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Effect of stage of lactation on transport of colloidal carbon or *Staphylococcus aureus* from the mammary gland lumen to lymph nodes in guinea pigs

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SUMMARY. Guinea pig mammary glands which were either lactating, involuting or dry were infused with colloidal carbon or killed staphylococci. At different time intervals following infusion, animals were killed and the superficial inguinal lymph nodes examined for the presence of carbon. Sides which had nodes with visible carbon were designated 'positive'. The time intervals from infusion to positive for the three groups were compared using logistic regression. The times required for 50 % of the sides to be positive were estimated to be ~4 h for lactating glands, 32 h for those in involution, and 13 min for dry glands. Histological differences in distribution of carbon in the mammary tissue suggest that differences in transit time may have been due to different mechanisms of transport through the glands in the three different physiological states. The distribution of bacteria was similar to that of the carbon in the corresponding tissues.

The route of administration of antigen has been shown to be important in the development of a local immune response in mammary glands of various species. Chang *et al.* (1981) found greater antibody levels and more antibody-producing cells in colostrum and milk from cows which were immunized by intramammary infusion of antigen than in secretions from animals immunized by either intrajejunal or subcutaneous injections near prescapular or external inguinal lymph nodes. Similarly, Bourne *et al* (1975) observed that the antibody response was greater in milk of sows following intramammary infusion of antigen than in animals immunized by the intramuscular route. Watson (1982) found that infusion of antigen into the lumen of mammary glands of ewes stimulated greater antibody levels than did interstitial intramammary administration. These findings are all in general accord with concepts of a secretory immune system reviewed in McGhee & Mestecky (1983). The mechanisms by which localization of antigen affects these immune responses are still largely unknown.

Infusion of foreign material, or even autologous milk, into the lactating udder is frequently followed by a leucocytosis in the milk which may be indicative of an inflammatory response (Derbyshire & Berman, 1968). The leucocytosis induced by intramammary infusion of antigens in primed animals becomes evident earlier, and reaches greater levels than that in unprimed animals, and has been interpreted as immune-mediated inflammation (Targowski & Berman, 1975; De Cueninck, 1979).

Such local inflammatory responses apparently can be generated in the mammary gland without prior destruction of the glandular epithelium. For example, Brooker *et al.* (1981) described a large outpouring of leucocytes into the milk, without loss of the integrity of the glandular epithelium, following intramammary infusion of endotoxin. It is not clear how material within the lumen affects responses in the gland, yet the intersection of the pathways of antigen and the cells involved in developing an immune response and their concomitant and subsequent interactions undoubtedly are important in the development of the local immune response.

In their investigation of involution of the mammary gland, Lee *et al.* (1969) observed transport of colloidal carbon from the lumen of the gland to the draining lymph nodes of ewes and cows, following intramammary infusion at the time of last milking. They interpreted the large foamy cells present within the ducts, in the interalveolar areas, lymphatics, and cortical regions of the draining lymph nodes to be macrophages, on the basis of the carbon which they had phagocytosed. They also considered them to be important in the removal of lipid from the involuting mammary gland.

In the experiments described here, colloidal carbon or killed staphylococcal cells were infused into lactating, dry or involuting mammary glands of guinea pigs, and their distribution within the mammary tissue and transport to the regional nodes was followed. The major objective was to elucidate the contribution of physiological state of the gland as a factor in the induction of a local immune response.

MATERIALS AND METHODS

Experimental design

In preliminary trials of infusions of lamp black and India ink into the mammary glands of guinea pigs, carbon was transported from the lumen of the gland to the draining lymph nodes regardless of the lactation status of the gland. Furthermore, there were differences in time required for transport of carbon depending on the physiological state of the gland. Based on these findings, studies were carried out in order to establish for each physiological state the earliest time at which carbon would be present in the draining lymph nodes, when carbon would be present in nodes draining 50 % of glands and when carbon would be present in lymph nodes of almost all glands.

Experimental animals

Primiparous Hartley strain guinea pigs, which were either lactating, in involution, or dry were used. The lactating group was made up of sows whose young had been removed at either 4 or 11 d post partum and the first glands infused immediately. The involuting group was made up of sows whose young were removed 10 or 20 d post partum and the first glands infused 24 h later. Sows of the dry group had lactated for 10 or 20 d before weaning, and after an additional 10 d their glands were infused.

Colloidal carbon

India ink (Pelikan 518 Fount India, Pelikan AG, D-300 Hannover 1, FRG) was sterilized by autoclaving at 121 °C before use.

Preparation of killed bacteria

The organisms used were subcultured from a strain of *Staphylococcus aureus* isolated from milk of a cow with clinically apparent mastitis. Organisms were grown in trypticase soy broth and killed by addition of formaldehyde to yield a 10 % concentration. Washed packed cells were resuspended in an equal volume of phosphate buffered saline for infusion.

Intramammary infusions

These consisted of 0·1 ml India ink or bacterial suspension, administered from a 1·0 ml syringe, via a blunted 1 cm, 30 gauge needle, through the teat meatus, into the left gland. After an appropriate time interval the right gland was infused.

For experiments on carbon transport, lactating glands were infused 1, 2, 3, 4, 5, 6, 7 and 9 h before killing the animals, involuting glands at 3, 6, 9, 12, 18, 24, 32, 48, 72, and 96 h, and dry glands at 5, 10, 20, 30, 40, and 50 min before killing.

Additional guinea pigs were infused with killed cells of *Staph. aureus*. Lactating glands were infused at 9 h, involuting glands at 60 h, and dry glands at 2 h before killing.

Animal and tissue procedures

The carbon infused guinea pigs were killed by exsanguination under ketamine/xylazine anaesthesia (for trials involving time intervals greater than 2 h), or by intraperitoneal injection of a barbiturate-containing euthanasia solution. The mammary glands were removed, examined for the distribution of carbon and presence of milk, and weighed. The superficial inguinal lymph nodes were removed, counted and each node examined grossly for the presence of carbon. Sides were scored as positive or negative on the basis of presence, or absence, of visible carbon in one or more lymph nodes.

Guinea pigs which had been infused with killed *Staph. aureus* were treated similarly, omitting scoring for carbon.

Three guinea pigs from each group, which had not been infused, were used as negative controls.

Mammary glands and the superficial inguinal lymph nodes from each guinea pig were preserved separately in 10 % phosphate buffered formaldehyde (pH 7·2), and processed for histological examination. Sections from mammary glands and lymph nodes from each group of guinea pigs, representative of the different time intervals, were stained with haematoxylin and eosin. Sections from guinea pigs which had received infusions of killed bacteria were also stained with Brown and Brenn stain.

Statistical methods

The proportion of positive sides to total sides in a group at each time after infusion was subjected to logistic regression. That is, $\lg [P/(1-P)]$ was regressed on time, where P was the proportion of positive sides at a particular time sampled. A hierarchy of models which were linear in time but could have different slopes and intercepts for the different groups was considered. The best model was determined using the G-statistic from the GLIM© package (Baker & Nelder, 1978). The 50 % positive point estimate (PP_{50}), i.e. that time at which half of the guinea pig sides in the group were estimated to be positive for carbon, could then be calculated. A confidence interval for each PP_{50} was computed using Fieller's theorem (Fieller, 1940). No formal test of differences of PP_{50} was performed.

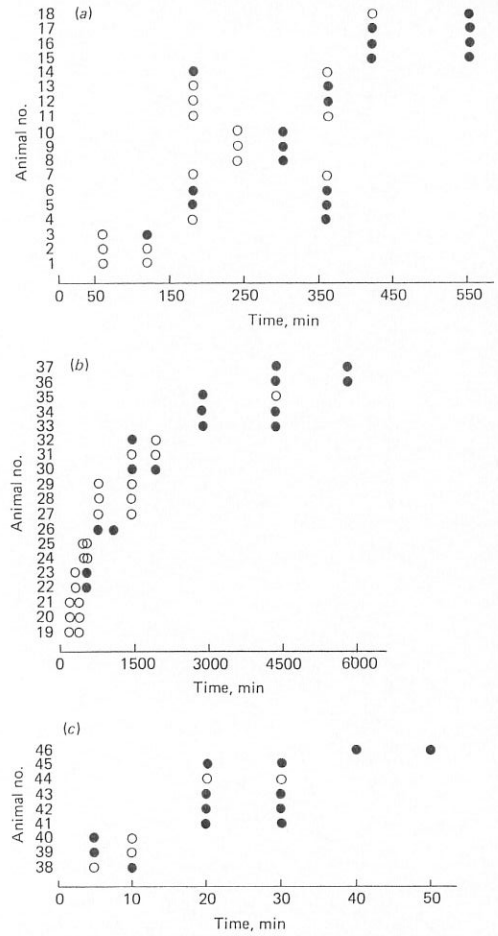

Fig. 1. Sides positive (●) or negative (○) for carbon in the inguinal lymph nodes of guinea pigs following intramammary infusion of 0·1 ml colloidal carbon suspension into lactating (*a*), involuting (*b*) or dry (*c*) mammary glands. Time is interval between infusion and killing. Both sides of each guinea pig were infused, and the presence or absence of carbon for each side is shown opposite the corresponding animal identification number.

RESULTS

In the lactating animals (Fig. 1*a*), lymph nodes positive for carbon were first observed from sides which had been infused 2 h before killing, and the lymph nodes were consistently positive at 9 h. With guinea pig no. 3, for example, there were positive lymph nodes draining the left gland, which had been infused 2 h before killing, whereas all nodes draining the right gland, which had been infused 1 h before killing, were negative.

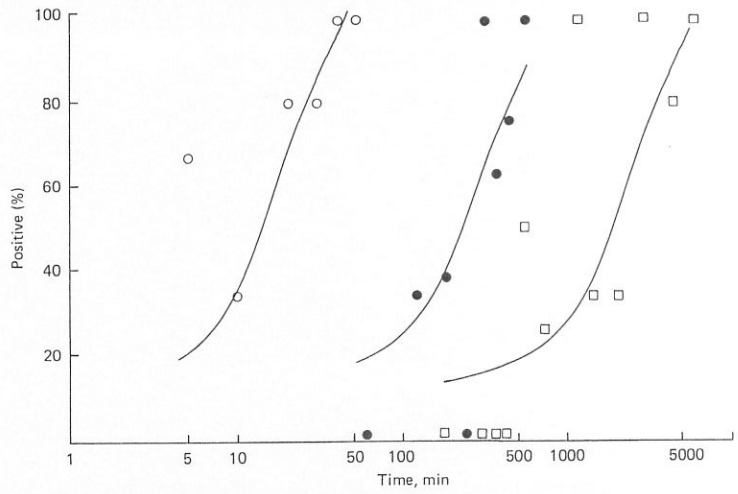

Fig. 2. Percentages of positive sides observed in the dry group (○), lactating group (●), and involuting group (□). Curves are estimates for the three groups from the best logistic model $\lg [P/(1-P)] = a+b_g t$ (see text). On a lg (time) scale, the three curves have the same shape with a simple location shift.

In contrast to the lactating group, lymph nodes of animals in the involuting group were not scored as positive for carbon until 9 h post infusion, and they were not consistently positive until 96 h (Fig. 1*b*).

In animals of the dry group, lymph nodes positive for carbon were observed as early as 5 min post infusion, and by 40 min, sides were consistently positive (Fig. 1*c*).

Statistical model

The logistic regression model generated, which best fitted the data, was $\lg [P/(1-P)] = a+b_g t$, where P was the proportion of positive sides at a particular time t after infusion, a was a common intercept for the three groups of data, b was the slope and was dependent on the particular group g. For these data $b_{(\text{lactating})} = 0{\cdot}007343$ (s.e. 0·001925), $b_{(\text{involuting})} = 0{\cdot}0009378$ (s.e. 0·0002880), and $b_{(\text{dry})} = 0{\cdot}1359$ (s.e. 0·03948), and $a = -1{\cdot}836$ (s.e. 0·4750). The model is represented graphically (Fig. 2). On a log (time) scale the curves have the same shape, with a simple location shift.

The PP_{50} for the lactating group was 4 h 20 min; for the involuting group, 32 h 37 min; and for the dry group, 13·5 min. The 95 % confidence intervals for PP_{50} for the lactating, involuting, and dry groups were, respectively, (3 h 15 min, 8 h 25 min), (25 h 9 min, 84 h 39 min), and (8·5 min, 33·8 min). These intervals do not come close to overlapping, corroborating the results from logistic regression which indicate that the response times are different for the three groups.

Histopathology

In sections from lactating glands, multiple foci of intralumenal carbon were observed with some association of the carbon with the alveolar epithelium (Fig. 3*a*).

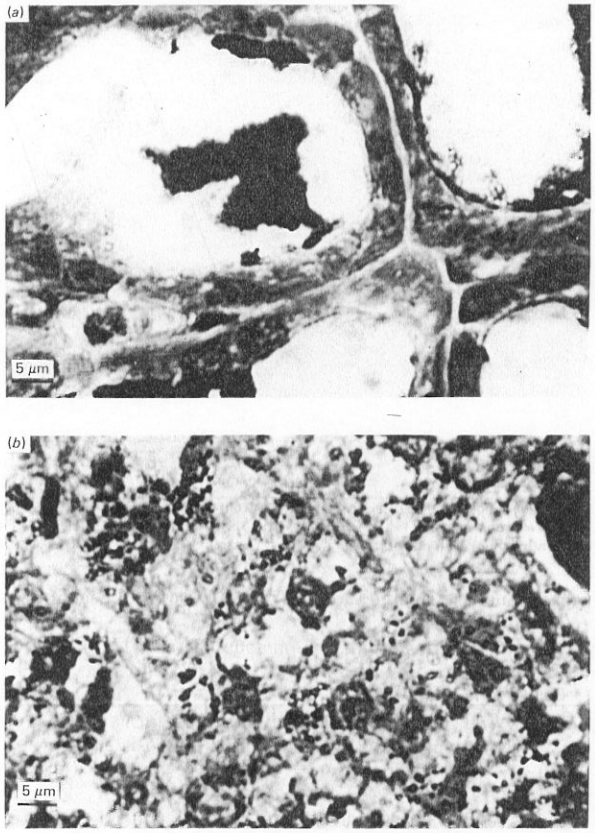

Fig. 3(*a*), (*b*). For caption see opposite page.

In sections of involuting gland, the disruption of the normal glandular architecture characteristic of involution was seen and carbon was dispersed throughout the tissue (Fig. 3*b*). The prominent and distinctive feature of the dry glands was the presence of carbon in the epithelium of many small ducts (Fig. 3*c*). Carbon was also seen within macrophages in the mammary tissue in all three groups.

In sections of lymph node which were positive for carbon on gross examination, carbon was observed within macrophages, particularly in the subcapsular sinuses, but also progressing into the nonfollicular medullary regions. In serial sections of afferent lymphatics from dry guinea pigs, most of the carbon was within the lumen and was extracellular.

Gram-positive cocci were found in lymph node sections of Brown and Brenn stained tissue after infusion of killed staphylococci in lactating, involuting and dry mammary glands. These cocci were located in the subcapsular sinuses, and some were

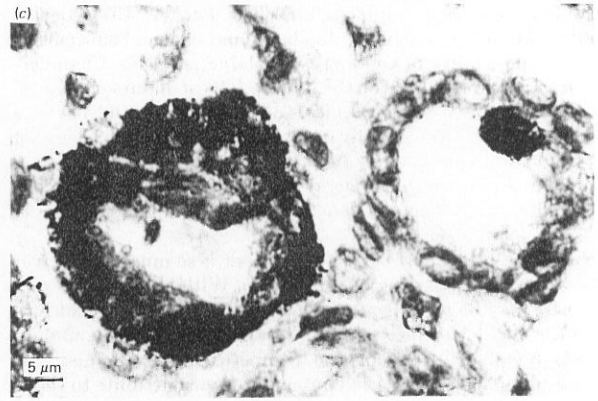

Fig. 3. Mammary tissue from (*a*) left gland of guinea pig no. 1 (lactating) showing carbon in the alveolar lumens and aggregated along the secretory epithelium, (*b*) left gland of guinea pig no. 29 (involuting), showing carbon dispersed throughout the field, and (*c*) right gland of guinea pig no. 46 (dry) showing a considerable amount of carbon within the epithelium of one small duct and within a macrophage in the lumen of another small duct.

obviously within the cytoplasm of macrophages. Cocci were less prominent in these lymph node sections than were carbon particles in the corresponding sections from India ink infused animals. The distribution of cocci in the mammary tissue was similar to that of carbon in the corresponding sections. In sections from lactating glands, intralumenal bacteria were prominent, some bactera were adherent to the secretory epithelium, and some appeared to be within the epithelium. In sections of involuting glands, the bacteria were dispersed throughout the tissue, and in sections from dry glands, bacteria appeared to be within the ductular epithelial cells. Macrophages, laden with cocci, were somewhat more prominent in these mammary tissues than were carbon laden macrophages in the corresponding mammary tissue sections from guinea pigs infused with India ink.

DISCUSSION

It is apparent that carbon or killed staphylococci can transit from the lumen of the guinea pig mammary gland to the superficial inguinal lymph nodes whether the gland is in involution, dry or lactating. The time necessary for carbon to reach the nodes varied markedly among the three groups, suggesting differences in the mechanisms of transport depending on the physiological state of the glands.

The transport of carbon to the draining lymph nodes from lactating mammary glands suggests that particulate material in the lumens of functional mammary glands is not entirely isolated from the rest of the body. Lee & Lascelles (1969) found that lactating mammary glands of ewes were unresponsive to levels of antigenic stimulation which were able to trigger local immune responses in both involuting and involuted mammary glands. To explain this difference they proposed a mechanism involving smaller numbers of lymphoid cells and less efficient antigen trapping, as well as an effect of removal of antigen by milking 24 h after its administration. Our results suggest that the lack of response of lactating glands is probably not a consequence

of their inability to take up antigen. Linzell & Peaker (1971) demonstrated the relative impermeability of mammary glands to ions and small molecules, but this does not preclude the possibility of endocytosis of large particles. Chandler *et al.* (1980) have done ultrastructural studies on the interactions of mammary tissue and bacteria infused into the mammary glands of mice. In their micrographs, bacteria can be seen within epithelial cells and occasionally in subepithelial locations. They concluded that streptococci and staphylococci may be phagocytosed and digested by the secretory cells of the mammary gland, but they also suggested that the intracellular location may be of benefit to some bacteria, shielding them from the effects of inflammatory cells.

It is not clear why transport to the lymph node is so much slower from involuting glands than from either lactating or dry glands. With the influx of macrophages at involution, there may be more local phagocytosis and a subsequent intraphagocytic transport which might be slower. Disruption of the regular glandular architecture might impede lymphatic flow, or the competition of mammary secretions for phagocytosis and removal from the gland might also contribute to the slower transit of carbon in this physiological state.

The rapidity with which large amounts of carbon reached the lymph nodes from dry glands suggests that transport in this group is not dependent on macrophage conveyance. This was supported by finding free carbon in the lumens of sections of afferent lymphatics within minutes after infusion. The high concentration of carbon observed in the ductal epithelium suggests that transepithelial transport is a potential mechanism of passage.

Transepithelial transport of carbon has been demonstrated both to the bursa of Fabricius in chicks (Naukarrinen & Sorvari, 1980) and to Peyer's patches in mammals (Bockman *et al.* 1983). Mammary ductal epithelial cells have been reported to be non-secretory, relatively devoid of organelles, primarily of structural importance (Pitelka, 1983) and unable to reabsorb secreted substances (Allen *et al.* 1984). Recently, however, non-secretory epithelial cells, which line the teat and lactiferous sinuses of the bovine mammary gland, have been shown to produce prominent pseudopodia which are able to ingest milk fat globules and casein micelles by phagocytosis (Brooker, 1983).This phagocytic activity was greater in involuting than in lactating glands. Alternatively, the mechanism for the rapid transport in the fully involuted state might be direct lymphatic drainage from the lumen of a gland in which the epithelium is no longer intact. Current investigations are designed to determine the mechanisms of transport at each physiological state.

If the carbon in the ductal epithelium represents an efficient phagocytic phenomenon, it may be necessary to re-evaluate the nature of the large foamy cells found in milk. These cells have been reported to be of probable macrophage origin primarily because of their phagocytic ability, and their ability to spread and adhere (Jensen & Eberhart, 1975). Mononuclear cells in the milk have also been classified as macrophages because of their enzyme activity, and because bovine immunoglobulins of the IgG class are cytophilic for them (Desiderio & Campbell, 1980). However, bovine mammary gland epithelial cells have been found to exhibit acid phosphatase activity (Brooker, 1983) and nonspecific esterase activity (D. I. Schenkman & D. T. Berman, unpublished observation). Bovine immunoglobulin of the IgG class also has an affinity for mammary epithelium; mammary epithelial cells are involved in the selective transfer of IgG from serum to colostrum (Brandon *et al.* 1971). The criteria which have been used so far to classify the foamy cells in mammary secretion as macrophages do not rigorously distinguish macrophages from epithelial cells.

The statistical model for the data presented here proposes that the differences among the three groups can be explained as a function of time. That is, by rescaling time the three groups of data can be described by the same curve. This suggests a relatively simple mechanistic difference in transport among the groups. The effect of differences in gland weight on diffusion phenomena could be postulated as such a mechanistic factor in the comparison between dry and lactating glands because lactating glands were $\sim 4{\cdot}5$ times heavier than dry glands. This hypothesis was rejected, as the lactating glands were also slightly larger than the involuting glands, and no weight effect trend was apparent when multiple regressions considering time, weight, group, and positivity for carbon were calculated.

The statistical analyses were based on an assumption of independence of left and right sides. Anatomically, the glands and lymphatic drainage are separate, and there was no apparent effect on a gland from infusing the contralateral gland. Gland weights, however, were not independent; left and right glands from the same animal were approximately the same weight. The experimental design in future studies should incorporate the paired dependence of left and right sides. Randomization of left and right sides would eliminate any possible bias due to bilateral asymmetry.

In summary, the physiological state of the gland influences the localization and transport of colloidal carbon particles and bacteria infused into the mammary gland. We hypothesize that these differences in transport of antigen will result in differences in the local immune responses in the mammary glands, and this experimental system should have practical implications for optimization of immunization methods for prevention of mastitis, as well as in the elucidation of mechanisms of induction of local immunity and immune-mediated inflammation.

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