

Annulate Lamellae: Comparison of Antigenic Epitopes of Annulate Lamellae Membranes with the Nuclear Envelope

Tai-Ying Chen* and Elaine M. Merisko‡

*Department of Anatomy, University of Kansas Medical Center, Kansas City, Kansas 66103; and

‡Eastman Pharmaceuticals, Great Valley, Pennsylvania 19355

Abstract. Annulate lamellae (AL) are a membranous structure frequently observed in differentiating gametes and tumor cells. In spite of numerous morphological studies, the function and biochemical composition of this membrane system are not well understood. In this study, we have examined the AL membrane system of vinblastine-treated mouse L cells using immunocytochemistry and Western blot analysis. Our results show that antibodies directed against nuclear envelope la-

mins, i.e., lamins A, B, and C, did not cross react with constituents of the AL membrane system. Furthermore an AL-specific antibody failed to react with the nuclear envelope and reacted minimally producing only a background stain over other cellular components. The data suggest that the AL membrane system has a distinct molecular make-up that is antigenically distinct from that of other subcellular structures.

ANNULATE lamellae (AL)¹ are a network of intracellular membranes that have been described in numerous studies over the past 30 years (11). These membranes are often observed in rapidly dividing cells, germ cells, and cells at the onset of differentiation (11, 12, 18, 19). However, the function served by this membrane system has yet to be elucidated. This organelle consists of a parallel stack of membranous cisternae trimmed with dense staining fibrillar plaques. Characteristically, the membranes contain pores. These intramembranous pores are morphologically similar to those found in the nuclear envelope. This has led to the speculation that these two membrane systems are structurally and perhaps functionally related (18).

In this study we have compared the antigenicity of the nuclear envelope and the AL membrane system with the hope of identifying epitopes common to both membrane systems. As a marker for the nuclear envelope we have used antibodies directed against the nuclear lamins. In recent years the nuclear lamins have been shown to be a highly conserved family of proteins associated with the inner aspect of the nuclear envelope (6, 15). Lamins A, B, and C have been purified and extensively characterized (5, 7). Each is believed to form part of a protein complex that structurally supports the envelope during mitosis (5). An immunocytochemical approach was used to determine whether the lamins are present in the AL membrane system. In addition an antibody specific for the AL membrane system was detected in a serum sample taken from a patient having lupus erythematosus. This AL specific antibody was used to determine the cellular distribution of AL antigens.

Materials and Methods

Reagents

Mouse connective tissue NCTC clone 929 of strain L (mouse L cells) was obtained from American Type Culture Collection (Rockville, MD). Serum samples of human lupus erythematosus patients were kindly provided by N. Abdou, M. D., Department of Medicine, University of Kansas Medical Center, Kansas City, KS. Guinea pig antisera against rat liver nuclear lamins were generous gifts from L. Gerace, Ph.D., Department of Molecular Biology, the Research Institute of Scripps, La Jolla, CA. Fluorescein-conjugated rabbit anti-human IgG, silver stain kit, and 4-chloro-1-naphthol were from Bio-Rad Laboratories (Richmond, CA). Fluorescein conjugated F (ab')₂ goat anti-guinea pig IgG was from Cooper Biomedical (Malvern, PA). Vectastain ABC anti-guinea pig IgG kit was purchased from Vector Laboratories (Burlingame, CA). Culture supplies and FCS were purchased from Hazleton Research Products, Inc. (Lenexa, KS). Other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Induction of AL Membranes

Mouse L cells were grown in RPMI 1640 supplemented with 10% FCS and 1% penicillin-streptomycin. Under standard conditions the cells were cultured to near-confluency. Afterwards, the cells were cultured in medium containing vinblastine sulfate (0.1 µg/ml) for 36 h. The cell cultures were then processed for either transmission electron microscopy (TEM) or immunocytochemistry.

TEM

Cell cultures were processed according to the method of Kessel et al. (13). Cells were rinsed once with 0.1 M cacodylate buffer, pH 7.4, containing 4% sucrose, 1 mM MgCl₂, and 1 mM CaCl₂ and then fixed with 3% glutaraldehyde in the same buffer for 30 min at room temperature. Postfixation was carried out with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h and the samples were processed for TEM.

Immunocytochemistry

For Immunofluorescence. Normal and vinblastine-treated mouse L cells were cultured on coverslips. Cells were rinsed twice with PBS (pH 7.4) for

1. *Abbreviations used in this paper:* AL, annulate lamellae; TEM, transmission electron microscopy.

5 min and then fixed in 3.7% paraformaldehyde in PBS for 10 min. After three rinses in PBS containing 0.02% sodium azide, the cells were permeabilized with 0.05% Triton X-100 in PBS. After two rinses, the cells were equilibrated with PBS containing 0.2% gelatin (reaction buffer) for 20 min. The cells were then incubated with 50 μ l of primary antisera, appropriately diluted in reaction buffer containing 10% goat serum, for 30 min at room temperature. After three rinses in reaction buffer, the cells were incubated for 30 min with either fluorescein-conjugated rabbit anti-human IgG or goat anti-guinea pig IgG. After three rinses in reaction buffer and two in PBS, the coverslips were mounted with 90% glycerol in PBS and examined with a Leitz microscope using epifluorescent optics. Routinely, for negative controls, cells were incubated with reaction buffer instead of the primary antibody.

For EM Immunocytochemistry. Indirect immunoperoxidase was used basically according to the method of Brown and Farquhar (3). Aliquots of $\sim 1 \times 10^7$ vinblastine-treated L cells were fixed with the periodate-lysine-paraformaldehyde mixture of McLean and Nakane (21) for 90 min. The cells were then rinsed (three times, 10 min each) in PBS containing 5% sucrose, 1% BSA, and 0.02% sodium azide, pH 7.4 (buffer A). After being permeabilized with 0.05% Triton X-100 in buffer A for 10 min, the cells were rinsed in buffer A (three times, 10 min each) and incubated with normal goat serum diluted in buffer A for 30 min. Afterwards, the cells were incubated with 200 μ l of the human lupus serum or normal human serum diluted (1:5) in buffer A for 14 h. The cells were then rinsed (six times, 20 min each) in buffer A depleted of sodium azide (buffer B). The immune reaction was detected using the method of Hsu et al. (10). The cells were incubated with biotinylated goat anti-human IgG for 30 min, rinsed in buffer B (three times, 20 min each), and incubated with avidin-peroxidase conjugate for an additional 30 min. After being rinsed in buffer B (six times, 20 min each), the cells were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer containing 5% sucrose, pH 7.4 for 20 min, rinsed in 0.1 M cacodylate buffer containing 7.5% sucrose, pH 7.4 (three times, 15 min each), followed by rinsing (three times, 15 min each) in 0.05 M Tris-HCl containing 7.5% sucrose, pH 7.4 (buffer C). The cells were then resuspended with 0.1% diaminobenzidine and 0.01% hydrogen peroxide in buffer C for 10–15 min. Subsequently the cells were rinsed in buffer C (three times, 15 min each) followed by postfixation in 1% OsO₄ and 1% KFeCN in deionized water for 15 min. The cells were then processed for TEM.

Subcellular Fractionation

Monolayers of vinblastine-treated cells, $\sim 4 \times 10^9$, were rinsed with 0.25 M sucrose and homogenized in 0.25 M sucrose containing vinblastine sulfate (0.1 μ g/ml) and 0.02% sodium azide using a glass-glass homogenizer. The homogenate was centrifuged at 600 *g* for 10 min at 4°C. The pellet containing nuclei and the postnuclear supernatant were separated, resuspended in 0.1 M cacodylate buffer, pH 7.4, and processed for TEM. For immuneoverlays, the nuclear and postnuclear fractions were treated as described below.

SDS-PAGE and Immuneoverlays

The nuclear fraction (sonicated) and postnuclear supernatant were assayed for protein content by the method of Bradford (2). Aliquots (100–400 μ g protein) from either nuclear fraction or postnuclear supernatant were precipitated in acetone at –20°C. Afterwards the pellets were resuspended and boiled for 3 min in solubilization buffer (20 mM Tris-HCl, pH 6.8, containing 1% SDS, 20 mM dithiothreitol [DTT], and 0.5 M urea). The samples were subjected to electrophoresis on 10% polyacrylamide gels containing 0.2% SDS for 14 h at 5 mA (17). Afterwards, the gels were either stained with silver (22) or proteins were electrophoretically transferred to nitrocellulose (8). The protein replicas were quenched in PBS containing 2% BSA and 0.2% gelatin for 2 h at room temperature and then incubated overnight with either an anti-lamin A/C, anti-lamin B, or human lupus serum. After washing in PBS for 2 h, the binding sites were detected using a biotin-avidin-peroxidase conjugate according to the method of Hsu et al. (10) using 4-chloro-1-naphthol as a substrate for peroxidase.

Screening of Sera From Human Lupus Erythematosus Patients

Sera from five lupus patients were stained with vinblastine-treated mouse L cells using indirect immunofluorescent microscopy. The majority of the serum samples tested stained primarily the nuclei and the nuclear envelope. However, we detected a unique cytoplasmic reaction with serum from one

patient; this reaction was found only in vinblastine-treated mouse L cells. The serum was further characterized and then used for the studies described below.

Results

AL Membranes in Mouse L Cells

Mouse L cells cultured under standard conditions in defined media exhibit features typical of normal fibroblasts. Rarely are AL membranes found in these cells (13, 14). As previously reported the incidence of AL membranes in mouse L cells is effected by exposing cells to sublethal doses of vinblastine sulfate (13, 14). After being cultured for 36 h in the presence of 0.1 μ g/ml of vinblastine, 30% of the cell population exhibited a well-defined system of AL membranes (Fig. 1). These membranes were morphologically similar to the AL network described in other cell types. The organelle consisted of a parallel array of three to four cisternae containing a periodic arrangement of pores. Routinely, AL membranes were detected in the peripheral area of the cell abutting the plasmalemma.

Distribution of Nuclear Lamins in AL-positive Cells

The ability to reproducibly generate AL membranes in mouse L cells provided a convenient means to study their make-up. Immunocytochemical studies were carried out to determine whether antigens associated with the nuclear envelope, nuclear lamin A/C and B, were present in the AL membrane system. Mouse L cells, incubated under standard conditions (normals) or in the presence of vinblastine sulfate (AL-positive cells) were reacted with antibodies directed against the nuclear lamins. As shown in Fig. 2, the lamins were localized on the nuclear envelope irrespective of how the cells were treated. When the immune reaction was carried out on vinblastine-treated cells, no signal was detected in the cytoplasm and there was no indication that the antigens either redistributed or were associated with AL system (Fig. 2, *c* and *d*). The only effect the vinblastine appeared to have on the localization of the nuclear lamins was that the fluorescent signal associated with the nuclear envelope intensified and the background staining observed in the nuclei of the control cells diminished. These results suggest that the nuclear lamins are not present in the AL complex. However, the approach is limited in sensitivity and does not rule out the possibility that the antigenicity of the lamins is masked in the AL network. To address these concerns and substantiate the observations described above, an AL-enriched fraction was obtained from a homogenate of vinblastine-treated mouse L cells (Fig. 3 *a*). As shown, the integrity of the AL system was maintained during the fractionation; the membranes appeared as a readily identifiable stack of rigid lamellae. This preparation and a nuclear fraction (Fig. 3 *b*) also isolated from vinblastine-treated L cells were used for the immuneoverlays shown in Fig. 4. Immuneoverlays were carried out using a highly sensitive avidin-peroxidase conjugate to detect a positive immune reaction. The results were consistent with our immunofluorescent studies: anti-lamin A/C and B only reacted with the nuclear fraction. No signal was detected in either the AL-enriched fraction (Fig. 4) or in nonimmune controls (data not shown).

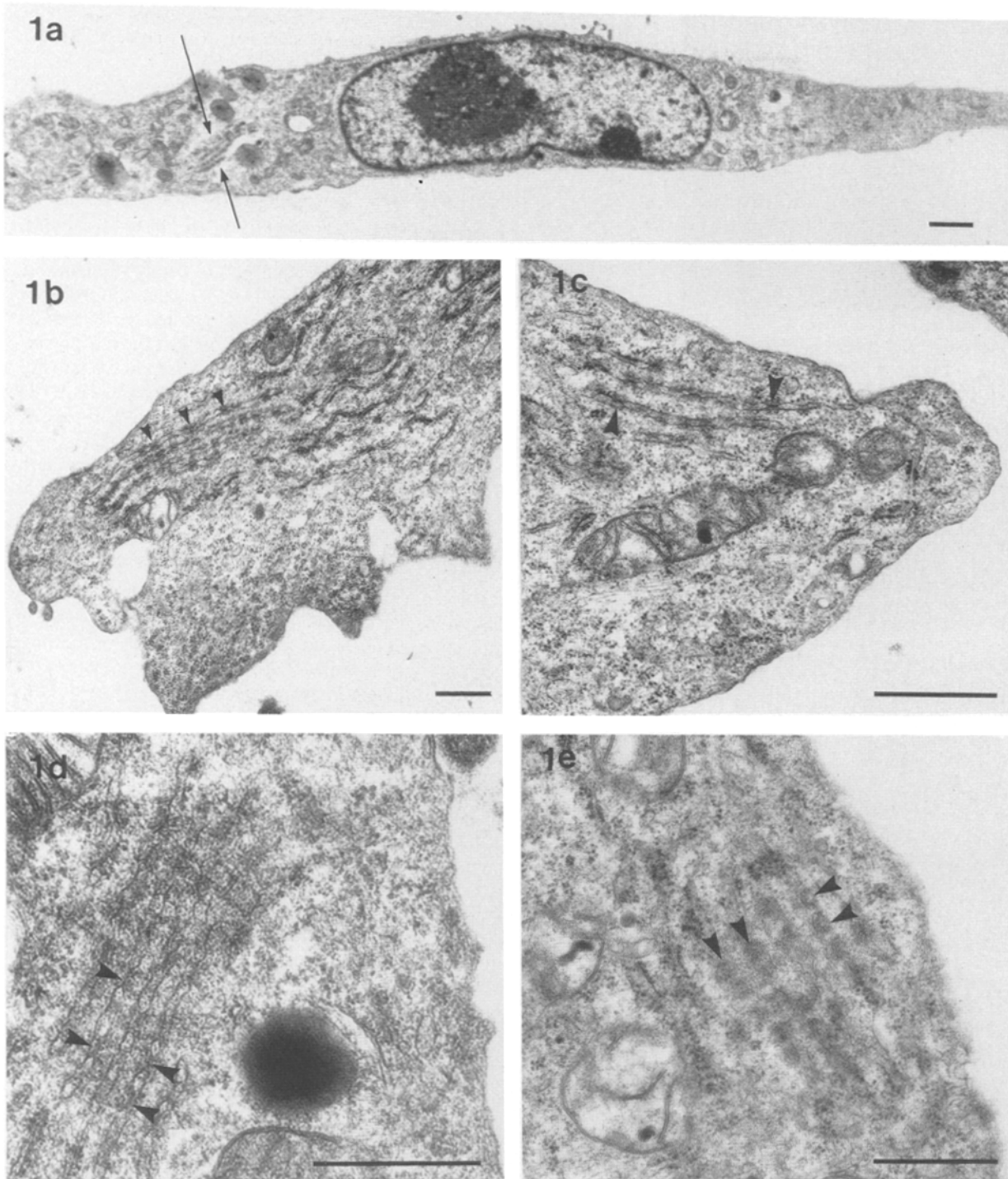


Figure 1. Stacks of AL membranes (*arrows*) are readily observed in mouse L cells treated with vinblastine sulfate (0.1 $\mu\text{g/ml}$). As judged by TEM, 30% of the cells examined have AL membranes. The membranes are usually located peripherally in the cytoplasm of these cells and exhibit features characteristic of the AL network. The membranes consist of stacked cisternae, contain pores (*b-e*, *arrow heads*), and are associated with a dense staining fibrillar material. In *e*, this tangential section shows a surface view of AL pores (*arrow heads*). Bars: (*a*) 1 μm ; (*b*, *c*, *d*, and *e*) 0.5 μm .

AL-specific Antibody

Sera from patients with lupus erythematosus have been used by many investigators as a source of antibodies for a wide variety of cellular antigens (1, 16, 20, 24, 25). With the inten-

tion of obtaining an antibody specific for antigens of the AL membrane system, serum samples from a number of lupus patients were assayed for AL-specific antibodies by indirect immunofluorescence carried out on vinblastine-treated

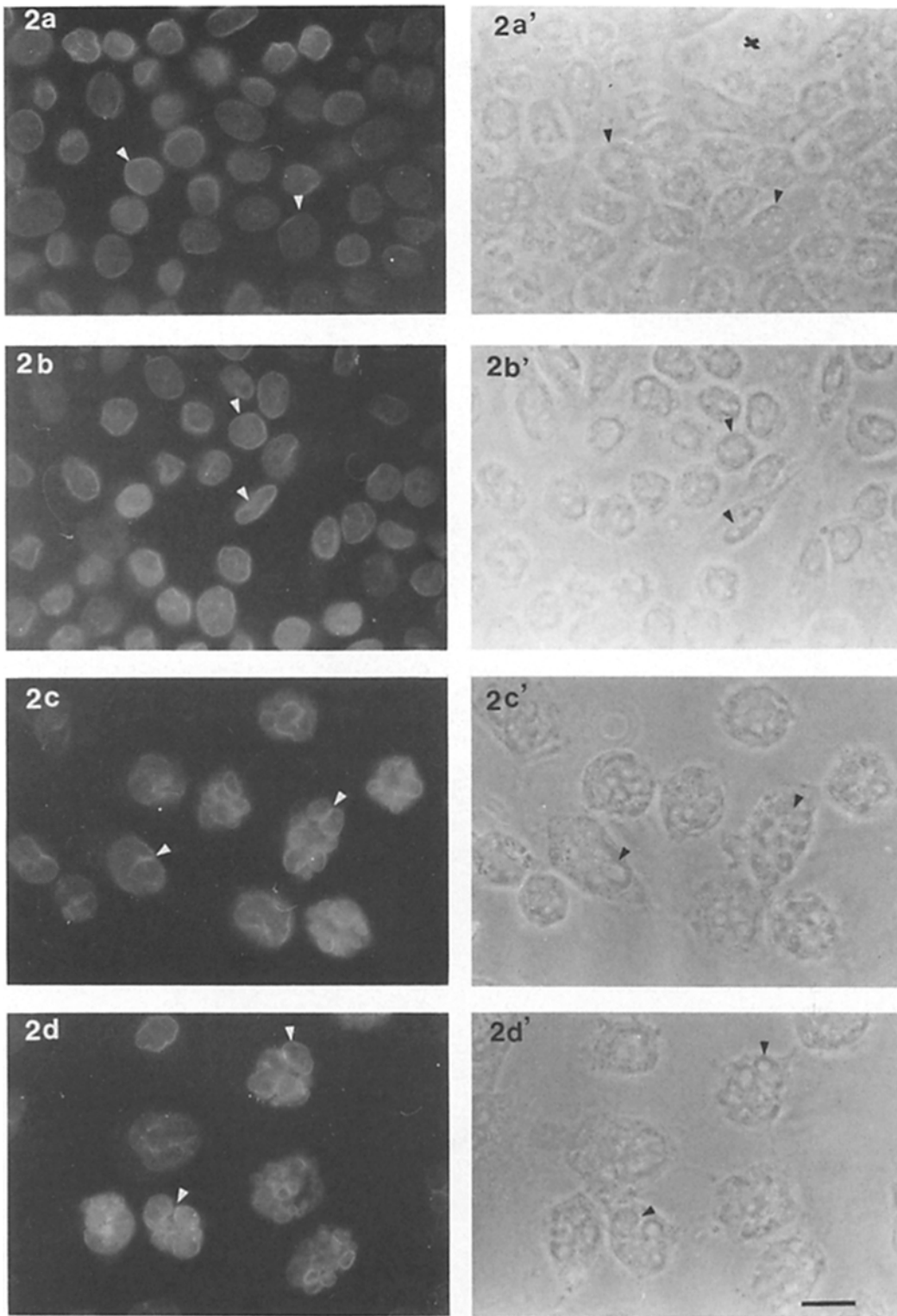


Figure 2. In the *left column*, the micrographs show indirect immunofluorescent labeling carried out on normal L cells (*a* and *b*) and vinblastine-treated L cells (*c* and *d*). The *right column* shows the corresponding phase image of these cells. The cells were incubated with anti-lamin A/C (*a* and *c*) and anti-lamin B (*b* and *d*), respectively, followed by a FITC-conjugated goat anti-guinea pig IgG. As shown in these micrographs, labeling is associated only with the nuclear envelope (*arrow heads*). Bar, 5 μ m.

mouse L cells. As shown in Fig. 5, *a*, *b*, and *c*, a serum sample was obtained in which a positive immune reaction was detected only in AL-positive cells. In addition, the cellular localization of this reaction corresponded to the area of the

cell where AL membranes were routinely observed (Fig. 1, *a-c*). All other regions of the cell and other subcellular structures were negative. Also, no reaction was seen in cells cultured under normal conditions (Fig. 5 *a*). It should be

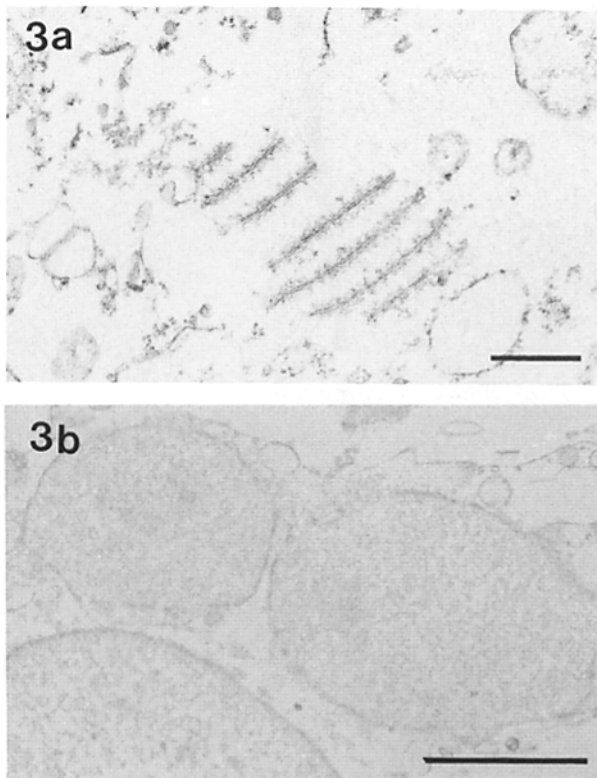


Figure 3. The micrographs show the predominant structural features of a postnuclear supernatant and nuclear fraction obtained from a homogenate of vinblastine-treated cells. Vinblastine-treated cells were homogenized in the presence of vinblastine (0.1 $\mu\text{g}/\text{ml}$). The homogenate was centrifuged at 600 g for 10 min at 4°C. The nuclear fraction (pellet) and postnuclear supernatant were separated and analyzed for the presence of AL membranes by TEM. AL membranes were found only in the postnuclear supernatant (*a*) and the pellet contained mainly nuclei (*b*). Bars, 0.2 μm .

noted that this immune reaction was not detected in all serum samples assayed from this particular patient. The response varied from bleed to bleed. In the majority of samples assayed, a cytoplasmic and the nuclear reaction characteristic of a lupus response were observed. For these studies, caution was taken to use serum from a specific bleed in which this cytoplasmic reaction was observed.

The specificity of this reaction was confirmed by immune TEM (Fig. 6). The lupus serum specifically stained stacks of AL membranes. In comparison to controls, cells that reacted with normal human serum (Fig. 6 *b*), using AL-specific anti-serum, were discernable even at relatively low magnifications (Fig. 6 *a*) and were definitely localized in association with only AL membranes (Fig. 6, *c* and *d*). The reaction appeared to be associated with the perimeter of pore complexes. Also in comparison to controls (Fig. 6 *d*), there was very little background staining and there was no reaction detected on other subcellular structures including the nuclear envelope, rough endoplasmic reticulum, and mitochondria. To identify the antigen(s) recognized by this AL-specific anti-serum, immuneoverlays were carried out using protein blots of the AL-enriched fraction described above (Fig. 3 *a*). As shown in Fig. 7, an immune reaction was associated with a number of proteins, however an intense signal

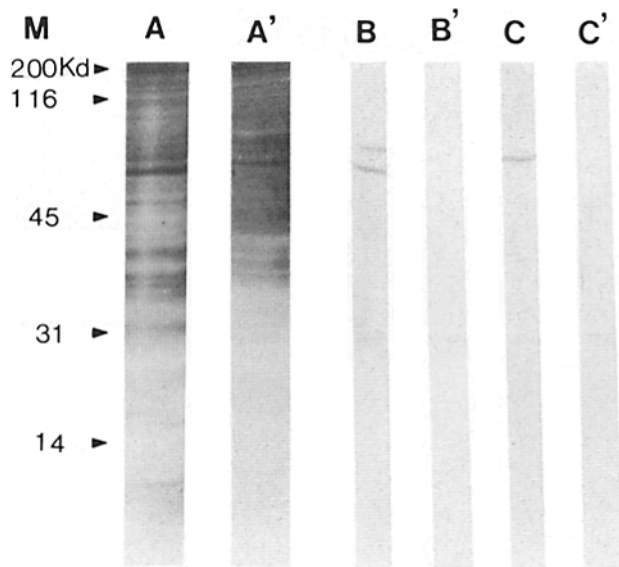


Figure 4. Immuneoverlays were carried out on the nuclear pellet (*A*, *B*, and *C*) and the postnuclear supernatant (*A'*, *B'*, and *C'*) described in Fig. 3. Aliquots (100–400- μg protein) from each sample were subjected to electrophoresis on a 10% SDS polyacrylamide gel and transferred to nitrocellulose. Protein replicas were quenched and incubated with anti-lamin A/C and B, as described in Materials and Methods. (Lane *A*) SDS-PAGE silver-stained protein profile of nuclear fraction; (lane *A'*) SDS-PAGE silver-stained protein profile of postnuclear supernatant; (lane *B*) immunoblot of nuclear fraction (100- μg protein) reacted with anti-lamin A/C antibody; (lane *B'*) immunoblot of postnuclear supernatant (200- μg protein) reacted with anti-lamin A/C antibody; (lane *C*) immunoblot of nuclear fraction (100- μg protein) reacted with anti-lamin B antibody; (lane *C'*) immunoblot of postnuclear supernatant (400- μg protein) reacted with anti-lamin B antibody. The molecular mass markers (*M*) used were myosin, B-galactosidase, ovalbumin, carbonic anhydrase, and lysozyme.

was associated with two proteins having relative molecular masses of 110 and 35 kD.

Discussion

The purpose of this study was to obtain an understanding of the biochemical make-up of the AL membrane system. With the expectation of identifying a marker for AL membranes, we were interested in comparing the antigenicity of the AL membrane system with the nuclear envelope. In this study we used immunocytochemistry to determine whether there are antigenic epitopes common to both of these membrane systems.

The results of our immunocytochemical studies using anti-lamin A/C and B on mouse L cells incubated in the presence or absence of vinblastine clearly showed that these nuclear envelope-specific antibodies stained only the nuclear envelope. An intense signal was associated with the envelope irrespective of whether or not the cells were AL positive or negative. No signal was detected in the cytosol or, in particular, the region of the cytosol abutting the plasmalemma where AL membranes are normally observed. It should be mentioned that to assure that AL membranes were present in the vinblastine-treated cells, a cell sample was routinely

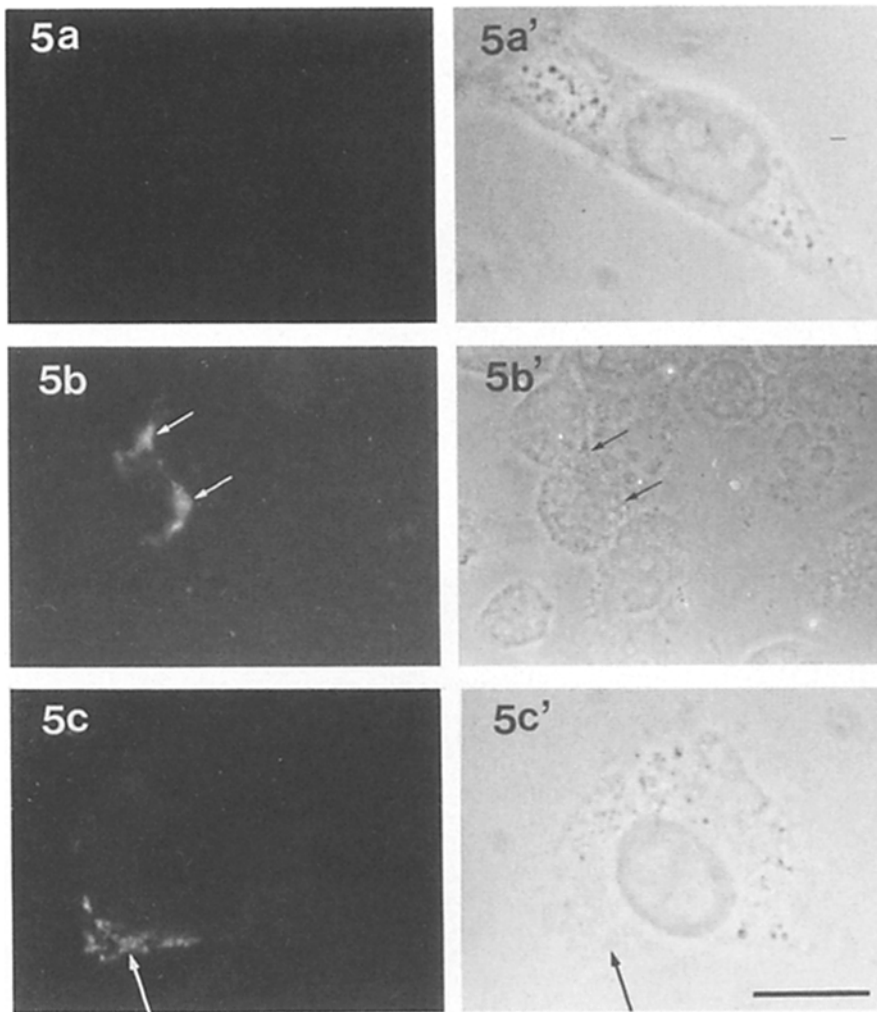


Figure 5. The micrographs shown are normal (*a*) and vinblastine-treated (*b* and *c*) L cells labeled with human lupus serum using indirect immunofluorescence. The phase image of these cells are shown in *a'*, *b'*, and *c'*. As shown in *a*, normal L cells were negative, no labeling was associated with either the nuclear envelope or the cytosol. The micrograph shown in *b* is a low magnification view of cells labeled with the human lupus serum. As shown in this micrograph, the signal was found only in peripheral region of the cytoplasm. The signal is punctate and located in the periphery of the cells (*arrows*). Bar, 5 μm .

saved and processed for TEM. In addition, our results addressed the possibilities that the lamins were present but in a concentration too low to be detected by immunofluorescence, and that they were masked by the structure of the AL membrane system; our immuneoverlays were carried out using highly sensitive conjugates on SDS-solubilized fractions.

Failure to detect the nuclear lamins in the AL membrane system raised the question of whether the nuclear lamins were an appropriate marker for the nuclear envelope. The lamins are a highly conserved set of proteins that are tightly associated with the nuclear envelope. However, they are not integral membrane markers of the envelope and, as demonstrated by Gerace et al., lamin A and C dissociate from the envelope during mitosis (5). With the hope of obtaining a nuclear envelope antibody that would react with the AL membrane structure a series of serum samples from patients with lupus erythematosus was assayed. Sera from lupus patients were tried because even though the primary autoimmune reaction is directed against DNA and RNA, investigators have obtained antibodies from such patients specific for nuclear envelope associated proteins (20, 24), and other cytoskeletal constituents (1). To our surprise we obtained a

serum sample that only reacted with vinblastine-treated mouse L cells. The reaction was localized in the cytosol in the area occupied by the AL membrane system. There was no reaction associated with other cellular organelles including nuclear envelope. The fact that the reaction could only be detected in vinblastine-treated mouse L cells and was localized in the area normally occupied by the AL membrane system strongly suggested that the antibody was directed against constituents of the AL network. The results from our EM immunocytochemistry confirmed these observations. The reaction was intense, stained only the AL membrane system, and appeared to be specifically associated with the outer perimeter of AL pore complexes. However, due to diaminobenzidine diffusion artifact and the fact that the serum recognized a number of antigens we have not ruled out the possibility that the reaction may also be associated with AL membrane components and/or the intracisternal space. The primary antigens recognized by our serum sample, $M_r = 110$ and 35 kD, remain to be characterized. It would be interesting to determine whether these proteins are bona fide pore-associated proteins. If so, in lieu of the recent work carried out on the "nucleoporins" (4, 9, 23, 26), perhaps these "cytoporins" belong to a similar family of glycoproteins that

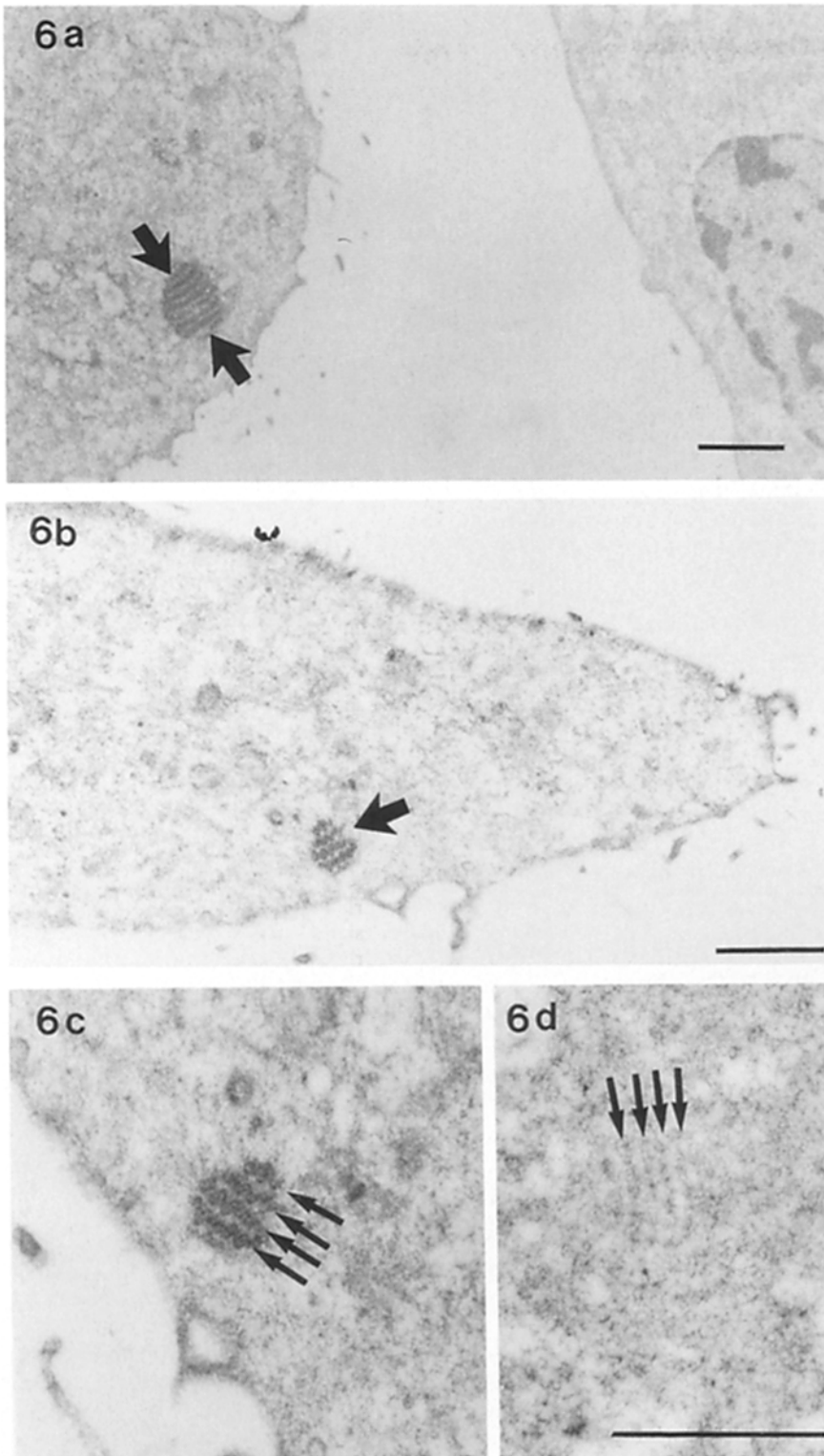


Figure 6. The micrographs show the immune electron microscopy of vinblastine-treated mouse L cells stained with human lupus serum (*a*, *b*, and *c*) and with normal human serum (*d*) using immunoperoxidase (see Materials and Methods). As shown in *a* and *b*, the immune reaction is discernable even at relatively low magnifications. An intense reaction is found only in association with the AL membrane system (*a*, *b*, and *c*). There is a minimal amount of background stain distributed throughout the cytoplasm but this is also observed in controls, cells that reacted with normal human serum (*d*). Also in these controls, due to their lack of reactivity the AL network is detected only with great difficulty (*d*). Bars, 0.5 μm .

contain *N*-acetyl-glucosamine attached in a O-glycosidic linkage to the peptide chain.

In summary our results suggest that the AL membrane system is a distinct subcellular organelle with respect to its antigenicity. At present we are attempting to further characterize these AL-specific antigens and to determine their distribu-

tion in other cell types. With a general marker for this organelle, it will be possible to address questions concerning the origin and more importantly the function of the AL membrane system.

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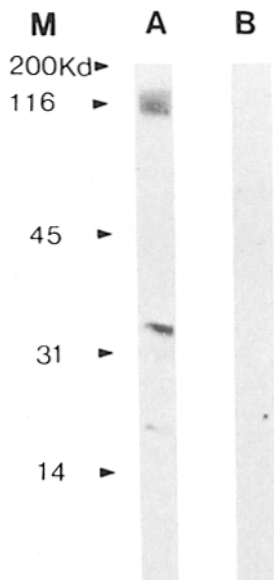


Figure 7. The immuneoverlays are of the AL-enriched postnuclear supernatant described in Fig. 3. The protein blots were reacted with either human lupus serum (A) or a normal human serum (B) using 4-chloro-1-naphthol as the peroxidase substrate (see Materials and Methods). (Lane A) Protein blot reacted with human lupus serum; (lane B) protein blot reacted with normal human serum. The molecular mass markers (M) were myosin, B-galactosidase, ovalbumin, carbonic anhydrase, and lysozyme.

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