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USE OF POLYMER CASTS OR METAL PARTICLE INFUSION OF DUCTS TO STUDY ANTIGEN UPTAKE IN THE GUINEA PIG MAMMARY GLAND

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Abstract

Microcorrosion casts were made of the duct system of guinea pig mammary glands by intramammary infusion of Mercox® polyester resin following involution of the glands after the first lactation. The acinar configuration of the involuted gland was apparent on examination of the casts by scanning electron microscopy (SEM). Surface features, which were readily identified as those of imprints of ductal epithelium, were visible at higher magnifications. The morphology of these casts corresponded to the patterns observed by SEM of ethanol cryofractured specimens of mammary tissue.

Cryofractured specimens of guinea pig mammary glands were also examined by SEM following intramammary infusion of tantalum. Tantalum particles were observed within the lumina of many ducts. Large phagocytic cells within the lumina were shown to contain tantalum by using back scatter imaging in conjunction with secondary imaging.

KEY WORDS: Antigen, duct morphology, cryofracture, guinea pig, mammary gland, mammary immunity, mastitis, microcorrosion cast, scanning electron microscopy, tantalum.

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Introduction

Intramammary infusion of antigen has been shown to result in greater specific antibody levels in milk than does intramuscular or subcutaneous administration, including interstitial intramammary injection (Bourne et al., 1975; Chang et al., 1981; Watson, 1982). Since the mechanisms for this phenomenon are largely unknown, distribution and transport of antigen following intramammary infusion have recently been studied (Schenkman et al., 1985). It was found that after infusion of colloidal carbon or killed Staphylococcus aureus organisms into the lactating, involuting, or dry mammary glands of guinea pigs, the particles accumulate in the superficial inguinal lymph nodes. Transport of carbon to the nodes took more than a day in the involuting glands and several hours in the lactating glands. In the dry glands, the ductal epithelium took up carbon quickly, and it was transported to the superficial inguinal lymph nodes within minutes following infusion. It is not clear whether phagocytosis by the ductal epithelial cells in dry glands is essential for carbon to reach the draining lymph nodes, or alternatively, whether the ductal epithelium is not intact, allowing direct drainage of carbon to the lymphatics. In order to test these possibilities, microcorrosion casting techniques have been developed to examine the integrity of the duct system during the dry period. These methods were derived from investigations in which Mercox® polymer was used for making detailed microcorrosion casts of blood vessels for investigation of the morphology of the endothelial surface structure and of the three dimensional structure of the vasculature (Hodde et al., 1977). We report here on the correlation of scanning electron micrographs of casts of mammary gland ducts with micrographs of mammary gland tissue, which has been prepared using ethanol cryofracture in evaluating the completeness of the barrier formed by the ductal epithelium. The distribution of inert metal particles infused into the mammary gland has also been studied by SEM.

Materials and Methods

Animal Procedures

Fifteen six month old primiparous Hartley strain guinea pigs were used in these experiments ten days following weaning of young.

Uninfused guinea pigs were sacrificed by intraperitoneal injection of Euthanasia Solution (Veterinary Laboratories, Inc., Lenexa, Kansas) prior to preparation of ethanol cryofracture specimens.

Intramammary tantalum infusions consisting of 0.1 ml suspension (approximately 50% tantalum, volume/volume), were administered from a 1.0 ml syringe, via a blunted 1 cm, 30 gauge needle, through the teat meatus, into the glands of unanesthetized animals. One hour following infusion, they were sacrificed, as described above, for preparation of cryofracture specimens.

For preparation of ductal casts, guinea pigs were anesthetized by intramuscular injection of ketamine (22 mg/kg) and xylazine (1 mg/animal). A one cm blunted 27 gauge needle was passed through the teat meatus into the lumen of each mammary gland, and secured with a number 1 silk ligature, placed around the teat to prevent leakage of Mercox. Each gland was infused gently, via a 1 ml glass syringe attached to the preplaced needle, with 0.10, 0.25 or 0.50 ml of Mercox. After 10 minutes the animals were sacrificed as described above.

Preparation of ductal casts

Five ml of Mercox® polymer (Ladd Research Industries, Inc., Burlington, Vermont) were prepared as described for making vascular casts (Hodde et al., 1977). Catalyst (Mercox MA, 0.2 gm) was added to methyl methacrylate monomer (1.3 ml). Resin (Mercox CL-2B, 3.7 ml) was added and rapidly mixed, approximately one minute prior to infusion.

Following infusion and euthanasia, animals were placed in a water bath at 40-50°C for 20 minutes. The mammary glands were excised, placed in jars, and the tissue was removed by alternating daily changes of 15% sodium hydroxide and 5% sodium dodecyl sulfate solutions. The casts were washed in three changes of distilled water and transferred to absolute ethanol (two changes) prior to drying by the critical point method using molecular sieve dried CO_2 as the transitional fluid. The casts were then sputter coated with 20-30 nm of gold and examined on a JEOL JSM 35C scanning electron microscope at 15-25 kV accelerating voltage.

Preparation of ethanol cryofractured mammary gland specimens

Following sacrifice, mammary glands were removed and specimens no larger than 0.75 cm^3 were fixed in 2.5% glutaraldehyde in phosphate buffered saline (PBS), 0.1M, pH 7.2. They were dehydrated using a graded series of alcohol to absolute ethanol, and fractured in a liquid nitrogen bath using a precooled single edge razor blade. The specimens were dried, coated and scanned as described above.

Preparation of tantalum

Tantalum powder was suspended in PBS (0.01M, pH 7.2) and allowed to settle. Tantalum was then gently aspirated into a 1.0 ml syringe from the upper layer of the sediment. Particles were approximately 0.5 -1.0 μm in diameter.

Results

Cryofractured mammary specimens

Examination of cryofractured tissue specimens of involuted mammary gland revealed concavities which were interpreted to be the terminal saccules of the ductal system. The surface morphology of many of these saccules included pit-like structures at the base of the concavity and deeper, pore-like structures in the neck region (Fig. 1).

Ductal casts

Infusions of 0.10 ml Mercox resulted in casts of the major ducts but only a few of the smaller ducts (Fig. 2), whereas infusions of 0.25 ml filled most of the smaller ducts and terminal saccules as well (Fig. 3). Infusions of 0.50 ml also filled the ducts and saccules, and additional Mercox, which did not correspond to the ductal system, was present between the ductal structures and appeared to emanate from the neck regions of the terminal saccules (Fig. 4).

Imprints which were interpreted as those of epithelial cell margins were visible on casts of the saccules. Knob-like structures, corresponding to the pit-like structures in the cryofractured tissue specimens, were also present on saccular portions of casts (Fig. 5). Not all casts contained these knobs, and there was considerable variation in their distribution and density on the casts, consistent with the distribution of the corresponding pits in the cryofractured specimens.

Tantalum-infused cryofractured specimens

Examination of the tantalum infused mammary glands revealed particles enshrouded in membrane in the lumina of many ducts (Fig. 6a). These structures were interpreted to be tantalum-engorged macrophages. Back-scatter imaging confirmed the high density composition of the particles (Fig. 6b).

Discussion

Vascular casting techniques have generally involved using a perfusion system with two portals to allow flushing of blood, fixative, and excess polyester resin (Hodde et al., 1977). In this study, casting of the unfixed, involuted mammary gland duct system was done via the teat meatus without benefit of a second portal and without flushing of endogenous intraluminal material. It was necessary to establish appropriate volumes of resin for infusion into the glands in order to approximate the volume of the duct system.

Despite these technical limitations, the acinar morphology of the casts corresponded to that of a ductal system. At higher magnification,

visualization of imprints of surface structure of the ductal epithelium was possible because of the relative paucity of intraluminal material in the nonlactating ducts. Features of the cast surface structure corresponded to those of the ethanol cryofractured tissue specimens. The knob-like protrusions (Fig. 5) on casts of terminal saccules corresponded to the pit-like depressions within the saccular concavities of the cryofractured tissue specimens (Fig. 1). This is of particular interest because "craters" in scanning micrographs of lactating mammary duct and alveolar specimens have been ascribed by others to extraction of lipid during preparation of tissue specimens (Nemanic & Pitelka, 1971). An advantage of the corrosion casting technique, which was used to study ducts of nonlactating glands, was that no prior tissue preparation was done, and therefore it is probable that the pit-like structures in the corresponding cryofractured tissue specimens were not artifactual.

The extruded cast material (Fig. 4) appears to emanate from the pore-like structures in the neck region of intact saccules (Fig. 1), and this may be the basis for rapid transport of small particles from the mammary gland lumen to the lymph nodes in nonlactating guinea pigs. The extruded material is prominent in casts of glands infused with 0.50 ml, but not with 0.25 ml. The volume of the duct system is probably approximately 0.25 ml. In the previous study (Schenkman et al., 1985), carbon or staphylococci were infused in volumes of 0.1 ml, and it is clear, from the casts produced with 0.10 ml of Mercox, that this volume does not overload the system.

Although colloidal carbon particles were rapidly taken up by ductal epithelial cells (Schenkman & Berman, unpublished observation), the infused tantalum remained predominantly within the lumina of the ducts, and much of it was within intraluminal macrophages. It is possible that tantalum is less liable to phagocytosis by the ductal epithelium because of its larger particle size. This hypothesis is currently being tested in experiments in which gold beads of different sizes are being infused into guinea pig mammary glands, and ethanol cryofractured tissue specimens examined using secondary and back-scatter imaging techniques on the scanning electron microscope.

Ductal casting and heavy metal infusions have proven to be useful techniques in studying the functional anatomy of the dry guinea pig mammary gland, particularly with respect to particulate antigen uptake in the gland.

Acknowledgements

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Discussion with Reviewers

T.W. Keenan: How do you propose that particles such as carbon or killed cells reach lymph nodes when presented by intramammary infusion? In involuting or dry glands one can envision that the ductal epithelium is not intact. In lactating glands ductal epithelia should be intact; otherwise one would expect to see "natural" particles (casein micelles, lipid globules) in lymph nodes. To my knowledge such has not been observed. Is it possible that infused materials disrupt epithelial integrity?

Authors: The rapid transport of carbon during the dry period, as reported in the previous study (Schenkman et al., 1985), in conjunction with the findings in this study, lead us to hypothesize that foreign materials may reach the lymph nodes by passing through epithelial pores of dry glands into the extracellular space, and from there, into the lymphatics. We have not investigated possible mechanisms of transfer of foreign materials during the lactating state. However, the phagocytic capabilities of ductal epithelial cells for carbon particles in the dry state (see text), and of epithelial cells for "natural" particles in the lactating gland (Brooker, B. (1983) Pseudopod formation and phagocytosis of milk components by epithelial cells of the bovine mammary gland, Cell and Tissue Research, 229, 639+), as well as the association of foreign particles with lactating epithelium (Schenkman et

al., 1985) makes the hypothesis of epithelial phagocytosis of foreign materials during lactation an attractive one. "Natural" particles might not be intact by the time their components reach the lymphatic system, with the possible exception of those in the milk hypersensitivity syndrome.

The inocuous nature of the carbon and tantalum, as well as the small volumes infused, lead us to believe that infusion of these materials is unlikely to have damaged the epithelium. There may have been epithelial damage following infusion of killed staphylococci, due to the toxic components present and possibly due to enzyme release from inflammatory cells.

S.C. Nickerson: The phenomenon of the pore-like structures in the neck region of saccules is interesting. Have the authors observed such pore-like structures in thin sections? It would be advantageous in subsequent studies to confirm this theory with transmission electron micrographs.
Authors: Studies are in progress to look for pore-like structures using transmission electron microscopy and high voltage electron microscopy. We decided to present our findings at this stage because the casting technique was effective, and microcorrosion casting of other nonvascular anatomic structures may be useful for other investigators.

E. Spring-Mills: Do the authors have any ideas about the biological importance of the pore-regions in normal animals?
Authors: Besides the possibility of particulate antigen uptake, which we mentioned, it is conceivable that residual milk constituents ("natural" particles) in the lumen of dry glands may be transported out of the gland through these structures. Questions still remain about the fate of milk and milk constituents following lactation.

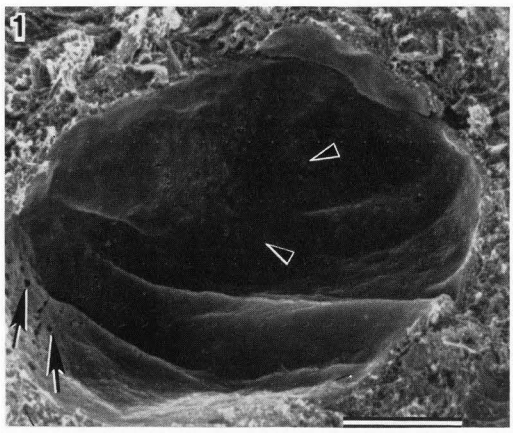
E. Spring-Mills: During which stage of the estrous cycle were the tissues obtained?
Authors: Unfortunately we do not have this information. Particularly in light of the report by Loeb & Hesselberg (Loeb L, & Hesselberg, C (1917) The cyclic changes in the mammary gland under normal and pathological conditions. J. E. M. 25 285+) in which mammary epithelial changes were characterized through the estrous cycle of lactating guinea pigs, it may be interesting to look for possible differences depending on the estrous cycle in the nonlactating glands.

S.C. Nickerson: Do the authors have convincing evidence that the structures in the ductal lumina as in Fig. 6 are engorged macrophages?
Authors: The metal dense particles are covered by membranes which contain ruffles and ridges characteristic of macrophages, and are of an appropriate size and shape. By light microscopy, the nuclei of some of these cells can be observed. Cells of this nature, in nonlactating glands have been reported to be macrophages. Although morphologically these cells resemble macrophages, and they are actively phagocytic, there is no compelling evidence to classify them as such.

B. Brooker: Might the extrusion of cast material from the neck region of intact saccules be due to overloading of the mammary glands with excessive perfusion fluid?
Authors: It is unlikely. First, as we showed in the uninfused cryofractured specimens, pore-like structures were observed in the neck regions. Second, the extruded material consistently appears to emanate from the neck of the saccules. If the epithelium has a uniform strength, pressure damage and extrusion would be anticipated in the depth of the saccules. This was not observed. Third, colloidal carbon particles, when infused in volumes shown to be considerably smaller than the volume of the duct system, reached the lymph nodes within minutes following infusion, indicative of an opening between the lumen and lymphatic system.

S.C. Nickerson: It does appear unlikely considering the method for cast preparation and tissue removal, but is there a possibility that the additional cast material in Fig. 4 (arrows) could be undigested epithelia?
Authors: Incompletely cleaned casts tend to have residual stromal material present, which is rather fibrous in nature. The material indicated by the arrows in Fig. 4 has a much more smooth consistency.

Figure 1. Terminal saccule of involuted mammary gland. Pit-like structures are visible (arrowheads) on the epithelial surface of this concavity. Larger and deeper pore-like structures (arrows) are present in the neck region. Ethanol cryofractured specimen. Bar=10 µm.
Figure 2. Cast of ducts of involuted mammary gland following infusion of 0.10 ml Mercox®. Larger ducts contain polymer, but only few of the smaller ducts (arrowheads) are filled. Bar=1mm.
Figure 3. Cast of ducts of involuted mammary gland following infusion of 0.25 ml Mercox®, with acinar morphology apparent and masking the underlying large ducts. Bar=100 µm.
Figure 4. Cast of small duct and its terminal saccules (arrowheads) of involuting mammary gland, following infusion of 0.50 ml Mercox®. Additional cast material present, which does not correspond to the ductal system, appears to emanate from neck regions of the terminal saccules (arrows). Bar=10 µm.
Figure 5. Polymer cast of terminal saccule. Imprints of epithelial cell borders (arrows) are present. Knob-like structures (arrowheads), present on some of the epithelial cell imprints, correspond to the pit-like structures (Fig. 1) of the cryofractured tissue specimens. Bar=10 µm.
Figure 6. (a) Mammary duct with engorged macrophage present in lumen following infusion of tantalum. Epithelial cell nuclei (n), cell borders (arrowheads) and macrophage membrane (arrow) are present. (b) Metal density of tantalum particles is apparent in this back-scatter image of same field. Bars=1 µm.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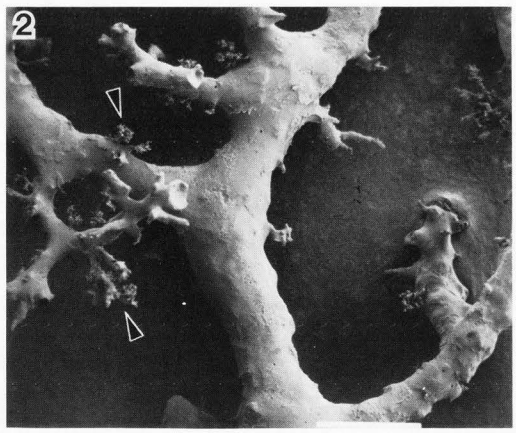
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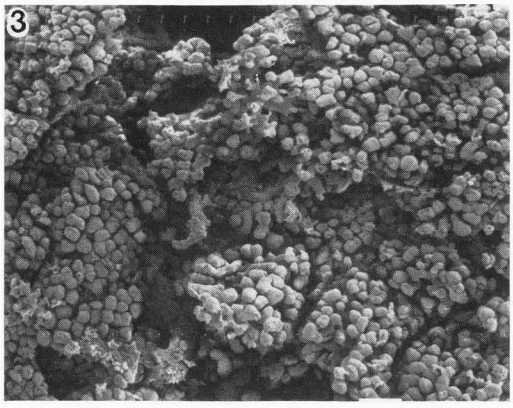
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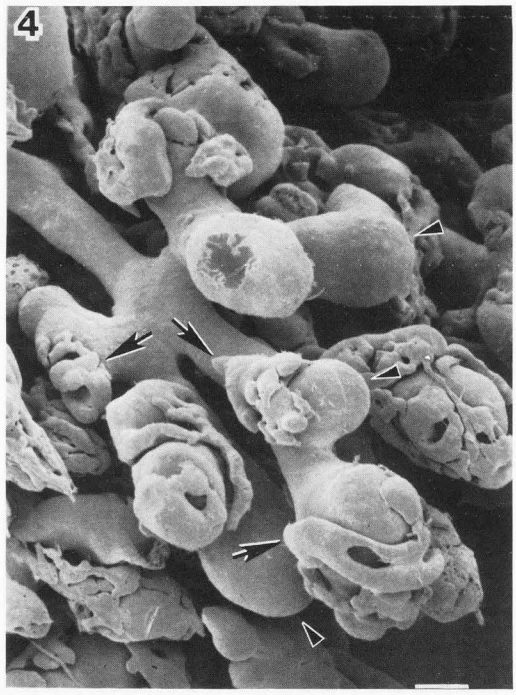
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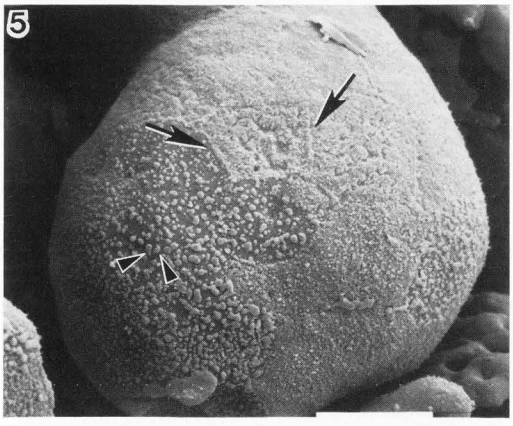
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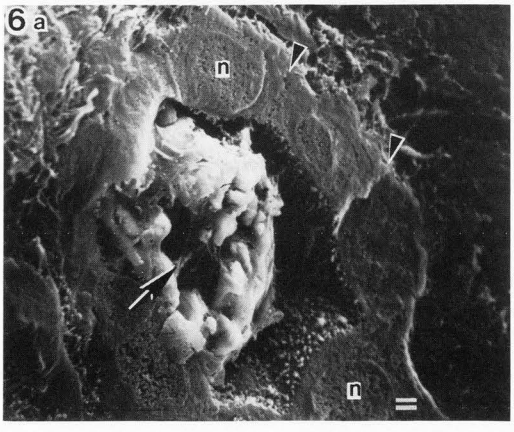
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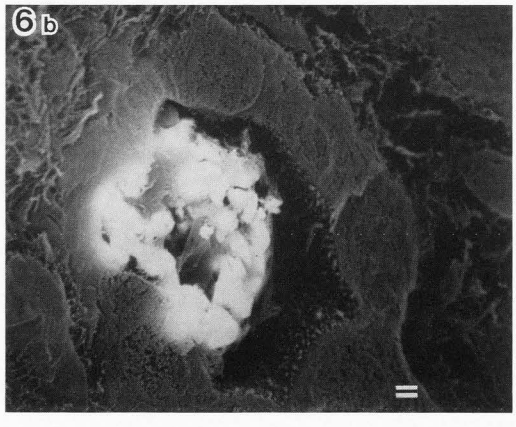
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S.C. Nickerson: The "craters" in the study of Nemanic & Pitelka (1971) would be much larger and far less numerous than the pit-like depressions of cryofractured tissue specimens, thus the two may not be analogous.
Authors: We agree.

S.C. Nickerson: Plicae are apparent in the cryofractured tissue. They do not appear on the photographs of the casts.
Authors: We have observed indentations on casts of some saccules which correspond to plicae. Conversely, not all of the saccules in the cryofractured tissue contain plicae.