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Immunology

Visualization of specific antibody and C1q binding to hapten-sensitized lipid vesicles

(freeze-etch electron microscopy/spin labels/complement/phospholipid vesicles)

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ABSTRACT **Specific IgG antibodies directed against the spin-label nitroxide group present as a lipid hapten in single-compartment lipid vesicles have been visualized by using freeze-etch electron microscopy. Individual "particles" with diameters of the order of 20 nm are identified as single IgG molecules bound to lipid hapten. No significant aggregation of these IgG molecules was observed over a period of 1 hr in a dipalmitoyl phosphatidylcholine vesicle at 22°. Binding of the human complement component Clq results in the formation of large (~50–100 nm) asymmetric particles having a partially resolved substructure that may arise from individual IgG molecules bound to the membranes as well as to Clq. The binding of Clq appears to result in a clustering of membrane-bound IgG molecules. Samples containing a serum factor (perhaps anti-IgG antibodies) exhibit some IgG clustering distinct from that produced by Clq.**

Previous work in this laboratory has been directed toward obtaining an understanding of the elementary molecular events at the membrane surface that are involved in complement activation (1–6). Related work has been reviewed by Porter (7). In a recent paper (5) we described a quantitative study of the binding of human complement component Clq to lipid hapten-sensitized phospholipid vesicles in the presence of specific antibody. The lipid hapten used in this earlier work as well as in the present work contains a nitroxide spin-label group in the polar head group region.

In our previous work it had been assumed that there is no spontaneous aggregation of hapten-bound IgG molecules in the vesicle membrane. It also had been assumed that Clq binding to vesicle membranes did not require the crosslinking of vesicles. The present study was undertaken to resolve these and related questions by using freeze-etch electron microscopy.

MATERIALS AND METHODS

Preparation of Vesicles. Dipalmitoyl phosphatidylcholine vesicles containing 0.2 mol % spin-labeled phospholipid were made by a modification of the method of Deamer and Bangham (8) as described (5).

Anti-Nitroxide IgG. Anti-nitroxide IgG was prepared by the following modifications of the procedure described by Brûlet and McConnell (1). In place of *N,N*-dimethyl-*N*-(2′,2′,6′,6′-tetramethyl-4′-piperidinyl-1-oxy)-2-hydroxyethylammonium chloride, methyl glutamate 2,2,6,6-tetramethylpiperidinyl-1-oxyamide was used to elute the antibodies from the affinity column. Also, the spin label was separated from the antibody by gel filtration on a Sephadex G-100 column equilibrated with 0.05 M diethylamine instead of dialysis against phosphate-buffered saline for 10 days. A sufficient quantity of 1.0 M phosphate buffer (pH 6) was added to the collecting tubes to restore immediately to neutrality the pH of each fraction from the Sephadex G-100 column. The antibody-containing fractions were combined, concentrated by vacuum dialysis, and passed through a Sepharose 6B column to verify that all of the binding activity was IgG. Unless indicated otherwise, the following step was added to remove anti-IgG (9) or any other serum proteins that might bind activated IgG molecules. After heat inactivation (56°, 30 min), the spin-labeled keyhole limpet hemocyanin antisera were titrated with enough nonlabeled keyhole limpet hemocyanin to form a maximum immune precipitate. The precipitate was allowed to form for 1 hr at 37° and an additional 4 hr at 4°. The sera were then centrifuged for 10 min at 10,000 rpm in a Lourdes 9RA rotor. All antibody preparations were centrifuged at 20 psi (138 kPa) for 20 min in a Beckman Airfuge immediately prior to use.

Preparation of Human Clq. Clq was prepared by the method of Assimeh *et al.* (10) but with an additional final pass through a Sepharose 6B column, at 4°, equilibrated with 0.1 M phosphate buffer (pH 7.2). The protein solution thus prepared was concentrated by vacuum dialysis to ~1 mg/ml and stored in liquid nitrogen. Just prior to use, the Clq solution was thawed at 4° and passed over a Sepharose 6B column equilibrated with barbital-buffered saline to remove any aggregates and hydrolysis products. Aliquots from the fractions containing the leading edge of the Clq peak were used directly in the binding incubations. The Clq from these preparations revealed a single precipitin band in immunodiffusion and immunoelectrophoresis (11) against goat anti-human whole serum and against rabbit anti-human Clq. We also obtained an identity reaction with immunodiffusion using a reference sample of Clq and anti-Clq provided to us by A. Esser. No such band was formed when the Clq preparation was heated at 56° for 30 min prior to electrophoresis. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis gave a single band of molecular weight 400,000 (11).

Preparation of Samples for Freeze-Etching. Vesicles, antibody, and Clq were mixed to give final concentrations of 1 mM phospholipid, 1 μM antibody, and 0.1 μM Clq. The concentrations used were chosen from the binding curve given by Parce *et al.* (5) so as to optimize the likelihood of seeing bound Clq. All solutions were in barbital-buffered saline, and barbital-buffered saline was used as a diluent in control experiments in which antibodies or Clq was absent. The mixture was allowed to stand at room temperature for 10 min unless otherwise indicated. This mixture was then layered on top of a 1:2 solution of 10% (wt/vol) sucrose in barbital-buffered saline and centrifuged at 1000 × *g* for 10 min in a swinging-bucket centrifuge. The supernate was then drawn off and the pellet was resuspended to a final concentration of 6 mM phospholipid.

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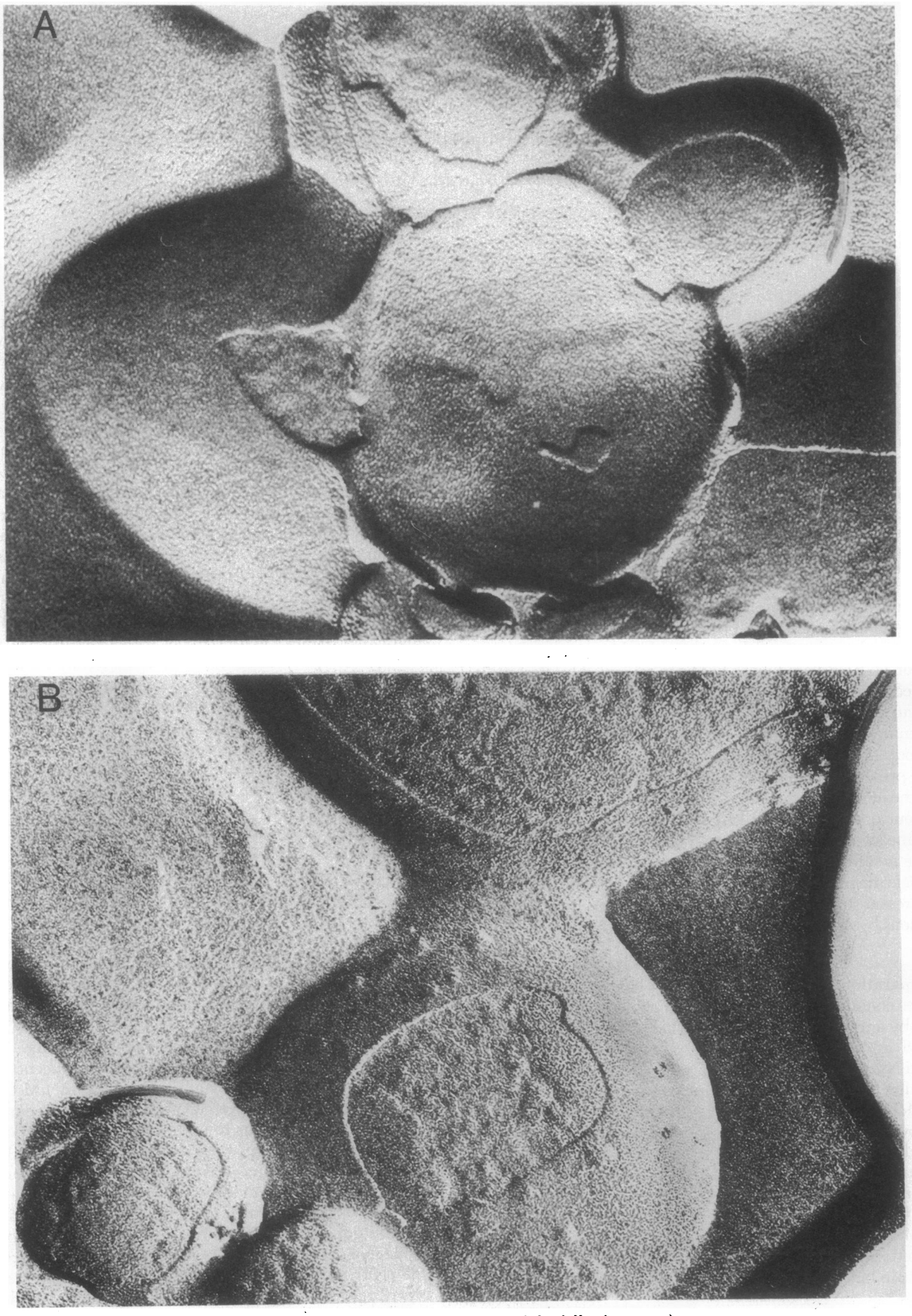

FIG. 1 *(Legend appears at bottom of the following page.)*

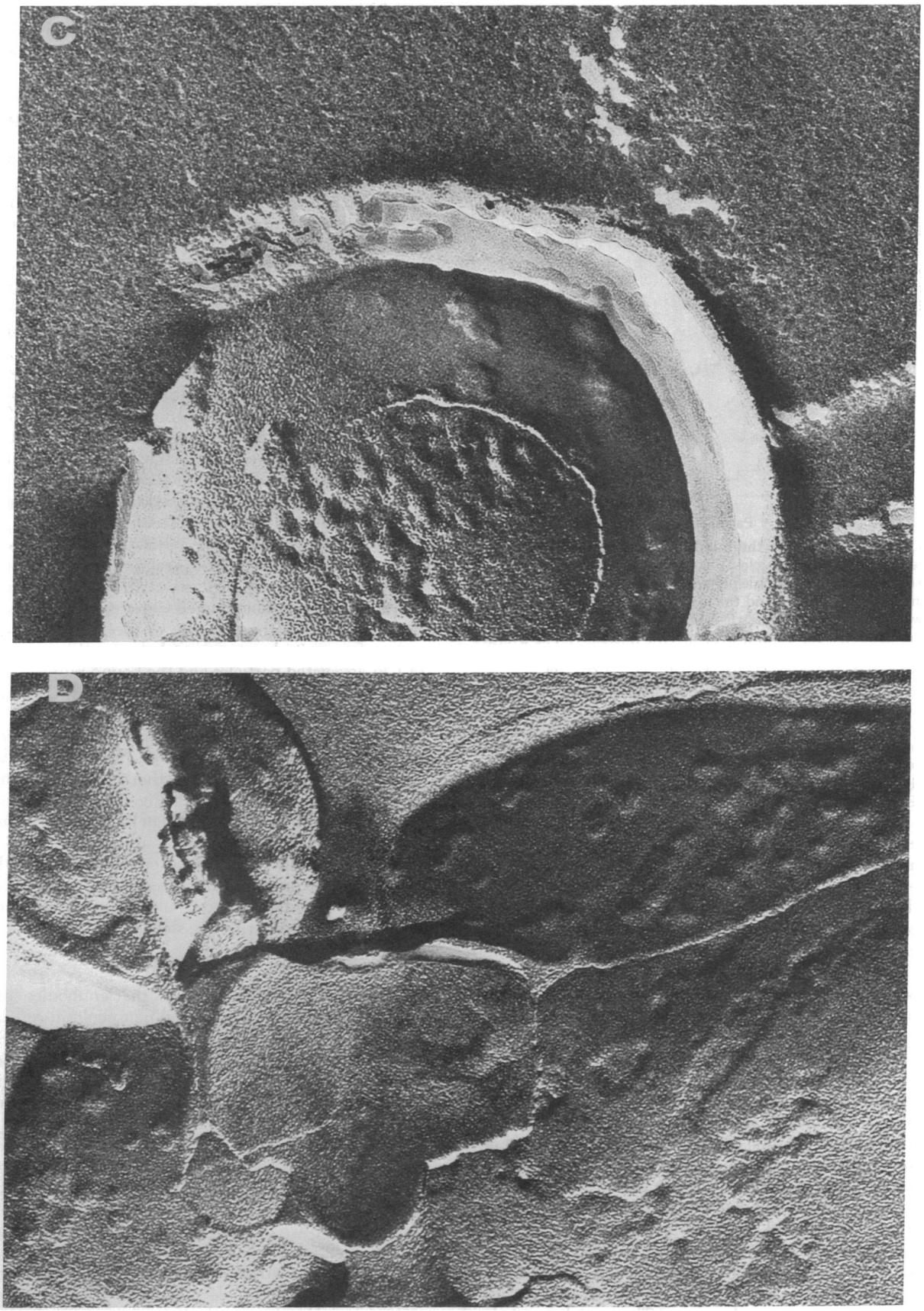

FIG. 1 (*on preceding page and above*). Freeze-etch electron micrographs of dipalmitoyl phosphatidylcholine vesicles incubated for 10 min under the following conditions. (×160,000.) (*A*) Vesicles containing 0.2 mol % spin-labeled hapten in a solution containing nonspecific rabbit serum. (*B*) Vesicles containing 0.2 mol % spin-labeled hapten with specific antibodies to spin label. (*C*) Same as *B* but with the addition of human Clq. (*D*) Same as *B* but with antibody prepared without precautions to remove serum factors that might bind to IgG molecules.

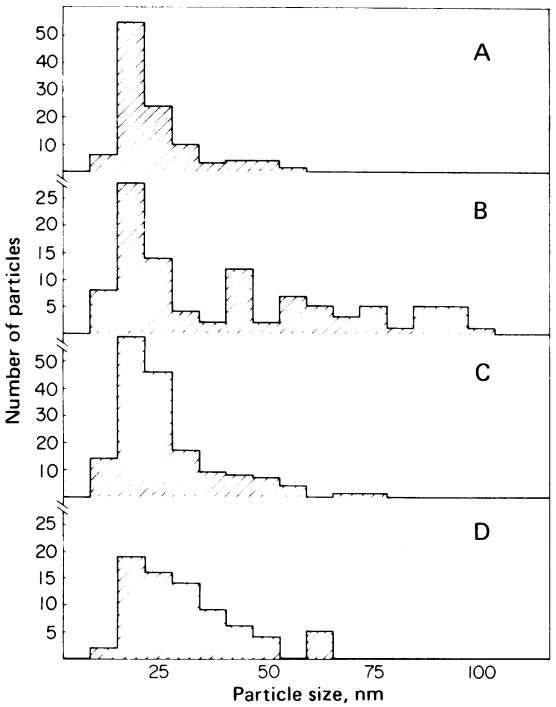

FIG. 2. Histograms of the number of particles on the vesicle surfaces versus size of particles. Sizes of particles were determined by the dimension of the particle perpendicular to the shadow direction for symmetrical particles and the largest dimension irrespective of shadow direction for asymmetrical particles. (*A*) Hapten-sensitized vesicles incubated for 10 min with specific antibody. The average particle density was 1.4 particles per 10^4 nm^2 of membrane surface (SD, ±0.5). (*B*) Same as *A* with the addition of Clq. The average particle density was 0.90 particle per 10^4 nm^2 of membrane surface (SD, ±0.35). (*C*) Same as *A* but incubation was carried out for 60 min. The average particle density was 1.5 particles per 10^4 nm^2 (SD, ±0.4). (*D*) Same as *A* but with antibody prepared without precautions to remove serum factors that might bind to IgG molecules. The average particle density was 2.68 particles per 10^4 nm^2 of membrane surface (SD, ±0.21).

Freeze-Etching and Electron Microscopy. One to 2 μl of this suspension was equilibrated at room temperature on 3-mm copper planchets before being rapidly quenched in Freon 22 cooled in liquid nitrogen. The freeze-fracture and etching were carried out at −106° in a Balzers BAF 301 instrument. The replicas were cleaned in commercial bleach. Micrographs were taken at a magnification of ×40,000 with a Siemens 1A electron microscope. The magnification was checked with a carbon grating replica (54,800 lines per inch).

RESULTS

Fig. 1*A* exhibits the etch face of a hapten-sensitized lipid vesicle incubated at room temperature in the presence of nonspecific rabbit serum. Essentially identical particle-free etch faces were obtained with all possible combinations of hapten, antibody, Clq, nonspecific serum, and specific serum, except those two combinations in which hapten and specific antibodies or hapten and specific antibodies and Clq were simultaneously present. Fig. 1*B* shows a typical etch face obtained from a vesicle suspension having 0.2 mol % lipid hapten, together with specific antibodies directed against the nitroxide group. Particles having an identical appearance were obtained when specific antiserum was used. (See *Discussion*, however.) The particles are judged to be individual antibody molecules from their size and monomodal size distribution. The average diameter of a particle considered to be single was 20 nm. From the distribution of particle sizes given in Fig. 2*A* it is clear that the great majority of particles are indeed single molecules. Some contribution to the apparent size distribution may arise from shadowing conditions.

The sample in Fig. 1*B* was obtained after an incubation period of 10 min at room temperature (22°). To test the possibility that a time-dependent aggregation of bound IgG molecules might take place on the membrane surface, we incubated identical samples for 1 hr prior to freeze-quenching. The observed distribution of particle sizes, illustrated in Fig. 2*c*, shows that no major dimerization or higher aggregation of bound IgG molecules takes place.

Fig. 1*C* shows hapten-sensitized vesicles in the presence of specific IgG and human Clq. Some of the observed particles are obviously different from those in Fig. 1*B*; they are much larger, have a broad size distribution, and are highly asymmetrical. This asymmetry is not unexpected in view of the molecular structure of Clq inferred from negative-staining electron microscopy (12). The broad size distribution is shown by the histogram in Fig. 2*B*; note the contribution of single IgG particles to the distribution. In many cases the Clq-associated particles show a partially resolved substructure, which may be individual IgG molecules bound to Clq molecules. If this interpretation is correct, we have often observed four to six IgG molecules associated with a single Clq molecule.

The surface density of Clq-associated particles given in the legend to Fig. 1*C* is in order-of-magnitude agreement with the Clq binding data given by Parce *et al.* (5). Freeze-etch micrographs in which radioiodinated Clq was bound to hapten-sensitized vesicles in the presence of specific IgG also showed order-of-magnitude agreement between the surface density of Clq-associated particles and the bound radioactivity. Both carrier-free ^{125}I-labeled Clq and ^{125}I-labeled Clq diluted with nonlabeled Clq gave similar results.

In our initial experiments, in which no precautions were taken to remove anti-IgG-like factors from the heat-inactivated antiserum, we observed the rather large and symmetrical freeze-etch particles shown in Fig. 1*D*. The corresponding particle size distribution is shown in Fig. 2*D*. We assumed that these particles were composed of anti-nitroxide IgG together with anti-IgG antibodies or some equivalent protein that binds to IgG (9). The procedure described in *Materials and Methods* was therefore adapted and resulted in the smaller and more uniform particle distribution shown in Figs. 1*B* and 2*A*.

DISCUSSION

The present paper demonstrates that antibodies specifically bound to lipid hapten-sensitized phospholipid vesicles can be visualized by using freeze-etch electron microscopy. The observed random distribution of bound IgG molecules settles one immunological problem: at low surface concentrations, IgG molecules bound to lipid hapten at a membrane surface do not spontaneously associate with one another in significant numbers (for a discussion of the significance of this result, see ref. 13). We have already shown (1) that vesicles having the compositions used in the present work are effective in depleting complement in the presence of specific antibodies. Close lateral proximity of two or more bound IgG molecules is almost certainly required for C1 activation (13). Our results obtained with Clq suggest to us that this molecule does result in some clustering of bound IgG molecules in this solid-phase membrane. This is based on the particle size measurements discussed above and on the particle density counts summarized in the figure legends. That is, the density of isolated IgG-size particles in membranes in the presence of Clq is one-third of the number

of bound IgG molecules in the absence of Clq. (Probably even more IgG is bound in the presence of Clq.)

One particular rabbit serum depleted of antibody against keyhole limpet hemocyanin contained a very high titer of anti-nitroxide IgG (3.7 mg/ml). Freeze-etch micrographs obtained with this serum showed a high density of bound IgG, of the order of 10% surface coverage. In some of these micrographs the IgG lateral distribution was determined to be random by statistical analysis, but in others the distribution was not random, sometimes showing clusters or linear arrays or both. We suspect that these nonrandom distributions are artifacts associated with the high density of bound IgG molecules and interactions of IgG-coated vesicles with one another or with ice. The essential points in the present work are that (*i*) at the lower densities of bound IgG, the lateral distributions of the single IgG-associated particles and the (Clq + IgG)-associated particles are always random, (*ii*) in all cases the (Clq + IgG)-associated particles are readily disiinguished from the single IgG-associated particles, and (*iii*) even in the solid-phase membrane, lateral redistribution of the hapten-bound IgG molecules does take place.

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