the proportion of cells with caps (<1/3 cell surface fluorescent) was counted.

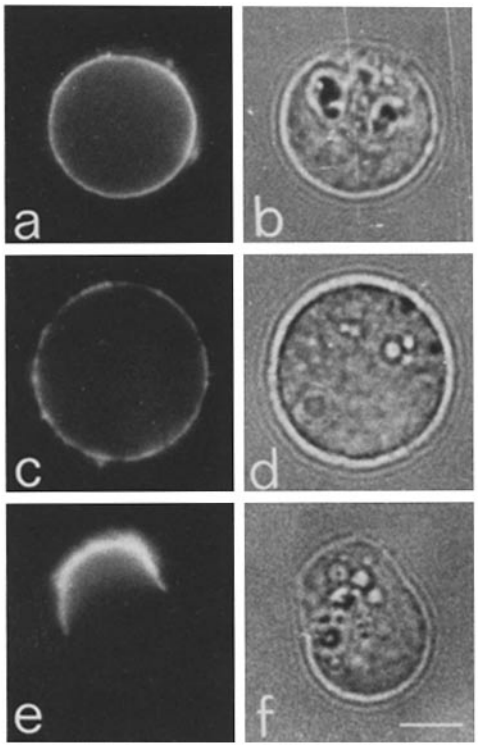

FIGURE 2 Distribution of inserted FA on P815 cells. (*a* and *b*) Fixed cells were indirectly labeled at 37°C. (*c* and *d*) Cells were indirectly labeled at 4°C in azide. (*e* and *f*) Cells were indirectly labeled at 37°C. Fig. 2*a*, *c*, and *e*: fluorescence; Fig. 2*b*, *d*, and *f*: bright field. Bar, 5 μm; × 1,700.

FA were indirectly labeled and visualized at the electron microscope level using ferritin-conjugated second layer antibodies. The distribution in embryonal carcinoma cells, a naturally FA-positive cell type (10), was examined for comparison under the same labeling conditions (azide, 4°C). This is shown in Fig. 3; both cell types, FA positive by incorporation (Fig. 3*a*) or biosynthesis (Fig. 3*c*), have ferritin molecules distributed in patches fairly evenly over the surface of the cell and in close apposition to its membrane. No labeling of P815 cells is seen without prior incubation with FA (Fig. 3*b*) or of embryonal carcinoma cells if M1/22.25 antibodies are omitted from the labeling procedures (Fig. 3*d*). When naturally or inserted FA-positive cells are prefixed and then indirectly labeled with ferritin-conjugated antibodies, the ferritin molecules are found uniformly distributed over the whole cell's surface (data not shown). These results suggest that the exogenously added glycolipid has inserted into the lipid bilayer of the cell.

When the inserted glycolipid is cross-linked by addition of M1/22.25 antibodies followed by R.anti-Rat Ig-Fl under conditions which allow the redistribution of cross-linked membrane antigens (no azide, 37°C), all the cell types examined exhibited cells on which the membrane fluorescence had moved to the pole of the cells and formed caps (Table I and Fig. 2*e* and *f*).

Antibody-induced redistribution of endogenous membrane antigens to form caps is known to be sensitive to metabolic inhibitors (azide, 2,4-dinitrophenol [DNP]) and to the combined effects of colchicin and cytochalasin B (11). P815 and EL4 cells, which had been incubated in FA, were tested for their ability to cap the antigen in the presence of various drugs. The cells were incubated with M1/22.25 at 23°C, washed, incubated with R.anti-Rat Ig-Fl at 37°C and washed, all these stages being carried out in the presence of the drug in

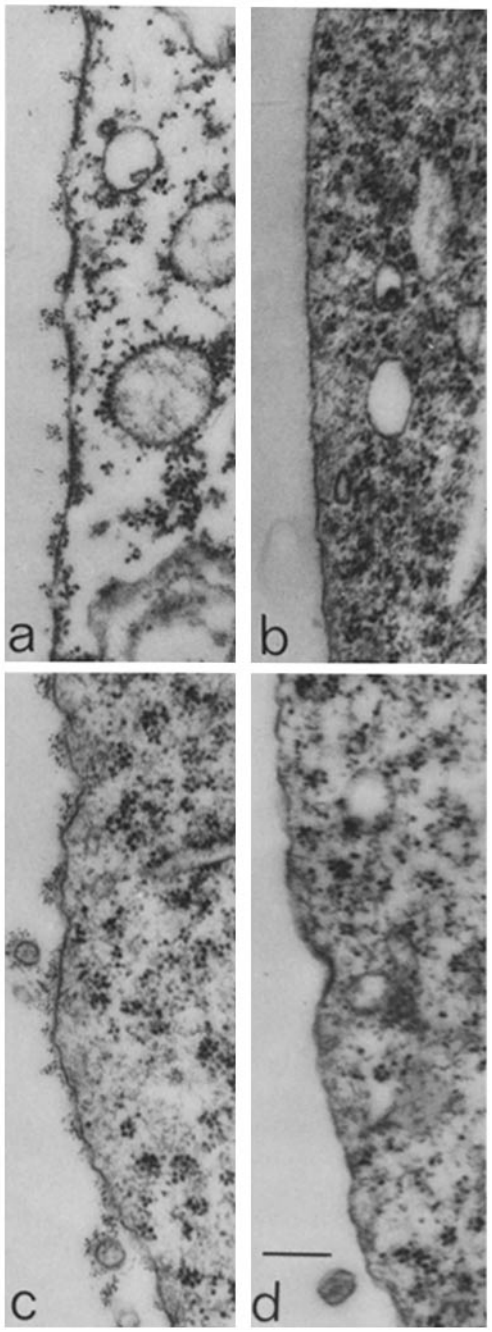

FIGURE 3 Immuno-electron microscopy: Labeling of cells with M1/22.25 antibodies and R.anti-Rat Ig-Ft. (*a*) P815 cells preincubated with 1 mg/ml FA, labeled at 4°C in azide. (*b*) P815 cells preincubated in RPMI-1640 and labeled as for Fig. 3*a*. (*c*) Nulli-SCCl embryonal carcinoma cells labeled as for Fig. 3*a*. (*d*) Nulli-SCCl embryonal carcinoma cells labeled as for Fig. 3*b* omitting M1/22.25 antibodies in the first stage. Ferritin molecules are seen in clusters close to the membrane on P815 cells with inserted FA (*a*). No labeling of P815 cells occurs without FA treatment (*b*). The distribution of ferritin molecules on the membrane of Nulli-SCC1 embryonal carcinoma cells, a naturally FA-positive cell type, is similar to that seen with P815 cells treated with exogenous FA (*c*). No labeling of the embryonal carcinoma cells occurs if M1/22.25 antibodies are omitted from the labeling procedure (*d*). Bar, 0.25 μm. × 35,000.

TABLE II

Inhibition of Capping of Inserted FA Glycolipid

Pretreatment	P815	EL4
	% capped cells	
None (1% DMSO)	26.8	73.0
Colchicin (10^{-4} M)	21.7	72.7
Cytochalasin B (10 μg/ml)	20.4	32.3
Colchicin (10^{-4} M) + cytochalasin B (10 μg/ml)	5.8	3.5
DNP (10 mM)	3.4	3.0

All drugs (except colchicin) were added from a 100 × stock solution in DMSO.

question. The data in Table II illustrate that these drugs affect capping of inserted FA glycolipid in a manner similar to their effects on the capping of protein antigens.

DISCUSSION

We have taken a glycolipid, FA, and added it to the membranes of a variety of motile cells. Although it is never possible to state with certainty that a newly added lipid becomes inserted into the lipid bilayer of the recipient cell's plasma membrane, our evidence—the stability of association and the electron microscope study—is compatible with correct insertion. This glycolipid, when cross-linked by antibodies, behaves like a normal membrane antigen with respect to ligand-induced redistribution. Previously, other workers have shown that the glycolipid, GM1, will redistribute on cells when cholera toxin, bound to it, is cross-linked (4, 9). It might be argued, in their case, that the glycolipid (which is normally present in their cells) or the toxin binds to an endogenous protein and that the redistribution reflects, in effect, the cross-linkage of these receptors. There are two reasons why we think this possibility is unlikely here. First, we have studied a variety of cell types (Table I), all normally FA negative. If capping were due to specific association of FA with a protein in the membranes of these cells, all these cell types would have to have such receptors. Second, it is improbable that the pentasaccharide head group of FA could bind to a specific antibody binding site and a separate protein receptor at the same time.

Because glycolipids can extend only into a bilayer but not across it, these results indicate that the process of capping of these antigens (and, possibly, other antigens) cannot be mediated by direct linkage of a cytoskeletal network to the patch. They do not rule out schemes involving hypothetical molecules X (2), which specifically recognize the cross-linkage of antigens on the outside of the cell, although we believe such models unlikely. That cross-linked glycolipids cap can be fitted into a scheme for capping in which either the membrane or the bulk phase lipids are in a continual state of directed flow (1, 3). The patched lipids would simply move along with the flow, away from the region of the cell where membrane or lipid is inserted.

There are three additional points we should like to make. First, a flow scheme requires a separation between the sites of insertion and withdrawal of membrane, but they need not both be unique sites. In a fibroblast, the site of insertion is most likely the ruffling membrane at the leading edge, and the site of membrane retrieval the whole cell's surface. Second, the two extreme schemes for capping (by direct interaction with the cytoskeleton or by a flow mechanism) are not the only viable models. For instance, it is conceivable that a motile cell continually rakes its surface, the teeth of the rake being situated across the plasma membrane and the handle inside the cell (perhaps being part of the cytoskeleton) (6). This is similar to a flow scheme, in the sense that no specific recognition of cross-linkage is involved. In both cases, any object of sufficient size (which gets caught in the teeth of the rake or cannot diffuse against the flow) would cap. Finally, it is widely assumed that the effects of cytochalasin B and colchicin on capping are due to their inhibition of the functions of microfilaments and microtubules, respectively. If this is in fact so, then the observations that neither drug alone eliminates capping and that they have a synergistic effect indicate that the role of microfilaments and microtubules in the capping process is not a simple one.

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