

Labeling of Animal Cells with Fluorescent Dansyl Cerebroside

Richard T. C. Huang

Institut für Virologie, Justus Liebig-Universität, Gießen

(Z. Naturforsch. 31 c, 737–740 [1976]; received September 9, 1976)

Fluorescence Labeling, Dansyl Glycolipids, Glycolipid Patches, Plasma Membrane, Myxoviruses

A dansyl (diaminonaphthalenesulfonyl)-derivative of cerebroside was prepared which could be effectively incorporated into the plasma membranes of tissue culture cells and erythrocytes. The cells which had assimilated the glycolipid fluoresced intensely and could be observed under a fluorescent microscope. Cells were initially labeled rather homogeneously over the whole surface. With longer incubation time organization of the fluorescent glycolipid took place and patches of the lipid in the membrane were formed. The redistribution and organization of the membrane lipid could be demonstrated most clearly when cells labeled with this fluorescent glycolipid were infected with myxoviruses. After infection of MDBK and BHK cells with fowl plague virus areas of dense fluorescence appeared at margins of neighboring cells. When BHK cells were infected with Newcastle disease virus fusion of the cells was accompanied by complete redistribution of the glycolipid. Erythrocytes could also easily incorporate dansyl cerebroside. Chicken erythrocytes which contain cytoplasmic and nuclear membranes incorporated the fluorescent glycolipid in both membranes.

Introduction

Fluorescent probes have been introduced into biological membranes in the past and changes in the intensity of fluorescence occurring have been measured to interpret the fluidity and polarity of the environment¹. The probes were thought to be incorporated in the lipid membranes. However, most probes thus far applied were ionic and they also reacted with proteins and other groups. Further, the probes were usually toxic and in addition did not cause emission of fluorescence sufficiently intense for microscopic observations. The lateral organization and dynamics of lipids in the membrane could be directly studied microscopically if a typical membrane lipid could be labeled with fluorescence and assimilated into the membrane by a biological process. For such an approach glycosphingolipids would be good candidates since they are specific components of plasma membranes^{2–4}. Thus, cerebroside, a typical glycosphingolipid, can be easily converted into a fluorescent dansyl derivative via psychosine, a deacylated cerebroside. The present work reports about the integration of this fluorescent glycolipid into plasma membranes of animal cells and about its potential use as a probe for studying various phenomena relating to dynamics of the lipids of plasma membranes.

Material and Methods

Preparation of dansyl cerebroside

Pure human brain cerebroside (5 g) was treated with barium hydroxide according to the procedure of Klenk⁵ to yield psychosine (3.2 g). The psychosine (1 g) was mixed with 1 g of dansylchloride (Serva, Heidelberg) and dissolved in 50 ml of ethanol. 10 ml of 1 M NaHCO₃ (preadjusted to pH 10 with NaOH) was added slowly while stirring and the pH of the mixture was maintained at 10 with an automatic titrator. After a period of 2 h the mixture was neutralized with HCl, evaporated to near dryness in a rotary evaporator under vacuum. The residue was then dissolved in 200 ml of Folch⁶ lower phase and washed twice with 100 ml of Folch upper phase. The lower phase which contained the dansyl sphingosine was evaporated to dryness and purified in a column of Kieselgel 60; mesh 70–320 (3 cm × 45 cm). 300 ml each of the following solvents, chloroform, 5% methanol in chloroform, 10% methanol in chloroform, 20% methanol in chloroform and 50% methanol in chloroform were used to elute the lipid. Dansyl sphingosine was eluted with 5% methanol in chloroform and dansyl cerebroside with 20% methanol in chloroform. In practice, 20 ml fractions were collected and fractions were monitored for purity by thinlayer chromatography, using chloroform/methanol/water 65/35/8 as the developing solvent⁷. Under these conditions dansyl sphingosine and dansyl cerebroside had *R_F* values of 0.96 and 0.79. Spots on thinlayer plates were easily visualized under UV-illumination.

Requests for reprints should be sent to Dr. R. T. C. Huang, Institut für Virologie der Justus-Liebig-Universität, Frankfurter Str. 107, D-6300 Gießen.

Chromatographically pure dansyl cerebroside fractions were then pooled and dried under vacuum.

Cell cultures

MDBK and BHK cells were grown in plastic petri dishes in reinforced Eagle's medium or Dulbecco's medium with 10% calf serum according to Choppin⁸.

Labeling of cells with dansyl cerebroside

To ease solubilization dansyl cerebroside was dissolved with the aid of Tween 20. The following procedure proved most satisfactory. 100 mg each of dansyl cerebroside and Tween 20 (Merck, Darmstadt) were mixed and dissolved in 2 ml of ethanol. After evaporation of ethanol in a rotary evaporator the residue was suspended in 1 ml of sterile PBS and sonified briefly till the solution became clear. This was then used as the stock solution of dansyl cerebroside. To label the cells, the solution of dansyl cerebroside was added to the culture medium in an amount equivalent to 50 μ g per ml of culture medium for cell cultures and 100 μ g or 200 μ g per ml of suspension for erythrocytes. Incubation was then continued for the time intervals as described. After the incubation period the dansyl cerebroside containing medium was replaced by appropriate culture media. The cells were then maintained in these media until observation. The labeled erythrocytes were washed three times with PBS.

Isolation of plasma membranes of labeled cells

The procedure of Atkinson and Summers⁹ was employed, except that the step for removal of nuclei was omitted. Plasma membrane ghosts which layered at the 30 to 45 percent sucrose interphase was collected for microscopic observation.

Infection of labeled cells with viruses

Fowl plague virus, strain Rostock and Newcastle disease virus, strain Italien were used to infect cells. The multiplicity was about 50 PFU/cell. After an adsorption period of 30 min, the inoculum was removed and cells washed and overlaid with 4 ml of Earle's medium. Cells were then observed microscopically (Zeiss UV-fluorescence microscope) at designated time intervals.

Analysis of fluorescent lipids

Cells which were labeled with the dansyl cerebroside were incubated further for 24 h. The monolayers were then washed with PBS and scraped off

the plates and lyophilized. The dried residue was extracted with chloroform/methanol 2/1 and aliquots were analysed thinlayer chromatographically using precoated Kieselgel 60 plates and the solvent system of chloroform/methanol/water 65/35/8. Spots of fluorescence were measured under UV-illumination.

Results

A suitable condition for labeling the cells with fluorescence was first tested. Confluent cells were incubated in appropriate culture media which contained varying concentrations of dansyl cerebroside. After different time intervals culture media were decanted and monolayers were washed with PBS and observed under a fluorescent microscope. It could be seen that the uptake of fluorescence was dependent on incubation time and concentration of the glycolipid. After a series of experiments it was found that the concentrations of 10–50 μ g per milliliter of culture medium in an incubation time of 5 h was optimal for labeling cells. Under these conditions the glycolipid was not hazardous to cells and MDBK and BHK cells were densely labeled with fluorescence. After replacing the glycolipid containing medium by appropriate culture media cells could be maintained for several days without losing the fluorescence. At higher concentrations of the glycolipid cells may round up and may finally detach from the plates. For the following experiments the condition of 50 μ g of glycolipid per milliliter and incubation time of 2–3 h were chosen.

Initially, cells took up the lipid rather uniformly (Fig. 1 a * and c). After the glycolipid containing medium was replaced by normal culture media and incubation was continued lateral organization of the glycolipid took place. Reorganization of the glycolipid in the membrane was a slow process. With MDBK and BHK cells clusters or patch like aggregates were formed (Fig. 1 b and d) after several hours of incubation. Experiments were undertaken to see if these labeled cells were viable. To do this labeled cells were passed in appropriate media. It was found that cells were fully capable of multiplying and they could be passed for several generations until the fluorescence became too dilute to be seen microscopically. Also, labeled cells were capable of producing normal amounts of viruses,

* Figs 1–3 see Plates on page 740 a and b.

providing additional evidence of their full viability.

Following studies were proceeded to determine whether the incorporated glycolipid was in fact assimilated into the plasma membranes. Firstly, labeled cells were fractionated to isolate the plasma membranes. It was found that appreciable amount of fluorescence was present only in the plasma membrane structure (Fig. 3 a). Secondly, labeled cells were infected with myxoviruses which were thought to specifically modify the plasma membranes during the budding process. The results are demonstrated in Fig. 2. With both cells infection with viruses caused marked rearrangement of the membranes. In non-fusing systems, such as when fowl plague virus was used to infect BHK or MDBK cells, areas of dense fluorescence appeared at cell margins (Fig. 2 a and b). With the only fusing system which we tested i.e. BHK cells infected with Newcastle disease virus, the onset of fusion was accompanied by distribution of the glycolipid throughout the membrane of the syncytia. At the maximum of fusion fluorescence gathered at certain areas and within the fluorescent areas lateral asymmetry was seen in patches or grains of densely packed glycolipids (Fig. 2 c and d).

The last piece of evidence showing the incorporated glycolipid was integrated into the plasma membranes came from experiments with dividing versus unrelated neighboring cells. Thus, when cells divided fluorescence was seen to be transferred to progeny cells, showing that daughter cells acquired lipid constituent of parent cells. However, when pre-labeled MDBK and unlabeled chick embryo cells were seeded together and cocultivated the fluorescence was not transferred from the former to the latter.

Erythrocytes could also be readily labeled with the dansyl cerebroside. Relatively high concentrations of dansyl cerebroside were used to ensure rapid incorporation of the glycolipid. Fig. 3 b and c show chicken erythrocytes labeled under different conditions. It can be seen that cytoplasmic as well as nuclear membranes were marked (Fig. 3 b). Nuclear membrane was more strongly labeled than cytoplasmic membrane and as yet undefined ring like structures were seen to be attached to the nuclear membrane. With increasing incubation time and glycolipid concentration structures suggesting fusion appeared (Fig. 3 c).

Investigation was carried out to examine whether the incorporated dansyl cerebroside could be metabolized by cells. To do this cells were labeled with dansyl cerebroside for 5 h in the usual manner. After this period the incubation medium was replaced by normal culture media and cells were maintained in these media for further 24 h. The lipids were then extracted and analysed by thinlayer chromatography. Approximately the same results were found for both cell types investigated. About 80% of the incorporated dansyl cerebroside remained unchanged (R_F 0.79). Nearly 10% of the total fluorescence appeared as dansyl-sphingosine (R_F 0.96) and the rest of the glycolipid was elongated to slow moving compounds corresponding to sulfatide (R_F 0.71), dihexoside (R_F 0.68) and higher glycolipids (R_F 0.29, 0.27 and 0.19). The chemical nature of the latter elongation products was not rigorously determined.

Discussion

The ready incorporation of dansyl cerebroside into the plasma membrane of cells was conveniently applied for observation of the dynamics of lipid membranes. After cells were labeled with dansyl cerebroside asymmetrical lateral distribution of the glycolipid took place and patches appeared. The process of rearrangement of the lipid in the membrane was time dependent and became very pronounced after overnight incubation (Fig. 1). The observation is in accordance with the concept of fluid mosaic model of the lipid membrane.

The glycolipid was thought to be incorporated into the plasma membranes because of the following reasons: 1. when labeled cells were homogenized and fractionated it was found that fluorescence was present in the plasma membranes, 2. when cells were infected with enveloped viruses the extensive redistribution of glycolipid during the budding process or migration of glycolipid during fusion could be most plausibly explained when the glycolipid was integrated into the plasma membranes, 3. the glycolipid could be transferred only between dividing cells and not between unrelated neighboring cells and 4. erythrocytes were clearly marked by the glycolipid only in the membrane structures.

During the virus multiplication the condensation of fluorescence at the periphery of MDBK and BHK cells is most interesting, although it is not

known whether areas of dense fluorescence actually corresponded to places of budding.

When erythrocytes were labeled it is not clear why the nuclear membrane was also labeled. It seems possible that a continuity exists between the cytoplasmic membrane and the nuclear membrane of avian erythrocytes and that the glycolipid initially inserted into the cytoplasmic membrane was eventually transferred into the nuclear membrane. However, with tissue culture cells nuclear membranes were not labeled by the glycolipid.

The present method provided an easy way to label the lipid of the plasma membrane under normal or specific conditions. The changes occurring could be observed directly under a fluorescent microscope. The method gave for the first time evidence of lateral asymmetry of the membrane lipids visually and showed that arrangement of the lipid in the membranes was liable to change under different circumstances.

From results obtained above dansyl cerebroside can be considered as a specific marker of the lipid of plasma membranes. In a recent work Sedlacek *et al.*¹⁰ also prepared fluorescent gangliosides and found that they could be incorporated into lymphocytes and functioned as receptors for tetanus toxin. Gangliosides are strongly negatively charged and might be involved in multi-ionic reactions with components of the membranes including proteins. For the purpose of studying dynamics of the lipid membrane neutral glycolipids such as the presently described dansyl cerebroside would appear more suitable than gangliosides.

The discussion and valuable suggestions of R. Rott and H.-D. Klenk are very much appreciated. Also the technical assistance of W. Berk and E. Otto is gratefully acknowledged. The work was supported by the Sonderforschungsbereich 47 (Virologie).

¹ L. Brand and J. R. Gohlke, *Ann. Rev. Biochem. Palo*, 843–868, Palo Alto, California 1972.

² E. D. Korn, *Science* **153**, 1491–1498 [1966].

³ J. S. O'Brien, *J. Theoret. Biol.* **15**, 307–316 [1967].

⁴ D. B. Weinstein and J. B. Marsh, *J. Biol. Chem.* **244**, 4103–4111 [1969].

⁵ E. Klenk, *Hoppel-Seyler's Z. Physiol. Chem.* **153**, 74–82 [1926].

⁶ J. Folch, M. Lees, and G. H. Sloan Stanley, *J. Biol. Chem.* **226**, 497–510 [1957].

⁷ R. J. Penick, M. H. Meisler, and H. McCluer, *Biochim. Biophys. Acta* **116**, 279–287 [1966].

⁸ P. W. Choppin, *Virology* **39**, 130–134 [1969].

⁹ P. H. Atkinson and D. F. Summers, *J. Biol. Chem.* **246**, 5162–5175 [1971].

¹⁰ H. H. Sedlacek, J. Stärk, F. R. Seiler, W. Ziegler, and H. Wiegandt, *FEBS Letters* **61**, 272–276 [1976].

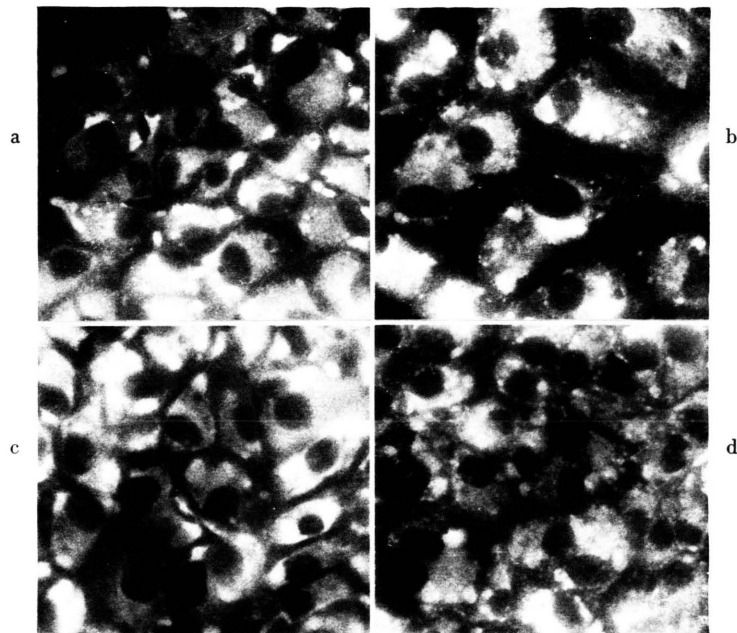


Fig. 1. Labeling of MDCK and BHK cells with dansyl cerebroside. a. MDCK, 2 h labeling; b. MDCK, 2 h labeling, then overnight incubation; c. BHK, 2 h labeling; d. BHK, 2 h labeling, then overnight incubation. Labeling conditions were as described in the experimental section. $\times 1200$.

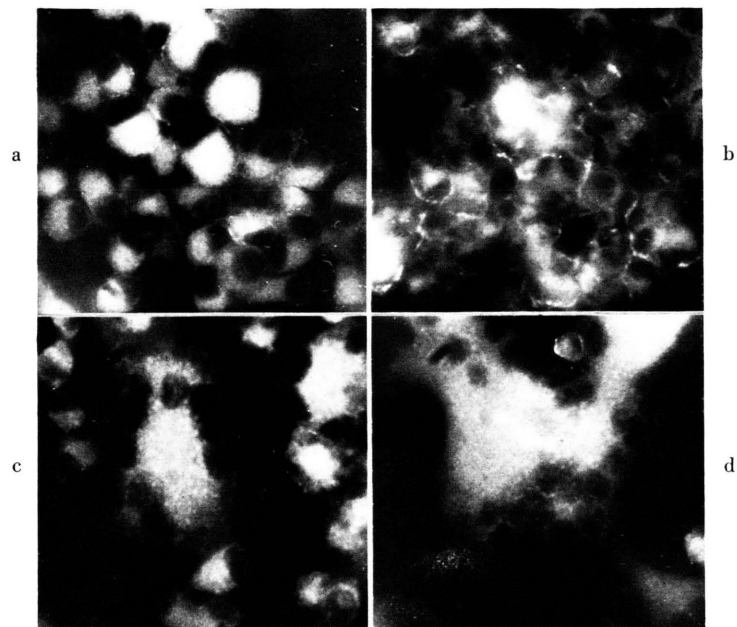


Fig. 2. Labeling of virus infected MDCK and BHK cells with dansyl cerebroside. All cells were labeled for 3 h with dansyl cerebroside, then infected with fowl plague or Newcastle disease virus. a. MDCK cells, infected with fowl plague virus (16 h p.i.); b. BHK cells, infected with fowl plague virus (16 h p.i.); c. BHK cells, infected with Newcastle disease virus (9 h p.i.) and d. BHK cells, infected with Newcastle disease virus (11 h p.i.). $\times 1200$.

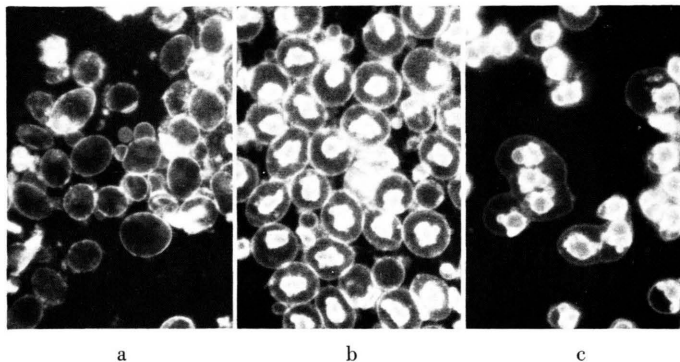


Fig. 3. Membrane structures labeled with dansyl cerebroside. a. Ghosts of plasma membrane of MDBK cells. b. Chick erythrocytes, labeled with $100\text{ }\mu\text{g}$ of dansyl cerebroside for 2 h. c. Chick erythrocytes, labeled with $200\text{ }\mu\text{g}$ of dansyl cerebroside for 5 h. Other labeling conditions as described in the experimental section. $\times 2500$.