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Interaction of Antibodies to Sheep Urothelial Plaque Regions with the Lumenal Plasma Membranes of Other Mammals*

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Abstract. The lumenal plasma membrane of most mammalian urothelia possesses discrete polygonal plaque regions which are characterized by particles projecting through the lumenal leaflet and bridging the membrane interior. Fractions enriched in plaque regions were prepared from sheep urinary bladders and were used as antigen. A 50% saturated ammonium sulphate fraction of immunized rabbit serum showed at least two antibodies to plaque region components in Ouchterlony diffusion test. Intact sheep urothelium was treated with the antibody preparation and labelling was visualized by treatment with peroxidase-conjugated goat anti-rabbit antibody followed by peroxidase reaction. Control tissue treated with non-immune globulin showed no reaction product. The plaque-specific antibody preparation was also shown to stain the lumenal leaflet of isolated sheep membranes. Further, cross reactivity of sheep plaque antibody was demonstrated with rat and rabbit membranes. The possibility that antibodies to specific components in sheep urothelial membranes may be used diagnostically to detect changes in urothelial plasma membranes of other species is discussed.

Key words. Immunocytochemistry - Peroxidase-labelled antibodies - Sheep - Rat and rabbit urinary bladder.

The lumenal plasma membrane of the urinary bladder of all mammalian species so far examined (7) is distinguished both morphologically and functionally. Approximately 75% of the lumenal plasma membrane surface consists of particle-bearing regions (plaques) separated one from another by smooth, particle-free interplaque regions (23, 24). In thin section the lumenal plasma membrane takes on a scalloped appearance (10), resulting from the alternating pattern of concave plaque regions and convex interplaque regions (24). Plaque regions, in profile, possess a thickened, asymmetric unit membrane and thus can be differentiated easily from the usual symmetric unit membrane structure of the interplaque regions (24). Functionally, the lumenal plasma membrane stands apart from most plasma membrane systems in its relative impermeability to water and small ions (11, 14). Together with the tight junctions present at the lumenal surface (26) the urothelial lumenal plasma membrane constitutes a physical and chemical barrier preventing loss of water into the hypertonic urine.

Hyperplastic and neoplastic changes in the urothelium involve the loss of characteristic ultrastructure (12, 15, 5, 16, 19) and increased permeability to water and small ions (6, 14, 2). In model systems (5, 16, 19) profiles of tumor lumenal membranes are devoid of the particle-rich plaque regions exhibiting asymmetric unit membrane structure. Instead the entire lumenal plasma membrane, in thin section, appears undifferentiated, is particle-free and uniformly has a symmetrical unit membrane structure. The significance of the morphological alterations for permeability changes is unknown.

To understand the pathogenesis of bladder cancer the search for markers that accompany neoplastic transformation is essential. Biochemical changes have been observed between normal and tumour-bearing urinary bladders in protein spectrum (17), in surface carbohydrate coat (19) and in glycolipid composition (9). Some of these changes have been correlated with the development of experimentally induced tumours in rats (5, 16). What is needed now is a method for detecting a biochemical change indicative of an ear-

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ly stage of bladder carcinogenesis. To this end we have investigated the use of the immunocytochemical technique in detecting the presence and the localisation of specific antigens on the lumenal surface of the urinary bladder. Further, we have shown interspecies cross-reactivity, i.e., antibodies produced against sheep bladder lumenal plasma membrane proteins were shown to interact specifically with the lumenal plasma membranes of rat and rabbit bladders. The possibility of extending this immunocytochemical assay to human biopsy material for early detection of bladder cancer is discussed.

MATERIALS AND METHODS

Isolation of the Lumenal Plasma Membrane and the Plaque Regions from Sheep Urinary Bladders. One hundred fresh sheep bladders, kept on ice, were inverted in order to expose the lumenal surface to 0.02 M sodium thioglycolate, pH 7.4, at room temperature for 30 minutes. The lumenal plasma membrane that had sloughed off into the thioglycolate solution was isolated according to the procedure outlined earlier (3), and the plaque regions were subsequently separated from the lumenal membranes by treatment with 2% deoxycholate (3). The latter preparation is referred to below as "plaques."

Preparation of Sheep Erythrocyte Ghosts. Fresh, citrated sheep blood, obtained from the Colorado Serum Co., Denver, CO, was washed three times with 0.155 M NaCl and care was taken to remove all of the buffy coat after each low speed centrifugation (1000 x g for 10 minutes). Washing of the erythrocytes was continued twice more in ghost buffer (92.9 mM NaCl, 3.6 mM KCl and 0.05 M sodium phosphate buffer, pH 7.4). The washed cells were then suspended in diluted (1:20) ghost buffer in a ratio of 1 to 20 (erythrocytes to buffer) and lysed for 1 hour. After centrifugation at 20,000 x g for 20 minutes, the ghosts were rewashed five times in diluted ghost buffer and then were suspended in ghost buffer.

SDS-polyacrylamide Gel Electrophoresis. Slab gels containing 3% (stacking gel) and 10% (separation gel) acrylamide were prepared and run as described by Ames (1). The running buffer, pH 8.3, contained 0.025 M Tris base, 0.192 M glycine and 0.1% sodium dodecyl sulfate (SDS).

Protein Determination. Protein was determined by the method of Lowry et al. (18).

Preparation of Antibodies. Before antibody induction, pre-immune globulin was collected from a New Zealand rabbit by bleeding from the ear vein. After clot formation, serum was collected and tested against isolated plaques, solubilized in 4 percent Triton X-100, by the Ouchterlony double diffusion method (22).

Rabbits exhibiting no cross-reactions with plaque proteins were immunized by the following regimen: 2.5 mg of plaques were emulsified in complete Freund's adjuvant (1:1) and injected subcutaneously. A similar injection of 2.5 mg was given three weeks later. After three more weeks a booster injection of 4.0 mg without Freund's adjuvant was given into the ear vein. Rabbits were bled one week after the final injection, and bleeding was continued once per week for one month. Sera were combined and the antibody fraction was precipitated upon exposure to an equal volume of saturated ammonium sulphate for 30 minutes at room temperature. After centrifugation in the Sorvall centrifuge at 12,000 x g for 10 minutes, the antibody fraction was dialysed against phosphate buffered saline (PBS), 0.01 M sodium phosphate buffer, 0.15 M sodium chloride, pH 7.6, for 2 hours at room temperature and then stored frozen. This 50% ammonium sulphate fraction will be referred to as "immune globulin." The comparable preparation from non-immunized rabbit will be called "control globulin."

Preparation of Peroxidase-Labelled Antibody. Horseradish peroxidase (HRPO) was conjugated with commercially prepared (Cappel Laboratories, Dowingtown, PA) goat anti-rabbit gammaglobulin according to the procedure described by Nakane and Kawaoi (21). The conjugated product was purified chromatographically on Sephadex-200, and the completion of the conjugation reaction was evaluated by reading the absorbance at 280 and 403 nm as suggested by Nikane and Kawaoi (21).

Immunocytochemical Detection of Interaction Between Specific Antibodies to Plaque Regions and the Lumenal Membrane with HRPO-Labelled Antibody Method. Fresh sheep urinary bladders, obtained from the slaughterhouse and transported to the laboratory, were fixed in Karnovsky's fixative (8) for 25 minutes at room temperature, rinsed three times in 0.2 M sodium cacodylate, pH 7.4, and placed in PBS. Pieces of bladder tissue showing maximal urothelial surface area were carefully excised and returned to PBS. The same procedure was followed for bladder tissue obtained from rats within 5 minutes of asphyxiation in CO_2. In experiments utilizing isolated membrane preparations instead of intact bladder tissue, the lumenal plasma membrane, isolated under standard conditions as described above, was fixed in 4% paraformaldehyde in PBS for 15 minutes, centrifuged in the Beckman L2-65B ultracentrifuge, type 65 rotor, at 20,000 rpm for 15 minutes, rinsed in PBS and recentrifuged. The pelleted membranes were resuspended in PBS.

The immune globulin, prepared as described above to the plaque regions of sheep urothelial plasma membranes, was used to test for specific interaction with the lumenal plasma membrane. Gammaglobulin protein prepared from a non-immunized rabbit (control globulin) was used in the control experiments. Both globulin preparations were used at a protein concentration of 20 mg/ml.

Fifty µl of the appropriate serum preparation were pipetted into individual wells in a titration plate. Bovine serum albumin (BSA) was added to a concentration of 2 mg/ml. Pieces of fixed bladder epithelium were placed lumenal side down into the serum from 5 to 20 minutes at room temperature. Intermittent, gentle stirring was accomplished by blowing softly through a pipette into the solution. The tissue pieces were removed and washed three times in PBS containing BSA at 2 mg/ml over the next 20 minutes. The lumenal surfaces of the tissue pieces were then exposed to 50 µl of HRPO-labeled goat anti-rabbit gammaglobulin containing BSA at a level of 2 mg/ml. The interaction was allowed to proceed with gentle stirring for 5 to 20 minutes at room temperature. At the appropriate time the pieces of tissue were removed and washed thrice with PBS containing BSA at 2 mg/ml for a total of 30 minutes.

When isolated membrane preparations were used instead of tissue pieces, the membranes were suspended in 0.4 ml of PBS, and an equal volume of either control globulin or immune globulin was added. After 20 minutes of interaction at room temperature, the suspension was spun in a clinical centrifuge for 5 minutes, washed thrice in PBS and then exposed to 0.5 ml of the HRPO-labeled goat anti-rabbit gammaglobulin for 20 minutes at room temperature. After centrifugation, the labeled membrane preparation was washed in PBS three times.

As suggested by Nakane (20) the antibody-reacted membranes were post-fixed in Karnovsky's fixative for 30 minutes at room temperature, washed three times in 0.05 M Tris HCl, pH 7.6, for 15 minutes per wash and stained for peroxidase activity by incubating for 1 hour, unless otherwise indicated, in 0.075% 3, 3' -diaminobenzidine 4 HCl (DAB) and 0.001% H_2O_2 as described by Graham and Karnovsky (8). The stained tissue was washed in 0.05 M Tris HCl, pH 7.6, re-washed in distilled water, exposed to 1% aqueous osmium tetroxide for 1 hour, dehydrated in acetone and embedded in Epon-Araldite.

Microscopy. For light microscopy, sections 1 - 2 µm in thickness were placed on a glass slide and stained in 1% aqueous toluidine blue. For electron microscopy blocks were sectioned with a diamond knife on an MT-1 Porter-Blum microtome, stained with uranyl acetate and lead citrate, and observed with a Phillips 300 electron microscope at 60 kV.

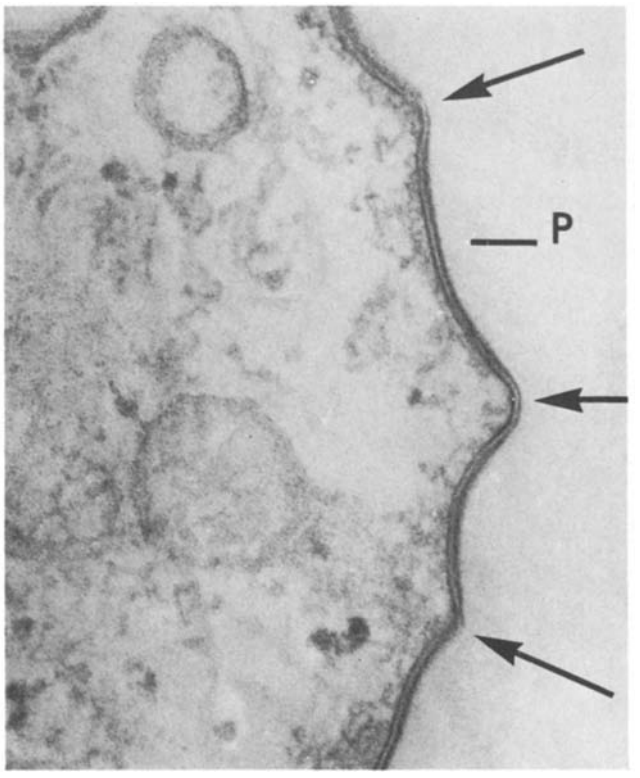

Fig. 1. Normal lumenal surface of urothelium in the sheep as visualized in thin section. The thickened outer leaflet of the lumenal membrane can be seen in concave (plaque) regions (P). This thickening is due to the presence of specific particles, which do not occur in interplaque regions. The latter are seen (arrows) as wave crests projecting into the lumen. (140,000X)

RESULTS

The distinctive morphology of the lumenal plasma membrane of normal sheep urothelium is shown in profile in Fig. 1. The scalloped appearance of the surface membrane results from the alternating pattern of the concave "plaque" and the convex "interplaque" regions. In the sheep the interplaque region measures approximately 10.0 nm in thickness and possesses a symmetrical unit membrane structure. In contrast, the thickened membrane of the plaque regions measures approximately 12.5 nm in thickness and is characterized by an asymmetric unit membrane in which the lumenal leaflet is approximately twice the thickness of the cytoplasmic one. The features characterize not only the sheep (3) and rat urothelia (23) but also those of most mammalian species (7). This characteristic morphology has been used diagnostically to identify the lumenal membrane during its separation and purification (13, 4, 25, 3).

Upon isolation the lumenal plasma membrane remains in large sheets rather than becoming

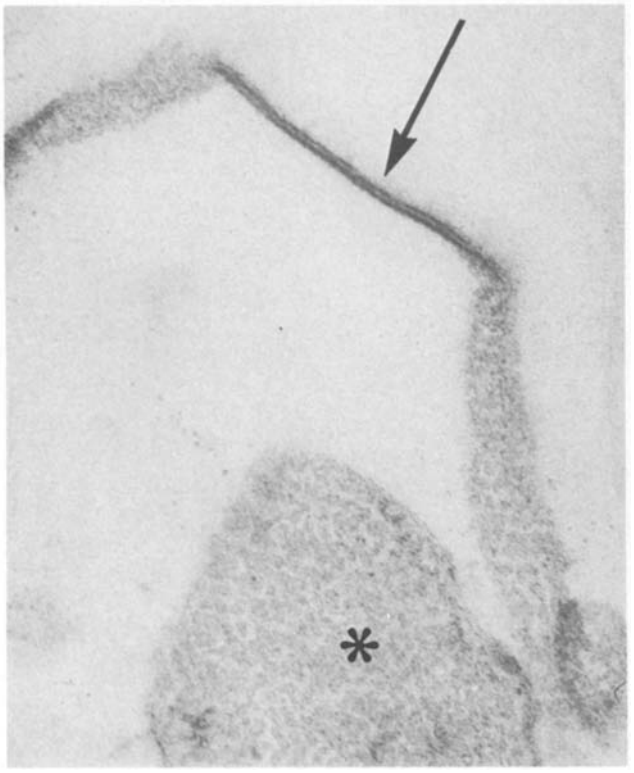

Fig. 2. Thin section through pellet of isolated plaque regions from lumenal membranes of sheep urinary bladder. Deoxycholate treatment has largely removed interplaque regions. When viewed in cross section (arrow), they retain the asymmetric unit membrane structure characteristic of the normal membrane in situ (compare Fig. 1). In oblique section, hexagonal arrays of particles (asterisk) are visible. (175,000X)

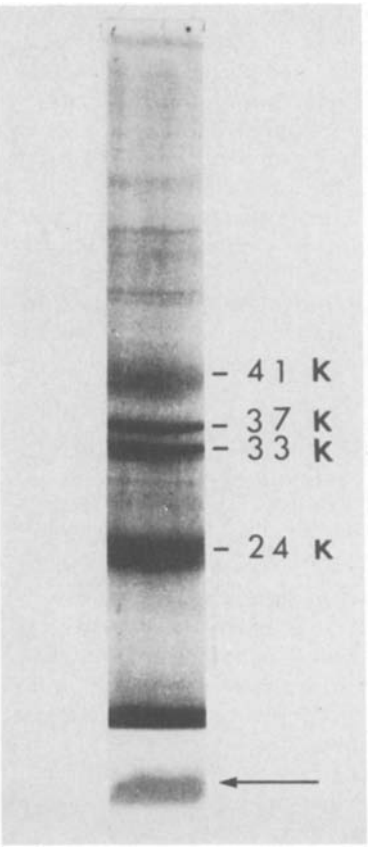

Fig. 3. SDS-polyacrylamide slab gel electrophoretic pattern of the isolated plaque regions. Arrow indicates glycolipid component

fragmented into small vesicles. When treated with deoxycholate the lumenal plasma membrane fragments and the plaque regions become separated from the interplaque regions (4, 3). With differential centrifugation a fraction enriched in plaque regions is obtained. In thin section (Fig. 2) the plaque region fraction consists of small concave pieces of membrane exhibiting an asymmetric unit membrane structure. It appears that the plaque regions, separated from the lumenal plasma membrane, retain their morphological identity.

In SDS-polyacrylamide gel electrophoresis (Fig. 3) the plaque regions fraction shows a rather simple protein spectrum. The most prominent bands present are those representing the 41,500, 37,000, 33,500 and the 24,000 dalton proteins. In rough estimation the 24,000 dalton species appears to be in highest concentration and may actually represent twin protein bands or two species of very similar molecular weight. Also dominant is a band running close to the anode. This band, which stains darkly in periodic acid-Schiff base (3) and which is sensitive to chloroform-methanol extraction, represents the glycolipid component of the plaque regions membrane.

In order to show that components of the urothelial plasma membrane can be used as antigens to probe the organisational structure of the lumenal plasma membrane, the fraction enriched in isolated plaque regions was used as antigen. As illustrated in Fig. 4, antibodies produced against the plaque regions from sheep membranes react positively in the Ouchterlony test. As would be expected, more than a single antibody is produced to the plaque regions. At least three precipitin lines characterized the interaction between the plaque regions antigens and the antibodies.

The specific interaction between the immune globulin (containing antibodies to plaque regions) and the sheep bladder urothelial cells was also followed with light microscopy. A time-dependent aggregation was easily observed between the immune globulin and the cells scraped from sheep bladder lumenal surface. In the control, where

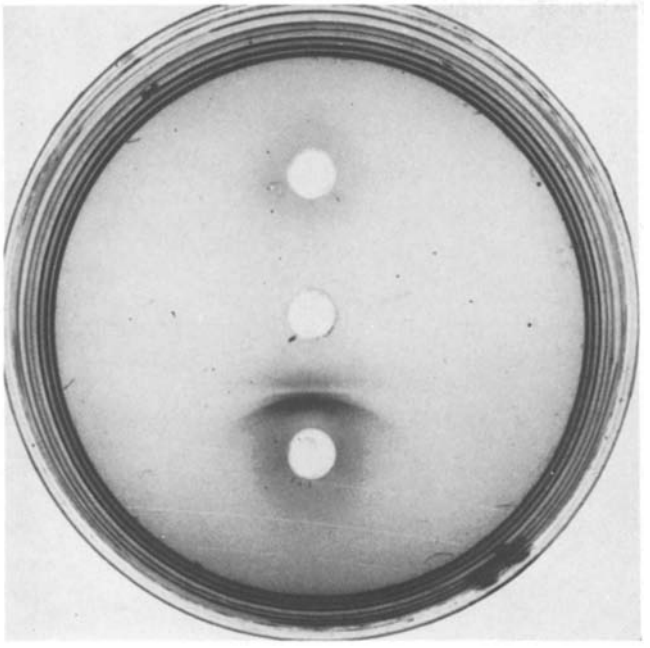

Fig. 4. Ouchterlony diffusion test. A 1% agar medium in PBS contained 0.1% sodium azide and 1% Triton X-100. Antigen was solubilized completely in PBS made 4% in Triton X-100. Three precipitin lines are observed between the plaque region antigen (center well) and the immune serum (bottom well). No reaction bands are seen between the antigen and control serum. (top well)

gammaglobulins from non-immunised rabbits were used, no such state of aggregation is observed. Aggregation can be discerned 5 minutes after initiation of the immunological reaction and an increased level of aggregation develops with time.

The antibodies to the plaque regions were then shown immunocytochemically to interact with isolated preparations of the lumenal plasma membrane. The indirect labeling technique (first exposing the isolated membrane preparation to the immune serum followed by exposure to HRPO-coupled antirabbit gammaglobulins) was used to visualize the interaction of the antibodies with the lumenal plasma membrane. As shown in Fig. 5a the membranes interacting with the immune globulin are heavily stained when compared with the control membranes which were interacted with serum from a non-immunized rabbit (Fig. 5b). The increase in density at the membrane surface is unmistakable, and it would appear that only the lumenal surface and not the cytoplasmic side of the plasma membrane interacts with the antibodies. Under the experimental conditions used, a thickness of 13.4 nm is measured for experimental membranes compared to 12.5 nm for control membrane. A value of 12.5 nm is also obtained for the thickness of plaque regions in situ (Fig. 1).

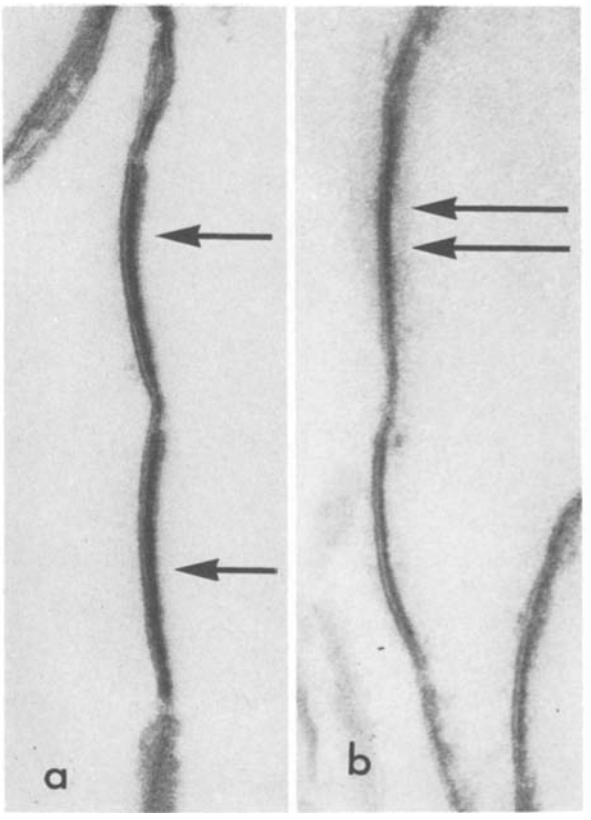

Fig. 5. Isolated lumenal plasma membrane from sheep urothelium. (a) Membranes labelled for presence of antibodies to plaque regions (interaction with serum from a rabbit immune to plaque regions followed by exposure to peroxidase-labeled goat anti-rabbit gammaglobulin). (b) Control preparation in which membranes were exposed to serum from a non-immune rabbit prior to application of the peroxidase-labeled anti-rabbit gammaglobulin. Notice the increase in density and thickness of the experimental preparation (a), in which the lumenal leaflet (arrows) in the plaque regions is clearly labelled. In contrast, the lumenal leaflet of plaque regions in the control preparation (b) is relatively low in density (double arrows), and the total thickness of the control membrane in plaque regions is about half that of the experimental. (162,000X)

Close inspection of the stained membranes in Fig. 5 suggests that it is primarily the plaque regions that are involved in the immune reaction. The interplaque regions appear to be stained to a much lesser extent than the plaque regions.

The specific interaction between the immune globulin and the lumenal plasma membrane was also demonstrated in intact tissue of sheep bladder urothelium (Fig. 6a). The increase in staining is localized to the lumenal surface of the urothelium. Control experiments (Fig. 6b) in which tissue pieces are exposed to control globulin show

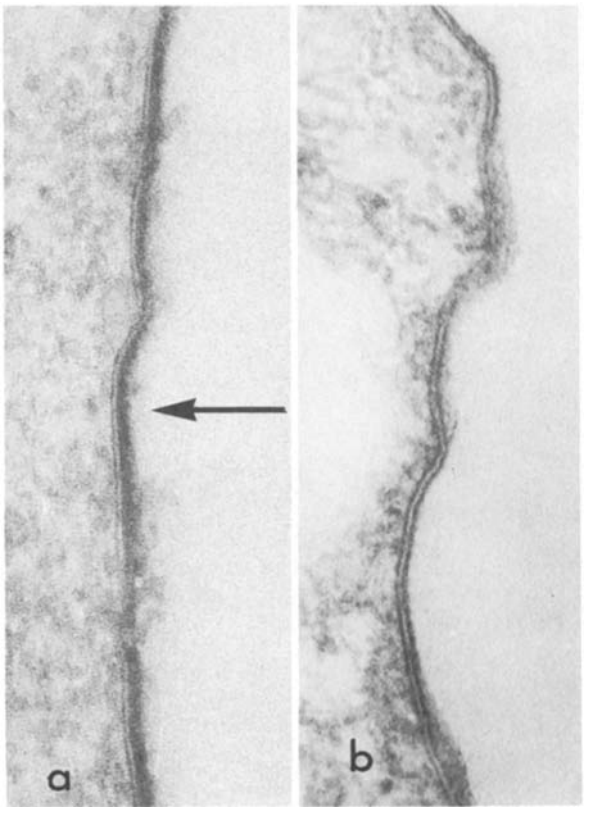

Fig. 6. Lumenal surface of intact sheep urothelium. (a) Interaction of surface membrane with antibodies to plaque regions. The indirect labelling method was carried out as described in Fig. 5a except that the incubation in DAB was 20 minutes. The plasma membrane (arrow) is noticeably stained along the lumenal surface of the plaque regions. (167,000X). (b) Control preparation does not show enhanced staining of the lumenal plasma membrane. (162,000X)

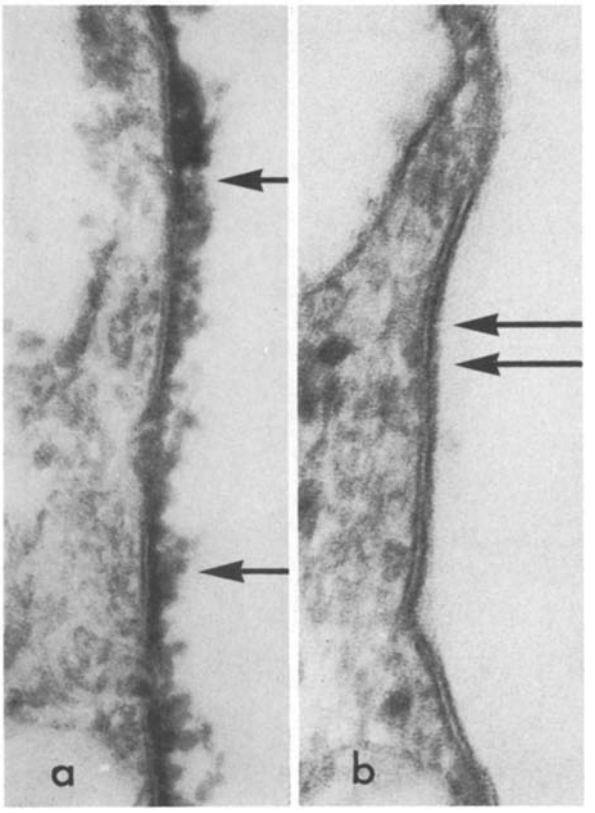

Fig. 7. Lumenal surface of intact sheep urothelium. (a) Localization of antibodies to plaque regions of lumenal plasma membranes isolated from sheep. Indirect labelling method comparable to that described in Fig. 5a. Notice appreciable deposition of stain associated with the lumenal surface of plasma membrane (arrows). (b) Control preparation in which the surface of the bladder was exposed to serum from a non-immune rabbit prior to treatment with peroxidase-labeled anti-rabbit gammaglobulin. In the control preparation, the lumenal membrane of the plaque regions (double arrows) has less than half the thickness of the experimental. (167,000X)

no increased staining due to indirect HRPO-labeling, and the lumenal plasma membrane in the control displays the morphology and the membrane dimensions (12.5 nm in diameter) of untreated tissue, (Compare Fig. 6b with Fig. 1).

The experimental results shown in Fig. 6 were obtained after incubation of the tissue pieces in DAB for 20 minutes at room temperature. If the peroxidase reaction is allowed to proceed for a longer time interval (e.g., 1 hour), there is an increased accumulation of reaction product at the membrane surface of the tissue interacted with immune globulin as exemplified in Fig. 7a.

Control experiments, after staining in DAB for 1 hour, do not show for the major part increases in staining due to HRPO-labeling, as illustrated in Fig. 7b where the membrane thickness measures 12.5 nm. However, it has been possible to find sporadically along the membrane surface of controls regions which show non specific staining. The appearance of stain in controls correlated with prolonged exposure to DAB, and by decreasing the time of incubation in DAB this problem can be circumvented.

Cross species interaction was demonstrated when the immune globulin produced to sheep membrane plaque regions was interacted with pieces of intact bladder urothelium from both the rat and rabbit. Figs. 8a and 9a are electron micrographs showing deposition of stain in association with the outer leaflet of the lumenal surface plasma membrane of the rat and rabbit urothelium, respectively. The intensity of staining under experimental conditions contributes greatly to the measurable thickness of the interacted membranes. The heavily stained lumenal plasma membranes mea-

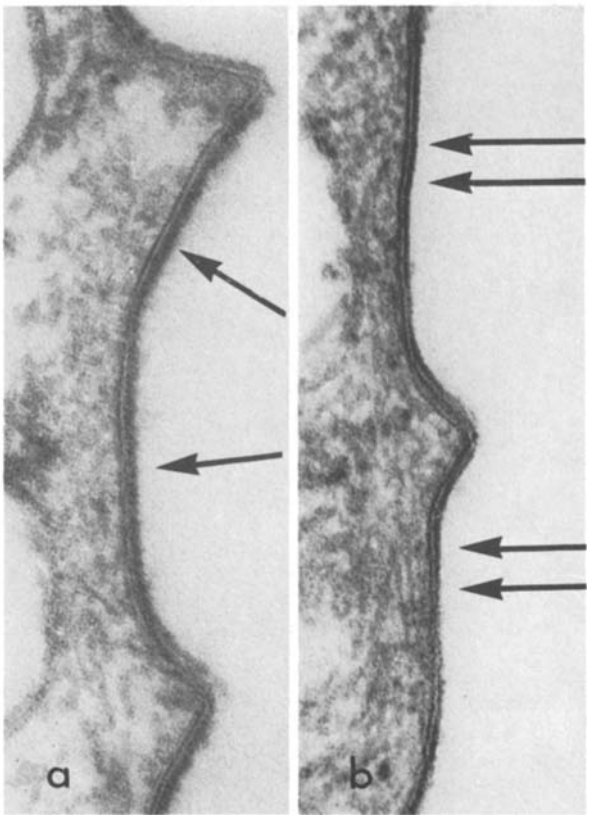

Fig. 8. Thin sections showing the free surface of the intact urothelium of the rat. (a) Intact tissue exposed to antibodies to plaque regions isolated from the lumenal plasma membranes of sheep. The indirect labelling technique employing horseradish peroxidase was used. The lumenal leaflet of the plaque regions (arrows) appears appreciably thicker than in control preparations (b, double arrows). (162,000X)

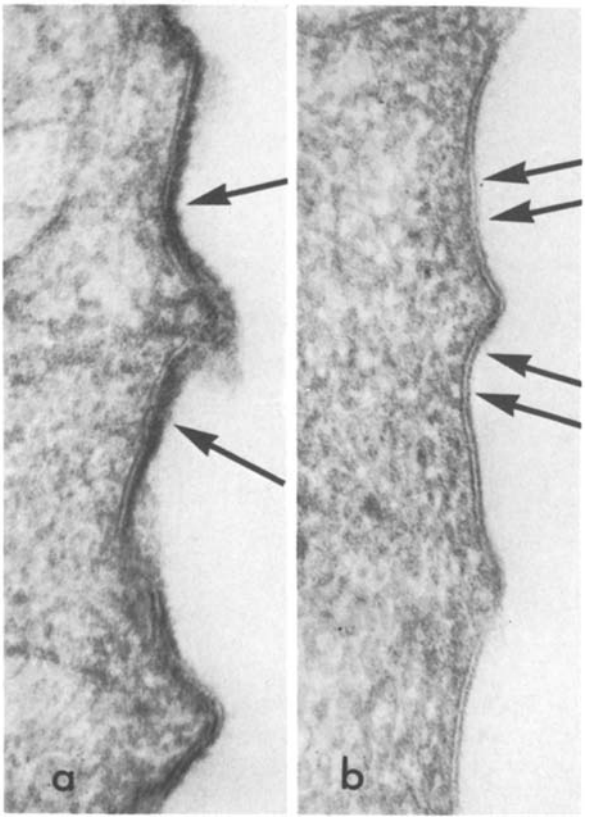

Fig. 9. Thin sections showing free surface of intact urothelium of the rabbit. (a) Intact tissue exposed to antibodies to plaque regions isolated from the lumenal plasma membranes of sheep. The indirect labelling technique employing horseradish peroxidase was used. The lumenal leaflet of the plaque regions (arrows) exhibits staining lacking in control preparation (b, double arrows). (162,000X)

sure approximately twice the thickness of membranes measured under control conditions (Figs. 8b and 9b) in which membranes were treated with serum from non-immunized rabbits. The lumenal plasma membranes of the controls appear similar to that of the untreated rat bladder.

To determine whether the preparation of sheep antibodies used in these experiments can interact with antigens common to plasma membranes of sheep in general, or whether they are specific for urothelium, both immune and control globulin were interacted with sheep erythrocyte ghosts. In neither case, (Figs. 10a and 10b) could staining, i. e. increased thickness of the membrane leaflets, be detected.

DISCUSSION

The study presented here was undertaken to ask whether an antigen from animal A which is available in large quantity can be used to localize specifically components in membranes of animal B which does not allow unlimited supply of tissue. Such cross-species interaction could then be used as a tool in exploring specific membrane alterations during developmental differentiation and carcinogenesis.

Plaque regions of sheep urothelial surface membranes were used as antigens in preliminary studies aimed at localizing components within the urothelial plasma membrane. Morphologically, the plaque regions are distinct, and biochemically, under dissociating conditions, they show particular enrichment in a protein subunit and in the glycolipid component (3). Antibodies produced to sheep plaque regions were shown immunocytochemically to interact specifically with the lumenal plasma membranes of sheep, rat and rabbit urothelia. Failure of antibodies raised to urothelial membrane components of sheep to interact with sheep erythrocyte ghosts indicates that the

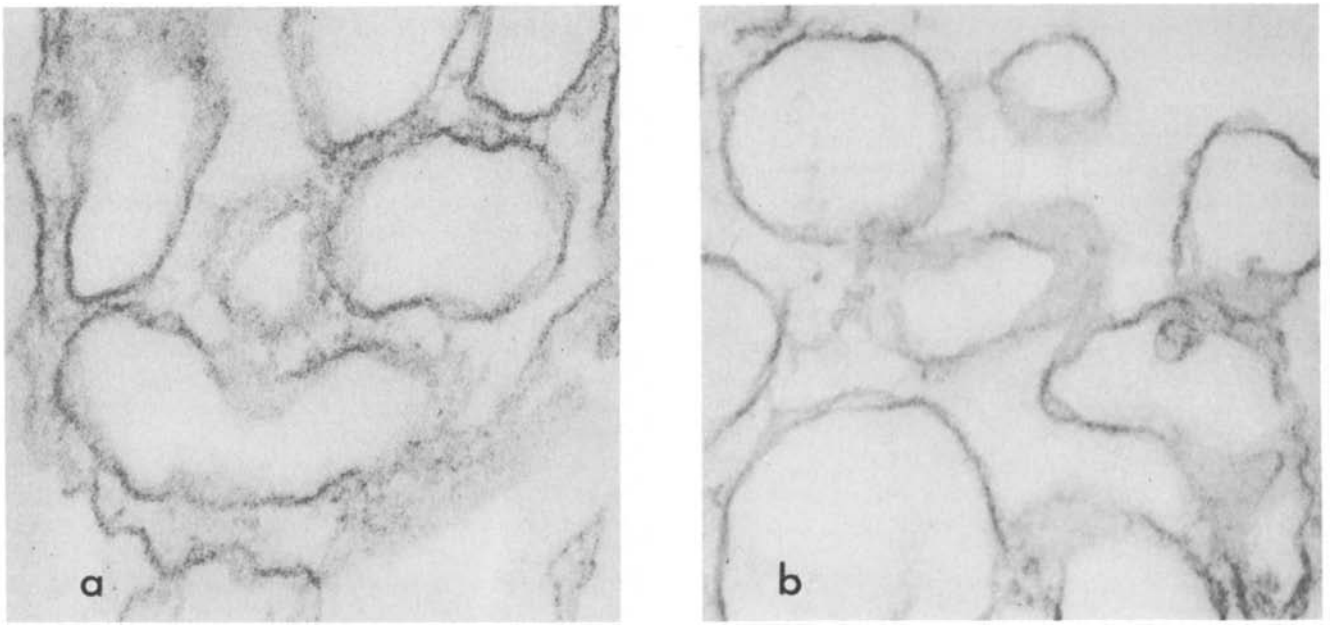

Fig. 10. Sheep erythrocyte ghosts (a) exposed to antibodies to plaque regions from lumenal membrane of sheep urothelium. Indirect labelling with horseradish peroxidase. No increase of unit membrane thickness can be observed in comparison with control (b) which was labelled after exposure to non-immune globulin. (125,000X)

antigens are probably organ specific and not found generally in sheep plasma membranes.

In many micrographs of immunocytochemically stained urothelia it appears that only the plaque regions -- and not the interplaque regions -- take on increased staining, i.e., interact with the immune globulin. Because the ratio of plaque to interplaque regions is approximately 4 to 1 (24) and staining of the plaque regions dominates the staining of the plasma membrane in general, it remains difficult to state unambiguously that there is no interaction at the interplaque regions. Indeed, it is possible that there is a slight immune reaction between antibodies and the interplaque regions of the lumenal plasma membrane. It is possible that some of the minor components of the particulate plaque regions are common to the non-particulate (24) interplaque regions.

Interaction between antibodies and the lumenal plasma membrane is visualized immunocytochemically as a build up of reaction product along the membrane surface. The accumulation of reaction product is dependent on the duration of the peroxidase reaction. When the peroxidase reaction is allowed to proceed for a long time (e.g., 1 hour) there is a heavy accumulation of reaction product at the membrane surface and the entire length of the surface membrane appears to be stained. Upon decreasing the duration of the peroxidase reaction not only the amount of stain but also the extent of membrane surface stained decreases. Furthermore, standardization of experimental conditions is necessary for urothelia from different species. The experimental conditions which are deemed ideal for immunocytochemical staining of the urothelial lumenal plasma membrane from sheep are not optimal for that from rat or other species. In our hands immunocytochemical staining of sheep urothelium could not tolerate long incubation times in DAB whereas no such restriction was observed for rat or rabbit urothelia.

Once specific components of the lumenal plasma membrane are purified to homogeneity, future investigations can be carried out to pinpoint their membrane localizations and describe their surface orientations. Because the lumenal plasma membranes in rat (16) and human (19) bladders undergo a de-differentiation under hyperplastic conditions wherein there is a complete loss of the specialized plaque particulate structure, an immunological test for the disappearance of a key membrane component is conceivable. With such immunocytochemical testing it may be possible to identify a change in the antigenic make-up of a membrane component and to correlate this change with a specific stage in the progression of carcinogenesis.

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