Induction of aggregation and fusion of cholesterol-containing membrane vesicles by an anti-cholesterol monoclonal antibody

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Abstract A monoclonal IgM antibody that reacts with cholesterol was able to aggregate small and large unilamellar lipid vesicles. Vesicles aggregated by the antibody could be dispersed by trypsin digestion. Inclusion of unsaturated phosphatidylethanolamine in the vesicle formulation lowered the relative amount of cholesterol necessary for aggregation, and prevented disaggregation by trypsin treatment. Fluorimetric assays indicated that membrane mixing occurred in aggregates resistant to trypsinization, but the vesicles did not mix or leak their aqueous contents. Analysis of the kinetics of lipid-mixing showed an increase in the aggregation and fusion rate constants with increasing antibody concentrations, indicating that the antibody reaction promotes both processes. An apparent inactivation process whose rate increased with antibody dose has been considered. We conclude that the simultaneous binding of antibodies to more than one vesicle at densities that allow the contact of membrane surfaces, induces first aggregation followed by hemifusion, and with excess of antibody also results in inactivation of the latter process.—Agirre, A., S. Nir, J. L. Nieva, and J. Dijkstra. **Induction of aggregation and fusion of cholesterol-containing membrane vesicles by an anti-cholesterol monoclonal antibody.** ***J. Lipid Res.*** **2000.** 41: **621–628.**

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The existence of antibodies (Ab) specific to cholesterol was initially reported in 1925. During the subsequent years, it has been shown that anti-cholesterol Ab can be induced in animals by conjugating or incorporating the cholesterol antigen (in)to a variety of structures (reviewed in 1, 2). Even cholesterol monohydrate crystals per se can be antigenic in mice (3). Moreover, both animals and humans appear to have naturally occurring Ab to cholesterol (1, 2).

Recently, we reported on the interaction of anti-cholesterol Ab with human lipoproteins and artificial phospholipid vesicles (liposomes) (4). Both, anti-cholesterol hyperimmune serum and a monoclonal anti-cholesterol Ab were found to aggregate isolated human low density lipoproteins and very low/intermediate density lipoproteins, but not high density lipoproteins. Similarly, small unilamellar liposomes prepared with a cholesterol/phospholipid ratio of 2.5:1 rearranged into structures with up to 300-times increased diameters upon treatment with anti-cholesterol Ab. Evidence for Ab-induced aggregation of the lipoproteins and liposomes was obtained using flow cytometric and microscopic techniques. The smooth, droplet-like or vesicular appearance of the obtained structures under the microscope suggested that the anti-cholesterol Ab were able to induce a fusion-like reaction of the cholesterol-containing lipid monolayers and bilayers of the lipoproteins and phospholipid vesicles, respectively.

In the present study, we investigated the interaction of the monoclonal anti-cholesterol Ab with phospholipid vesicles using fluorimetric fusion assays. This approach makes it possible to measure the relative contribution of aggregation and lipid–membrane fusion in the Ab-induced formation of the large structures. Our results demonstrate that the anti-cholesterol mAb is able to induce aggregation of phosphatidylcholine liposomes containing cholesterol, a process that can be reversed by trypsin digestion. However, when phosphatidylethanolamine is included in the membrane composition, membrane mixing occurs and the aggregation is not

Abbreviations: ANTS, 8-aminonaphtalene-1,3,6-trisulfonic acid; CHOL, cholesterol; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPX, p-xylenebis(pyridinium) bromide; HEPES, N-(2-hydroxyethyl)-piperazine-*N′*-2-ethanesulfonic acid; mAb, monoclonal antibody; N-NBD-PE, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; N-Rh-PE, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPG, 1-palmitoyl-2-oleoyl-phosphatidylglycerol; R_{18}, octadecylrhodamine B.

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reversed by the treatment with trypsin. We conclude that binding of the antibody to cholesterol and phosphatidylethanolamine-containing vesicles promotes aggregation and lipid mixing, the latter resulting from a hemifusion process.

MATERIALS AND METHODS

Materials

Cholesterol (CHOL), dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and the fluorescent probes, N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine (N-NBD-PE) and N-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE) were purchased from Avanti Polar Lipids (Birmingham, AL). 8-Aminonaphthalene-1,3,6-trisulfonic acid sodium salt (ANTS), p-xylenebis(pyridinium)bromide (DPX), and octadecylrhodamine B (R_{18}) were from Molecular Probes (Junction City, OR). Triton X-100 and Trypsin (bovine pancreas-type III) were obtained from Sigma (St. Louis, MO). All other reagents were of analytical grade.

Monoclonal antibody to cholesterol

The hybridoma cell line 2C5-6 (ATCC 8995) was used as the source of the monoclonal Ab to cholesterol. As described previously (4), the Ab were isolated from ascites fluid of Balb/c mice by affinity chromatography with immobilized mannan-binding protein, using an IgM purification kit (ImmunoPure®, Pierce, Rockford, IL). The biological activity of purified Ab was checked with the cholesterol ELISA on PVDF membranes (5). An irrelevant isotype murine IgM mAb was utilized as a negative control for CHOL recognition by the anti-cholesterol mAb.

Vesicle preparation

All liposomal preparations were freshly made on the day of the experiment. For the preparation of small unilamellar vesicles (SUV), dried lipid films were dispersed in buffer, 5 mm HEPES, 100 mm NaCl (pH 7.4) and sonicated in a probe sonicator. The obtained opalescent suspension was subsequently centrifuged to remove large aggregates and titanium particles. This procedure is essentially similar to the procedure described by Collins and Phillips (6) for the preparation of sonicated vesicles containing cholesterol/phospholipid molar ratios exceeding 1. Morphological characterization by electron microscopy of DMPC:DMPG:CHOL (9:1:25 mole ratio) samples (high cholesterol levels) confirmed that the main elements in our sonicated dispersions consisted of spherical particles between 40 nm and 230 nm in diameter. In samples containing DMPC:DMPG:CHOL (9:1:5 mole ratio) a comparable morphology could be observed (diameters ranging between 45 nm and 200 nm) (not shown).

Large unilamellar vesicles (LUV) were prepared according to the extrusion method of Hope et al. (7). Dispersed lipids in buffer were subjected to 10 freeze–thaw cycles prior to extrusion 10 times through two stacked polycarbonate membranes of a nominal pore size of 0.1 μm (Nuclepore, Inc., Pleasanton, CA). Internal and external osmolarities were adjusted by adding NaCl. The osmolarities of all solutions were measured in a cryoscopic osmometer (Osmomat 030, Gonotec, Berlin, Germany). Lipid concentrations of liposome suspensions were determined by phosphate analysis (8).

Assays for vesicle aggregation, fusion, and leakage

Vesicle aggregation was estimated by measuring the increase in turbidity of the samples (absorbance at 500 nm) in an Uvikon 860 spectrophotometer (Kontron, Zürich, Switzerland).

Membrane lipid mixing was monitored using the resonance energy transfer (RET) assay, described by Struck, Hoekstra, and Pagano (9). The RET assay is based on the dilution of N-NBD-PE and N-Rh-PE. Dilution due to membrane mixing results in an increase in N-NBD-PE fluorescence. Vesicles containing 0.6 mol % of each probe were mixed with unlabeled vesicles at 1:1 ratio for kinetic analysis or 1:4 for the rest of lipid-mixing experiments. The NBD emission was monitored at 530 nm with the excitation wavelength set at 465 nm. A cutoff filter at 515 nm was used between the sample and the emission monochromator to avoid scattering interference. The fluorescence scale was calibrated such that the zero level corresponded to the initial residual fluorescence of the labeled vesicles and the 100% value to complete mixing of all the lipids in the system. The latter value was set by the fluorescence intensity of vesicles, labeled with 0.3 mol % each of the fluorophores (labeled-to-unlabeled 1:1 ratio) or 0.12 mol % (labeled-to-unlabeled 1:4), at the same total lipid concentration as that in the fusion assay.

Lipid-mixing was also assayed in asymmetrically labeled membrane vesicles produced according to two different methods. Outer-leaflet labeling was carried out by incubating vesicles with externally added R18 (4 mol % with respect to total lipid in vesicles) from an ethanolic solution as described by Stegmann et al. (10). Lipid-mixing was measured as the increase in fluorescence intensity after dilution into unlabeled membranes of the previously self-quenched probe (labeled-to-unlabeled vesicle ratio, 1:4) (11). Rhodamine emission was monitored at 590 nm with the excitation wavelength set at 560 nm. A cutoff filter at 570 nm was placed before the emission monochromator. Complete mixing of all the lipids (100% value) was estimated from the fluorescence intensity of vesicles labeled with 0.8 mol % of fluorophore. Inner-leaflet labeling was accomplished as in (12) following the method initially described by McIntyre and Sleight (13). Vesicles symmetrically labeled with N-NBD-PE and N-Rho-PE were incubated with dithionite 10 mm for 1 min at 25°C. Further exchange of external buffer at 4°C through gel filtration on a Sephadex G-75 column rendered vesicles specifically labeled in their inner monolayer with the fluorescent NBD probe. These vesicles were used inmediately after gel filtration for lipid-mixing experiments as described in the RET assay.

Vesicle contents mixing and leakage were monitored by the ANTS/DPX assay (14). LUV containing either *a*) 25 mm ANTS, 40 mm NaCl, and 5 mm HEPES, *b*) 90 mm DPX and 5 mm HEPES, or *c*) 12.5 mm ANTS, 45 mm DPX, 20 mm NaCl, and 5 mm HEPES were obtained by separating the unencapsulated material by gel filtration on a Sephadex G-75 column eluted with 5 mm HEPES, 100 mm NaCl (pH 7.4). Osmolarities were adjusted to 200 mosm as described above. Fluorescence measurements were performed by setting the ANTS emission at 520 nm and the excitation at 355 nm. A cutoff filter (470 nm) was placed between the sample and the emission monochromator. The 0% vesicle contents mixing was set by using a 1:1 mixture of ANTS and DPX liposomes. The 100% mixing of contents or 0% leakage corresponded to the fluorescence of the vesicles containing coencapsulated ANTS and DPX at time zero. The 100% leakage was the fluorescence value obtained after addition of Triton X-100 (0.5% v/v).

Analysis of fusion

Analysis followed the mass-action kinetic model (15–17) which views the overall fusion reaction as a sequence of two gross steps: *1*) aggregation and *2*) fusion, i.e., membrane destabilization and merging. Dissociation of aggregates was explicitly considered. The calculations were performed by introducing certain modifications into the program described by Nir, Stutzin, and Pollard (18) which was used here for aggregation-fusion products consisting of up to eight particles. The program calculates

the distribution of aggregation fusion products and the corresponding values of increase in fluorescence intensity, I, due to probe dilution arising from membranes mixing. The rate constants of aggregation, fusion, and dissociation are $C_{ij}(M^{-1}\cdot s^{-1})$, $f_{ij}(s^{-1})$, and $D_{ij}(s^{-1})$, respectively, where i = j = 1 refers to aggregated dimers and fused doublets. An elaborate discussion of the determination of the rate constants and the pattern of fluorescence increase is given in Nir (19).

In the analysis of the data, we first determined approximately the aggregation rate constants by focusing on intermediate times, where the fluorescence increase corresponded to a situation where aggregation was the rate-limiting step. A minimal number of parameters was used by assuming that higher order rate constants were equal to the first order ones.

In the second stage, the rate constants f and D were fixed. This procedure was adequate for the analysis of fluorescence increase in dilute liposome suspensions at a given ratio of antibody/lipid, where the increase in fluorescence was almost linear in time. As will be elaborated in the Results section, an extension was needed for the analysis of results obtained with more concentrated suspensions.

RESULTS

The interaction of polyclonal or monoclonal anti-cholesterol antibodies with small unilamellar phospholipid vesicles (SUV) containing a 71 mol % of cholesterol transforms these SUV into vesicular structures with up to 300-times increased diameters (4). The morphological appearance of the obtained structures suggested that the Ab induced a fusion-like reaction between the vesicles. To investigate in more detail the Ab-mediated vesicle–vesicle interaction, we determined in our first experiment the physical parameters of the aggregation process by measuring changes in turbidity.

As shown in **Fig. 1A**, incubation of anti-cholesterol mAb with SUV suspensions composed of DMPC:DMPG:CHOL (9:1:25 mole ratio) induced a dose- and time-dependent increase in turbidity, indicating an increase in the size of the particles in the solution. Incubation of the same SUV suspension with an irrelevant IgM mAb that does not recognize cholesterol (see Materials and Methods) did not induce any increase in turbidity under the same experimental conditions.

Results in panel B demonstrate that the mAb effect was dependent on the cholesterol molar ratio. To induce detectable aggregation, the DMPC:DMPG vesicles had to contain over 50 mol % of cholesterol. A similar cholesterol dependence was previously observed for binding of this mAb to DMPC:DMPG multilamellar vesicles (MLV) as assessed by a complement-dependent immune assay (20), and for the stimulation of anti-cholesterol Ab secretion in mice by DMPC:DMPG containing MLV (4, and Fig. 1B).

Because vesicle aggregates may consist either of individual vesicles brought to close apposition or fused vesicles that have mixed their lipids and/or aqueous contents, we decided to investigate the nature of the aggregates formed as a consequence of Ab reaction with membrane cholesterol. Trypsin treatment can induce IgM fragmentation (21). As shown in **Fig. 2A**, anti-cholesterol mAb preincubated with trypsin was unable to promote vesicle aggregation. Therefore, we used trypsin digestion as a method to distinguish vesicle aggregates cemented by the Ab from aggregates containing fused vesicles. In the former case trypsinization would reverse the increase in turbidity, whereas in the latter it would not. When DMPC:DMPG:CHOL (9:1:25) SUV aggregated for 1 h by the action of the anti-cholesterol mAb were further incubated with trypsin at 37°C, the suspension recovered the turbidity level at time 0, i.e., the absorbance of the SUV prior to the incubation with Ab (Fig. 2B). This observation suggests that the aggregates were composed of unfused vesicles. Trypsin digestion of the Ab yielded three fragments whose electrophoretic mobilities are compatible with sizes between 20 and 30 kDa (Fig. 2C). This indicates that, under our incubation conditions, the IgM μ heavy chains ($M_r \sim 75$ kDa) had been digested by trypsin. Fragmentation of heavy chains would cause the disassembly of the pentameric structure of the Ab.

The results in Fig. 2 were further confirmed by the ab-

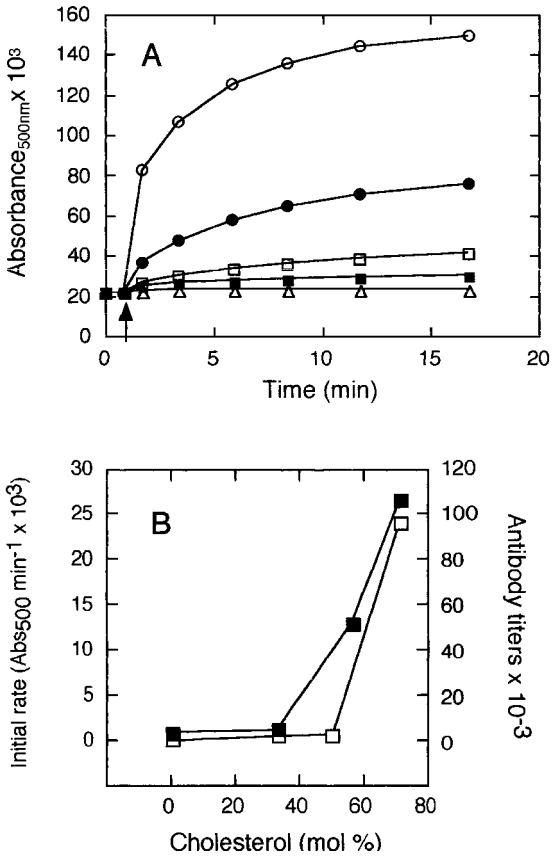

Fig. 1. Cholesterol-dependent, antibody-mediated aggregation of DMPC:DMPG:CHOL (9:1:25) SUV. A: Turbidity increase induced by adding different amounts of anti-cholesterol mAb to a vesicle suspension (addition-time indicated by the arrow). The samples contained the following amounts of Ab: (empty circles) 30 μg ml^{-1} (filled circles) 15 μg ml^{-1}, (empty squares) 7.5 μg ml^{-1}, (filled squares) 3.75 μg ml^{-1}, (triangles) 40 μg ml^{-1} of irrelevant mAb that does not recognize CHOL. Lipid concentration was 250 μm. B: Effect of CHOL mole ratio on mAb-induced SUV aggregation process (empty squares) in comparison with the ability of liposomes to induce Ab against CHOL (filled squares). Aggregation was monitored as in panel A, and Ab production in sera of immunized mice was determined by PVDF-ELISA.

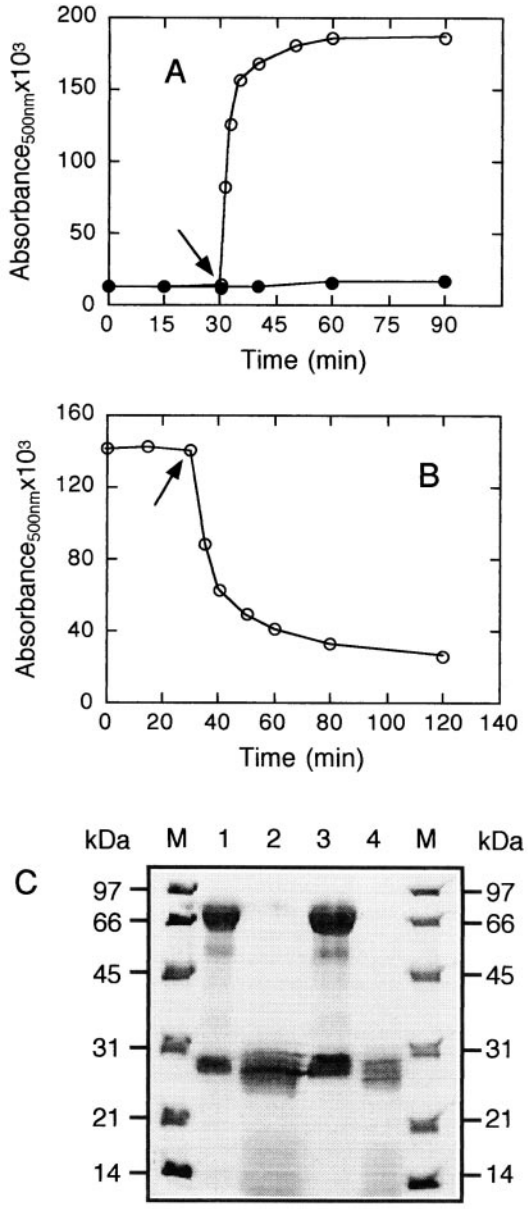

Fig. 2. Trypsin effect on anti-cholesterol mAb-induced aggregation of DMPC:DMPG:CHOL (9:1:25) SUV. A: Turbidity increase induced by anti-cholesterol mAb (empty circles) as compared to that induced by anti-cholesterol mAb preincubated with trypsin (300 μg ml^{-1}, 3 h, 37°C) (filled circles). Antibody concentration was 30 μg ml^{-1}, and lipid concentration 250 μm. Time of antibody addition indicated by the arrow. B: Turbidity decrease induced by trypsin treatment. SUV (250 μm) aggregated for 1 h by the action of the anti-cholesterol mAb (30 μg ml^{-1}) were further incubated with trypsin (300 μg ml^{-1}) at 37°C. Time of trypsin addition indicated by the arrow. C: Trypsin digestion of the anti-cholesterol mAb. Products were precipitated in cold acetone and separated on 12.5% SDS/PAGE (staining with Coomassie brilliant blue). M: molecular weight markers; lane 1: untreated mAb; lane 2: mAb incubated with trypsin as described in panel A; lane 3: untreated mAb incubated with SUV for 60 min; lane 4: mAb successively incubated with SUV (60 min) and trypsin (3 hours) as described in panel B.

sence of lipid-mixing in the Ab-aggregated DMPC:DMPG:CHOL (9:1:25) SUV samples as detected using the RET assay (data not shown). It has been described that membrane fusion may be promoted by certain configuration of both polar head groups and aliphatic moieties of constituent phospholipids (22–24). In particular, membranes made of phospholipids bearing large and highly hydrated polar head groups, such as phosphocholine, and saturated acyl chains are unlikely to support fusion. Therefore, we decided to explore liposome formulations that would simultaneously sustain antibody binding and membrane fusion.

Table 1 summarizes the results obtained with different membrane compositions using SUV and LUV as target vesicles. From the results displayed in the table it can be concluded that CHOL was the major requirement for the aggregation phenomenon. Both SUV and LUV could be aggregated by the antibody. Negatively charged DMPG was unnecessary. Unsaturated DOPC could substitute saturated DMPC as well. However, DOPC:CHOL (1:2 mole ratio) vesicle aggregates produced by the antibody reaction could be equally dispersed by trypsin digestion.

When DOPE was included in the membrane composition, vesicles could be aggregated by the antibody action and the resultant aggregates were mostly resistant to the trypsin digestion. The turbidity of DOPC:DOPE:CHOL liposomes aggregated by the antibody decreased only 15% after incubation with trypsin. It is noteworthy that the presence of DOPE also reduced the relative amount of CHOL necessary for the Ab-induced vesicle aggregation. Membrane fusion of SUV and LUV within the DOPE-containing aggregates was further confirmed by a fluorimetric lipid-mixing assay (RET assay).

In the following we will describe the specific characteristics of the Ab-promoted fusion process in the DOPC:DOPE:CHOL (1:1:1 mole ratio) LUV system. Merging of boundary vesicle membranes can lead to the mixing of their aqueous contents. Alternatively, merging of mem-

TABLE 1. Anti-cholesterol mAb-induced aggregation and fusion of vesicles of several compositions

Type of Vesicle	Aggregation[a]	Trypsinization[b]	Lipid-mixing[c]
DMPC:DMPG:CHOL (9:1:0)	–	nd	nd
DMPC:DMPG:CHOL (9:1:5)	–	nd	nd
DMPC:DMPG:CHOL (9:1:10)	±	nd	nd
DMPC:DMPG:CHOL (9:1:25)	+	+	–
DMPC:DMPG:CHOL (9:1:25)[d]	–	nd	–
DMPC:DMPG:CHOL (9:1:25)[e]	+	+	–
DOPC:CHOL (1:2)	+	+	nd
DOPC:DOPE:CHOL (1:1:1)	+	–	+
DOPC:DOPE:CHOL (1:1:1)[d]	–	nd	–
DOPC:DOPE:CHOL (1:1:1)[e]	+	–	+

Mol ratios represent the stoichiometric concentrations of lipid and cholesterol in the initial organic solutions from which vesicles were made.

[a] Aggregation was detected as increase in turbidity of vesicle-suspensions.

[b] + indicates that vesicle-aggregates were susceptible to trypsinization.

[c] Lipid-mixing was monitored by means of the fluorescence RET assay.

[d] Vesicles were incubated with an irrelevant mAb that does not recognize CHOL.

[e] LUV were used instead of SUV.

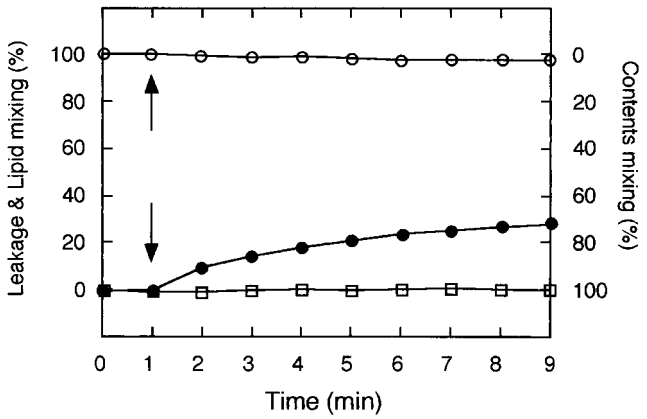

Fig. 3. Fusogenic effect of anti-cholesterol mAb on DOPC:DOPE:CHOL (1:1:1) LUV. Mixing of aqueous contents (empty circles) in comparison with lipid-mixing (RET assay) (filled circles) and leakage (empty squares). Lipid and mAb concentrations were 50 μm and 10 μg ml^{-1}, respectively. The mAb was added at the time indicated by the arrows.

branes can occur in the absence of mixing of the aqueos contents. In this case, 'hemifusion' can take place, a phenomenon consisting in the merging of the outer-leaflets of membranes without mixing of the internal ones (24). Consequently, this latter process will be characterized by the mixing of the lipids residing in external monolayers of vesicle membranes and the lack of leakage or mixing of contents.

The results in **Fig. 3** show that the Ab induced membrane mixing (filled circles), without mixing of the aqueous contents (empty circles), and leakage (squares). Thus, aggregated vesicles mixed their lipids but retained the integrity of their internal aqueous compartments.

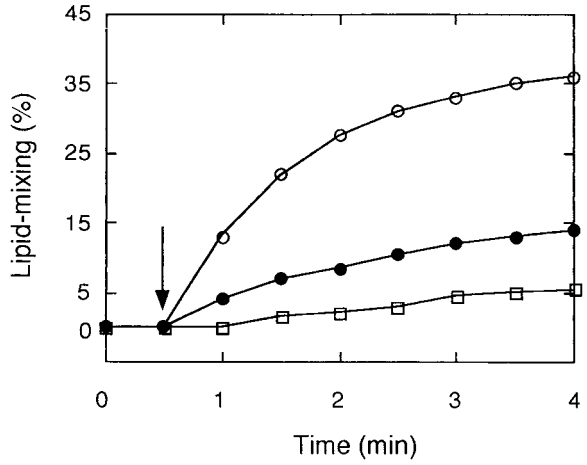

Fig. 4. Lipid-mixing in vesicles asymmetrically labeled as compared to symmetrically labeled vesicles (see Materials and Methods); (empty circles) vesicles labeled in their external monolayers with R18; (filled circles) symmetrically labeled vesicles with N-NBD-PE and N-Rho-PE; (empty squares) asymmetrically labeled vesicles with N-NBD-PE and N-Rho-PE in the internal monolayer. Other conditions were similar to those in Fig. 3. The mAb was added at the time indicated by the arrow.

This observation would be compatible with the existence of a "hemifusion" process. To further test this possibility, we devised lipid-mixing experiments using asymmetrically labeled liposomes. In **Fig. 4**, lipid-mixing results obtained with asymmetrically labeled vesicles are compared with the ones obtained using symmetrically labeled LUV. When only the outer membrane leaflets were labeled, approximately a double amount of lipid mixed per time unit as compared to the case in which symmetrically labeled vesicles were used. On the other hand, consistent with the existence of a hemifusion process, vesicles labeled in their internal membrane monolayers only marginally mixed their lipids.

The rates and extents of the lipid-mixing were very sensitive to the amount of anti-cholesterol mAb present in the mixtures. In order to investigate the degree of involvement of the triggering agent in the whole fusion reaction, we next analyzed the kinetics of the lipid-mixing process. The results in **Fig. 5A** show an almost linear increase in fluores-

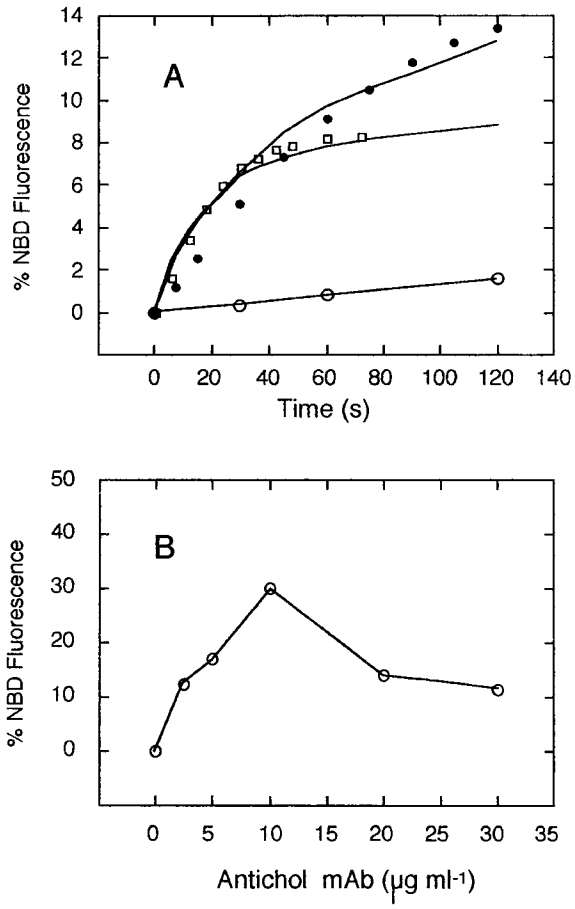

Fig. 5. Analysis of the lipid-mixing process induced by the anti-cholesterol mAb on DOPC:DOPE:CHOL (1:1:1) LUV. A: Lipid-mixing as a function of time at 3 different lipid concentrations. Lipid-to-mAb ratio was kept constant. Experimental results are given by solid lines. Symbols indicate values obtained from the results using a mass-action kinetic model (empty circles) 10 μm, (filled circles) 25 μm, (empty squares) 50 μm lipid. B: Extent of mAb-induced membrane lipid mixing (RET assay) as a function of antibody concentration. NBD fluorescence was determined 2 min after addition of mAb. Lipid concentration was 50 μm.

TABLE 2. Rate constants used in calculations of fluorescence increase

Lipid Concentration	C($M^{-1}s^{-1}$)	f(s^{-1})	D(s^{-1})	γ(s^{-1})
μM				
10	4×10^6	0.045	0.1	0
25	10^7	0.4	0.1	0.02
50	1.1×10^7	0.48	0.1	0.1
100	1.1×10^7	0.5	0.1	0.19

The concentration of anti-cholesterol mAb was 5 μg ml^{-1} for a lipid concentration of 10 μm. The same ratio mAb/lipid was used in all cases. The calculations followed the procedure in Nir et al. (18), using "γ" as defined in Eq. 2. The calculated values are shown in Fig. 5A.

cence intensity for the case where the DOPC:DOPE:CHOL LUV suspension included 5 μg/mL of mAb and the lipid concentration was 10 μm. In this case, the best fit was relatively uniquely determined, and the results correspond to a situation where aggregation is the rate limiting step (16, 17), i.e., $C_{11}L_o << f_{11}$, where $L_o = 1.2 \times 10^{-10}$ m is vesicle concentration. In this case, the product $C_{11}L_o$ equals $4.8 \times 10^{-4}s^{-1}$, whereas $f_{11} = 0.045\ s^{-1}$. Based on a molecular mass of roughly 1.0×10^6 Da of the pentamer Ab, this case corresponds to 40 mAb molecules per vesicle, but fewer are bound. By keeping the same ratio of mAb/vesicle, an increase in lipid concentration from 10 μm to 100 μm resulted in 15- to 50-fold larger rates of fluorescence increase, or fusion, depending on the times of observation. The analysis expressed this increase in overall rate of fusion as due to 2.5-fold increase in the rate constant of vesicle aggregation, and about an order of magnitude increase in the rate constant of fusion (**Table 2**). In fitting the data for the larger lipid concentrations, the calculated values overestimated the experiment values at later times. The analysis revealed that this overestimate could not be fully explained as due to reduction in the fusion rate constants of the higher orders, f_{ij}, i.e., corresponding to fusion of fused vesicles. Hence, we attempted to explain phenomenologically the apparent decrease in fusion activity of the mAb with time as due to some inactivation mechanism. Such a behavior was previously observed in synexin-induced fusion of chromaffin granule ghosts (18). Following the latter study, we considered the expressions

$$f(t) = f(o)\ \exp(-\gamma t), \text{ or} \qquad \textit{Eq. 1}$$

$$f(t) = f(o)/(1 + \gamma t)^2, \qquad \textit{Eq. 2}$$

in which $\gamma(s^{-1})$ is the rate constant of inactivation, and f(t) denotes the apparent fusion rate constant at time t.

The calculated values in Fig. 5A were based on using equations 2 with the rate constants listed in Table 2, but equation 1 gave similar fits. The values of the rate constants of inactivation increase with mAb (or lipid) concentration, while keeping the ratio mAb/lipid constant. We rule out an inactivation process due to self-aggregation of the mAb molecules in solution, as addition of mAb molecules prior to that of the vesicles had no effect on the outcome of fusion.

Figure 5B further illustrates the inactivation effect. The results in this figure demonstrate that at t = 2 min and for a given concentration of lipid (50 μm), a maximal extent of fusion is observed for mAb concentration of 10 μg/mL. Increasing the amount of Ab resulted in a reduction of the extents of lipid-mixing measured at that time.

DISCUSSION

Antibodies to cholesterol, such as the mAb 2C5-6 used in this study, belong to a group of natural autoantibodies which are predominantly of the IgM class, and are produced by self-sustaining primary B cells (25). Natural autoreactive Ab most probably participate in the innate branch of the immune system. The specificity of the mAb 2C5-6 is to cholesterol and structurally similar sterols containing an unmodified 3β-hydroxyl group (4). This mAb is able to recognize cholesterol in the lower density lipoproteins, in small and large unilamellar vesicles and in multilamellar vesicles (4, 20). It is therefore conceivable that this Ab can be used as a general aggregation device useful to bring cholesterol-containing particles into close apposition. Our findings in this work demonstrate that trypsinized anti-cholesterol mAb is unable to induce vesicle aggregation (Fig. 2), indicating that preservation of the overall structure of the IgM is essential for this phenomenon.

Our results also indicate that, besides the Ab concentration, the vesicle aggregation process primarily depends on the cholesterol content and phospholipid composition of the membrane. This is consistent with the existence of a threshold CHOL concentration in the membrane above which the mAb recognizes and interacts with this lipidic compound (4). It has been proposed that a direct interaction of membrane cholesterol with certain integral proteins may be possible (26). Stereospecific recognition of CHOL in the membrane activates pore-forming cytolysins (27), a property that requires preservation of the secondary and tertiary protein structure (28). In addition, stereospecific binding has also been invoked to explain the CHOL-dependence of alphavirus envelope glycoprotein-mediated membrane fusion (29). In both cases, a minimal CHOL mole fraction, that depends on the lipid composition, is required in the membrane to obtain optimal activity.

The incorporation into the membrane of unsaturated PE, a phospholipid bearing a small and poorly hydrated polar head group and a bulky hydrophobic moiety, decreases the amount of cholesterol necessary for the Ab-mediated aggregation process and promotes fusion (Table 1). The cholesterol molecule is oriented perpendicular to the membrane surface, or parallel to the acyl chains of the phospholipids, with its polar hydroxyl in contact with the aqueous solvent (26). Molecular recognition by the mAb might be facilitated by the spacing between small PE polar head groups. Alternatively, lower miscibility with PE due to the stronger intermolecular headgroup interactions occurring in this phospholipid might cause a local enrichment of CHOL in PE-containing bilayers (26).

The anti-cholesterol Ab used in this study might be uti-

lized as a fusogen of membranes that are initially refractory to fusion due to the effects of repulsive electrostatic and hydration forces that prevent aggregation. The Ab may aggregate those membranes and locate them close enough as to allow fusion to proceed. The latter process will be promoted when the membrane is inherently unstable due to the presence of non-lamellar lipids such as DOPE (24). As Ab-induced membrane mixing of DOPC: DOPE:CHOL (1:1:1) liposomes occurs in absence of contents mixing or leakage, we designate the process as hemifusion (Figs. 3 and 4). The observed increase in fluorescence is not due to diffusion exchange of the probe molecules, because it is also accompanied by resistance to trypsinization (Table 1). Furthermore, at later times, in the presence of larger concentrations of lipid and antibody, i.e., when massive aggregation has occurred, we observed a significant reduction in the rate of fluorescence increase, or inactivation of fusion (Fig. 5), whereas diffusion exchange is expected to be promoted.

Below we discuss the explanations for the "inactivation" observed. An inactivation according to equation 2 might suggest that it arises because of interaction between bound mAb molecules, as this equation gives the time dependence of the concentration of remaining monomers, due to aggregation in a system consisting initially of dispersed particles. It is feasible that the rate of vesicle fusion would be larger for a larger number of antibody molecules bound to a vesicle, which form contacts with neighboring vesicles, promoting membrane contacts and membrane destabilization. However, this statement is valid to the extent that the antibodies permit direct contact between the lipid bilayers. Larger total antibody and lipid concentrations imply larger numbers of mAb molecules bound per vesicle, which in turn yield initially larger rates of fusion. A larger number of bound mAb molecules per liposome results in increased fractions of mAb molecules interacting with other mAb molecules in the same and in neighboring vesicles. Consequently, at later times there is a reduction in the number of antibody molecules available for the fusion process. The rate of this reduction depends on the surface concentration of mAb molecules and on the total concentration of vesicles. Another explanation for the results in Fig. 5(A, and B) might be that with a large number of antibody molecules bound to the membrane, their aggregates can form pillared-like structures, in which the lipid bilayers are kept at a distance.

Although not studied in the present report, it is possible that the anti-cholesterol Ab-induced increase in size of the lipoproteins LDL and VLDL (4) results from a fusion process, rather than from mere aggregation. The lipid core of lipoproteins is surrounded by a lipid monolayer, which is composed of various phospholipids (PC, SM, and minor ones such as PE) and cholesterol (30). The hemifusion process described in the present study may result in true fusion in the case of the lower density lipoproteins, i.e., mixing of both the membrane components and the core contents of these particles. It is of interest that true fusion of LDL has been reported upon treatment of the lipoprotein with proteolytic enzymes or sphingomyelinase (31, 32).

Our work finally establishes the basis for the development of a new membrane fusion model system, in which the binding of a single Ab to a lipidic component from two different bilayers can lead to their merging. This model system might be useful to gain insight into the molecular mechanism underlying the membrane fusion phenomenon. Inasmuch as the overall membrane fusion process is limited by the preceding aggregation step, the use of membranes aggregated by lipid-specific Ab might allow better kinetic, structural, and thermodynamic characterization of the subsequent merging steps, e.g., exploring the regulatory effects of different membrane components.

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