Monoclonal Antibody Covalently Coupled with Fatty Acid

A REAGENT FOR IN VITRO LIPOSA_ME TARGETING*

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Monoclonal antibody to the mouse histocompatibility antigen, H-2^*, was derivatized with palmitic acid using an activated ester of N-hydroxysuccinimide. About 70% of the resulting amphiphatic antibody could be incorporated into liposomes by a detergent-dialysis method. The antibody-bound liposomes were about 900 Å in diameter and were heterogeneous in terms of the number of antibody molecules per liposome. These liposomes showed specific binding affinity to mouse L-929 cells (H-2^*), but not to A-31 cells (H-2^*), whereas native liposomes showed no detectable binding to either cell type. The specific binding of anti-H-2^*-bound liposomes to L-929 cells could be blocked by a preincubation of cells with an excess of free, undervatized anti-H-2^*-antibody but not by normal mouse IgG. Using a fluorescent phospholipid, liposomes containing anti-H-2^* specifically labeled L-929 cells but not A-31 cells in a mixed culture. Liposomes containing normal mouse IgG did not significantly label either cell type. These results clearly demonstrated the effectiveness of the monoclonal antibody for liposome targeting.

Targeting of liposomes to specific cell types has been attempted previously (1-4). Among different targeting molecules tested, immunoglobulins are best adapted for this purpose due to their relative ease of preparation and the versatility of their antigen specificity. Immune IgG used in the previous studies contained relatively small amounts of specific antibody. Consequently, the number of antibody molecules that could be bound to the liposome surface was diluted with nonimmune IgG. Monoclonal antibody preparations are now available which contain a homogeneous population of specific antibody. This greatly increases the possibility of incorporating a large number of target-specific molecules into each liposome and thus provides the liposomes with the potential for multivalent binding (5). Furthermore, covalent coupling of the antibody to the liposomal lipids is important for a stable association of the antibody to liposomes and this is crucial in liposome targeting attempts. We report here the use of a monoclonal antibody to the mouse major histocompatibility antigen as a model system. We have developed a method with which antibody can be derivatized with fatty acid. Specific binding of liposomes containing these amphipathic antibody molecules to target cells was demonstrated.

MATERIALS AND METHODS

Antibody—The hybridoma cell line 11-4.1 was a generous gift from the Cell Distribution Center, The Salk Institute, La Jolla, Calif., and was originally isolated by Ob et al. (6). The cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum and 1 mM Na-pyruvate. The monoclonal anti-H-2^* antibody in the culture fluid and MlgG from normal mouse ascites fluid (7) were purified by a protein A affinity column as described (8). The immunoglobulins were radiolabeled with [125] using chloramine-T to a specific radioactivity of 3 to 5.5 x 10^6 cpm/mg.

Lipid and Radioabeled Lipid—Phosphatidylcholine was purified from egg yolk as described (9). Hexadecyl [14C]cholesterol ether was synthesized from [14C]cholesterol (New England Nuclear) and hexadecyl methane sulfoxide and purified by silicic acid chromatography (10). This radio labeled lipid is a faithful marker for the bulk of the liposomal lipids and it is neither exchangeable with cellular lipids nor metabolized by cells (11). Trace amounts of hexadecyl [14C]cholesterol ether were added to phosphatidylcholine in chloroform to obtain a final specific radioactivity of 2.5 x 10^6 cpm/mg of lipids. N-Hydroxysuccinimide ester of palmitic acid was synthesized and purified by recrystallization from C,H,OH (12). Samples were counted in a dual channel liquid scintillation counter.

Coupling of Antibody with Palmitic Acid—Two milligrams of purified [125]labeled anti-H-2^* or MlgG was added to 44 µg of N-hydroxysuccinimide ester of palmitic acid in PBS containing 2% deoxycholate. The mixture was incubated at 37°C for 9 to 15 h and then chromatographed on a Sephadex G-75 column (1.3 x 45 cm) in PBS containing 0.15% deoxycholate to remove excess palmitic acid. The void volume peak which contained the immunoglobulin was collected and concentrated to one-sixth of the original volume on an Amicon ultrafiltration apparatus (XM50 filter). The concentration of deoxycholate in the concentrated antibody solution was about 0.3 to 0.4% as determined using [14C]deoxycholate.

Liposome Formation—H-lipid in PBS (16 mg/ml) was sonicated for 20 min in a probe type sonifier (Heat Systems, W-375) at 1°C under argon. Derivatized antibody was then added to the sonicated lipid at a lipid to protein ratio of 20:1 (w/w). Concentrated deoxycholate was also added to obtain a final concentration of 0.7%. The slightly turbid mixture was then dialyzed in Spectrapor-2 dialysis tubing (Spectrum) against three changes of 3 liters of PBS for 4 h at room temperature. The turbid liposome suspension was then loaded on a Sepharose 4B column (26 x 100 cm), pre-equilibrated, and eluted with PBS. The void volume peak which contained both H and [125]counts was collected and referred as antibody-bound liposomes (Fig. 1). The control liposomes were identically prepared with H-lipid alone.

Liposome Density Gradient Centrifugation—Analytical linear sucrose gradients (0 to 20% with a 65% sucrose cushion) were centrifuged in an SW 50.1 rotor at 200,000 x g for 5 h at 4°C and then fractionated from the bottom using a peristaltic pump.

* The abbreviations used are: MlgG, mouse immunoglobulin G; PBS, phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH,PO4, 1 mM Na,HPO4, pH 7.4); NBD-PE, N-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine.

† G. L. Pool, M. E. French, R. A. Edwards, L. Huang, and R. L. Lumb, unpublished observation.

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Liposome-Cell Incubation—L-929 (H-2k, a fibroblast line from C57 mouse) and A-31 (H-2d, a subclone of BALB/c 3T3 fibroblast line) cells were grown as monolayer cultures on 35-mm multiwell Linbro plates in McCoy’s medium with 10% fetal calf serum. Cultures near confluency (about 5 × 10⁶ cells/well) were used. Liposome-bound antibody or M IgG was added together with 1 ml of culture medium. Cells were incubated at 4°C for 3.5 to 4 h with gentle rocking. At the end of the incubation, cells were washed three times in cold PBS, scraped from the plates using a rubber policeman, and further washed with 1 ml of PBS by centrifugation. The final cell pellet was solubilized in 0.2 ml of 2% Na dodecyl sulfate, transferred to scintillation vials, and counted. Controls including free antibody or M IgG, with and without free liposomes, were incubated and processed identically. Measurements were done in duplicate.

Liposomes Labeled with Fluorescent Phospholipid—Fluorescent-labeled liposomes containing either fatty acid-derivatized anti-H-2k or M IgG were prepared identically as above, except that a mixture of phosphatidylcholine and NBD-PE (Avanti Biochemicals, Birmingham, Ala.) at a 95:5 molar ratio was used. L-929 and A-31 cells were grown on glass coverslips for 2 to 3 days either separately or as a mixed culture. The washed coverslips were transferred to a clean 35-mm dish into which 1 ml of IgG-bound liposomes (5 μg of IgG/ml in McCoy’s medium) was added. Incubation was done at 4°C for 3.5 to 4 h. Cells were washed three times in PBS and were kept in cold PBS until immediately before examination in a fluorescence microscope. A Leitz Orthoplan epiluminescence microscope equipped with an Orthomat-W camera was used. All fluorescence pictures were taken with the same exposure time.

RESULTS

Binding of Derivatized Antibody to Liposomes—A mixture of palmitate-derivatized antibody and sonicated lipids in deoxycholate was dialyzed extensively. When the dialysate was applied to a Sepharose 4B column, more than 70% of the derivatized antibody co-eluted with liposomes in the void volume (Fig. 1), suggesting their association. To confirm the association of the derivatized antibody and liposomes, a sample of the pooled void volume fractions was analyzed by linear sucrose density gradient centrifugation. Both lipid and protein floated on the top of the gradient (Fig. 1, inset). In control experiments not shown, aggregated and free antibody sedimented at the bottom and the middle (shown by arrow) of the gradient, respectively. These results indicated that the fatty acid-derivatized antibody can be incorporated into liposomes by the method used. The native, derivatized antibody did not incorporate into liposomes with an identical procedure, as the protein and liposomes were eluted separately on Sepharose 4B (data not shown). An identical sedimentation profile on sucrose density gradient was obtained for liposomes which have been stored at 4°C for 3 weeks, indicating that the association of antibody with liposomes was stable.

Liposome Characterization—Antibody-bound liposomes were quite homogeneous in size. Negative stain electron micrographs of antibody-bound liposomes showed that the average diameter of these liposomes was about 900 Å, similar to that of the protein-free liposomes prepared with an identical procedure (micrographs not shown). Although these liposomes were uniform in size, they were rather heterogeneous in terms of the amount of antibody molecule bound per liposome. Sucrose gradient analysis (Fig. 1, inset) showed that the ratio of 125I-labeled antibody to 3H-lipid varied across the fractions containing these labels, indicating that liposomes containing higher concentrations of protein sedimented further into the gradient. To study this heterogeneity further, the antibody-bound liposomes were precipitated by goat antiserum to mouse IgG. About 66% of the total anti-H-2k, but only 28% of the total lipid, in the liposome was precipitable. These results again confirmed the heterogeneous distribution of antibody molecules per liposome.

Liposome Binding to Target Cells—The antibody-bound liposomes were incubated with either L-929 (H-2k type) or A-31 (H-2d type) cells, and the uptake of both 125I-labeled antibody and the 3H-lipid were determined. The controls were (a) M IgG bound liposomes, (b) free antibody with and without free liposomes, (c) free M IgG with and without free liposomes, and (d) free liposomes alone. As can be seen in Table I, A-31 cells did not show significant binding of either liposomes or protein. However, significant binding of antibody to target cells, i.e., L-929 cells, was evident. Furthermore, liposomes bound to L-929 cells only when they were antibody-bound:

| Addition            | L-929 cells (H-2k) | A-31 cells (H-2d) |
|---------------------|---------------------|-------------------|
|                     | Lipid (counts/min)  | Protein (counts/min) |
| Free liposome       | <25                 | <25               |
| Anti-H-2k-liposome  | >25                 | >25               |
| M IgG-liposome      | >25                 | >25               |
| Free anti-H-2k      | <25                 | <25               |
| Free M IgG          | <25                 | <25               |
| Free anti-H-2d +    | >25                 | >25               |
| free liposome       | <25                 | <25               |
| Free M IgG + free   | <25                 | <25               |
| liposome            |                     |                   |

* Ratio of counts per min of 125I/counts per min of 3H was 3.17. The ratio in the applied liposome was 0.75.

| Table II Inhibition of binding of antibody-bound liposomes to L-929 cells |
|-----------------------------|-----------------------------|-----------------------------|
| Anti-H-2k* Pretreatment     | Lipid (counts/min) 3H | Protein (counts/min) 125I |
| Liposome-bound              | None                        | 753 ± 160                   | 3102 ± 500                 |
| Anti-H-2k                   | <25                        | 50 ± 16                     | 3525 ± 559                 |
| M IgG                       | 732 ± 186                  | 6037 ± 576                  |
| Free*                       | None                        | 6390 ± 863                  | 128 ± 37                   |
| Anti-H-2d                   | <25                        | 50 ± 16                     |
| M IgG                       | 732 ± 186                  | 6037 ± 576                  |

* Protein (100 μg/ml) was added 3.5 h before the addition of radiolabels.

* Anti-H-2k (1 μg/ml) and 32 μg/ml of lipid were added.

* Anti-H-2d (1 μg/ml) was added.
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FIG. 2. Binding of fluorescent liposomes to cells. Anti-H-2\(^{k}\) antibody-bound liposomes labeled with NBD-PE were used to label L-929 cells (a and b), A-31 cells (c and d), or a mixed culture of the two types of cell (e and f). Panels a, c, and e are phase-contrast photographs; Panels b, d, and f are fluorescence photographs. In e, A indicates an A-31 cell colony, which has the characteristic large nucleoli; L indicates L cell colony. Bar, 10 \(\mu\)m.

free liposomes with or without free antibody showed no binding. In controls, MIgG, whether liposome-bound or free, did not bind to either type of cell nor did it facilitate the binding of liposomes to cells. These results clearly demonstrated the specific binding activity of the antibody-bound liposomes to the target cells.

In order to demonstrate that the binding of antibody-bound liposomes to L-929 cells was mediated by specific antigen-antibody binding, cells were pretreated with excess free, unlabeled anti-H-2\(^{k}\) before the addition of liposomes. As shown in Table II, a 100-fold excess of free antibody completely inhibited the cellular uptake of both lipid and protein from the anti-H-2\(^{k}\)-associated liposomes. Only anti-H-2\(^{k}\) could inhibit; MIgG at the same concentration had no effect. In controls, the binding of free \(^{125}\)I-labeled anti-H-2\(^{k}\) was blocked by a pretreatment of free, unlabeled anti-H-2\(^{k}\), but not by MIgG, as would be expected.

Specific Labeling of Target Cells with Fluorescent Liposomes—The binding of the antibody-bound liposomes to target cells was also demonstrated by the use of fluorescent phospholipid. Liposomes containing the derivatized anti-H-2\(^{k}\) or MIgG were prepared using NBD-PE as a fluorescence marker for liposomes. Previous studies have demonstrated that the transfer of this fluorescent lipid from liposomes to plasma membranes by lipid exchange is insignificant (13). Therefore, the appearance of this lipid on the cells would most likely indicate the association of the entire liposome structure with cells, rather than the uptake of the individual fluorescent lipid. The IgG-bound fluorescent liposomes were incubated with cells. No significant fluorescence was found on A-31 cells no matter what type of liposomes was used (Fig. 2, c and d). However, the L-929 cells were brightly fluorescent after incubating with anti-H-2\(^{k}\)-liposomes (Fig. 2, a and b). The fluorescence appeared mostly on the cell periphery with a somewhat patchy distribution. Furthermore, all cells examined were fluorescent. On the contrary, L-929 cells incubated with MIgG-liposomes showed only background fluorescence (data not shown). The most convincing evidence of the target-specific binding of liposomes came from the experiment in which a mixed culture of L-929 and A-31 cells was incubated with anti-H-2\(^{k}\)-liposomes. Only L-929 cells were fluorescent (Fig. 2, e and f). These results directly indicated that the targeting of liposomes to the L-929 cells was achieved by the use of the fatty acid-derivatized anti-H-2\(^{k}\).
DISCUSSION

The present study has demonstrated two novel aspects of antibody-mediated liposome targeting: (a) the covalent coupling of antibody to liposomal lipids, and (b) the use of monoclonal antibody. The necessity of covalent coupling stems from the requirement of stable binding of antibody to the liposome surface. Furthermore, immune IgG noncovalently bound to liposomes by sonication (14) showed poor binding activity to cell surface antigens. Purified monoclonal antibody can provide an immunoglobulin population which is maximally enriched with high affinity antibody. Monospecific antibody bound to liposomes should promote multivalent binding to the target cells (5). Immune IgG preparations which contain nonantibody IgG cannot generate liposomes with maximally enriched high affinity antibody. Monospecific binding to the target cells (5). Immune IgG. We have used a monoclonal antibody to the mouse H-2\(^k\) antigen as a model system. Although the IgG produced by the hybridoma line, 11-4.1, is not homogeneous (the light chains of both parental types are produced), the antibody population shows high affinity to the cell surface H-2\(^k\) antigen (6). The apparent dissociation constant, \(K_d\), is about \(10^{-10}\) M. Furthermore, it can be readily coupled to fatty acid without losing its antigen-binding activity. It is therefore a good reagent for in vitro liposome targeting. The characterization of the fatty acid-derivatized anti-H-2\(^k\) will be published elsewhere.

Although the antibody-bound liposomes were rather homogeneous in size, the amount of antibody per liposome was quite heterogeneous. As a consequence, one would predict that only those liposomes which had enough antibody bound on the surface would bind to target cells. This was indeed the case; the ratio of antibody to lipid in the fraction of antibody-bound liposomes adhering to cells was significantly higher than that of the applied preparation (3.17 versus 0.75, Table 1). On the average, it was estimated that there were 67 antibody molecules/liposome for those liposomes that did bind, as compared to only 16/liposome for those liposomes in the original population.

We have deliberately carried out the liposome-cell incubation experiments at 4°C to avoid any possible complicated interactions after the initial binding of liposomes, such as endocytosis, fusion, and molecular exchange. Therefore, the present experiments do not address the question of whether the contents of liposomes are transferred to cells after liposome binding. Furthermore, the orientation of antibody in the liposome membrane is unknown. We are currently investigating these problems, which are of vital importance for the purpose of liposome targeting for therapy.

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\(^1\) S. J. Kennel and L. Huang, unpublished observation.

\(^2\) P. T. Naylor and L. Huang, unpublished observation.