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Binding of Antibodies in Liposomes to Extracellular Matrix Antigens

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We have incorporated antibodies against fibronectin or laminin into liposomes and studied their interaction with insoluble forms of these antigens. The antibodies, after modification by palmitoylchloride, were incorporated into the lipid bilayer by the cholate dialysis method. The antibodies in the liposomes recognized their specific antigen with little reaction to the alternative attachment protein or to albumin (<2%). The binding of antibody-containing liposomes to insoluble antigen was inhibited by soluble antibodies to the respective antigens but not by antibodies to other antigens. The affinity constant of the liposome-antibody complex with the antigen was estimated at $1–10 \times 10^{-9}$ M liposomes. Thus, antibodies in liposomes retain their reactivity and specificity, and the reaction constant is comparable to that observed for immune complexes.

Key words: fibronectin, laminin, liposomes

We are investigating the possibility of using antibody-liposome constructs to localize substances to different extracellular matrices. Such matrices are chemically heterogenous, containing diverse collagens, glycoproteins, and proteoglycans. The relative amount and types of these components, however, are specific to each tissue [1,2]. For example, stromal tissues, such as tendon, dermis, and bone, contain type I collagen, fibronectin, and a small chondroitin sulfate proteoglycan. Basement membranes, the thin extracellular sheets separating epithelial cells from stroma, are formed of different components, including type IV collagen, laminin, and a heparan sulfate proteoglycan [3]. These components are unique gene products that are chemically and immunologically distinct. Laminin and fibronectin are large glycoproteins that link cells to extracellular matrices. Laminin occurs only in basement membranes, whereas fibronectin is widely distributed in fibrous tissues and is also present as a soluble protein in the blood [1–4].

We have incorporated antibodies to laminin and to fibronectin into liposomes and studied their ability to recognize their respective antigens. Such investigations are a first step in determining whether liposomes can target to specific matrices. Lipo-

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somes can be constructed as small, spherical vesicles with an outer phospholipid membrane enclosing a central aqueous domain [5–7]. The advantage of liposomes that contain antibody over soluble antibody is that drugs, hormones, or other factors can be introduced into their central aqueous domain. After the liposomes are targeted to the specific antigens, their contents may be released. Since liposome incorporation of specific antibodies to tumor cells has shown promise for application to cancer chemotherapy [8], it might be possible to direct liposomes to specific matrices by using antibodies to tissue-specific matrix components. In previous studies, we showed that liposomes prepared with antibodies to type I collagen and fibronectin bind to the matrix deposited by endothelial cells in culture [5]. Here we have utilized antibodies to laminin and to fibronectin in the preparation of liposomes and have found that the antibodies, when incorporated into liposomes, retain their specificity for these matrix components. Similar specificity of antibodies bound to liposomes has been reported to other systems [8–10].

MATERIALS AND METHODS

Materials

Pure egg lecithin was obtained from the Kharkov plant (USSR), and palmitoylchloride from BDH (Germany). Cholic acid was obtained from Calbiochem (La Jolla, CA) and was further purified by recrystallization from ethanol. [^{3}H]-Cholesterol (52 Ci/mmole) and cholesteryl-[^{14}C]-oleate (25.7 mCi/mmole) were obtained from Amersham (Arlington Heights, IL), and Sepharose CL4B and Ficoll 400 from Pharmacia (Uppsala, Sweden). Fibronectin was isolated from serum by gelatin affinity chromatography [11]. Highly purified laminin was isolated from the Engelbreth-Holm-Swarm (EHS) basement membrane producing tumor [12]. Antibodies against fibronectin and laminin were prepared in rabbits [13], purified by affinity chromatography, and their specificity was confirmed by enzyme-linked immunosorbent assay (ELISA) and by immunoprecipitation [14].

Methods

Antibodies, including a small amount of [^{125}I]-labeled rabbit IgG (nonspecific) (2×10^5 cpm/μg), were modified by incubation with palmitoylchloride [6]. Ten microliters of a 10% solution of palmitoylchloride in water-free acetone was added to 0.5 ml of the antibody solution, which contained 2% sodium cholate in 0.1 M phosphate buffer, pH 8.0. The mixture was briefly sonicated and to it was quickly added 0.5 ml of antibody solution (1.2 mg/ml) in 0.15 M NaCl plus 0.02 M sodium phosphate, pH 7.4 (phosphate buffered saline [PBS]). To prepare liposomes with bound protein, this solution was incubated for 1 hr, centrifuged at 12,000g for 10 min, mixed with an equal volume of a lipid solution composed of either 5 mg/ml egg lecithin plus trace quantities of [^{14}C]-cholesteryl oleate or of [^{3}H]-cholesterol dissolved in 2% sodium cholate, and then dialyzed against PBS. All procedures were performed at 4°C. Subsequently, liposomes were separated from protein aggregates and from non-bound antibodies by successive contrifugation for 12 min at 12,000g and by gel filtration on a Sepharose CL4B (Uppsala, Sweden) column [15] or by flotation after centrifugation in 10% Ficoll 400 (Uppsala, Sweden) [16]. The quantity of protein bound in liposomes, in moles of protein per mole lipid, was estimated in a separate experiment by measuring the liposome-associated [I^{125}]-immunoglobulin in a gamma counter Ractigamma II (LKB)(Gaithersburg, MD).

Experiments on liposome binding to protein monolayers were performed as

follows. Polystyrene plates (Falcon 3008 (Oxnard, CA) Multiwell: area 2 cm^2 and volume 1 ml) were incubated for 24 hr at 4°C with either laminin, fibronectin, or bovine serum albumin (4 μg of protein per well in 0.4 ml carbonate buffer, pH 8.3). Subsequently, the coated wells were rinsed with water and incubated for 1 hr in PBS containing 2 mg/ml of bovine serum albumin. Then the liposome preparations were added to the coated wells in PBS containing 2 mg/ml of bovine serum albumin. Incubation of liposomes was performed at 20°C for 1 hr on an orbital incubator at 100 rpm with 0.3 ml of liposome per well. In inhibition experiments, free antibodies were added to the liposome preparations at various concentrations and incubated at 20°C, with shaking, in the coated wells. Subsequently, non-bound liposomes were removed with PBS containing 2% bovine serum albumin, and the wells were washed five times with PBS alone. The bound liposomes were liberated from the wells with a hot (70°C) 10% solution of sodium dodecyl sulfate (SDS) in water. The quantity of monolayer-bound liposome was determined by counting the bound radioactivity of the liposomal lipid in a liquid scintillation counter.

Enzyme-linked immunosorbent assays [14] were carried out to confirm the specificity of the antibodies used. Microtiter polystyrene plates were coated overnight with 500 ng/well of protein in carbonate buffer. The unbound antigen was removed by three successive washes with PBS containing 0.02% Tween 20 (PBS-Tween). Various concentrations of each antibody in PBS-Tween (Sigma Chemical Co, St. Louis, MO) were added to the wells and incubated for 1 hr. After three washes with PBS-Tween, a second antibody, peroxidase-conjugated goat anti-rabbit, was added to the wells for 1 hr. After the substrate was added, the amount of bound second antibody was measured spectrophotometrically using a Titertex Multiskan (Flow Labs, Rockville, MD).

RESULTS

The specificity of the antibodies to be incorporated into liposomes in this study was assayed by measuring their binding to plastic surfaces coated with laminin, fibronectin, or albumin. The antilaminin antibodies showed a strong reaction with laminin substrates and no reaction with the fibronectin substrates (Fig. 1). Some binding to the albumin-coated surface was observed, but this represented less than 2% of that observed with the laminin substrate. The anti-fibronectin antibody also showed considerable specificity for the fibronectin coat and little or no reaction with either laminin or albumin substrates.

The modification of proteins with hydrophobic residues has been used successfully to bind antibody molecules to liposome membranes [17,18]. The antibody molecules were first modified by incubation with palmitoylchloride, which allows the preparation of mixed micelles of protein, lipid, and detergent. We found that as much as $3 - 4 \times 10^{-4}$ moles of protein were incorporated per mole of lipid. These estimates are based on measurements of incorporation of [^{125}I]-labeled immunoglobulin into the liposome and correspond to the binding of 30–40 protein molecules per liposome (assuming that the liposomes have an average diameter of 100 nm and that protein is equally distributed among the liposomes).

In a typical experiment, the [^{125}I]-immunoglobulin/[^{14}C]-lipid ratio before liposome separation from unbound protein was 3.64×10^{-2}. It corresponded to 4.72×10^{-4} moles protein per mole lipid. After separation of unbound protein by the Ficoll flotation method, the [^{125}I]/[^{14}C] ratio was 2.39×10^{-2}. This corresponds to

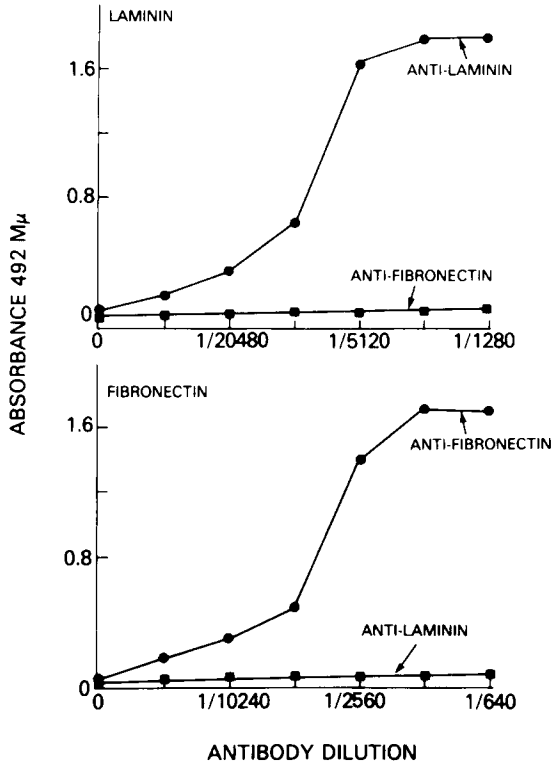

Fig. 1. Enzyme-linked immunosorbent assay (ELISA) determining the specificity of the anti-fibronectin antibodies for fibronectin and the anti-laminin antibodies for laminin. Laminin antibody shows a strong reaction with the laminin substrate (upper panel) and little or none with fibronectin. Antibody to fibronectin (lower panel) shows a strong reaction with fibronectin but little or none with laminin.

TABLE I. Binding of Antibody Containing Liposomes With Protein Monolayers*

Liposome bound antibodies	Added lipid (dpm) per well × 10^5	Protein in substrate[a]		
		Laminin	Fibronectin	Albumin
Anti-laminin	8.5	12,797	Not determined	260
Anti-fibronectin	7.1	257	16,718	Not determined

*Incubation was performed in 300 μl PBS containing 2 mg/ml albumin for 1 hr on an orbital incubator at +20°C. Excess liposomal lipid (135 μg anti-laminin and 113 μg anti-fibronectin liposomal lipid) was added for each incubation.

[a]Data are expressed as dpm of liposomal [^{3}H]-cholesterol (specific activity 6,300 dpm per microgram of liposomal lipid) bound to the monolayer.

3.1×10^{-4} moles protein per mole lipid. In the control experiments, it was found that for the preparation of protein treated in the same way but containing no lipid, 55 times less protein was present than for the liposome-protein sample (ie, there was a negligible amount of free protein in the liposome-protein sample).

The liposome-bound antibody (i) preserved its affinity and (ii) maintained its specificity (Table I). Anti-laminin-antibody-bearing liposomes specifically recognized laminin monolayers with up to 64 times higher binding of liposomal lipid to laminin

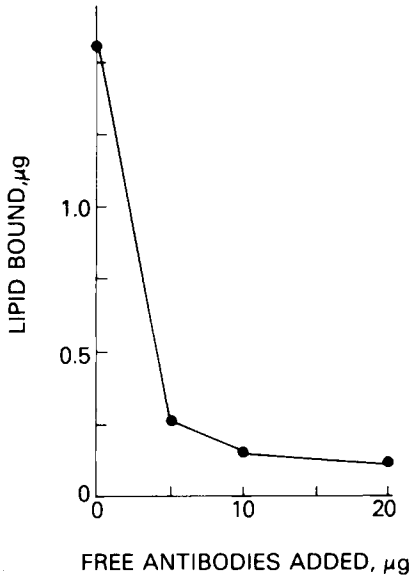

Fig. 2. Inhibition of anti-fibronectin antibody modified liposome binding to fibronectin monolayers in the presence of increasing amounts of free antibodies. The free antibody, at the concentrations designated, was added to the monolayer 5 min before the liposomes were added. Liposomal lipid (37.7 μg) was added to each well in a final volume of 0.3 ml.

than to either fibronectin or albumin monolayers. Binding of liposomes containing no protein was nearly the same as nonspecific binding of antibody liposomes (data not shown). Also, the addition of anti-fibronectin antibodies blocked the binding of liposomes carrying anti-fibronectin to fibronectin substrates, assayed in this case by the binding of [^{3}H]-labeled liposomes (Fig. 2). As expected, anti-laminin antibodies had no effect on the binding of the anti-fibronectin containing liposomes to fibronectin substrates (data not shown). When the converse studies were conducted on liposomes with antibody to laminin, competition was observed with anti-laminin antibodies but not with anti-fibronectin antibodies. These results indicate that these antibodies retain their specificity when incorporated into liposomes.

An estimate of the dissociation constant for liposome-antibody binding to the target was made by varying the amount of liposomes over a range of 5–150 μg lipid incubated with antigen monolayers. The typical data obtained are expressed as the reciprocal of the amount bound versus the reciprocal of the amount initially added (Fig. 3). Three such studies gave binding constants in the range $1 - 10 \times 10^{-9}$ M liposomes. These data were obtained assuming liposomes to be single-particle-containing lipid molecules. Values obtained correspond to binding constants observed in the reaction of antigens with free antibody molecules.

DISCUSSION

Various extracellular matrices contain diverse components that are antigenically distinct. This specificity may be useful in the targeting of liposomes to extracellular matrices. In this report, antibodies to laminin and to fibronectin were tested for their

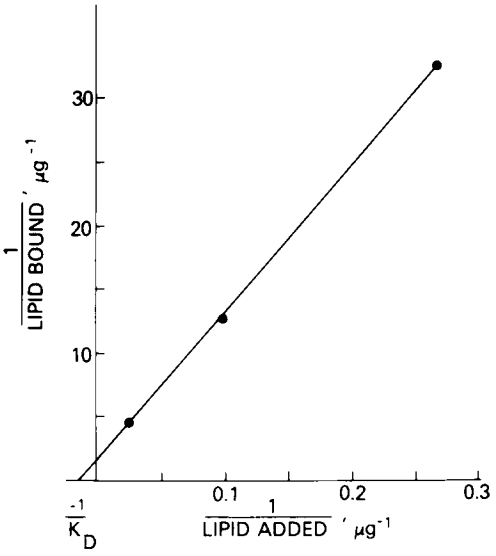

Fig. 3. Binding of various amounts of anti-laminin antibody modified liposomes to laminin monolayers. The amount of liposome bound was determined as described in Materials and Methods.

reaction with immobilized laminin and fibronectin, either directly or after incorporation into liposomes. Modification of the antibody molecules was achieved with palmitoylchloride, allowing the protein to be incorporated into the liposome membrane with retention of antigenic activity and specificity.

In principal, these antigen-liposome complexes offer the possibility of delivering drugs directly to target tissues. Accumulated fibronectin could provide a marker for clots and wounds. Indeed, a recent study [19] showed differences in the organ distribution after the injection of free or liposome-bound fibronectin. Free fibronectin was removed by the liver, whereas liposome-bound fibronectin accumulated in lung. Laminin, however, is found only in basement membranes [3]. Anti-laminin could be used to target specifically to the exposed basement membranes of blood vessels. Injection of anti-laminin antibodies into animals has shown that it concentrated along basement membranes [20–22]. Furthermore, the antibody was cleared from the membranes over a few days and elicited little or no toxic response. Studies with anti-laminin liposomes carrying anticoagulants would be of interest for experimental models of atherosclerosis, where platelets accumulate on areas of exposed basement membranes and are believed to elicit a transarterial migration of smooth muscle cells [23]. Similarly, thrombus-lysing enzymes could be delivered to such areas in liposomes.

The present report establishes the activity and specificity of the antibody-liposome complexes to matrix proteins. Their value as a drug delivery system is under investigation. Given the wide variety of matrix collagens, glycoproteins, and proteoglycans, it is likely that one could develop specific delivery systems to bones, teeth, tendons, cartilage, and many other unique matrix sites where growth, repair and maintenance are impaired by disease.

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