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VITAMIN A IN LIPOSOMES

INHIBITION OF COMPLEMENT BINDING AND ALTERATION OF MEMBRANE STRUCTURE

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SUMMARY

Incorporation of vitamin A aldehyde (retinal) into liposomes had an inhibitory effect on the amount of human complement protein bound in the presence of specific antiserum. The total membrane-bound protein was directly measured on liposomes which were washed after incubation in antiserum and fresh human serum (complement). At every concentration of complement, decreased protein binding was found with liposomes which contained retinal. Binding of the third component of complement (C3) was also measured directly on washed liposomes and was found to be decreased in the presence of retinal.

The diminution in protein binding due to retinal was not caused by differences in the amount of antibody bound and this was shown by two experiments. First, specific antibody protein binding to liposomes was directly measured and was essentially unaffected by retinal. Second, liposomes were prepared from lipid extracts of sheep erythrocytes. These liposomes were used as as immunoadsorbants to remove antisheep erythrocyte antibodies. The immunoadsorbant capacity was the same in both the presence and the absence of retinal. A further conclusion from these experiments was that retinal did not change the number of liposomal glycolipid antigen molecules available for antibody binding and thus presumably did not change the total number of lipid molecules present on the outer surface of the liposomes.

Retinal did have an effect on the geometric structure of the liposomes. Size distribution measurements were performed in the diameter range of 1–6.35 μm by using an electronic particle size analyzer (Coulter Counter). Liposomes containing retinal were shifted toward smaller sizes and had less total surface area and volume.

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It was suggested that retinal-containing liposomes may have had a tighter packing of the molecules in the phospholipid bilayer. This effect of retinal on liposomal structure may have been responsible for the observed decreased binding of C3 and total complement protein.

INTRODUCTION

Extensive work has shown that liposomes prepared from pure lipids may undergo membrane damage in the presence of a specific antibody and complement (for review, see ref. 1). In most ways, liposomal immune reactions have been found to parallel the immune reactions at the surface of cell membranes. In this context liposomes have served as an effective model in the study of immune cytolysis. Liposomes have also been particularly useful as a model in determining the influence of membrane composition on complement-dependent immune reactions. Thus, recent work using liposomes has demonstrated that the membrane lipid composition may profoundly affect the antigen-antibody interactions [2, 3]. Because of the simplicity and effectiveness of liposomes, it was felt that they might also be a useful model system in which to study the effects of membrane-bound vitamin A on immune cytolysis.

In previous reports it was shown that vitamin A aldehyde (retinal) may exert an influence on immune complement reactions at the surface of erythrocytes. Retinal inhibited immune hemolysis of sheep erythrocytes by guinea pig complement and several different stages in the complement cascade were affected [4–6]. The inhibitory effect of retinal on immune hemolysis was not reversed by washing the cells [5], thus indicating that retinal directly interacted with the membrane. In contrast to the above findings, retinal was found to enhance the hemolysis of sheep erythrocytes in the presence of human complement [7]. The reason for this latter finding is not completely understood. β-Ionone, an analogue of retinal which lacks nutritional vitamin A activity, gave similar results to those found with retinal [8].

In the preceding paper in this series, it was demonstrated that incorporation of retinal into liposomal model membranes caused an inhibition of immune damage, resulting in decreased glucose release in the presence of guinea pig complement [9]. In contrast to erythrocytes, inhibition of liposome damage also occurred in the presence of human complement. The inhibitory effect of vitamin A on complement-dependent damage to liposomes was not changed by washing the liposomes free of unincorporated retinal. Fluid phase retinal, which was added to the medium after formation of the liposomes, also did not cause inhibition. Because of this it was suggested that retinal influenced only membrane-associated reactions in the liposome system. This could have been through an effect on (1) the antigen-antibody interaction, (2) the complement cascade or (3) the membrane itself. In the present study, each of these three possible sites of action of retinal were examined in detail.

MATERIALS AND METHODS*

Lipids

The sources of all of the lipids have been given elsewhere [9]. Lipids derived from sheep erythrocytes (Fraction IIa) and which were capable of reacting with rabbit antisheep erythrocyte serum (hemolysin), were prepared as previously described [3, 10].

Preparation of liposomes

Liposomes were prepared on a daily basis with a vortex mixer and using the same concentrations of lipids as described before [9, 10], with the exception that they were swollen in 0.15 M NaCl instead of 0.3 M glucose. The lipids used in each case are given in the appropriate figure or table legend.

Sera

Fresh human serum and rabbit antigalactocerebroside serum were obtained as previously described [9]. Rabbit antisheep erythrocyte serum (hemolysin) was purchased from either Difco Laboratories (Detroit, Mich.) or Cappel Laboratories (Downington, Pa.). The antisera were routinely heated at 56 °C for 30 min before use.

Washing of liposomes and measurement of bound protein

The incubation and washing procedures were similar to those employed before [11]. Briefly, 50 μl of liposomes were incubated in 15-ml centrifuge tubes for 15–45 min at room temperature (approx. 22 °C) with various quantities of fresh human serum (complement) and/or rabbit antiserum. Each tube also contained 10^{-3} M $MgCl_2 \cdot 6H_20$ and $1.5 \cdot 10^{-4}$ M $CaCl_2$ in a total volume of less than 1 ml. The tubes were cooled in an ice bath and the liposomes were washed four times by centrifugation at 15 000 rev./min ($27\,000 \times g$) after adding 10 ml of ice-cold 0.15 M NaCl (containing Ca^{2+} and Mg^{2+} as above). The final washed pellets were homogenized in a total volume of 1.0 ml of 0.15 M NaCl, using tuberculin syringes and 26 gauge needles, and aliquots were dispensed for phosphate and protein analyses. Phosphate and protein were measured, respectively, by modifications of the methods of Gerlach and Deuticke [12] and Lowry et al. [9, 13]. Rabbit gamma globulin was used as a standard in the protein assay. As previously defined [9], specific binding refers to μg of protein bound per μmol of liposomal phosphate.

Adsorption of hemolytic antibodies from hemolysin

Sheep Fraction IIa liposomes (5.53 μl), which either contained or lacked retinal, were incubated in 15-ml centrifuge tubes for 3 h at 37 °C with either 15, 30, 50 or 100 μl of hemolysin, and appropriate quantities of veronal-buffered saline in a total volume of 1.0 ml. The antiserum contained $8.32 \cdot 10^{11}$ hemolytic anti-

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* In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences-National Research Council.

body molecules per ml as determined by the Cl fixation and transfer test [14]. The liposomes were removed by centrifugation at 10 000 rev./min (12 000 × *g*) and the supernatant was assayed for hemolytic activity remaining against sheep erythrocytes [15]. The number of molecules of antibody adsorbed by liposomes in each case was calculated based on the decrease of hemolytic titer compared to unadsorbed serum. Specific hemolytic antibody adsorption is defined as the number of molecules of hemolytic antibody adsorbed per μmol of liposomal phosphate.

Detection of the third component of complement (C3) bound to liposomes

Liposomes (50 μl), either containing or lacking retinal, were incubated in 15-ml centrifuge tubes for 30 min at room temperature (approx. 22 °C) with either 300 μl of antigalactocerebroside serum or 30 μl of hemolysin, as appropriate, and 500 μl of fresh human serum (complement). The liposomes were washed four times by adding 10 ml of ice-cold 0.15 M NaCl containing Ca^{2+} and Mg^{2+} and centrifuging at 15 000 rev./min for 10 min at 2 °C. The pellet was resuspended in a total volume of 1.0 ml using 0.15 M NaCl. C3, which was present on the washed liposomes, was then measured by inhibition of anti-C3 antibody, as described by Borsos and Leonard [16].

Measurement of liposomal size distribution

The size distribution of liposomes was determined with an electronic particle size analyzer (Coulter Counter^R, model T, Coulter Electronics, Hialeah, Fla.). This device simultaneously enumerates the number of particles in each of fifteen different sizes based on average volume [17]. Nine of the fifteen sizes were employed in this study and, using a 70-μm aperture, this spanned a total diameter range of 1 – 6.35 μm. Diameter size calibration was performed with latex particles having an average diameter of 3.49 μm (Coulter Electronics). The size of the latex particles was confirmed by photographing a suspension of the particles over a hemocytometer grid. Lantern slides of the photographs were made and the diameters of the latex particles measured on the projected image using a ruler. Analysis was performed on 16 different liposome preparations, half of which contained, and half of which lacked, retinal. The liposomes were swollen and diluted 1:432 525 in a modified Eagle's solution (Isoton^R, Coulter Electronics). Duplicate determinations were made with each liposome preparation, using 500 μl of diluted liposome suspension for each determination. The number of liposomes of each diameter size was corrected by subtracting the number of particles of corresponding size counted in a sham preparation. The sham consisted of Isoton^R carried through all the steps of liposome preparation and dilution. For each of the nine diameter sizes measured, the total surface area and total volume of liposomes in the original 500-μl sample were calculated as follows: area = $\pi d^2 n \cdot$ dilution factor, and volume = $1/6\,\pi\, d^3 n \cdot$ dilution factor, where d is the average diameter and n is the number of particles counted.

RESULTS

Inhibition of complement fixation by retinal

In a previous study with liposomes it was demonstrated that large quantities

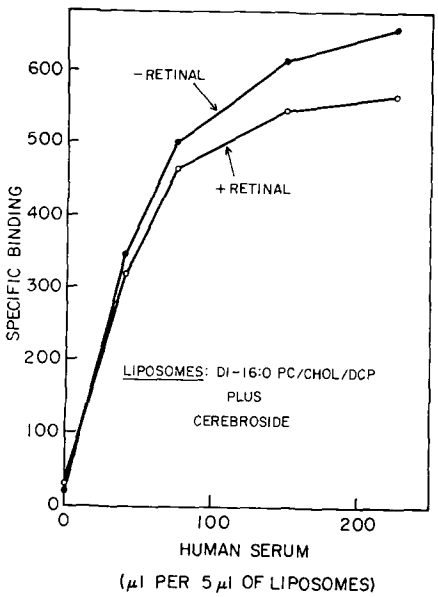

Fig. 1. Effect of retinal on binding of total complement protein. The liposomes contained dipalmitoyllecithin, cholesterol and dicetyl phosphate (in molar ratios of 2 : 1.5 : 0.22) plus galactocerebroside in the amount of 150 μg/μmol of dipalmitoyllecithin. The dipalmitoyllecithin and retinal (where appropriate) were present, respectively, in concentrations of 10 mM and 1 mM compared to the final aqueous volume of swollen (i.e. dispersed) liposomes. The liposomes (50 μl) were incubated for 45 min with 350 μl of clarified (27 000 × *g*, 10 min, 20 °C) rabbit antigalactocerebroside serum and the amounts of fresh human serum shown on the abscissa. Following this the liposomes were washed and protein was measured as described in Materials and Methods. Specific binding refers to the μg of protein bound per μmol of liposomal phosphate. Each curve represents the mean of two experiments performed a month apart. The same batches of antiserum and human serum were used for each determination. Except for one point on one curve which had 2.8 % variation, the range of variation at each duplicated point of human serum was never greater than 0.9 % of the mean.

of complement protein were bound in the presence of specific antibody [11]. The influence of retinal on complement protein binding under similar conditions is illustrated in Fig. 1. The amount of protein bound was measured as a function of the amount of complement (i.e. fresh human serum) which was initially added. At every concentration of complement, liposomes containing retinal bound significantly less total protein. Similar results (not shown) were also found when sheep Fraction IIa liposomes were substituted for cerebroside-containing liposomes.

The experiments in Fig. 1 measured total protein binding in the presence of antiserum and fresh human serum. It was presumed that this mainly corresponded to complement protein binding, as it was almost completely dependent on the presence of liposomal antigen, specific antiserum and fresh, rather than heated, human serum [11]. Direct demonstration of inhibition of complement protein binding by retinal was provided in the experiments shown in Table I. In the presence of specific antiserum, binding of the third component of complement (C3) to liposomes containing retinal was markedly decreased compared to liposomes lacking retinal. Control experiments demonstrated that only about 7% as

TABLE I

EFFECT OF RETINAL ON C3 BINDING

The liposomes were prepared either from pure lipids, as described in the legend to Fig. 1, or from sheep Fraction IIa. In the latter case the sheep phospholipid was 10 mM compared to the final aqueous dispersion of swollen liposomes. Specific C3 binding is defined as μg of C3 bound per μmol of added liposomal phosphate.

Liposome composition	Specific C3 binding to liposomes	
	−Retinal	+Retinal
Sheep Fraction IIa	61.4	44
Dipalmitoyllecithin, cholesterol, dicetyl phosphate and galactocerebroside	61.4	37.5

much C3 was bound to liposomes lacking antigen or when heated complement was used.

Antibody binding

Complement protein binding to liposomes and complement-dependent liposomal membrane damage require the presence of specific antibodies to initiate the complement cascade [10, 11]. If retinal were to interfere with antibody binding this would result in decreased complement activation. Two types of experiments were performed to test the possibility of inhibition of antibody binding. First, the amount of antibody protein bound to liposomes was directly measured. Second, the liposomes were used as an immunoadsorbant to remove hemolytic antibodies directed against sheep erythrocytes. In both cases, liposomes containing or lacking retinal were compared, and the results of these experiments are

TABLE II

EFFECT OF RETINAL ON ANTIBODY BINDING

Liposomes were prepared as described in the legends of Fig. 1 and Table I. To measure protein binding, 50 μl of liposomes were incubated, as described in Materials and Methods, with either 50 μl of rabbit antisheep erythrocyte serum or 500 μl of antigalactocerebroside serum, as appropriate. The conditions for adsorption of hemolytic antibody are given in the text. Specific protein bound is defined in the legend of Fig. 1. Specific antibody adsorption is defined as the number of hemolytic antibody molecules adsorbed per μmol of liposomal phospholipid.

Liposome composition	Specific protein binding to liposomes		Specific antibody adsorption by liposomes ($\times 10^{12}$)	
	−Retinal	+Retinal	−Retinal	+Retinal
Sheep Fraction IIa	38.1	41.0	8.66	8.66
Dipalmitoyllecithin, cholesterol, dicetyl phosphate and galactocerebroside	43.7	39.6	—	—

shown in Table II. Incorporation of retinal into either sheep Fraction IIa or cerebroside-containing liposomes had no appreciable effect on the amount of antibody protein bound. The absorption experiments (Table II) showed that sheep Fraction IIa liposomes removed hemolytic antibodies with equal effectiveness either in the presence or absence of retinal. Thus, it was concluded that retinal did not have a detectable effect on antibody binding to liposomes. These results also suggest that retinal did not influence the number of glycolipid antigen molecules available for antibody binding.

Liposomal geometry

In previous experiments [9], based on the measurements of glucose trapping, light scattering and recoverable phosphate, it was concluded that retinal may not have had a significant influence on liposomal geometric structure. The parameters used in that study, however, were relatively insensitive to size changes, especially in the lipid bilayer. Because of this, the liposomal size distribution was measured directly by using a Coulter Counter. The surface area (Fig. 2b) and volume (Fig. 2c) at each diameter size of liposomes were calculated from the frequency data which is given in Fig. 2a (see Materials and Methods). In terms of both the total surface area (Fig. 2b) and total volume (Fig. 2c), the distribution of liposomes containing retinal was shifted toward smaller diameter sizes and decreased surface area and volume compared to liposomes lacking retinal. These results are the summary of eight experiments each for liposomes containing or lacking reti-

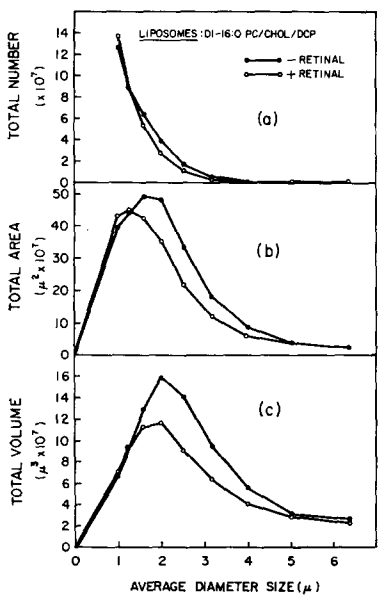

Fig. 2. Effect of retinal on liposomal size distribution. Liposomes were prepared from dipalmitoyllecithin, cholesterol, dicetylphosphate and galactocerebroside as described in the legend of Fig. 1. The bottom two frames (b and c) were drawn from calculations derived from the data shown in the top frame (a) as described in Materials and Methods. The data were adjusted to correspond to 5 μl of liposomes (containing 50 nmol of liposomal phospholipid).

nal and the differences shown were highly significant. Separate calculations, in which the data of Figs. 2b and 2c were each summated, showed that, over the average diameter range of 1–6.35 μm, retinal-containing liposomes had 14.5% less total area ($P < 0.01$) and 19.8% less total volume ($P < 0.01$) than did liposomes lacking retinal.

DISCUSSION

Our results demonstrate that retinal exerted an inhibitory effect on the overall binding of complement protein to liposomes in the presence of specific antibody. At least one early complement component (C3) was also inhibited. It is likely that these effects were at least partly responsible for the previous observation that retinal inhibited complement-dependent glucose release from liposomes [9].

We have shown in this study that the liposomal complement inhibition by retinal was not due to decreased binding of antibody or to decreased availability of antigenic sites. This latter observation strongly suggests that retinal did not influence the total number of lipid molecules present in the outer membrane of the liposomes.

By means of size distribution analysis with a Coulter Counter, we have also demonstrated that incorporation of retinal caused a shift toward smaller liposomes. Liposomes containing retinal had less total surface area and volume. Previously, we found that the aqueous volume of the liposomes, as determined by the amount of trapped glucose, was not affected by retinal [9]. The shift toward smaller liposomes must therefore have been due to diminution of the area and volume of the lipid bilayer, per se. In the absence of loss of molecules from the bilayer (see above) this could only be accomplished by a retinal-induced tighter packing of the lipid components of individual lamellae. The decreased volume could be explained if each individual bilayer were thinner. When summated over all of the lipid lamellae in the liposomes this would result in less total liposomal volume but would not affect the liposomal aqueous volume. In this context, it should be noted that electron micrographs have indicated that at least half of the total liposomal volume is apparently occupied by the concentric lipid membrane phases [18]. We are suggesting that the decrease due to retinal in the total liposomal volume occurred exclusively in the lipid phase, rather than the aqueous phase, and that it was due to a geometric reorientation of the bilayer molecules but was not accompanied by any net loss in the number of molecules. These conclusions are consistent also with the results of a recent electron spin resonance study in which it was demonstrated that retinal improved the degree of order of lipid bilayers [19].

Direct evidence for an interrelationship between the retinal-induced changes in the geometric structure of the membranes and the inhibition of complement binding is not available. It is clear that the surface area of the liposomes was decreased and, therefore, the total effective substrate for complement binding also may have been diminished. Inhibition of binding was observed, however, at low levels of complement when membrane-substrate was presumably still in excess (see Fig. 1).

It is concluded that retinal in liposomes caused inhibition of the complement

binding mechanism which occurred at least as early as the C3 step. Retinal also caused a change in the liposomal structure which was manifested by decreased liposomal size, probably caused by tighter packing of the molecules. These phenomena may have been associated. Thus, retinal inhibition may have been due either to a decrease in the availability of effective membrane substrate for complement or to a decrease in the efficiency of binding of one or more of the complement components.

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