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Identification of mouse mammary epithelial cells by immunofluorescence with rabbit and guinea pig antikeratin antisera

(myoepithelial cells/intermediate filaments/dysplasia/neoplasia)

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ABSTRACT **Few markers are available to identify the three types of mammary epithelial cells—ductal, alveolar, and myoepithelial—especially in pathological conditions and in cell cultures. We have used antisera to human keratins in immunofluorescence to facilitate the identification of the three mouse mammary epithelial cell types. In frozen tissue sections and primary cell cultures, a rabbit antikeratin antiserum specifically stained cytoplasmic filaments in all three types of epithelial cells. A guinea pig antiserum against the same keratin preparation, however, reacted preferentially with filaments in myoepithelial cells and readily detected this cell type in normal, dysplastic, and malignant mammary tissues and cell cultures. Neither antisera reacted with fibroblasts or any other mesenchymal cells. The combined use of the two antikeratin antisera thereby permits rapid surveys of tissue sections and cultures for the localization of not only all epithelial cells but also the subpopulation of myoepithelial cells. Moreover, when mammary cultures established from late-pregnant or lactating mice were stained simultaneously with guinea pig antikeratin and rabbit anticasein antisera, three populations of epithelial cells were mutually exclusive: those stained by anticasein antiserum, those stained by guinea pig antikeratin antiserum, and those stained by neither, consistent with properties of alveolar, myoepithelial, and ductal cells, respectively. These antisera thus offer a tool for studying different epithelial cell types during mammary development, tumorigenesis, and malignant progression.**

The fully developed mammary gland contains, in addition to stromal cells, three types of epithelial cells: ductal epithelial, alveolar (secretory) epithelial, and myoepithelial. A major problem in studies of the development, function, and pathogenesis of the mammary gland, both *in vivo* and in cell cultures, is the scarcity of markers for identifying the various cell types. This presents serious difficulties in the interpretation of experiments involving these heterogeneous cell populations. Markers that are needed include those that would discriminate between epithelial and stromal cells and those that would distinguish among the three types of epithelial cells. Ideally, these markers should be applicable to cells *in vivo* and in culture, valid regardless of the reproductive state of the animal, and useful in surveying large populations of cells rapidly and easily.

Recent studies have suggested that expression of intermediate filaments may facilitate classification of different cell types (for review, see ref. 1). Keratin filaments occur in almost all epithelial cells and can be used as a marker for these cells (for review, see refs. 1 and 2). Sun and Green have described the preparation of a rabbit antiserum against human keratins that reacts strongly with myoepithelial, alveolar, and ductal epithelial cells of human sweat gland but not with mesenchymal cells (3). Such an antiserum is thus useful for distinguishing epithelial cells from mesenchymal cells in tissue sections and in culture (4). However, to differentiate among the three epithelial cell types in sweat or mammary gland, additional markers are required. Relevant to this problem, Franke *et al.* (5) made an important observation when they found that guinea pig antisera to bovine keratins preferentially react with a filament system of myoepithelial cells in frozen sections of rodent mammary gland. Ductal and alveolar secretory cells showed minimal or negligible reactivity. This result suggests that certain antikeratin antisera may be uniquely suitable for the specific identification of myoepithelial cells.

In the present study, we demonstrate that, by the combined use of rabbit and guinea pig antisera against human keratin proteins and antisera to vimentin and casein, one can identify ductal, alveolar, and myoepithelial cells in both frozen sections and cell cultures from both normal and abnormal mouse mammary tissues.

MATERIALS AND METHODS

Mammary Tissues. All mammary tissues were obtained from BALB/cCrl female mice generously provided by D. Medina. Normal mammary glands were taken from virgin mice, pregnant mice at 16–18 days of gestation, and lactating animals. Preneoplastic mammary tissues were obtained from the D2 hyperplastic alveolar nodule (HAN) line (6) that was propagated by serial transplantation in the mammary fat pads of syngeneic mice. Mammary adenocarcinomas arose as primary neoplasms in mice bearing transplants of the D2 HAN line. Ductal carcinomas developed as primary tumors in animals fed 1 mg of 7,12-dimethylbenz[*a*]anthracene. Tissues were excised from the animals immediately before use.

Mammary Cell Cultures. The dissociation of mammary tissues with collagenase and the initiation of primary cultures with monodispersed mammary cells were performed as described (7). The cells were grown on 18-mm^2 glass coverslips in Dulbecco's modified Eagle's medium (GIBCO)/13% fetal calf serum (Reheis, Kankakee, IL)/15 mM Hepes containing gentamicin at 50 μg/ml (Schering).

Antisera. The preparation and characterization of rabbit antiserum against keratin isolated from human stratum corneum (callous) has been described (3). Guinea pig antikeratin antiserum was similarly prepared as follows. The total epidermal keratin fraction was isolated from human callous as described (3). An aliquot containing 1 mg of protein (in 8 M urea) was

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Abbreviation: HAN, hyperplastic alveolar nodule.

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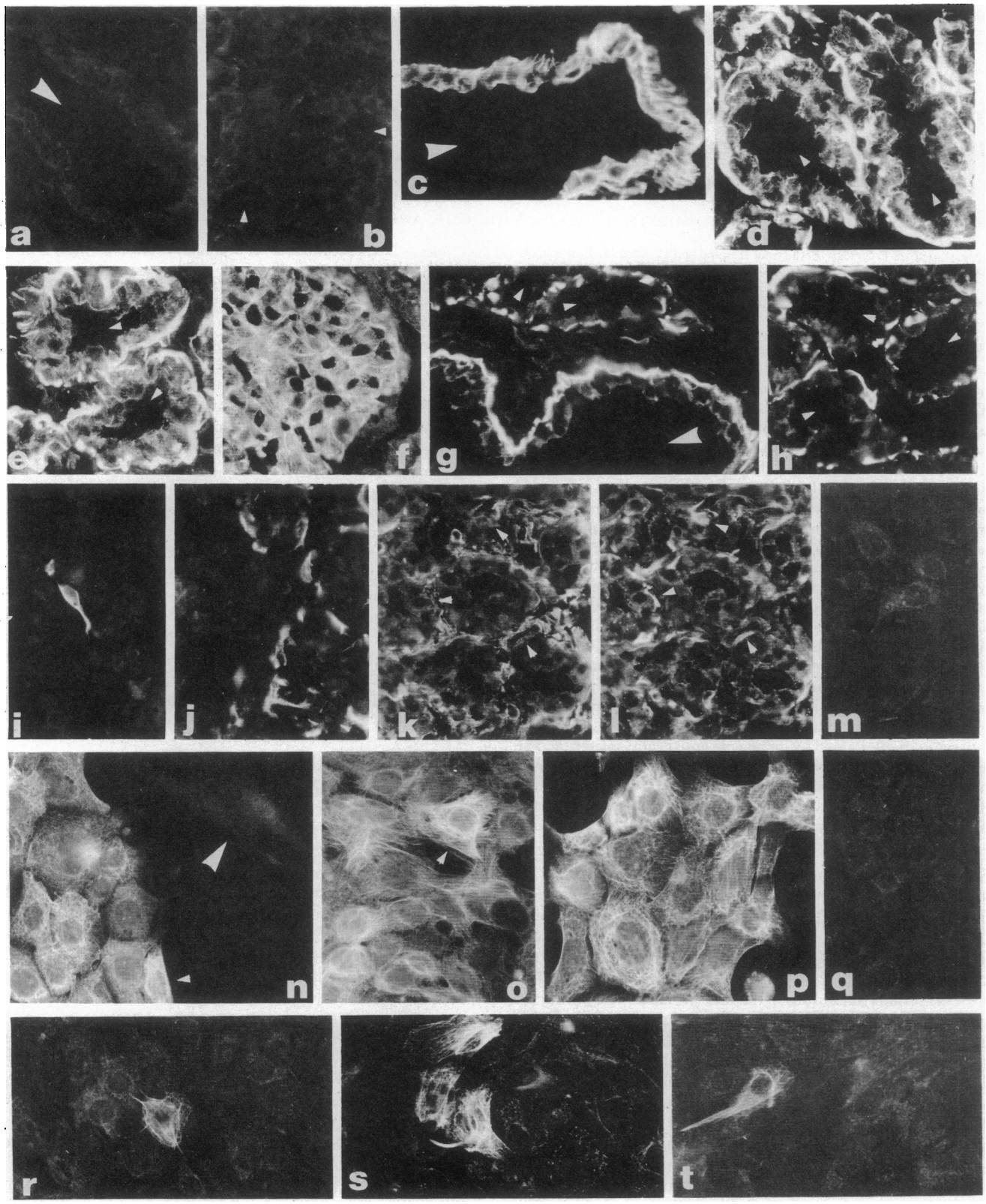

FIG. 1. Indirect immunofluorescent staining patterns of mouse mammary tissues and cell cultures by antisera to human epidermal keratins (*a–j*). Frozen sections (4 μm) were treated with rabbit or guinea pig antiserum and fluorescein-conjugated goat anti-rabbit or goat anti-guinea pig IgG. (*a* and *b*) Normal gland from an animal in late pregnancy incubated with rabbit (*a*) or guinea pig (*b*) preimmune serum. (*c–f*) Tissues stained with rabbit antikeratin antiserum. (*c*) A large duct in a virgin mouse; (*d*) alveoli in tissue from a pregnant animal. (*e*) Alveoli in preneoplastic HAN tissue. (*f*) Adenocarcinoma. (*g–j*) Tissues stained with guinea pig antikeratin antiserum. (*g*) Normal gland from a lactating mouse. (*h*) HAN tissue. (*i* and j) Adenocarcinoma. Note the brightly stained myoepithelial cells and the unstained tumor cells. Large arrows in *a–h* indicate lumina of ducts; small arrows indicate lumina of alveoli. (*k* and *l*) Double staining of a section of normal tissue from a pregnant animal with rabbit antivimentin (*k*) and guinea pig antikeratin antisera (*l*) in conjuction with rhodamine- and fluorescein-conjugated secondary antisera, respectively. Arrows indicate examples of myoepithelial cells that are unreactive with antivimentin but strongly stained by guinea pig antikeratin antiserum. (*m–t*) Pri-

dialyzed against 5 mM Tris·HCl, pH 7.4, to reconstitute the tonofilaments. This material was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into multiple body sites of guinea pigs, including the footpads. Four and 8 weeks later, the animals were boosted with 0.5 mg of protein treated as above but with Freund's incomplete adjuvant. Animals were bled 3 weeks after the last injection. Antikeratin antiserum from a single animal of each species was used in all studies reported here.

Rabbit antivimentin antiserum was a gift from R. O. Hynes. Its preparation, characterization, and specificity have been described (8). The properties of the rabbit anti-mouse casein antiserum, generously donated by J. M. Rosen, have been reported (9). Fluorescein- and rhodamine-conjugated goat anti-rabbit immunoglobulin and fluorescein-conjugated goat anti-guinea pig immunoglobulin were purchased from Cappel Laboratories (Cochranville, PA).

Indirect Immunofluorescence Studies. The preparation and single and double staining for indirect immunofluorescence study of frozen sections of mouse mammary tissues has been described (10). For fixation of cultured cells growing on glass coverslips, two procedures were compared. In the first, cells were fixed with 3% formaldehyde in Hanks' balanced salt solution for 30 min at room temperature and then immersed in acetone at −20°C for 5 min (11). In the second, cells were fixed in absolute methanol at −20°C for 5 min and then in acetone for 5 min. After fixation, the cells were stained for indirect immunofluorescence study as described in (10, 11). Both methods of fixation gave good results with the rabbit antikeratin antiserum, although the staining was more prominent after the methanol fixation. However, no staining was detected with the guinea pig antikeratin antiserum when formaldehyde-fixed cells were used. Therefore, the methanol/acetone procedure was used for all the experiments reported here.

RESULTS

Immunofluorescent Staining Patterns of Mammary Tissue Sections. Frozen sections of normal and abnormal mouse mammary tissues were stained for indirect immunofluorescence study with antikeratin antisera. Cells were identified by histological examination of adjacent sections stained with hematoxylin/eosin. Neither the rabbit nor the guinea pig preimmune serum produced any staining of mammary tissues (Fig. 1 *a* and *b*). Although the rabbit and guinea pig antisera were raised against the same preparation of human keratin proteins, they resulted in different immunofluorescent staining patterns. The rabbit antiserum stained a dense filament network in myoepithelial cells (Fig. 1 *c* and *d*). A sparser but clearly stained filament system also was shown in ductal and alveolar epithelial cells (Fig. 1 *c* and *d*), with the ductal cells usually exhibiting a brighter fluorescence. The reaction of the preneoplastic (HAN) tissue was similar. The rabbit antikeratin antiserum decorated a filamentous network in both the alveolar and myoepithelial cells (Fig. 1*e*). In primary adenocarcinomas and ductal carcinomas, all tumor cells exhibited filamentous cytoplasmic arrays (Fig. 1*f*).

The guinea pig antikeratin antiserum also recognized myoepithelial cells in sections of normal mammary tissue (Fig. 1*g*). However, in contrast to the rabbit antiserum, the guinea pig antiserum produced little or no staining in the majority of ductal and alveolar cells (Fig. 1*g*), even at high concentrations of antiserum (1:10). Only an occasional ductal or alveolar cell was found exhibiting one or two weakly fluorescent strands. Likewise, in sections of HAN tissue, reactivity was localized mainly in myoepithelial cells (Fig. 1*h*). Although myoepithelial cells were difficult to identify in routine sections of carcinomas stained with hematoxylin/eosin due to the loss of their normal location and configuration, intensely fluorescent myoepithelial-like cells were readily detected in the tumor sections with the guinea pig antiserum (Fig. 1 *i* and *j*).

Evidence for the distinction of myoepithelial cells and the surrounding mesenchymal cells was obtained from double-staining experiments. Vimentin is the major intermediate filament protein of mesenchymal cells such as fibroblasts and endothelial cells (8, 12–14). Sections of normal mammary gland from a mouse in late pregnancy therefore were treated simultaneously with a rabbit antiserum against vimentin and a rhodamine-conjugated secondary antibody (Fig. 1*k*) and with guinea pig antikeratin antiserum and a fluorescein-labeled secondary antibody (Fig. 1*l*). As shown in Fig. 1 *k* and *l*, cells stained by the two primary antisera were mutually exclusive. The antivimentin antiserum reacted with the filament system in fibroblasts, endothelial cells, and lipocytes, but not with the myoepithelial cells, which were strongly stained by the guinea pig antikeratin antiserum.

Immunofluorescent Staining Patterns of Primary Mammary Cell Cultures. Although the three types of epithelial cells in sections of normal mammary tissues can be identified on a histological basis, no reliable marker is currently available for identification of the three cell types in cultures. To determine whether the differential staining of mammary cells by the two antikeratin antisera was applicable to cells in dispersed cultures, the antisera were used to stain primary cultures established from normal preneoplastic and neoplastic mammary tissues. The results closely paralleled those in the mammary tissues. No staining occurred with rabbit preimmune serum (Fig. 1*m*), whereas the rabbit antikeratin antiserum decorated an elaborate filamentous array present in all epithelial-like cells derived from the three types of tissues (Fig. 1 *n–p*). Moreover, a minority population of epithelial cells exhibited very intense staining that distinguished them from the rest of the keratin-positive cells (Fig. 1 *n* and *o*). No reaction was observed in the few fibroblastic cells located in areas between the epithelial colonies (see arrow in Fig. 1*n*). Such keratin-negative, and thus nonepithelial, cells represented only 5–10% of the total cell population, thereby demonstrating that all three types of mammary cultures (normal, dysplastic, and tumor) were highly enriched in epithelial cells.

Cultures treated with guinea pig preimmune serum had a dull granular-appearing fluorescence (Fig. 1*q*). As might be expected in view of the data from the tissue sections, the guinea pig antikeratin antiserum stained only a small proportion of cells in the mammary cultures (Fig. 1 *r–t*). The rest of the cells in the cultures showed little or no fluorescence. These results suggest that the cells recognized by the guinea pig antikeratin antiserum are myoepithelial in nature. Although cultures from all three types of mammary tissues contain such cells, consistent with the *in vivo* situation, they were most numerous in normal and preneoplastic cultures.

Double-staining experiments showed that, in the primary mammary cultures, as in mammary tissues, there were no cells that stained with both the rabbit antivimentin and the guinea

mary mammary cell cultures. (*m*) Normal cells treated with rabbit preimmune serum. (*n–p*) Cultures stained by rabbit antikeratin antiserum. (*n*) Normal—note unstained fibroblast indicated by large arrow and the very bright cell indicated by small arrow; (*o*) HAN cells—arrow indicates especially bright cell; (*p*) tumor cells. (*q*) HAN cells treated with guinea pig preimmune serum. (*r–t*) Cultures stained by guinea pig antikeratin antiserum. (*r*) Normal cells; (*s*) HAN cells; (*t*) tumor cells. (*a*, *b*, *k*, and *i*, ×230; *c*, *f–j*, ×260; *d* and *e*, ×300; *m–t*, ×360.)

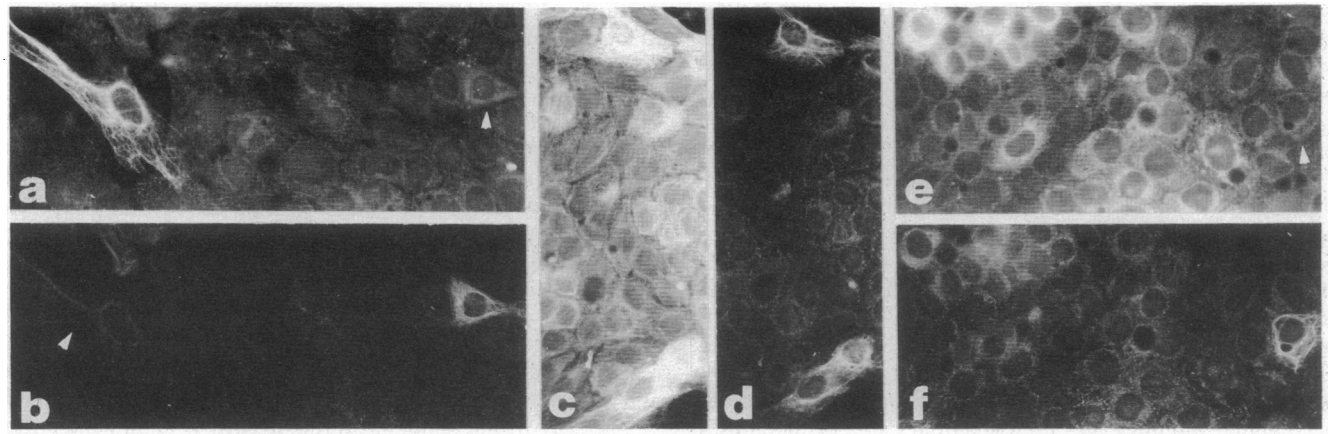

FIG. 2. Two-color indirect immunofluorescent staining patterns of primary cultures from normal mammary tissues of mice in late pregnancy. Cells were simultaneously treated with two primary antisera and then with two secondary antisera. In all examples, the secondary antisera were a rhodamine-conjugated goat anti-rabbit IgG and a fluorescein-conjugated goat anti-guinea pig IgG. (*a* and *b*) Culture treated simultaneously with rabbit antivimentin (*a*) and guinea pig antikeratin (*b*) antisera. The arrow in *a* indicates unstained myoepithelial cell that is stained in *b*, and the arrow in *b* indicates unstained fibroblastic cell that is stained in *a*. (*c* and *d*) Culture treated simultaneously with rabbit antikeratin (*c*) and guinea pig antikeratin (*d*) antisera. Note that the four brightest cells in *c* are also detected in *d*. (*e* and *f*) Culture treated simultaneously with rabbit anti-mouse casein (*e*) and guinea pig antikeratin (*f*) antisera. Arrow in *e* indicates an unreactive myoepithelial cell that is stained in *f*. Note the three cell populations present—those stained by the anticasein (*e*), by the guinea pig antikeratin (*f*), or by neither (*e* and *f*). These reactions are consistent with the properties of alveolar epithelial, myoepithelial, and ductal epithelial cells, respectively. (×320.)

pig antikeratin antisera (Fig. 2 *a* and *b*). This supports the hypothesis that the cells stained by the latter antiserum were myoepithelial cells and not mesenchymal derivatives.

Additional double-staining experiments showed that the cells that reacted most strongly with the rabbit antikeratin antiserum were identical to the ones recognized by the guinea pig antikeratin antiserum (Fig. 2 *c* and *d*). We have tested antisera from five more rabbits and two more guinea pigs to see whether the differential staining was due to species differences or variations among individual animals. The reactions of the additional antisera with mouse mammary cells were similar to those obtained with the antisera used in the present study (unpublished observations). Although the number of antibody-producing animals surveyed so far is relatively small, it seems that the differential staining of mammary cells by guinea pig and rabbit antikeratin antisera is due more to species differences between antibody-producing animals than to variations among individuals.

Finally, double staining of the cultures was conducted by using a combination of the guinea pig antikeratin antiserum and a rabbit antiserum to mouse casein (9) to distinguish among the three types of epithelial cells—alveolar, ductal, and myoepithelial. The synthesis of milk proteins, which is often used as a specific marker of differentiated function in the mammary gland, is performed mainly by alveolar cells (15, 16). These cells can be detected in cultures by immunocytochemistry with antisera to milk proteins such as casein (9, 17). Ductal epithelial cells do not produce milk (15, 16) and do not react with the guinea pig antikeratin antiserum *in vivo*. Therefore, in culture, they should possess neither of these properties. Myoepithelial cells demonstrable by the guinea pig antikeratin antiserum should be distinct from the alveolar and ductal cells. As shown in Fig. 2 *e* and *f*, when cultures prepared from mice in late pregnancy were double stained with a rabbit antiserum to mouse casein (9) and the guinea pig antikeratin antiserum, three cell populations were recognizable. Those that stained positively with the guinea pig antikeratin antiserum but negatively with anticasein antiserum were identified as myoepithelial cells (10–20% of the cells). As reported previously (9, 17), the presumptive alveolar cells identified by the anticasein antiserum usually occurred in clusters located in the central region of epithelial colonies (35–55%). A third group of cells in the epithelial islands was unreactive with both antisera, suggesting that they were primarily ductal epithelial cells (30–40% of the cell population). They usually occupied the cortical zone of the colony surrounding the cells that were positive for casein. This type of experiment thus permits a reasonable estimate of the types of epithelial cells present in cultures derived from mammary tissues.

DISCUSSION

We have shown that intermediate filaments are valuable markers for characterizing cell types in mammary tissues and cultures. Antisera to keratins not only provided the means for distinguishing between epithelial and mesenchymal cells but also facilitated specific identification of myoepithelial cells: a rabbit antikeratin antiserum recognized filaments in all three types of mammary epithelial cells whereas a guinea pig antikeratin antiserum, in agreement with (ref. 5, reacted preferentially with filaments in myoepithelial cells. Of particular interest was the finding that the staining properties of the mammary cells *in vivo* were apparently retained under *in vitro* conditions in primary cultures.

As reported previously (9, 17), alveolar epithelial cells producing milk components were detectable by their reaction with an anticasein antiserum. The main disadvantage of using a milk protein such as casein as a marker for alveolar cells is that not all alveolar cells may be engaged in milk production all the time, even during pregnancy and lactation. Moreover, there may be some ductal cells, particularly in the terminal portion of the ducts, that also synthesize milk proteins (18). These cells would obviously be misclassified if a milk protein was used as a criterion for identifying cell types in mammary cultures. Thus, when this type of marker, which is dependent on the reproductive state of the animal, is used, there is probably a certain amount of overlap in the estimated numbers of ductal and alveolar cells.

The basis for the differential staining obtained with the rabbit and guinea pig antisera raised against the same human keratin immunogen has not yet been determined. Several factors may have contributed to the differences observed: (*i*) qualitative or

quantitative differences in the keratin content of the myoepithelial cells versus that in the other mammary epithelial cells, (*ii*) differences in keratin composition between species (mouse versus human) and between tissues (mammary gland versus epidermis), and (*iii*) recognition of different antigenic sites in the keratin polypeptides by the immune systems of the rabbit and guinea pig. Keratins from several species have been studied and shown to consist of multiple numbers of subunit polypeptides (3, 19–21). Differences in keratin composition are known to occur during the course of differentiation of epidermal cells (22–24), among different epidermal (21, 25) and stratified squamous (26) epithelial cells of the same organism, and between fetal and adult tissues (27). Therefore, it would not be surprising if variations in keratins exist among the three types of mammary epithelial cells.

Differences also exist between keratins of the same tissue in different species (21). Preliminary results have shown that all three epithelial cell types in human mammary tissues react with the guinea pig antiserum (unpublished observations). A similar finding was made by Franke *et al.* (5), who showed that guinea pig antisera against bovine keratins reacted primarily with myoepithelial cells in rat mammary gland but with all epithelial cells in cow udder. Biochemical analysis of the intermediate filaments in the various mammary cell types and monoclonal antibodies against the different keratin polypeptides should help in elucidating the basis for the different staining properties of the cells. An especially intriguing question that remains to be answered is whether or not guinea pig antisera raised against keratins from a species such as mouse or rat might produce differential reactivity with various human mammary epithelial cells.

Differences in the immune response of different species to the same immunogen are well documented (28). We found that the antigenic sites detected by the two antikeratin antisera differed in their sensitivities to formaldehyde fixation and were therefore not identical. Although formaldehyde fixation of the cultured mammary cells only slightly affected staining by the rabbit antikeratin antiserum, staining by the guinea pig antiserum was abolished by such treatment. The same reactions were observed when the antisera were used to stain cultured epidermal and other epithelial cells (unpublished data), indicating that this effect is not limited to mammary cells.

Similar to other epithelial cell types (4, 11, 14), dysplastic and neoplastic alterations of the mammary cells did not noticeably affect expression of the keratin filaments demonstrable by immunofluorescence patterns. These results agree with recent reports that the presence of keratin filaments may help to determine whether a particular tumor is derived from epithelial or mesenchymal cells (29, 30). At the same time, these findings do not eliminate the possibility that differences in intermediate filaments may exist between normal and neoplastic mammary epithelial cells that have not been detected in the immunofluorescence patterns. In fact, analysis of intermediate filament proteins extracted from normal and neoplastic mammary cells by two-dimensional gel electrophoresis has suggested that differences are present (31).

In summary, we have shown that different antikeratin antisera can be used to distinguish different types of epithelial cells. Antisera to intermediate filaments thus provide a tool for studying mammary epithelial cells both in tissue sections and in culture during normal development, tumorigenesis, and malignant progression.

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