Title

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Journal

Journal of Cell Biology, 101(6)

ISSN

0021-9525

Author

Brodsky, FM

Publication Date

1985-12-01

DOI

10.1083/jcb.101.6.2047

Peer reviewed

Clathrin Structure Characterized with Monoclonal Antibodies. I. Analysis of Multiple Antigenic Sites

FRANCES M. BRODSKY
Becton Dickinson Immunocytometry Systems, Mountain View, California 94039

ABSTRACT Three monoclonal antibodies that react with previously undefined antigenic determinants on the clathrin molecule have been produced and characterized. They were isolated from a fusion between myeloma cells and popliteal lymphocytes from SJL mice that had received footpad injections of human brain clathrin. This protocol was chosen to favor the production of antibodies to poorly immunogenic proteins and thereby increase the repertoire of anti-clathrin monoclonal antibodies. One antibody (X16) reacts preferentially with the heavier of the two clathrin light chains (LC_a) when it is not associated with heavy chain. This specificity is different from that of the anti-LC_a antibody, CVC.6, which has preferential reactivity with heavy chain-associated LC_a. In addition, X16 and CVC.6 bound simultaneously to LC_a, confirming that they react with different sites. The other two antibodies produced, X19 and X22, react with two different determinants on the clathrin heavy chain, based on immunoprecipitation, Western blot, and binding studies. Competitive binding studies with anti-clathrin monoclonal antibodies showed that they define a total of five distinct antigenic determinants on bovine clathrin.

Endocytosis and biosynthesis of membrane-bound and secreted proteins are accompanied by membrane translocation (1). During these processes, closed membrane vesicles that contain internalized receptors and ligands or newly synthesized glycoproteins are formed within the cytoplasm (2, 3). These vesicles are surrounded by a polyhedral network of the protein, clathrin, and are therefore known as coated vesicles (4, 5). At the cell surface, assembly of clathrin into a membrane-associated network occurs simultaneously with membrane invagination, forming a coated pit (6). This circumstantial evidence, combined with the fact that purified clathrin will form a closed polyhedral structure under certain ionic conditions, suggests that clathrin assembly is essential to the mechanics of membrane translocation. (7–11). Clathrin is composed of three polypeptides, a heavy chain of 180 kD and two light chains, LC_a and LC_b, of 32–38 kD, depending on the tissue of origin (5, 9–11, 12). The LC_a light chain is 2–3 kD greater than the LC_b light chain. The light chains of brain clathrin are both of higher molecular weight than those in nonbrain tissue such that brain LC_b has approximately the same molecular weight as LC_a from other tissues (12). Clathrin can be removed from isolated coated vesicles by treatment with high pH or high concentrations of Tris or urea (8–10). Further fractionation by size-exclusion chromatography has demonstrated that clathrin heavy and light chains co-purify in a unit composed of three heavy chains and three light chains. Electron microscopy studies have shown that this unit has a distinct morphology. It resembles a three-legged pinwheel, known as a triskelion, which is geometrically favorable for forming the pentagons and hexagons characteristic of assembled clathrin polyhedra (9–11).

Production of monoclonal antibodies (MAbs)[1] to clathrin has already proved an informative approach to understanding structure–function relationships between the clathrin molecule, clathrin assembly, and membrane translocation. MAbs to a determinant on the LC_a light chain were used by Kirchhausen et al. to localize the position of this light chain on the clathrin triskelion to the portion of the triskelion leg proximal to the vertex (13). The same MAbs were used by Brodsky and Parham (12) to investigate the polymorphism of the light chains in different tissues and by Holmes et al. (14) to purify separated LC_a and LC_b from brain and adrenal glands and compare their primary structure by peptide mapping. Louvard et al. (15) have produced a MAb that reacts with clathrin heavy chain which has been used in immunofluorescence studies to demonstrate the existence of a pool of unpolymer-

[1] *Abbreviations used in this paper:* MAb, monoclonal antibody; RIA, radioimmunoassay.

ized clathrin within a cell. Kelly et al. (16) have used MAbs to bovine brain clathrin LC_a and to chicken coated vesicles to demonstrate the association of tubulin with coated vesicles. One noticeable feature of these studies is the limited number of antibodies used. Even more limited is the number of different antigenic determinants that have been defined on the clathrin molecule, suggesting that clathrin may be a poorly immunogenic antigen. This is further supported by the weak binding properties of the MAbs that have been produced. The anti–heavy chain MAb is of the IgM isotype characterized by low affinity individual binding sites (15). Its broad species cross-reactivity is probably a result of enhanced avidity acquired from the 10 antigen combining sites on an IgM molecule. The anti-LC_a MAbs are of the IgG isotype and have a binding site affinity that makes them useful in biochemical studies (13). However, their avidity is low and they react only with bovine clathrin.

This paper describes the production and characterization of IgG monoclonal antibodies that react with two newly defined antigenic determinants on clathrin heavy chain and one new determinant on the LC_a light chain. These three MAbs react with both human and bovine clathrin. They were produced following an immunization procedure designed to overcome poor antigenicity. Human clathrin was used as an antigen so that resulting antibodies might eventually be used to study clathrin-mediated processes in human cell lines in which many receptors have also been defined with MAbs. During the characterization of these new MAbs, their specificity was compared with the specificity of two existing anti-clathrin antibodies to determine the total number of antigenic sites thus far defined on the clathrin molecule.

MATERIALS AND METHODS

Monoclonal Antibody Production (17)

Five female SJL mice were immunized with purified human brain clathrin by the injection into each footpad of 5 μg in complete Freund's adjuvant on day 17, 5 μg in incomplete Freund's adjuvant on day 14, and 10 μg in 0.5 Tris, 0.05 M 2-(*N*-morpholino)ethane sulfonic acid (pH 6.2), 0.5 mM EGTA, 0.25 mM $MgCl_2$ on days 10, 7, 4, and 1 before fusion. On day zero the popliteal lymph nodes were removed, and the lymph node cells were fused with the myeloma cell line SP2/0-Ag14 (18) in a ratio of 2:1, according to the method of Galfrè et al. (19). Resulting hybridoma cell lines were cloned twice by limiting dilution. Cell lines were grown in ascites fluid of irradiated (300 rad) BALB/c mice, injected with 0.5 ml 2,6,10,14-tetramethyl-pentadecane (pristane) before cell passage. Antibody was purified from ascites fluid by ammonium sulfate precipitation and chromatography on Sephadex G-200, as previously described (20). Isotyping was carried out with a kit from Southern Biotechnology Associates (Birmingham, AL).

Radioimmunoassays (RIA)

IODINATION: Monoclonal antibodies or affinity purified anti-immunoglobulin antibody (25 μg in 100 μl 0.3 M phosphate buffer, pH 7.4) were labeled with 1 mCi ^{125}I-NaI (IMS.30, Amersham Corp., Arlington Heights, IL) by incubation with one Iodo-bead (No. 28660, Pierce Chemical Co., Rockford, IL) for 5 min at room temperature. Free iodine was removed by gel filtration over a 10-ml column of Sephadex G-50 (medium) equilibrated in phosphate-buffered saline, pH 7.4, with 0.5% bovine serum albumin (PBS/BSA).

INDIRECT SOLID-PHASE RIA (21): Clathrin, clathrin components, or coated vesicles (50–500 μg/ml in PBS) were applied to the wells of polyvinyl chloride microtiter plates, 20 μl/well. After 1 h at room temperature, plates were washed twice in PBS/BSA, and 200 μl PBS/BSA was left in the wells for 1 h at room temperature and removed. Antibodies as hybridoma culture supernatant or purified at 20 μg/ml in PBS/BSA were then added to the wells (20 μl/well) and incubated for 1 h at 4°C. After three washes in PBS/BSA, 20 μl of ^{125}I-F(ab')$_2$ of rabbit anti-mouse immunoglobulin was added to the wells and incubated for 1 h, 4°C. Plates were then washed four times, and wells were counted for radioactivity bound.

SANDWICH RIA (22): Monoclonal antibody (100 μg/ml in PBS) was applied to the wells of polyvinyl chloride microtiter plates, 20 μl/well, for 1 h at room temperature, and binding sites were blocked with PBS/BSA as described above. Serial dilutions of purified antigen in PBS/BSA were then added, 50 μl/well, along with a second ^{125}I-labeled monoclonal antibody (6×10^5 cpm/20 μl per well). After overnight incubation at 4°C with agitation, plates were washed four times with PBS/BSA, and wells were counted for radioactivity bound.

COMPETITION RIA (20): Purified clathrin was applied to the wells of polyvinyl chloride plates as described for the indirect solid-phase RIA, and then blocked with BSA. Monoclonal antibodies, starting at concentrations of 100 μg/ml, were serially diluted, and 20 μl of each dilution was added to clathrin-coated plates. After incubation for 1 h at 4°C, ^{125}I-labeled monoclonal antibodies were added directly to these wells and incubated for 30 min at 4°C. Plates were then washed four times and wells were counted for radioactivity bound. ^{125}I-labeled monoclonal antibodies were titered in advance for binding to clathrin in a solid-phase RIA to select a dilution that did not saturate the clathrin bound and would therefore be sensitive to blocking by unlabeled antibody.

Electrophoresis

SDS PAGE was carried out according to the method of Laemmli (23). All gels shown contain 10% acrylamide. Gels were stained with 0.2% Coomassie Brilliant Blue R in 9.5% acetic acid, 45.5% methanol in water, and destained with 10% acetic acid, 40% methanol in water. Gels were treated with Enlightening (New England Nuclear, Boston, MA), dried, and exposed to XAR-5 film using an intensifying screen (Dupont Lightening Plus, DuPont Photo Products Division-X-Ray, Burbank, CA) when autoradiography was required.

Western blotting was carried out as previously described with one modification (20). Whole gels with duplicate sets of samples were transferred to nitrocellulose. After transfer, the nitrocellulose was stained with Amido black and then cut into strips for separate incubation with antibodies or retention as a control stained for protein. Cell lysate samples were prepared by incubation of 2×10^7 cells/ml in 0.5% Nonidet P-40, 10 mM Tris, pH 7.3, 1 mM $MgCl_2$ for 30 min at 4°C and then centrifuged in a microfuge for 10 min (24). 20 μl of the resulting supernatant was applied to the gel.

Molecular weight marker proteins used on all gels were from Pharmacia Fine Chemicals (Piscataway, NJ). They include phosphorylase b (94 kD), BSA (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), soybean trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD).

Immunochemistry

Immunoprecipitation using fixed *Staphylococcus aureus* bacteria was carried out as described by Parham and Ploegh (24). For each reaction, 5 μg purified monoclonal antibody was incubated with 10^6 cpm of ^{3}H-labeled clathrin or ^{3}H-labeled light chains. Rabbit anti-mouse Ig serum was added to facilitate fixed *S. aureus* bacteria binding.

Affinity chromatography was according to the method of Parham using 50 mM diethylamine, pH 11.5, to elute the affinity columns (25). Monoclonal antibodies were coupled to CNBr-activated Sepharose 4B from Pharmacia Fine Chemicals according to the instructions except that the coupling reaction was carried out for 10 min. MAbs were coupled to CNBr-activated Sepharose 2B at pH 7.0 following the procedure of March et al. (26).

Purification of Clathrin, Clathrin Components, and Coated Vesicles

Clathrin was purified from human and bovine brain by extraction of a crude membrane preparation with 0.75 M Tris, 0.025 M 2-(*N*-morpholino)ethane sulfonic acid (pH 6.2), 0.25 mM EGTA, 0.12 mM $MgCl_2$, and then ammonium sulfate precipitation and chromatography on Sepharose 4B-CL were performed as previously described (27).

Clathrin light chains were isolated by heating purified clathrin to 100°C for 10 min. The denatured heavy chain was removed by centrifugation at 35,000 *g* for 30 min (14). Purified light chains were dialyzed into PBS, and LC_a was separated from LC_b by affinity chromatography on a CVC.6-Sepharose 4B column (1.5 × 5 cm). CVC.6 is an LC_a-specific MAb (13). The column retained ~30 μg LC_a/ml, which was eluted using 50 mM diethylamine, pH 11.5, then dialyzed into PBS, and centrifuged before use. The effluent consisted of ~80% pure LC_b.

Clathrin heavy chain was isolated after chromatography of clathrin in 1.3 M potassium thiocyanate on a column of AcA 34 (1 × 25 cm), according to the method of Winkler and Stanley (28). Fractions were dialyzed into 50 mM

Tris (pH 8.0), 50 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, and analyzed by SDS PAGE and indirect RIA for reactivity with the anti-LC_a MAb, CVC.6. The heavy chain–containing fractions with no antibody reactivity were pooled for use.

Human lymphoid coated vesicles were purified from 10^9 LB cells as described by Kinnon and Owen (29) using the deuterium oxide gradient method of Pearse (30).

Purified clathrin and purified clathrin light chains were radioactively labeled by reductive methylation according to the method of Tack et al. (31) as described by Schlossman et al. (32). Specific activities were 10^6 cpm/μg for clathrin and 6×10^5 cpm/μg for the light chains.

Cells, Media, and Antibody Reagents

The Anti-Leu 10 antibody (22) and the anti-Igh5b antibody (33), used as control antibodies of the IgG_1 isotype, react with HLA-DQ glycoproteins on the surface of human lymphocytes and mouse IgD, respectively. They were obtained in purified form from Becton Dickinson Immunocytometry Systems. The retinoblastoma cell line, Y79, and the neuroblastoma cell line, 134H, were a gift from D. Buck (Becton Dickinson Monoclonal Center, Mountain View, CA) and were maintained in RPMI 1640 with 10% fetal calf serum and passaged with 0.25% trypsin-EDTA (No. 610-5300, Gibco Laboratories, Grand Island, NY). The adenocarcinoma cell line LS184T and the 3T3 mouse cell line were obtained from the American Type Culture Collection (Bethesda, MD) and maintained according to instructions. The LB cell line, an Epstein-Barr virus–transformed human B cell line, a gift from V. Engelhard (University of Virginia, Charlottesville), was maintained in RPMI 1640 with 10% fetal calf serum.

RESULTS

Strategy for Anti-Clathrin MAb Production

To produce MAbs that reacted with human clathrin, clathrin was purified from human brain tissue by the same method used to isolate bovine brain clathrin. Purified human brain clathrin was then injected every 3 d into the footpads of SJL mice (5–10 μg/footpad, six injections). Lymphocytes from the popliteal lymph nodes of the immunized mice were fused with the SP2 myeloma cell line. 10 MAbs were isolated from this fusion. Initial specificity tests included solid-phase RIA, immunoprecipitation and Western blotting. These suggested that two of the MAbs (X19 and X22) had specificity for different determinants on the clathrin heavy chain. Three (represented by X16) had similar reactivity with the LC_a light chain and probably originated from the same expanded B cell clone. These MAbs all expressed the IgG_1 isotype. The remaining five antibodies reacted with minor contaminants in the human clathrin preparation that did not correlate with any of the known clathrin-associated proteins and were not investigated further in this study. Fig. 1 shows the results obtained when the X16, X19, and X22 MAbs were tested for reactivity with different types of clathrin and separated clathrin polypeptides in solid-phase RIA. They were compared with CVC.4 and CVC.6, representing the two types of anti-clathrin MAbs produced in a previous set of experiments. Interpretation of the chain specificities of these MAbs is slightly complicated by the fact that small amounts of LC_a were present in the LC_b preparation. However, it is clear that CVC.6 and X16 are specific for the LC_a light chain and that X19 and X22 react with heavy chain. The polypeptide specificity of the CVC.4 antibody is still ambiguous.

LC_a-specific Antibodies

Western blot analysis (Fig. 2) showed that X16 reacts with bovine and human clathrin LC_a, as suggested by the RIA in Fig. 1. The specificity of CVC.6 for bovine clathrin LC_a has been previously demonstrated by Western blotting (13). The

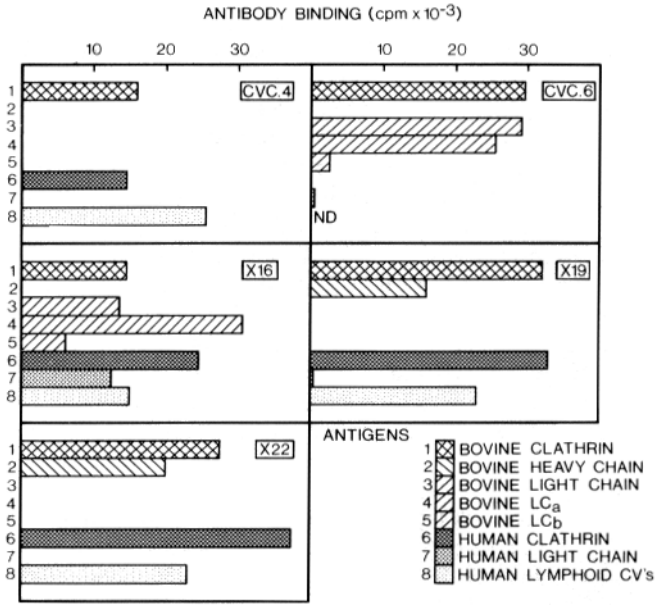

FIGURE 1 Test of MAb reactivity with clathrin, clathrin polypeptides, and coated vesicles (*CV's*) by indirect solid-phase RIA. The antigens listed at the lower right corner of the figure were applied to the wells of a polyvinyl chloride plate. The antibodies indicated in each panel were incubated in the wells of these plates, and antibody binding was detected by subsequent binding of ^{125}I-anti-immunoglobulin. Each bar represents antibody binding to the antigen numbered at the left. Bars are the mean of duplicate assays. Background binding (the mean of duplicate assays with a control antibody binding to the same antigen) is subtracted. Antigens 3 and 7 are pools of LC_a and LC_b produced by boiling and centrifugation of triskelions. Antigens 4 and 5 were separated by immunoaffinity chromatography.

clathrin light chains have been shown to be extremely sensitive to proteolysis, and proteolytic fragments of LC_a are frequently present in clathrin preparations (9, 14). One of these LC_a fragments co-migrates upon SDS PAGE with LC_b and has been confirmed by peptide mapping to be derived completely from LC_a (14). Another LC_a fragment migrates slightly faster than LC_b and reacts with the CVC.6 MAb (13). The Western blots in Fig. 2 show that X16 reacts with LC_a and both of these LC_a fragments in preparations of bovine and human brain clathrin (Fig. 2, lanes *A* and *B*). It also reacts with four or five smaller proteolytic fragments of LC_a. Partially purified clathrin in lymphoid coated vesicles and clathrin in crude tissue extracts has been subjected to less manipulation and consequently less opportunity for proteolysis. In such preparations X16 binding detects LC_a at its native molecular weight (Fig. 2, lanes *C, E–H, K*). Note that the LC_a in these preparations is of the lower molecular weight typical of nonbrain tissue. This is expected for the cells of lymphoid and endocrine origin but somewhat surprising for retinoblastoma and neuroblastoma cells, which are derived from brain-like tissue. This suggests that these tumor cells no longer make brain-type clathrin or that the tumors arose from a minor population of non-neuronal cells in the tissue. The Western blot in Fig. 2 also shows that X16 reacts with LC_a from a mouse cell line (lane *K*) and is, therefore, an autoantibody. This LC_a is of slightly higher molecular weight than human clathrin LC_a in nonbrain tissue.

To confirm that X16 reacts exclusively with LC_a from brain

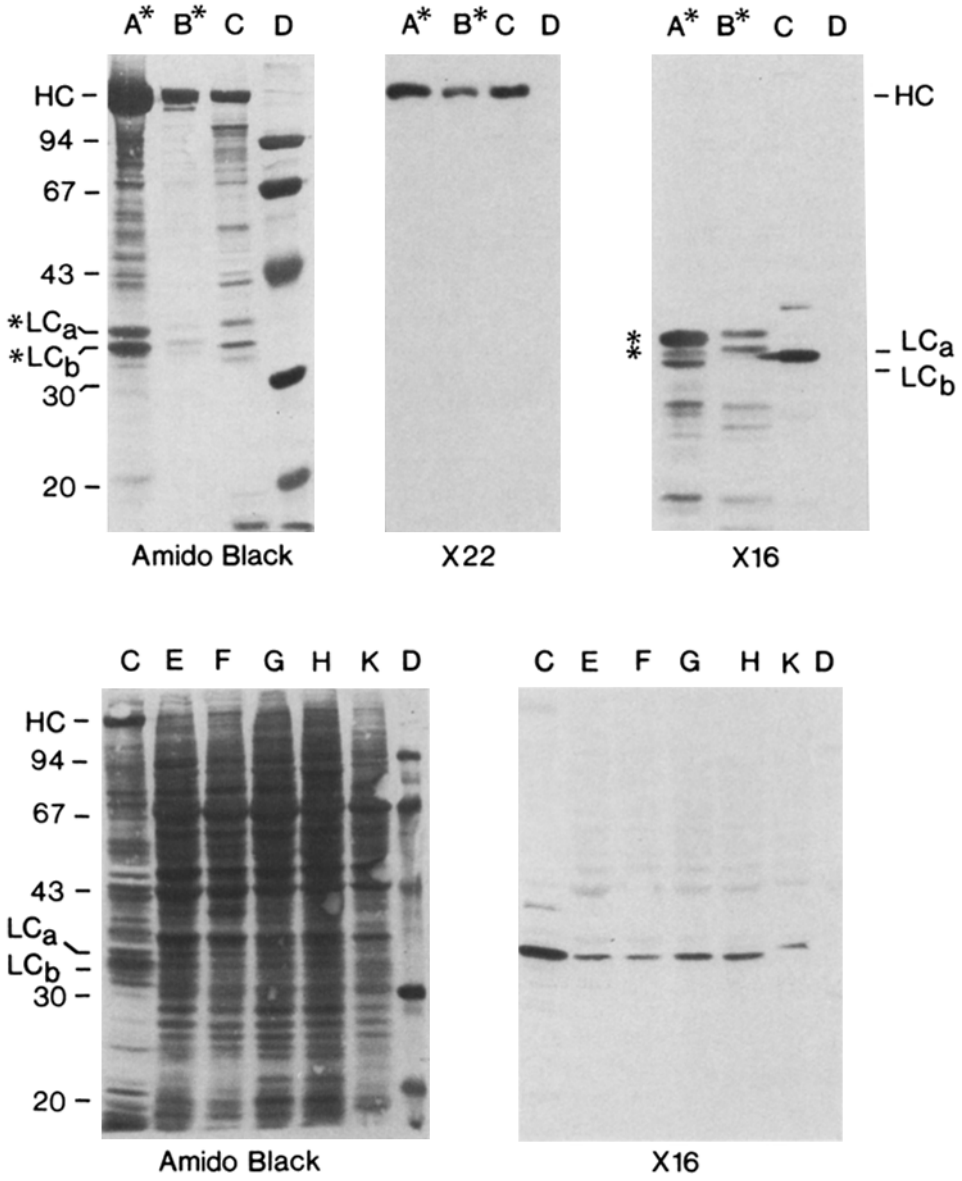

FIGURE 2 Test of MAb reactivity with purified brain clathrins, lymphoid coated vesicles, and cell line extracts by Western blot. Protein samples were applied to polyacrylamide gels in multiple identical sets and subjected to SDS PAGE. After electrophoretic transfer to nitrocellulose, one set of samples was stained with Amido black to detect total protein. After the other transferred samples were reacted with the antibody indicated, ^{125}I-anti-immunoglobulin was added. Antibody binding was detected by autoradiography. Samples analyzed are as follows: lane *A*, bovine brain clathrin; lane *B*, human brain clathrin; lane *C*, human lymphoid coated vesicles; lane *D*, molecular weight marker proteins; lane *E*, human B cell line; lane *F*, human adenocarcinoma cell line; lane *G*, human retinoblastoma cell line; lane *H*, human neuroblastoma cell line; and lane *K*, mouse 3T3 cell line. Those samples and markers with the higher molecular weight light chains typical of brain tissue are indicated by an asterisk. Other samples and markers show light chain mobilities characteristic of nonbrain tissue. Samples in lanes *E–H* and *K* are cell lysates prepared by solubilization in 0.5% Nonidet P-40. Identical panels were reacted with control monoclonal antibodies of the same isotype, and no binding was detected.

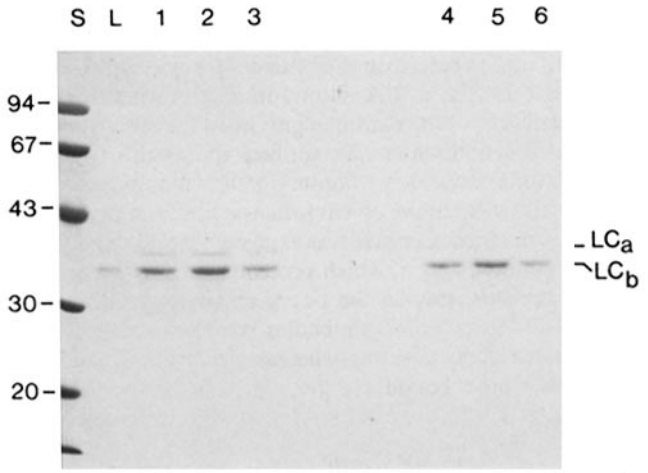

FIGURE 3 Specificity of X16-Sepharose 2B for human brain LC_a. Human brain light chains were prepared by boiling and centrifugation of purified human brain clathrin. A 1-μg sample was analyzed electrophoretically in lane *L*. A 16-μg sample of light chains was applied to a 0.5-ml column of X16 coupled to Sepharose 2B or anti-Igh5b coupled to Sepharose 2B. The latter is a control antibody of the IgG_1 isotype. Fractions (250 μl) were collected during sample application, and the protein was precipitated from them with 1 ml cold acetone. The protein content of these fractions was analyzed by SDS PAGE. Lanes *1–3* are effluent fractions from the control column. Lanes *4–6* are effluent fractions from the X16-Sepharose 2B column. Lane *S* shows standard molecular weight markers.

clathrin, purified human brain clathrin light chains were applied to an X16-Sepharose 2B column and to a column of Sepharose 2B coupled to a nonspecific MAb for comparison (Fig. 3). The effluent from the nonspecific column (Fig. 3, lanes *1–3*) contained both LC_a and LC_b whereas the effluent from the X16 column (lanes *4–6*) contained only LC_b. The amount of effluent LC_b from the X16 column appears slightly lower than that from the nonspecific column. This is due to removal of the proteolytic fragment of LC_a that co-migrates with LC_b and reacts with the X16 antibody. Similar X16 affinity columns have subsequently been used to remove LC_a from preparations of brain clathrin light chains. Resulting LC_b preparations were completely devoid of contaminating LC_a, as assessed by indirect solid-phase RIA.

It is evident from the experiment in Fig. 1 that CVC.6 and X16 have different species cross-reactivity although they both react with bovine clathrin LC_a. Results of several additional experiments confirm that these two MAbs react with different antigenic sites on the LC_a molecule. The X16 and CVC.6 MAbs were tested for their ability to immunoprecipitate bovine brain clathrin (triskelions) or isolated bovine brain clathrin light chains that were labeled by reductive methylation. As seen in Fig. 4, the CVC.6 antibody immunoprecipitated heavy chain in association with light chain, when reacted with ^{3}H-triskelions, and showed a weak immunoprecipitation reaction with ^{3}H-light chains. Note that the ^{3}H-light chains have degraded to yield an LC_a fragment that was also precipitated by CVC.6. The CVC.6 reaction with triskelions was weaker than that of X19 or X22, and the autoradiograph was somewhat overexposed in order to detect the triskelion-associated light chains in the CVC.6 immunoprecipitate. This resulted in exaggeration of the nonspecific binding seen with the control antibody, Anti-Leu 10. Compared with this control antibody, the X16 MAb showed no specific reactivity with ^{3}H-triskelions. In contrast, X16 gave a strong immunoprecipitation reaction with ^{3}H-light chains, isolating LC_a and the LC_a fragment. These results suggest that in immunoprecipitation X16 reacts preferentially with LC_a when it is not associated with heavy chain. This is supported by the properties of X16 and CVC.6 antibody affinity columns. An X16 affinity column bound free LC_a but not triskelions or coated vesicles. In contrast, a CVC.6 affinity column bound LC_a in all three forms of clathrin.

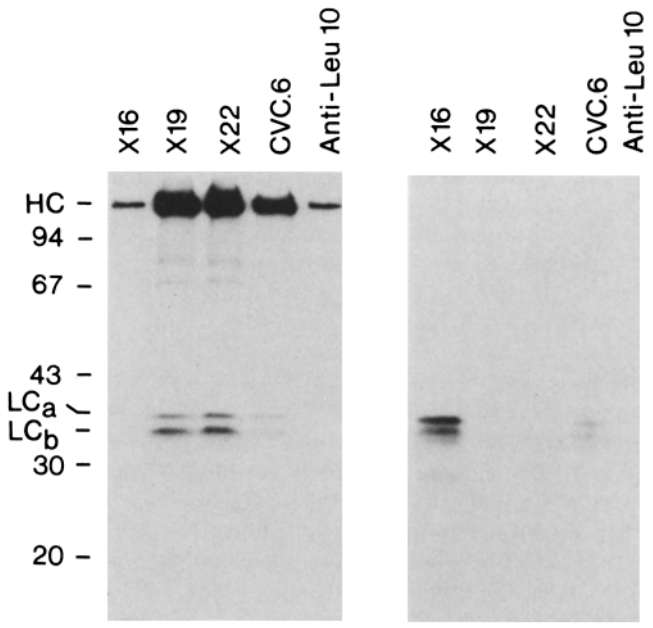

FIGURE 4 Immunoprecipitation of ^{3}H-clathrin triskelions and ^{3}H-clathrin light chains with anti-clathrin MAbs. Clathrin (triskelions) and clathrin light chains were purified from bovine brain and labeled by reductive methylation. A purified MAb (5 μg) was combined with each antigen preparation (10^6 cpm in 25–30 μl). Antigen–antibody complexes were isolated by immunoprecipitation with fixed *S. aureus* bacteria, analyzed by SDS PAGE, and immunoprecipitated antigen was detected by autoradiography. Anti-Leu 10 is a negative control with the same isotype as the test antibodies (IgG_1). The anti-clathrin antibodies used are indicated above the lanes that show their immunoprecipitated antigens. The left-hand panel shows immunoprecipitates obtained with ^{3}H-triskelions. The right-hand panel shows immunoprecipitates obtained with ^{3}H-light chains.

In solid-phase RIA (Fig. 1), X16 bound to coated vesicles and to LC_a in immobilized triskelions, apparently as well as to free light chain. To investigate the discrepancy between X16 reactivity in solid-phase RIA and immunoprecipitation, X16 and CVC.6 were tested quantitatively for binding to triskelions and free light chain in a solid-phase RIA (Fig. 5*A*) or a sandwich RIA (Fig. 5*B*). In the solid-phase RIA, X16 and CVC.6 showed little difference in their reactivity with either target antigen. The sandwich RIA is more sensitive to differences in antibody affinity because the reactions between the iodinated antibody and antigen take place in solution. These antibody-antigen complexes are "captured" by the antibody on the plate only if they are sufficiently stable (34). Iodinated X16 antibody, complexed with free light chain, was bound by CVC.6. Complexes formed between iodinated X16 and triskelions were not stable, and no binding to CVC.6 was detectable. In the reverse situation, iodinated CVC.6 complexed with triskelions bound to X16. The iodinated CVC.6-free light chain complexes were less stable, and significantly fewer complexes bound to X16. These results suggested that, in solution, X16 has a greater affinity for free LC_a than for heavy chain–associated LC_a; CVC.6 binding demonstrates the opposite preference. The fact that X16 and CVC.6 could form a sandwich with antigen between them confirmed that they were binding to distinct sites on LC_a. This was also supported by the blocking experiments described below.

Heavy Chain–specific Antibodies

The two MAbs, X19 and X22, that demonstrated binding to separated clathrin heavy chain in solid-phase RIA (Fig. 1) were tested for reactivity with clathrin in Western blots. The X22 antibody reacted with heavy chain from bovine brain, human brain, and human lymphoid clathrin as shown in Fig. 2. The X19 antibody did not react in Western blots. In the immunoprecipitation experiments shown in Fig. 5, X19 reacted with ^{3}H-clathrin triskelions, producing an immunoprecipitate similar to that obtained with X22, and did not immunoprecipitate antigen from a preparation of ^{3}H-light chains, as seen with CVC.6. This further suggests that the X19 antigenic site is present on the clathrin heavy chain.

In the solid-phase RIA shown in Fig. 1, clathrin heavy chain was prepared by chromatography in the presence of 1.3 M potassium thiocyanate. To confirm the specificity of X19 and X22 for heavy chain, a second method for obtaining light chain–free preparations of clathrin was used, and antibody binding was tested. Clathrin was exposed to mild digestion by elastase or thermolysin, which proteolyze the light chains to small fragments, leaving the heavy chain essentially intact. Both the X19 and X22 antibodies retained reactivity with clathrin treated in this way whereas the anti-LC_a antibody, CVC.6, no longer bound.

Quantitation of Antigenic Sites

To investigate the question of how many antigenic sites on the clathrin molecule are recognized by the MAbs described above, a competition experiment was carried out. The Mabs were iodinated, and an appropriate dilution was tested for reactivity with clathrin in the presence of increasing concen-

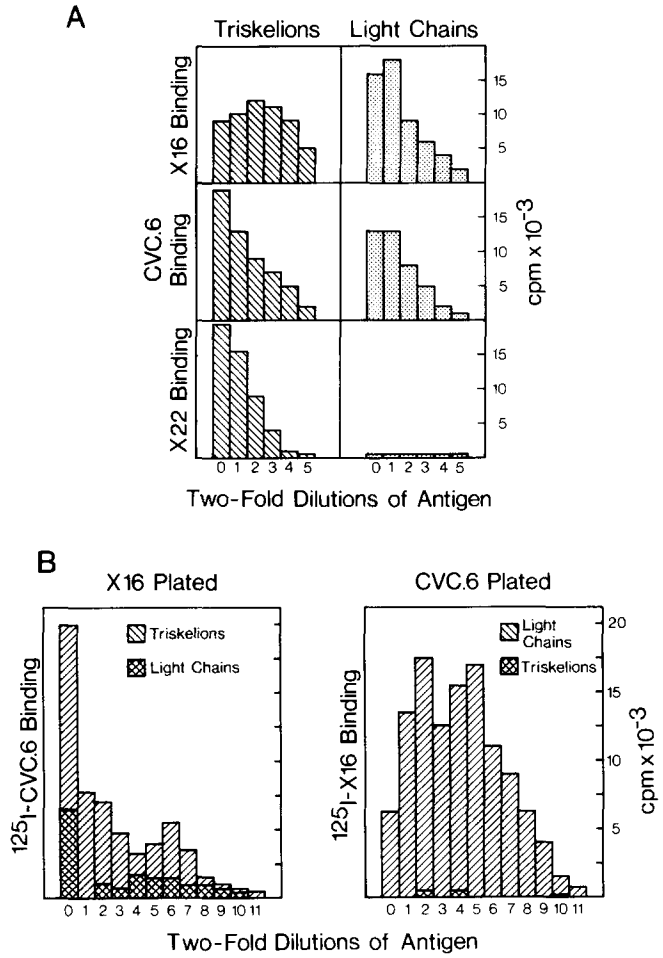

FIGURE 5 Comparison of X16 and CVC.6 reactivity with clathrin triskelions and clathrin light chains. (*A*) Indirect solid-phase RIA: Purified clathrin triskelions (1mg/ml) and light chains, prepared by boiling triskelions (1 mg/ml), were diluted 1/8 in PBS, then five further twofold dilutions were made. Samples of each dilution were applied to polyvinyl chloride plates. These were tested for reactivity with the MAbs indicated (20 μg/ml) by indirect RIA, using ^{125}I-anti-immunoglobulin to detect antibody binding. X22 was used as a control to demonstrate that no clathrin heavy chain remained in the light chain preparation. (*B*) Sandwich RIA: Purified X16 or CVC.6 was diluted to 100 μg/ml and used to coat the wells of polyvinyl chloride plates. The preparations of triskelions (1 mg/ml) and light chains derived from them, used in *A*, were diluted twofold in PBS/BSA 11 times. Dilutions of each antigen were added to wells of the antibody-coated plates. ^{125}I-labeled X16 antibody was added to the antigen-containing wells of plates coated with CVC.6. ^{125}I-labeled CVC.6 antibody was added to the antigen-containing wells of plates coated with X16. After overnight incubation plates were washed, and the binding of the iodinated antibodies was measured. Bars indicate the binding of the iodinated antibody to the coated plate in the presence of either triskelions or light chains. Background binding of an iodinated control antibody of the same isotype was subtracted in each assay. Note that in the left-hand panel cross-hatched bars indicate binding in the presence of light chains, and in the right-hand panel cross-hatched bars indicate binding in the presence of triskelions.

trations of unlabeled antibodies (Fig. 6). As predicted above, the X16 and CVC.6 antibodies did not substantially block each other in binding to LC_a, although CVC.6 binding caused a slight reduction in X16 binding at high concentrations. X22 and X19 did not block each other in binding to heavy chain. All four of these antibodies effectively blocked their own binding to clathrin. The CVC.4 antibody was most affected by the binding of the other antibodies. Thus far, the polypeptide specificity of this antibody has been unclear. It binds to bovine and human clathrin in solid-phase RIA. When coupled to Sepharose and used as an immunoaffinity column it retains coated vesicles and clathrin triskelions. It does not bind to free clathrin light chains, although its binding to clathrin is sensitive to mild proteolysis. The antibody does not show any specificity in immunoprecipitation or Western blot, and titration curves suggest that its affinity is quite low. This, perhaps, explains its sensitivity to the binding of other antibodies in the competition assay. CVC.4 binding was partially inhibited by CVC.6 and completely inhibited by X19 binding. In the reciprocal combination it partially inhibited CVC.6 binding but barely affected X19 binding. These results do not clarify the chain specificity of CVC.4, since CVC.6 reacts with LC_a and X19 seems to be specific for heavy chain. These competition studies indicate that five different antigenic determinants on bovine clathrin are recognized by the MAbs under investigation.

DISCUSSION

Experience with making monoclonal antibodies to clathrin has suggested that the molecule is not very immunogenic. Reports in the literature have described antibodies with relatively low affinity binding sites that detect a limited number of antigenic sites (13, 15, 16). The first MAbs reported to react with clathrin were produced by initial intraperitoneal immunization in adjuvant followed by intravenous hyperimmunization before fusion (13). This immunization protocol and subsequent fusion were repeated eight times using bovine and human clathrin as antigens with no success in producing additional clathrin-specific MAbs. The only MAbs produced from these fusions reacted with minor contaminants in the clathrin preparations used as antigen. These antibodies were isolated because they bound to the clathrin preparations in solid-phase RIA. This experience emphasized two points relevant to producing anti-clathrin MAbs. First, it provided further evidence for the poor antigenicity of the clathrin molecule since even minor contaminants were more immunogenic. Second, it demonstrated the necessity of using several independent tests to assay for clathrin-specific MAbs. With these points in mind, the footpad immunization protocol described in this paper was chosen for production of anti-human brain clathrin MAbs. This protocol has been previously shown to be effective for producing antibodies that react with poorly immunogenic antigenic sites (17, 35). SJL mice were selected to be immunized because they have an abnormal immune system, which might favor production of antibodies to highly conserved proteins (36). Initial screening of the fusion included tests for antibody reactivity by solid-phase RIA, Western blot, and immunoprecipitation. This approach resulted in the production of three anti-clathrin MAbs recognizing previously undetected determinants. One MAb (X16) reacts with the LC_a light chain and in immunoprecipitation and soluble binding assays has preferential affinity for free LC_a. The other MAbs (X19 and X22) react with two different clathrin heavy chain determinants. All three MAbs are of the IgG_1 isotype and react with both human and bovine clathrin. The X16 MAb was compared with the CVC.6 MAb shown previously to be specific for LC_a (13). In this study it was

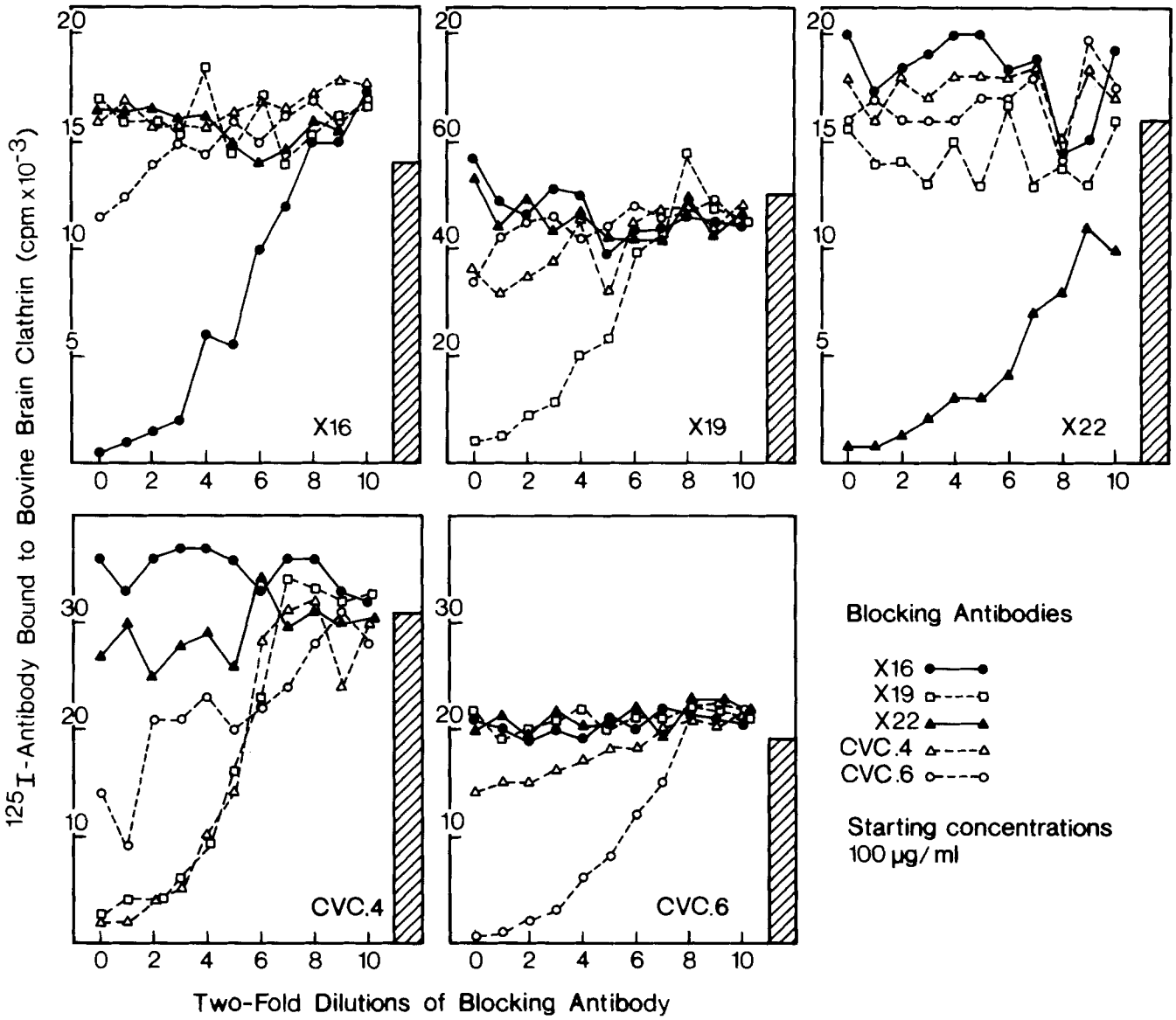

FIGURE 6 Competitive binding of five anti-clathrin MAbs to bovine brain clathrin. The CVC.4, CVC.6, X16, X19, and X22 antibodies were iodinated and titered to determine a concentration sensitive to inhibition. ^{125}I-antibody at this concentration was tested for binding to bovine brain clathrin in the presence of decreasing amounts of unlabeled preparations of each antibody. The ^{125}I-antibody tested is shown at the lower right of each panel. The symbols used to indicate binding in the presence of each unlabeled antibody are listed under Blocking Antibodies in the lower right-hand corner of the figure. The starting concentrations of blocking antibodies were 100 µg/ml, and 11 twofold dilutions were made. The bar at the right in each panel shows ^{125}I-antibody binding in the absence of blocking antibody and represents the mean of duplicate assays.

demonstrated that, in solution, CVC.6 reacts with heavy chain–associated LC_a preferentially over free LC_a. X16 and CVC.6 also react with physically distinct sites on LC_a, since they did not block each other in binding to LC_a and could bind simultaneously to the antigen. The X19 and X22 antibodies show similar specificity for clathrin heavy chain except for their reactivity in Western blot. The X19 antibody recognizes a determinant that is labile after denaturation in SDS PAGE and transfer to nitrocellulose and is therefore distinct from the X22 determinant, which is still reactive under these conditions. Competition experiments with X19, X22, CVC.4, and CVC.6 confirm that X19 reacts with an antigenic site on the clathrin heavy chain distinct from the X22 site. X19 binding influences CVC.4 binding, which is also affected by CVC.6 binding. Since CVC.6 binds to LC_a, this provides indirect evidence that the heavy chain determinant detected by X19 may be close to the region where LC_a binds, in the proximal portion of the triskelion arm (13, 37, 38).

MAbs that detect five different antigenic determinants on the clathrin molecule have been described in this paper. If the MAbs reported by Louvard et al. (15) and Kelly et al. (16) are taken into account, at least seven different antigenic sites on the clathrin molecule have now been defined. It is interesting that an MAb to the LC_b light chain has not yet been isolated. Nor has an antibody that reacts with both LC_a and LC_b been found. Even though LC_a and LC_b have a very different primary structure, as indicated by peptide maps, they both bind to clathrin heavy chain and should have some antigenic features in common (14, 38, 39). However, since heavy chain binding is an essential property of light chains,

the sequences responsible for mediating this interaction may be conserved and not very immunogenic. The high degree of conservation of clathrin structure and function between species as distant as yeast and humans is the most likely explanation for its overall poor antigenicity (40, 41). For this reason, production of anti-LC_b MAbs and additional anti-heavy chain MAbs may require immunization with separated clathrin polypeptides or with chemically modified clathrin. With the production of the three anti-clathrin MAbs described in this paper, it has become possible to carry out the accompanying study of the forms of clathrin in vivo. Future expansion of the antibody panel will make it possible to identify antigenic sites critical for clathrin assembly, look at the complete biosynthesis of the molecule, and gain further insight into the regulation of clathrin-mediated processes.

I thank B. Abrams and L. Kelley for technical assistance, D. Buck and V. Engelhard for supplying cell lines, B. Summey for artwork, O. Huff for manuscript preparation, and P. Parham for helpful discussions.

Received for publication 17 April 1985, and in revised form 12 July 1985.

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