Different Concanavalin A Binding Patterns of Malignant and Nonmalignant Mouse Mammary Epithelia in Monolayer Culture

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ABSTRACT

Concanavalin A (Con A) binding of fixed malignant and nonmalignant C3H/He mouse mammary epithelia in monolayer cultures was examined with markers and labels of various sizes. In Con A-mediated hemadsorption, high epithelial cell densities resulted in the adsorbance of more guinea pig red blood cells per culture dish but fewer per cell. Malignant epithelia adsorbed twice as many red blood cells as did nonmalignant cells at the same cell density. Ferritin-Con A tagged the budding particles of mouse mammary tumor virus and other parts of the epithelial surface evenly. Hemadsorption occurred at the particle-free part of the apical membrane. The amount of 125I-Con A bound per dish, however, was not related to cell density but was almost identical for cells from the same source. Nonmalignant cells bound twice as much 125I-Con A as did malignant cells. The calculated association constants of Con A-binding sites on malignant cells, however, were twice as large as those on nonmalignant cells. This explains, at least partly, the greater number of red blood cells adsorbed by malignant cells. Con A-labeled Sepharose 4B beads were not useful for our purposes, and fluorescein isothiocyanate-Con A did not distinguish between malignant and nonmalignant epithelia.

INTRODUCTION

Malignant transformation of cells is usually accompanied by a number of in vitro phenotypic markers: morphological change; loss of contact inhibition; and a higher growth rate and saturation density. For mouse mammary gland epithelia, however, little is known of markers that distinguish between normal and malignant cells. The only report is that of Voyles and McGrath (24) who used Con A-mediated hemadsorption (7) to demonstrate that cultured malignant epithelia could bind Con A-coated RBC, whereas normal cells could not. Their results were not confirmed in a similar but slightly different system (1, 2). Con A-mediated hemadsorption has also been successfully used with human breast cancer cells to identify patients who had high chances of an early recurrence of cancer (6). In these reports, however, the results were only semiquantitative.

We report here, on a quantitative basis, different patterns in the Con A binding of malignant and nonmalignant mouse mammary epithelia. Guinea pig RBC, Sepharose 4B beads, ferritin, FITC, and 125I were used as markers or labels to trace the interaction between mammary epithelia and Con A. A part of this study has been reported in preliminary form (22).

MATERIALS AND METHODS

Cells and Culture. All experiments were performed with primary cultures of malignant and nonmalignant mammary gland epithelia derived from multiparous female C3H/He mice. Each experiment consisted of a pair of cultures from a single tumor and mammary glands of a single normal female, instead of a mixture of cells from several individuals. A peak of mammary tumor incidence among our female mice was observed at 8 months of age, while females from which midpregnant mammary epithelia were obtained were 6 to 8 months old.

Spontaneous mammary adenocarcinomas were minced and treated at 37°C for 15 min on a magnetic stirrer with 10 ml of PBS containing 0.05% trypsin and 0.02% EDTA, pH 7.2, per g of wet tissue. Large cell clumps were removed by filtration through 2 sheets of gauze. Nonmalignant epithelia were removed, minced, and treated at 37°C for 1 hr with 10 ml of 0.1% collagenase in Hanks’ balanced salt solution per g of wet tissue, after which they were processed as above. When gauze filtrate contained small cell aggregates, it was treated at 37°C for 30 min with 0.05% Pronase in Hanks’ balanced salt solution. The single-cell suspension obtained was washed with PBS, then seeded at various densities in 30-mm Falcon petri dishes, and fed 2 ml of Dulbecco’s modified minimal essential medium containing 100 units penicillin, 100 μg streptomycin, 10 μg insulin, and 2 μg dexamethasone per ml and supplemented with 10% fetal calf serum. The culture was kept at 37°C in 5% CO2-95% air, and the medium was changed twice a week. Confluent monolayers with scattered areas called domes or mounds (14) were formed 4 to 7 days later; these consisted mainly of epithelia and a few fibroblasts.

Con A-mediated Hemadsorption. Epithelial monolayers were fixed with 2% paraformaldehyde in PBS for 1 hr; the remaining free aldehyde residues were blocked with 0.1 M glycine for 1 hr. Monolayers were treated with Con A at various concentrations for 1 hr; they were then washed with PBS and overlaid with 1 ml of 1% guinea pig RBC suspension. The overlaid monolayers were kept for 1 hr at room temperature; then they were washed very carefully with PBS so as not to mechanically dissociate the adsorbed RBC. At this step, some specimens were used for conventional thin-section electron microscopy.

For the quantitative study, specifically adsorbed RBC were dissociated with 1 ml of 0.15 M a-methyl-D-mannoside for 20 min. This treatment was repeated twice more, and all the washes were combined. The number of RBC specifically dissociated was counted in a hemocytometer. Each experimental group included at least 2 untreated dishes which were used to determine the number of epithelial cells per dish.

Con A-conjugated Sepharose 4B Beads. CNBr-activated Sepharose 4B beads were conjugated with Con A according to the manufacturer’s instructions. Conjugated beads were applied to the fixed monolayers. Concentrations were adjusted so that the sedimented beads would cover the entire surface of a monolayer; after being kept for 1 hr, the nonadsorbed beads were washed away.

FITC-conjugated Con A. Con A was conjugated with FITC, and this conjugate was purified by the method of Kawamura (11). Mammary epithelia grown on a coverslip were fixed with cold acetone and stained with the conjugate at 37°C for 30 min.

Con A-conjugated Ferritin. Purified horse ferritin was conjugated with Con A in the presence of 0.1 M a-methyl-D-mannoside by the method of Maylie-Pfenninger and Jamieson (13). The ConA-ferritin was applied to fixed monolayers at a concentration of 1 mg ferritin per ml for 1 hr at...
Route temperature in the presence of 1% BSA. These monolayers were washed with PBS and were then postfixed with 1% OsO₄ and dehydrated with ethanol, after which step propylene oxide was used to detach the cell from the plastic. These detached cells were processed for electron microscopy by the conventional method. In the control experiments, monolayers were treated with unconjugated ferritin or with Con A-ferritin in the presence of 0.1 M α-methyl-D-mannoside.

125I-labeled Con A. Con A was labeled with 125I by the chloramine-T method (20). The specific activities were 1.2 to 2.1 × 10⁷ cpm/mg Con A. Immediately before application to the fixed monolayers, the 125I-Con A solution was mixed with BSA (final BSA concentration, 0.1%) and then centrifuged at 20,000 × g for 15 min to remove aggregates. Monolayers were covered with 0.4 ml of this labeled Con A at concentrations of 5, 15, 50, and 100 μg/ml and were shaken continuously for 1 hr at room temperature. The treated monolayers were washed 3 times with 1 ml of PBS and then 3 times with 1 ml of α-methyl-D-mannoside. These PBS and mannoside washes were combined separately and counted in a gamma counter. The former represented free 125I-ConA, the latter in the bound form. For comparison, 125I-Con A binding to live guinea pig RBC was examined. RBC (3 × 10⁶) at a final volume of 250 μl were exposed to 125I-Con A and then treated as above.

Reagents. Con A and horse ferritin were purchased from Sigma Chemical Co. (St. Louis, Mo.). FITC was obtained from BBL (Cockeysville, Md.). Sepharose 4B beads were purchased from Pharmacia Fine Chemicals (New Market, N. J.) and 125I was from the Radiocchemical Centre (Amersham, England).

RESULTS

Epithelial Nature of the Monolayers. As Fig. 1 shows, monolayers derived from malignant and nonmalignant mouse mammary glands consist predominantly of epithelial cells. Although different dissociation protocols were used for these cultures, specific effects on lectin binding of various enzymes used for dissociation are only transient (1, 3); hence, these monolayers are appropriate materials with which to compare Con A binding by malignant and nonmalignant mammary epithelia in culture.

Con A-mediated Hemadsorption. Other authors (1, 2, 7, 24) have carried out this test with live epithelial monolayers, but we found that live cells often were detached from the dish during the experimental procedure which made observations and quantitative analysis, in particular, impossible. We therefore used fixed monolayers throughout the study. This allowed us to examine the properties of Con A-binding sites on epithelia without the secondary biological modifications of the cell surface such as pseudopod formation, endocytosis, and clustering of Con A-binding sites. It was also found that precoating of the monolayers with Con A (1, 2) was a much better system than not coated with Con A. Both malignant and nonmalignant epithelia seemed to adsorb the beads to some extent (Fig. 3), but it was almost impossible to keep beads adsorbed when the mildest shearing force was exerted by careful washing. Thus, our results with Con A-Sepharose 4B beads were neither reproducible nor reliable, although other workers (9, 10) have reported their successful application. The reason for this difference is discussed later.

FITC-conjugated Con A. Malignant and nonmalignant mammary epithelia grown on coverslips showed a similar pattern of the bound FITC-Con A distribution (Fig. 4). The specific fluorescence was concentrated linearly along the border of adjacent takes place at the tips of microvilli (Fig. 2e) or on the flat surface of epithelia (Fig. 2f). Deformation of live RBC seemed to facilitate hemadsorption (Fig. 2e). None of the micrographs obtained convincingly showed that budding particles of MMTV play a major role in the adsorption of RBC.

In quantitative studies, it was immediately evident that the number of adsorbed RBC varied with the cell density of the epithelial monolayer. The higher the cell density, the more RBC are adsorbed per dish (Chart 1A). At the same time, a high cell density resulted in the adsorbance of fewer RBC by individual epithelial cells (Chart 1B). Therefore, in the comparison of malignant and nonmalignant cells, only cases in which the final cell densities were almost identical were considered. Under this condition, malignant epithelia always adsorbed more RBC per dish and per cell than did normal epithelia (Chart 2). At moderate Con A concentrations (30 to 100 μg/ml), twice as many RBC were adsorbed by malignant epithelia. The difference was less prominent, however, at a high (200 μg/ml) and low (10 μg/ml) concentrations of Con A.

Con A-Sepharose 4B Beads. To see whether differences between malignant and nonmalignant epithelia held for particulates other than RBC, we conjugated Sepharose 4B beads with Con A and applied them to epithelial monolayers that had been fixed but not coated with Con A. Both malignant and nonmalignant epithelia seemed to adsorb the beads to some extent (Fig. 3), but it was almost impossible to keep beads adsorbed when the mildest shearing force was exerted by careful washing. Thus, our results with Con A-Sepharose 4B beads were neither reproducible nor reliable, although other workers (9, 10) have reported their successful application. The reason for this difference is discussed later.

FITC-conjugated Con A. Malignant and nonmalignant mammary epithelia grown on coverslips showed a similar pattern of the bound FITC-Con A distribution (Fig. 4). The specific fluorescence was concentrated linearly along the border of adjacent...
cells and appeared in granular forms within the cytoplasm. These observations are similar to those reported (1, 2) and are not significant for distinguishing between malignant and nonmalignant epithelia.

**Con A-conjugated Ferritin.** Con A-ferritin bound diffusely and evenly to the apical surface of cultured malignant and nonmalignant epithelia (Fig. 5). Budding particles of MMTV also were tagged with Con A-ferritin, which is consistent with the report that MMTV binds Con A (4). These results are in good agreement with those of another report (8) and with our previous observations made in vivo (23) except that the basolateral surfaces of cultured epithelia were ferritin free because their accessibility to Con A-ferritin was hindered by the intact tight junction.

A preliminary examination showed that the number of bound ferritin molecules per µm² of the plasma membrane was 132 for malignant and 102 for nonmalignant epithelia. This difference could not be considered significant without further investigation because a constant section thickness is difficult to maintain and fractions of Con A-conjugated apoferritin in respective experimental conditions were not known. In control experiments in which unconjugated free ferritin or Con A-ferritin was applied in the presence of α-methyl-D-mannoside, no binding of ferritin molecules to the apical surface was found.

**125I-Con A Binding.** The binding of 125I-Con A by the apical surfaces of monolayers is shown in Chart 3A. Solid circles represent malignant (9.7 x 10⁸ cells/dish); open circles represent nonmalignant (9.5 x 10⁹ cells/dish) epithelia. Bars drawn across each circle show the ranges of 125I-Con A binding with cells of different cell densities: 5.7 x 10⁶; 17.5 x 10⁶; and 31.0 x 10⁶ malignant cells/dish and 17.5 x 10⁶ and 21.9 x 10⁶ nonmalignant cells/dish.

Although the amount of bound 125I-Con A per dish increased with increasing concentrations of 125I-Con A applied, the overall binding pattern was independent of cell density and almost identical for cells from the same source. Furthermore, nonmalignant epithelia always bound more 125I-Con A than did malignant ones. These results were unexpected because the number of adsorbed RBC was dependent on cell density (Chart 1), and more RBC were adsorbed by malignant epithelia than by nonmalignant epithelia (Chart 2).

The above data are plotted in Chart 3B according to the method of Steck and Wallach (21). Straight lines were drawn by the method of least squares. Association constants and the number of Con A-binding sites per cell (and per dish), as estimated from Chart 3B, are given in Table 1. The number of sites per dish are almost identical for cells from the same source, as indicated in Chart 3A. Sites on nonmalignant cells are approximately twice the number found on malignant cells per dish, and per cell when the cell density was the same. In contrast, association constants of binding sites on malignant epithelia are about 10 x 10⁶ M⁻¹, whereas those on nonmalignant epithelia are slightly less than 5 x 10⁶ M⁻¹. Similar figures were obtained repeatedly in experiments with other malignant and nonmalignant mammary epithelia. Considerably different and conflicting figures for Con A-binding sites have been reported for live mouse mammary tumor cells in ascites form (5, 12). Interestingly, the

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Table 1

*Table 1: Association constants (Kₐ) and the number of Con A-binding sites on the apical surface of cultured mammary epithelia and on the whole surface of guinea pig erythrocytes.*

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value of the association constant of the binding sites on live guinea pig RBC is intermediate between the values for malignant and nonmalignant epithelia; we think that this fact is a favorable factor for successful Con A-mediated hemadsorption.

**DISCUSSION**

The markers and labels used to examine the interaction of Con A and the apical surface of cultured mammary epithelia were Sepharose 4B beads (40 to 190 μm), guinea pig RBC (6 to 9 μm), ferritin (10 nm), FITC (M, 389.4), and 125I. Of these, only RBC and 125I showed different quantitative behavior with malignant and nonmalignant mammary epithelia.

For Con A-mediated hemadsorption, we used fixed epithelial monolayers instead of the usual live ones (1, 2, 7, 24). Fixation has been reported to disturb and even eliminate lectin-induced cell agglutination by inhibiting such biological modifications of the cell surface as pseudopod formation and the clustering of lectin-binding sites, although it does not affect lectin binding itself (for review, see Ref. 15). Our study, however, has shown that there are few inhibitory effects of fixation on Con A-mediated hemadsorption. This can be explained by observations from the elegant cell-to-cell binding system reported by Rutishauser and Sachs (17, 18). On examination of lectin-induced binding between free cells and cells immobilized to nylon fibers, they found that glutaraldehyde fixation of either group of cells which were coated with lectin enhanced cell-to-cell binding whereas fixation of both groups of cells resulted in no binding. Their findings also provide the reason why Con A-Sepharose 4B beads failed to be adsorbed on fixed monolayers (Fig. 3); the nonliving rigid nature of the beads makes them comparable to fixed cells. The failure of Con A-Sepharose beads to adsorb fixed free cells also has been reported (9).

There are likenesses and differences between the results obtained with Con A-mediated hemadsorption and those obtained with 125I-Con A binding. Application of high concentrations of Con A resulted in more RBC adsorbed per cell and per dish (Chart 1) and larger amounts of 125I-Con A bound (Chart 3). However, high epithelial cell densities were accompanied by more RBC adsorbed per dish (Chart 1), whereas a fairly constant amount of 125I-Con A was bound among cells from the same source at various cell densities (Chart 3A). This means that a factor(s) other than Con A-binding sites (such as the density of the microvilli) participates in hemadsorption even with fixed monolayers. Budding particles of MMTV which bind Con A, as reported here (Fig. 5) and elsewhere (8, 23), cannot be such a factor as illustrated in Fig. 2, e and f. It is interesting that epithelial cells that grow in monolayer maintain a constant number of Con A-binding sites per unit area of the apical surface regardless of the cell density. Finally, more RBC are adsorbed on malignant epithelia (Chart 2), but there are fewer Con A-binding sites on these cells than there are on nonmalignant cells (Table 1). This difference may be due, at least partly, to appearance of the novel class of Con A-binding sites with high association constants on malignant epithelia (Table 1). A similar phenomenon has been reported with human lymphocytes (16).

Hemadsorption is a system that depends on a delicate balance between 2 forces: the binding force between epithelia and RBC mediated by Con A; and the shearing force exerted on RBC by washing or other treatment (19). The greater the shearing force or mass of the label, the less label remains adsorbed on the monolayer. This is why epithelia constituting domes were often RBC free (Fig. 2b). The large mass of the Con A-Sepharose is also one reason why they fail to be adsorbed tightly. Labels of much smaller masses such as ferritin, FITC, and 125I easily survive the heavy shearing force of vigorous washing. RBC are intermediate between these 2 extremes; also differences between the association constants of Con A-binding sites on RBC and on malignant and nonmalignant epithelia are relatively small (Table 1). Therefore, RBC must be treated with great care so that their adsorption is not disturbed. The conflicting results reported with Con A-mediated hemadsorption (1, 2, 24) may be at least partly due to these factors. A new method devised to dissociate Con A molecules differentially from binding sites of low and high association constants would make observations based on Con A-mediated hemadsorption less ambiguous. The use of labels with smaller masses, such as latex particles that are several hundred nm in size, even though of rigid structure, might facilitate studies of interaction between lectins and cells, but our investigations thus far have not yet been successful.

**ACKNOWLEDGMENTS**

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**REFERENCES**

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Fig. 1. Monolayer culture of mouse mammary gland epithelia. a, malignant cells. × 150. b, nonmalignant cells. × 150. Both cultures consist predominantly of epithelial cells.

Fig. 2. Con A-mediated hemadsorption. a, malignant mammary epithelia. RBC are massively and diffusely adsorbed on the apical surface of epithelia. × 150. b, the same as a. Epithelia that make up the "dome" structure (arrow) are RBC free. × 150. c, nonmalignant epithelia. A few RBC are adsorbed in this area. × 150. d, the same as c. Many RBC are adsorbed in a mottled pattern in this area. × 150. e, electron micrograph of Con A-mediated hemadsorption with malignant epithelia. A deformed RBC (f) is adsorbed at the tip of several microvilli of an epithelial cell. × 16,740. f, the same as e. Hemadsorption is present at the flat surface free of microvilli. × 27,000.

Fig. 3. Adsorption of Con A-labeled Sepharose 4B beads on the surface of mammary epithelial monolayers. a, malignant epithelia. × 75. b, nonmalignant epithelia. × 75. In both, beads of various sizes seem to have been adsorbed, but it was almost impossible to keep them in position.

Fig. 4. Staining of mammary epithelial monolayers with FITC-Con A. a, malignant epithelia. × 480. b, nonmalignant epithelia. × 480. Staining pattern in both is the same; specific fluorescence is concentrated linearly along the border of adjacent cells and in granular forms in the cytoplasm.

Fig. 5. Tagging of the apical surface of cultured mammary epithelia with Con A-ferritin. a, malignant cell. × 52,500. b, nonmalignant cell. × 59,400. In both a and b, budding particles of MMTV, some of which are indicated by arrows, and the particle-free surface are evenly tagged with Con A-ferritin.
Con A Binding and Mouse Mammary Epithelial Cancer

1a

1b

2a

2b

2c

2d

2e

2f
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