Biochimica et Biophysica Acta, 1023 (1990) 357–364
Elsevier

BBAMEM 74815

Giant liposomes as model membranes for immunological studies: spontaneous insertion of purified K1-antigen (poly-α-2,8-NeuAc) of *Escherichia coli*

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(Received 26 September 1989)

Key words: Giant liposome; Liposome; Model membrane; Flow chamber; Antigen insertion; Capsular polysaccharide; Immunofluorescence; ELISA

A flow chamber has been constructed to use giant liposomes (diameter 5–50 μm) as model membranes for immunological studies and other experiments involving the interaction with water-soluble compounds. As an example of immunological importance, the insertion of purified K-antigen from *Escherichia coli* K1 has been studied. Despite its large hydrophilic part (poly-α-2,8-NeuAc), which is capped at its potential reducing end with phosphatidic acid acting as a lipid anchor group, this water-soluble material is readily incorporated into liposomal membranes of dimyristoylphosphatidylcholine (DMPC). The incorporation has been proven by immunofluorescence using a FITC-labeled monoclonal anti-K1-IgG. Without the lipid residue, however, no binding of poly-α-2,8-NeuAc to the liposomes has been observed. This could be shown by using colominic acid, an oligomeric form of α-2,8-NeuAc with free reducing ends instead of purified K1-antigen. The possibility for further manipulation of this model system has been shown by using a poly-α-2,8-NeuAc cleaving enzyme (endoneuraminidase). The function of the endoneuraminidase has been proven by showing no binding of the antibody after enzyme treatment of K1-bearing liposomes as well as by rapid loss of fluorescence of a previously bound FITC-antibody.

Introduction

The structure and function of biological membranes as well as their interaction with various signal molecules have attracted the interest of cell biologists, biochemists, and biophysicists for a long time. In order to study the structure-property relationships of individual components of biological membranes at a supramolecular level, investigations have relied increasingly on model membranes [1].

One of these model membrane systems consists of lipid monolayers at the air/water interface, which supply precise information about the molecular packing and orientation of the amphiphiles. Also, the interaction of the monolayer with substances (e.g., proteins) dissolved in the subphase has been studied [2]. Using fluorescence microscopy [3,4], it is possible to directly visualize the specific recognition of proteins at monolayers as well as protein crystallization [5,6].

Planar lipid bilayers resemble much more the structure of biological membranes. Apart from the free-standing black lipid membranes (BLM) [7], supported planar membranes have been developed in the last few years. Especially when combined with the technique of fluorescent excitation in an evanescent radiation field (TIRF), substrate supported membranes became very attractive for surface recognition studies [8].

A more simple and more realistic model membrane system are spherically-closed lipid bilayers (liposomes or vesicles), which, in analogy to the cell membrane, enclose an aqueous compartment. For reconstitution

Abbreviations: cps, capsular polysaccharide; DMPC, dimyristoylphosphatidylcholine; ELISA, enzyme-linked immunoadsorbant assay; FITC, fluorescein isothiocyanate; GUV, giant unilamellar vesicle; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulphonic acid; mAb, monoclonal antibody; NeuAc, *N*-acetylneuraminic acid; PBS, phosphate-buffered saline.

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experiments and recognition reactions, mostly small liposomes (diameter 100–500 nm) have been used so far [9]. If, however, the interaction of water-soluble components with liposomal membranes is to be followed directly under the light microscope to observe phenomena like aggregation, patching [10], or other morphological changes [1,11,12], giant unilamellar vesicles (GUVs) of 5–50 μm in diameter must be used. Especially when working with fluorescence-labeled compounds, a separation of the unbound components (background fluorescence) from the actual model membrane is an important step. Gel permeation chromatography as well as several centrifuge/washing cycles are problematic due to the GUVs inherent mechanical instability. Furthermore, these procedures do not allow the observation of all stages of the interaction under the microscope. It is desirable, therefore, to exchange the medium of the liposomes in situ while they are on the microscope stage. One very elegant method to do this is the technique developed by Evans to hold an individual giant liposome with a micropipette [13].

Here, a more convenient method is presented, the GUVs are formed by swelling a dry lipid film in a special flow-chamber. Since there is no transfer step or agitation involved, the liposomes are not freely suspended. In fact, most of them remain connected to the residual lipid film at the glass slide of the chamber. Thus, they can be kept in place during a gradual exchange of the medium or injection of additional components. Using this method, the morphological changes of giant liposomes due to their interaction with synthetic amphiphilic polymers have already been studied [12]. Here, one example of immunological importance using this method is presented: the insertion, recognition and manipulation of the K1-antigen of *Escherichia coli* in liposomal membranes.

Among Gram-negative bacteria, *Escherichia coli* K1 and *Neisseria meningitidis* B strains, whose capsules are chemically and immunologically identical, cause serious infections. The capsular polysaccharides (cps) of these strains have been shown to be an important pathogenicity factor [14]. Strong humoral immune response against the bacterial capsules as major cell surface structures of the bacteria is not achieved since their chemical structures resemble those of host determinants (embryonic neural cell adhesion molecule) and thus are not recognized as foreign, a phenomenon known as antigenic mimicry [15]. As a consequence, the poor immunogenicity results in escape of the pathogen from antibody-mediated host defenses, including certain complement-dependent bactericidal mechanisms and phagocytotic events. Because of its poor immunogenicity, antibodies against the K1-antigen have been difficult to produce. Recently, by using the hyperreactive NZB autoimmune mouse it has been possible to obtain a monoclonal antibody of subclass IgG against poly-α-2,8-NeuAc of *Neisseria meningitidis* B [16].

The K1-antigen consists of a large hydrophilic part, a homopolymer of about 200 α-2,8-linked NeuAc residues [17], which is connected to a 1,2-diacylglycerol through a phosphodiester linkage (Fig. 1). It has been proposed that the phospholipid may play a role in linking the capsule to the membrane, although no direct evidence has been presented [18,19]. The problem addressed here is whether the readily water-soluble K1-antigen incorporates spontaneously via its lipid anchor group into liposomal membranes, and if, in addition, the K1-antigen-bearing giant liposomes in the flow-chamber present a suitable model for studying functions of the K1-antigen.

Materials and Methods

Chemicals. DMPC (puriss.) was obtained from Fluka and used without further purification. Colominic acid was purchased from Sigma, poly-α-2,8-NeuAc from *Neisseria meningitidis* from Connaught Lab. Inc., Swiftwater, U.S.A. Water was purified by using a Millipore Milli-Q system (> 18 Mohm).

mAb735. The production of the hybridoma cell line 735D4 excreting a monoclonal antibody specifically recognizing poly-α-2,8-NeuAc of *Neisseria meningitidis* B or *Escherichia coli* K1 has been described [16]. For production of ascites fluid, hybridoma cells were injected intraperitoneally into Pristane-pretreated BALB/c AnN mice previously irradiated with 300 rad (3 Gy). For covalent coupling of FITC, mAb735 was purified from ascites fluid by affinity chromatography on protein A-Sepharose and dialyzed against carbonate buffer (0.3 M, pH 9.5). FITC (Serva, Heidelberg) was dissolved in DMSO to a final concentration of 2 mg/ml and added to the antibody solution (10 mg protein/ml)

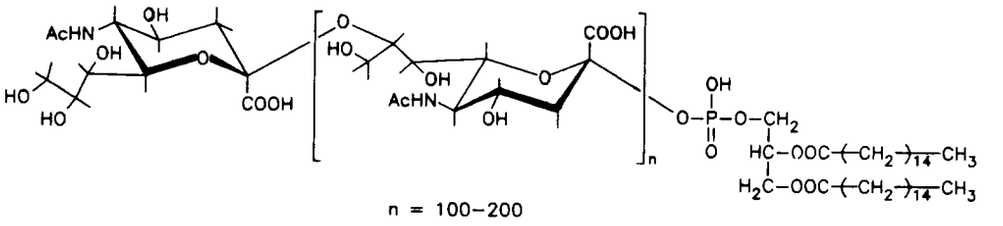

Fig. 1. Chemical structure of K1-antigen of *Escherichia coli.*

to a maximal concentration of 10% DMSO (v/v). The coupling mixture was separated from excess FITC by passage over a desalting column (PD10, Pharmacia, Freiburg) and purified by ion exchange chromatography on DE52 (Whatman) (1.5 × 5 cm). The conjugate mixture was applied in 17.5 mM sodium phosphate buffer (pH 6.3) and the column washed with three column volumes. FITC-mAb735 was eluted in sodium phosphate buffer containing 0.125 M NaCl or 0.25 M NaCl.

Isolation of poly-α-2,8-NeuAc from Escherichia coli K1. A heavily encapsulated strain of *Escherichia coli* K1 (B2032/82, a clinical isolate) was grown in a 6 l laboratory fermenter (HWS, Mainz) in 5.5 l phosphate-buffered rich medium containing 60 g bactotryptone, 60 g yeast extract, 130 g K_2HPO_4, 32 g KH_2PO_4, and 80 g glucose. Growth was promoted with an oxygen supply of 12 l/h until late-logarithmic phase ($A_{600} = 8–9$). Cells were collected by centrifugation (8000 × *g*, 10 min) and Cetavlon (Sigma, Munich) was added to the supernatant to reach a final concentration of 0.1%. The pellet was resuspended in 500 ml 1 M $CaCl_2$, 0.1% Cetavlon and extracted by treating the suspension with a Polytron (Model PT 10 OD, Bachhofer, Reutlingen) two times for 3 min under cooling (ice/water). The suspension was combined with the Cetavlon precipitate of the culture supernate and purification of K1-antigen was performed according to Jann [20]. Final purification was achieved by chromatography on Sephacryl S-300 (0.9 cm × 60 cm) in 1 M NH_4Ac. The K1-antigen was precipitated by 80% ethanol and stored in solution (10 mg/ml 20 mM Tris-HCl, pH 8) at −70 °C. The purity of the polysaccharide preparation was controlled by determining the nucleic acid, protein, lipopolysaccharide and enterobacterial common antigen content as described under analytical methods. The impurities proved to be lower than 0.1% of the chemically determined NeuAc content.

Isolation of endoneuraminidase. Isolation of a poly-α-2,8-NeuAc specific endoneuraminidase was performed according to Tomlinson and Taylor [21] with slight modifications. In short, an *Escherichia coli* K1 wild-type strain (U9/41) was grown in a 6 l laboratory fermenter (HWS, Mainz) in phosphate-buffered rich medium (see above). After inoculation (15%), growth was continued until an optical density (600 nm) of 3 (approx. 10^{13} bacteria) was reached. Then, a freshly prepared lysate of K1 Phage E (10^{12} pfu) was added to give a m.o.i. of about 0.1. After addition of 1 ml antifoam concentrate (Sigma, Munich) incubation was continued at 37 °C under slight stirring until the optical density had decreased to 0.2. The crude lysate was kept frozen at −60 °C until further use. Portions of 1 l crude lysate were used to isolate endoneuraminidase as described by Tomlinson and Taylor [21]. Final purification was achieved by replacing the ion exchange chromatography on DEAE-Sephadex by FPLC on Mono Q (Pharmacia, Freiburg). Activity of endoneuraminidase was monitored using poly-α-2,8-NeuAc isolated from *Escherichia coli* K1 by determining the amount of NeuAc oligosaccharides liberated by the enzyme as described [22].

Analytical Methods

Poly-α-2,8-NeuAc by sandwich fluorescence ELISA. Polystyrol microtiter plates (Greiner, Nürtingen) were coated with 20 µl protein A purified monoclonal antibody mAb735 (50 µg/ml) overnight at 4 °C. After saturation and washing with PBS (pH 7.4) containing 1% bovine serum albumin (BSA), 20 µl of the sample were added and the plate was incubated for 1.5 h at room temperature. The microtiter plates were washed three times with PBS and incubated with galactosidase-labeled monoclonal antibody mAb735 in PBS-BSA for 1 h. After washing, 50 µl of a 4-methylumbelliferyl-β-D-galactoside solution (Sigma, Munich) at a concentration of $5 \cdot 10^{-5}$ M (dissolved in PBS, pH 6.9) were added and galactosidase activity was measured as relative fluorescence units in a Titertek Fluoroskan I (Flow Lab., Meckenheim). Poly-α-2,8-NeuAc concentration was determined via 50% values using purified poly-α-2,8-NeuAc of *Escherichia coli* K1 as a standard.

Assays. Protein was determined by the modified Lowry assay (BCA, Pierce, Rodgau), NeuAc in poly-α-2,8-NeuAc was determined after acid hydrolysis (0.1 M H_2SO_4, pH 2; 80 °C, 4 h) by a modified thiobarbituric acid assay [23]. DNA was determined by UV spectroscopy (OD_{260}). Lipopolysaccharide was determined by ELISA using mAb786, a monoclonal antibody against the outer core of lipopolysaccharide as described [24]. Enterobacterial common antigen was determined by sandwich fluorescence ELISA using mAb898 as described [25].

Construction of the measuring chamber. For the experiments with giant liposomes reported here, a special flow chamber has been constructed (Fig. 2). This chamber allows the gradual exchange of the medium as well as the injection of additional water-soluble components to a preparation of giant liposomes. The main brass body (1) is connected to a circulator to provide temperature control. For assembling the chamber the lower brass part (6) is screwed into the main body tightly in order to hold the upper cover glass (2), the teflon spacer (3) with the inlet and outlet capillaries (4, 7), and the lower cover glass (5) carrying the dry lipid film. A similar chamber has been already described by Fricke and Sackmann [26] for experiments with erythrocytes. Our design has the advantage that the inner part (Teflon spacer (3) with capillaries) can be easily exchanged; additionally the sample is in contact only with glass, teflon and stainless steel. This construction also allows the use of various spacer parts different in size and positioning of the capillaries. The best results were

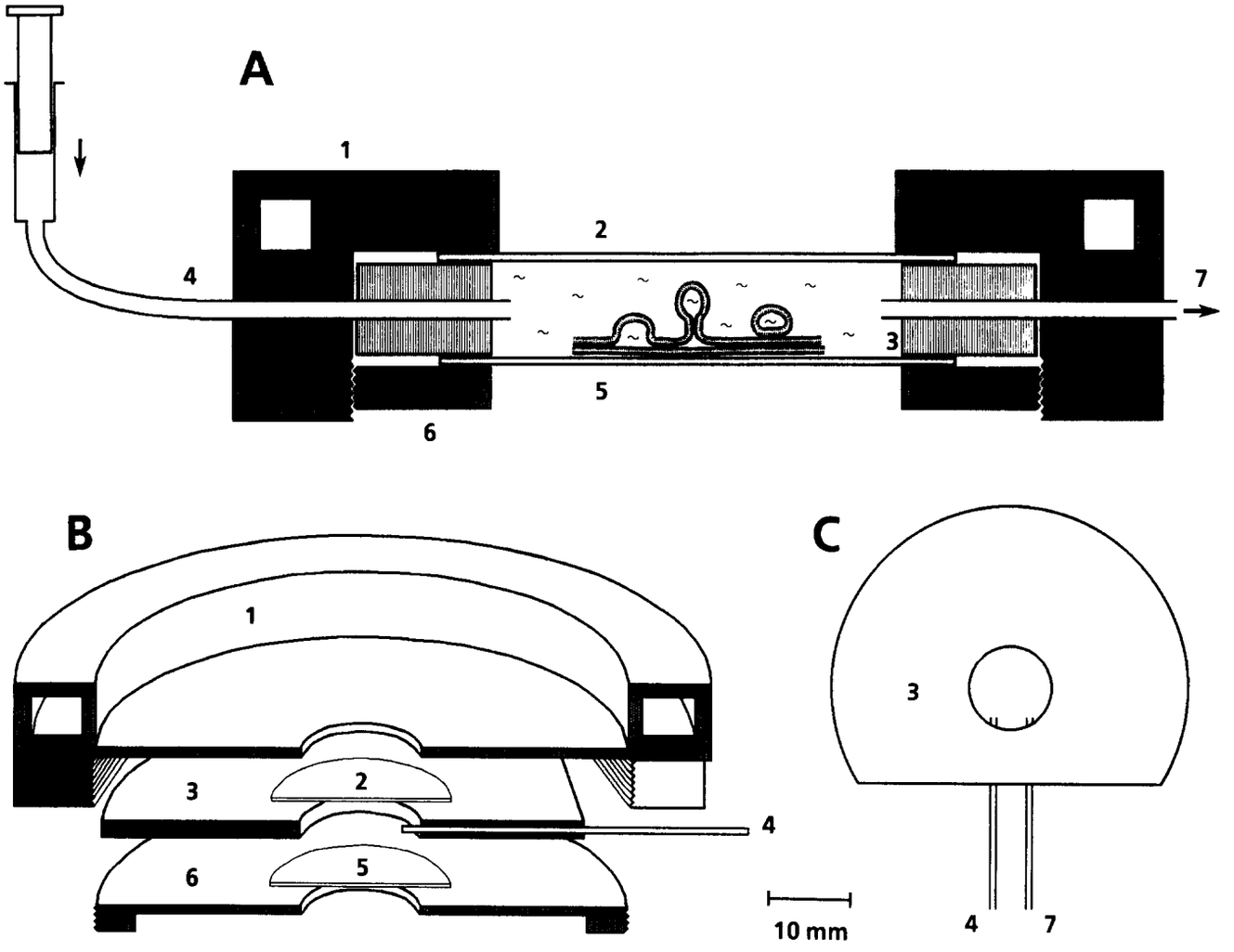

Fig. 2. Chamber for experiments with giant liposomes. The liposomes are prepared directly in the probe volume between the two cover glasses (2, 5) which are fixed by the temperature-controlled brass bodies (1, 6). The height of the probe volume is adjusted by a Teflon spacer (3) holding the inlet and the outlet capillary (4, 7). (A) Schematic cross sectional view of the liposome preparation (not to scale). (B) Cross sectional view of the measuring chamber. (C) Top view of the Teflon part.

obtained when a motor-driven syringe was used (injecting speed 0.5 ml/min) and the inlet and the outlet capillary were close together (Fig. 2C) thus causing less disturbances in the liposome preparation due to shear flow.

Microscope equipment. The observations were made using an inverse microscope Zeiss IM35 with phase contrast and epifluorescence equipment (phase contrast objectives 16 × and 40 ×). The documentation has been done by using a reflex camera as well as a video system (Low Light Level CCD-video camera (Proxitronic) with U-matic video cassette recorder) attached to the microscope. Note: An inverse microscope is necessary for simple optical reasons: the working distance of the 40 × phase contrast objective is 0.7 mm, the distance between the cover glasses is 3 mm, and due to gravity, it does not help to prepare the liposomes at the upper cover glass.

Liposome preparation. Giant liposomes (GUVs) were prepared directly on the cover glass in the flow chamber by hydration of a dry lipid film similar to the procedure first described by Reeves and Dowben [27]. The lipid films were cast from 5 μl of a chloroform solution of DMPC (1 mg/ml) with a subsequent removal of the last traces of the solvent in vacuum. After assembling the chamber, it was filled with cold Hepes-buffer (50 mM, pH 7.0). The hydration of the lipid film was then performed at 28° C (above the phase transition temperature of DMPC) without any agitation to yield preferentially unilamellar giant liposomes of a diameter between 5 and 50 μm.

Results and Discussion

Giant liposomes of DMPC as the model membrane system

In all experiments reported here, GUVs consisted of

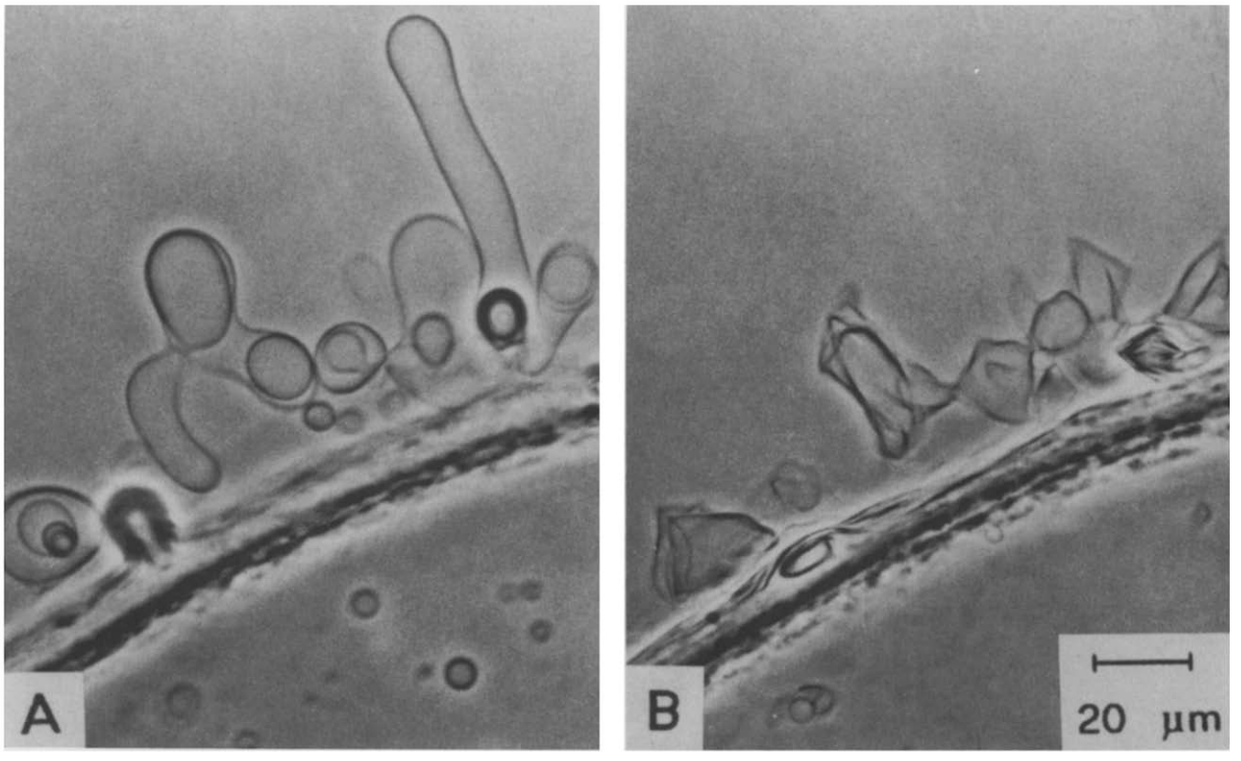

Fig. 3. Giant liposomes of DMPC (A) above (25°C) and (B) below (21°C) the phase transition temperature of the lipid (23°C) (50 mM Hepes pH 7.0).

DMPC as the matrix lipid. The advantage of using this synthetic lipid instead of a natural mixture (e.g., egg phosphatidylcholine) is that DMPC has its phase transition at a convenient temperature (23°C). This phase transition becomes also apparent in the morphology of the GUVs: In the fluid state (above 23°C), the GUVs are flexible and shape fluctuations are clearly visible [28] (Fig. 3A). In the gel state (below 23°C), however, they are stiff, polygonally shaped and no membrane movements can be observed (Fig. 3B). These shape changes can even be used to determine the phase transition temperature of any lipid able to form GUVs, simply by microscopic observation. Thus it is possible to examine the influence of the physical state of the membrane without changing the composition of the model system, just by varying the temperature. In addition, there is another practical reason: By using a lipid with a phase transition temperature in a practical temperature range, all injections can be done in the gel state of the GUVs. This is important because GUVs in the fluid state are extremely sensitive to shear forces resulting in a transformation of the fluid GUVs into very long (up to 500 µm) tube-like structures, even if the injection speed is very slow (e.g., 1 ml/h).

Insertion of K1-antigen into giant liposomes

In a typical insertion experiment, the GUVs were prepared by swelling a lipid film at 28°C for 2 h and were then cooled to 18°C. Subsequently, 3 µg of K1-antigen in 100 µl buffer were injected. After incubation at 28°C for 30 min, the preparation was again cooled to 18°C and washed with 2 ml of buffer. Then 8 µg of the FITC-labeled mAb735 in 100 µl of buffer was added to detect whether the K1-antigen was attached to the liposomal membranes. Because of the background fluorescence of unbound, excess antibody, the result of the recognition reaction has been hard to evaluate directly after addition of antibody. Therefore, after incubation at 28°C, the preparations have been washed again at 18°C with at least 3 ml of buffer to remove free dissolved antibody. A typical result of such an experiment is presented in Fig. 4. The comparison of the phase-contrast micrograph (A) with the corresponding epifluorescence one (B) shows that the fluorescence of the antibody is associated with the membranes, i.e., K1-antigen can be detected at the membranes. In one experiment using a different poly-α-2,8-NeuAc from *Neisseria meningitidis,* the fluorescence of the bound antibody was, after approx. 30 min, no longer homogeneously distributed at the liposome surface (Fig. 5) [1,12]. Such patching has never been observed with the K1-antigen used in the experiments reported here.

To distinguish whether antibody binding to liposomes is caused by incorporated K1-antigen or by unspecific adsorption [29] of antibody, control experiments were done using GUVs without K1-antigen. No

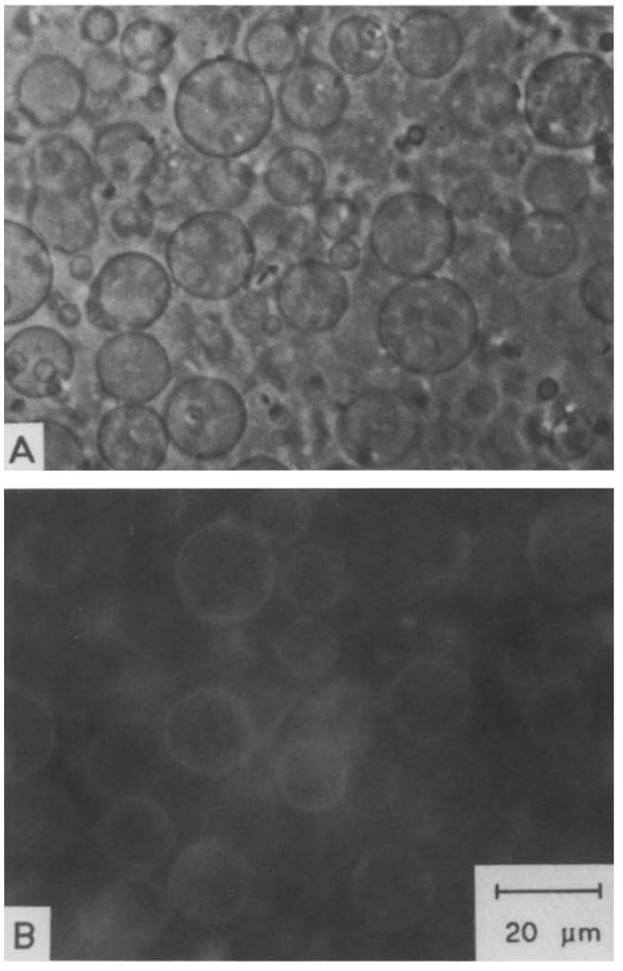

Fig. 4. Binding of FITC-mAb735 to giant liposomes of DMPC incubated with K1-antigen of *Escherichia coli*: (A) phase contrast, (B) epifluorescence (50 mM Hepes (pH 7.0), 18° C).

detectable fluorescence was observed when liposomes were incubated with antibody alone, assuring that unspecific adsorption of the antibody is not relevant in these experiments.

Importance of the lipid anchor group

Since incorporation of the K1-antigen is caused either by insertion of the lipid anchor or by an unspecific (e.g., ionic) interaction of the polysaccharide with liposomal membranes, colominic acid has been used in place of K1-antigen to check for unspecific adsorption of poly-α-2,8-NeuAc to liposomal membranes. Colominic acid consists of oligomers of poly-α-2,8-NeuAc, isolated from weakly buffered medium of *Escherichia coli* cultures [30] where the pH has dropped to about 5. These oligomers are derived from larger membrane associated NeuAc polymers by acid catalyzed hydrolysis as shown by Troy and McCloskey [31]. Thus, the ketosidic linkages are identical to the ones in K1-antigen, yet oligomers are of shorter chain length and have free reducing ends, i.e., do not have a lipid anchor group [32]. Attempts to incorporate colominic acid into lipid membranes did not result in any antibody binding to the liposomes, even when 60 µg of colominic acid (20-fold of the normal amount of K1-cps used) per liposome preparation were used. It has to be noted that the oligomers still have the ability to bind mAb735. This has been shown by sandwich-ELISA as described in Materials and Methods.

An additional proof for the lipid anchor insertion came from experiments using pH-treated K1-antigen. Since the phosphodiester bond by which the lipid is bound to the polysaccharide is acid labile [18,19], purified K1-antigen was treated at 60° C for 1 h at pH 5. The control incubation was performed at pH 7. Treatment at pH 5 resulted in complete loss of incorporation of K1-antigen into liposomes, whereas pH 7-treated material showed no detectable decrease in liposome fluorescence after incorporation and antibody staining. No significant difference was observed by analysis in sandwich-ELISA demonstrating that recognition by mAb735 was retained after treatment at pH 5. Therefore, cleavage of the lipid-polysaccharide bond under mild acid conditions results in loss of K1-antigen insertion into liposomes. For this reason all experiments were performed in buffered medium (pH approx. 7.0). When the insertion experiments were performed in an unbuffered, isotonic (5% w/w) sorbit solution (pH 5.5), complete loss of the membrane bound fluorescence was observed after a few hours. This was probably caused again by the acid catalyzed cleavage of the lipid-polysaccharide bond of the incorporated material.

When considering the chemical structure of K1-antigen, it can be regarded as an amphiphilic polymer: the poly-α-2,8-NeuAc is the large hydrophilic part and the lipid residue is the hydrophobic part. Therefore, the K1-antigen molecules form micelles in water [18,19] which are even stable enough to be separated from monomeric K1-antigen by gel permeation chromatography. Thus, the driving force for the insertion of the lipid residues into membranes is the gain in free energy resulting from the transition of a dynamic, micellar system with partially solvent-exposed alkyl chains to a system in which the hydrophobic residues are situated in the hydrophobic interior of the membrane. This explanation is supported by the finding that the insertion of the lipid anchor group seems to be irreversible: even after extensive washing with buffer (17 ml in 5 h, 18° C) no loss of the antibody binding ability could be observed. Other examples of such an interaction between synthetic amphiphilic polymers and giant liposomes have been described before [12].

Enzymatic manipulation of K1-cps

In order to verify the interpretations of the experiments described above, the incorporated K1-antigen has been treated with a poly-α-2,8-NeuAc cleaving enzyme

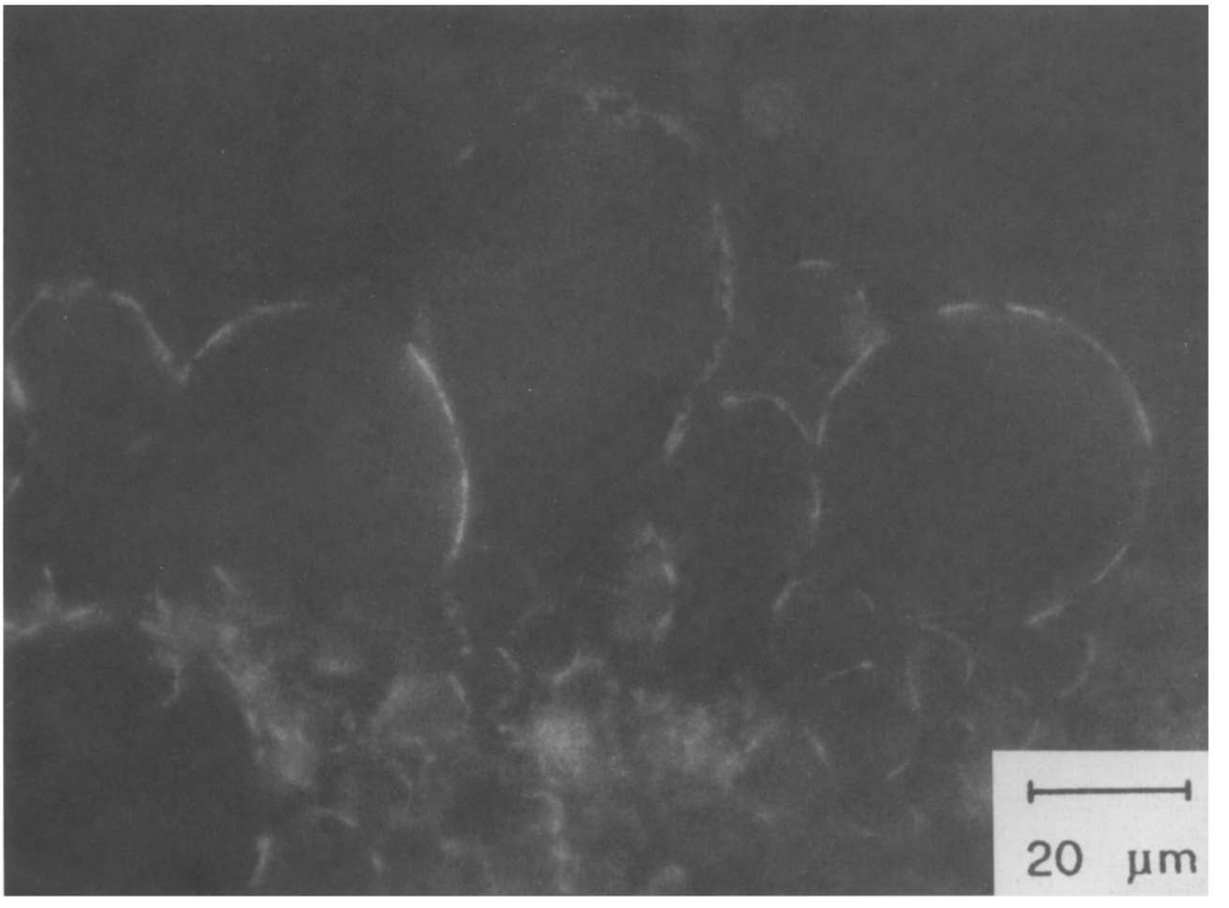

Fig. 5. Patching of FITC-mAb735 on giant liposomes of DMPC bearing poly-α-2,8-NeuAc from *Neisseria meningitidis* (epifluorescence) (5% (w/w) sorbit, pH 5.5, 35 °C).

(endoneuraminidase). These manipulations also show the potential and the versatility of the model membrane system presented. Bacteriophages infecting encapsulated bacteria frequently carry tail spikes as an attachment factor that possess capsule degrading enzymatic function. Isolating the neuraminidase associated with Coliphage E provides a means to selectively depolymerize poly-α-2,8-NeuAc [21].

Two series of experiments were considered: addition of the endoneuraminidase to the K1-antigen-bearing liposomes before and after binding of the mAb735. In both cases, an equal amount of endoneuraminidase (1.3 µg protein) dissolved in 100 µl of buffer has been used. Since the activity of the enzyme was determined to be 0.5 u/mg (1 u = liberation of 1 µmol NeuAc from K1-cps per minute [22]), all K1-antigen present in the liposome preparation should be cleaved theoretically within 10 min.

In the first set of experiments the endoneuraminidase was added to a typical preparation of K1-antigen-bearing GUVs (5 µg DMPC + 2 µg K1-antigen) at 18 °C. After incubation for 90 min at 30 °C and subsequent cooling to 18 °C, 8 µg of FITC-labeled mAb735 were added as usual. After incubation and washing with 2 ml of buffer, no membrane-associated fluorescence was detected. Since no antibody binding after the enzyme treatment could be detected, the chain length of the liposome associated poly-α-2,8-NeuAc has been reduced by the enzyme action to less than 10 NeuAc groups, which is the critical chain length for binding of mAb735 [33].

In a second set of experiments, the endoneuraminidase has been added after the antibody was allowed to bind to the K1-antigen-bearing liposomes. In this case, too, the concentrations of K1-antigen and mAb735 remained as described above. After removing the background fluorescence by washing, the enzyme was added at 18 °C. Apparently independent of the incubation temperature, a complete loss of the membrane-bound fluorescence could be observed within a few minutes. This indicates that the endoneuraminidase is able to cleave the K1-cps even when the mAb735 is bound. The question then arises whether there is a critical amount of bound mAb735 preventing the enzymatic cleavage. The giant liposomes in the flow chamber are not suited for such a quantitative investigation because the num-

ber and size of liposomes swelling out of the lipid film giving the total membrane area and therefore the total amount of membrane-standing K1-antigen is unknown and difficult to reproduce.

In conclusion, it could be shown in these experiments that the insertion of the K1-antigen in giant liposomes occurs spontaneously via its lipid residue. With the help of this model system, it could be clearly seen whether binding of the fluorescent antibody occurs or not. This is an advantage compared to the direct observation of monolayer binding with an epifluorescence microscope [34]. In monolayers it is sometimes difficult to discriminate between bound fluorescence and bulk fluorescence because one looks at the plane of the monolayer. By using the giant liposomes in the flow chamber there is always the contrast between the membranes and the bulk which allows a fast and reliable evaluation of the binding experiment.

Acknowledgements

This work has been supported by the Bundesminister für Forschung und Technologie (H.R.) and the Deutsche Forschungsgemeinschaft (We 1018/4-1).

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