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HISTOLOGICAL STUDY OF MAMMAE IN OESTROGENIZED RATS AFTER MAMMARY ISOIMMUNIZATION

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Summary.—Inoculation of 0·1 ml of an emulsion of a saline suspension of allogeneic lactating mammae and Freund's complete adjuvant into oestrone-primed female Wistar rats evoked in their mammary glands a progressively increasing focal mononuclear leucocytic infiltrate that was found in 25·0% of the animals 28 days after inoculation. Comparable changes were not seen during the experimental period in rats administered oestrone alone, or in those inoculated with other combinations of saline, lactating mammary or liver homogenates and Freund's complete or incomplete adjuvants. The mononuclear inflammatory lesions were substantially unaffected by the site of the intradermal inoculations, or by the addition of pertussis vaccine to the inocula, but were suppressed by antilymphocyte serum. It is suggested that some examples of human periductal mastitis may have an autoallergic basis.

THE pathogenesis of human idiopathic periductal mastitides is obscure. Morphologically they present a varied picture, which is reflected in the different descriptive terms that have been applied to them (Ingier, 1909; Adair, 1933; Tice, Dockerty and Harrington, 1948; Haneveld, 1967). However, as they are widely felt to represent the same disease process (Rodman and Ingleby, 1939; Bonser, Dossett and Jull, 1961), the generic term periductal mastitis (Foote and Stewart, 1945) is used here.

The sterility of the lesions, in the absence of fistulation, does not support an infective origin (Schultz, 1933; Bonser *et al.*, 1961). Though chemical irritation by retained duct secretions has been suspected (Bertels, 1913; Rodman and Ingleby, 1939; Cutler, 1962), it has been postulated that bovine (Payne, 1961) and human (Bonser *et al.*, 1961; Mackay and Burnet, 1963) periductal mastitides may have an autoallergic basis.

The conflicting experimental results obtained after auto- and iso-immunization in lactating (Sell and Weigle, 1961) and oestrogenized (Shinohara, 1968) rabbits prompted the experiments described here, using oestrogenized rats. The procedure adopted was to examine the mammae of oestrogenized intact female rats, together with appropriate control animals, at intervals after administration of a single dose of a saline homogenate of allogeneic lactating mammae emulsified with Freund's complete adjuvant.

MATERIALS AND METHODS

Animals.—Colony-bred female albino Wistar rats housed in the Department of Pathology, St Bartholomew's Hospital, London, were fed tap water and PRM dietary pellets *ad libitum*. Those examined histologically were aged 12–20 weeks at sacrifice.

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Experimental procedure.—When 6 weeks old (mean body weight ± S.D., 100 ± 13 g) the rats were etherized and 10 mg of oestrone crystals (B.D.H.) were implanted s.c. in the dorsal interscapular region. Six weeks later 0·1 ml doses of water-in-oil emulsions (stability tested by water flotation) of Freund's adjuvants (Difco), and saline or organ homogenates were injected intradermally into the base of the tail or a foot pad. To some animals 0·05 ml of pertussis vaccine (Burroughs Wellcome, 20,000 × 10^6 organisms/0·5 ml, Lot no. 67753) was administered s.c. close to the intradermal site of inoculation. Thereafter the animals were sacrificed by ether anaesthesia and cervical fracture 3, 7, 14, 21, 28, 35, and 56 days after the time of inoculation.

Preparation and quantitative examination of mammae.—Immediately *post mortem* the major part of the thoracic, abdominal and inguinal mammae were removed intact attached to a skin pelt. The pelts were resected by means of 3 consecutive incisions: a midline ventral mandibulo-pubic incision, a lateral incision from the posterior end of the ventral incision to pass posterior to the hind inguinal teat thence close to the anterior aspect of the thigh. The third incision passed anteriorly through the hind and lateral wing of the abdominal mammary fat pad to include the medial bodies of the thoracic mammae in the pelt. The pelts were then fixed for upwards of 24 hours in 10% formalin in saline. In all cases the entire hind mammae on the pelt were separated, processed and impregnated in paraffin; in some instances the thoracic mammae were similarly treated. From the centre of each block a single nominally 5 μm-thick section was taken for scoring the number of leucocytic foci in that hind mamma. Assessments of the sections were performed in large batches without knowledge of their origins.

A focus of leucocytes was defined as containing at least 30 cells, and measuring at least 40 μm × 40 μm in 2 perpendicular planes. Foci adjacent to lobules, ducts or in the adipose mammary connective tissue were acceptable, but those within the lumina of ducts, fibrous septa, fascial margins of the mammae, in the vicinity of larger vessels and adjacent to the intramammary lymph nodes were excluded.

Preparation of organ homogenates.—The mammary homogenates were prepared freshly on each occasion from a pool of lactating mammae derived from 3 to 6 Wistar rats, which were 3 weeks *post-partum*. These animals were rested for 2 to 4 hours after weaning and then sacrificed. The hind mammae were resected aseptically, haemorrhagic contamination being reduced by tension on the large posterior mammary vessels. The mammae were delivered into sterile saline (0·85 g/100 ml), the volume of which was adjusted to equal that of the mammae. The glands were finely chopped and homogenized for 45 seconds in stout wide-necked Pyrex tubes by an Ultra-Turrax homogenizer (Janke & Kunkel TP 18/2, RPM 20,000, 50 HZ). Within 15 minutes of homogenization water-in-oil emulsions were effected with an aliquot of Freund's adjuvant. The liver homogenates were similarly prepared having first bled the animals *post mortem* from the inferior vena cava.

Immunological procedures.—Antilymphocyte serum was raised in rabbits and absorbed according to the method of Levey and Medawar (1966). It was administered i.v. via alternate tail veins (0·5 ml one week before, at, and one week after inoculation of the emulsion containing Freund's adjuvant, and 1·0 ml 2 weeks after that inoculation) and i.p. (1 ml one week after inoculation of the Freund's adjuvant). Precipitin reactions were performed in Petri dishes containing 1·5% purified agar in tap water, to which was added 1 part in 10^4 merthiolate.

RESULTS

Effect of experimental inoculations in the tail base on prevalence of leucocytic foci in hind mammae three weeks later

The proportion of hind mammae containing 7 or more foci of leucocytes 21 days after inoculation of Freund's complete adjuvant emulsified with mammary homogenate was significantly higher ($\chi_c^2 = 24{\cdot}5$, 1 d.f., $P < 0{\cdot}001$) than was seen in uninoculated oestrogenized animals or in those in which Freund's complete adjuvant and saline or liver homogenate, or Freund's incomplete adjuvant and mammary homogenate were given (Table I). Up to 20 foci were found in hind mammae of the rats inoculated with Freund's complete adjuvant and mammary homogenate. The difference between the proportions of mammae containing 7

or more foci after inoculation with mammary homogenate and either Freund's complete or incomplete adjuvants was also significant ($\chi_c^2 = 4{\cdot}30$, 1 d.f., $P < 0{\cdot}05$).

No substantial difference was found between the body weights or the size of the granulomata at the base of the tail in the animals with or without more than 7 leucocytic foci in the hind mammae. Granulomatous foci were found in many local lymph nodes, and there were some in the liver and spleen of animals administered Freund's complete adjuvant. There was, however, no difference in these features between the groups given either saline, mammary or liver homogenate with the complete Freund's adjuvant. Few animals developed arthopathies and those joints examined histologically displayed only minor inflammatory infiltrates.

TABLE I.—*Effects of Inoculations at Base of Tail on Numbers of Leucocytic Foci in Hind Mammae of Oestrogenized Female Rats at 3 Weeks*

Inoculum	No. mammae examined	No. mammae with specified no. foci							
		0	1	2	3	4	5	6	7+
None	50	12	17	6	5	6	2	1	1
FCA-saline	49	15	16	4	3	5	3	2	1
FCA-liver	48	17	14	8	3	4	2	0	0
FIA-mamma	32	11	9	2	4	3	1	1	1
FCA-mamma	75	7	15	12	9	7	5	4	16

FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant

Chronological development of lesions

The numbers of leucocytic foci in the hind mammae in control, oestrogenized rats and in those given an emulsion of Freund's complete adjuvant and mammary homogenate are shown in Tables II and III. Though the control animal's hind

TABLE II.—*Numbers of Leucocytic Foci in Hind Mammae of Uninoculated Oestrogenized Female Rats*

Time (from inoculation in other groups)	No. mammae examined	No. mammae with specified no. foci							
		0	1	2	3	4	5	6	7+
7 days	24	12	5	6	0	0	0	1	0
14 days	12	5	7	0	0	0	0	0	0
21 days	50	12	17	6	5	6	2	1	1
28 days	30	16	11	2	1	0	0	0	0
35 days	26	5	9	5	3	1	0	2	1
56 days	18	10	6	2	0	0	0	0	0

EXPLANATION OF PLATE

FIG. 1.—Compact perivenular exudate of mononuclear leucocytes beside lobule of oestrogenized rat mamma 14 days after inoculation mammary homogenate and Freund's complete adjuvant (FCA). H. and E. ×400.

FIG. 2.—Larger perilobular exudate of mononuclear leucocytes 21 days after inoculation FCA-mamma. H. and E. ×250.

FIG. 3.—Destructive lobular and periductal mononuclear leucocytic infiltrate 28 days after inoculation FCA-mamma. H. and E. ×250.

FIG. 4.—Lobular infiltrate composed almost entirely of eosinophil and neutrophil granulocytes 21 days after inoculation FCA-mamma with concurrent administration of antilymphocyte serum. H. and E. ×400.

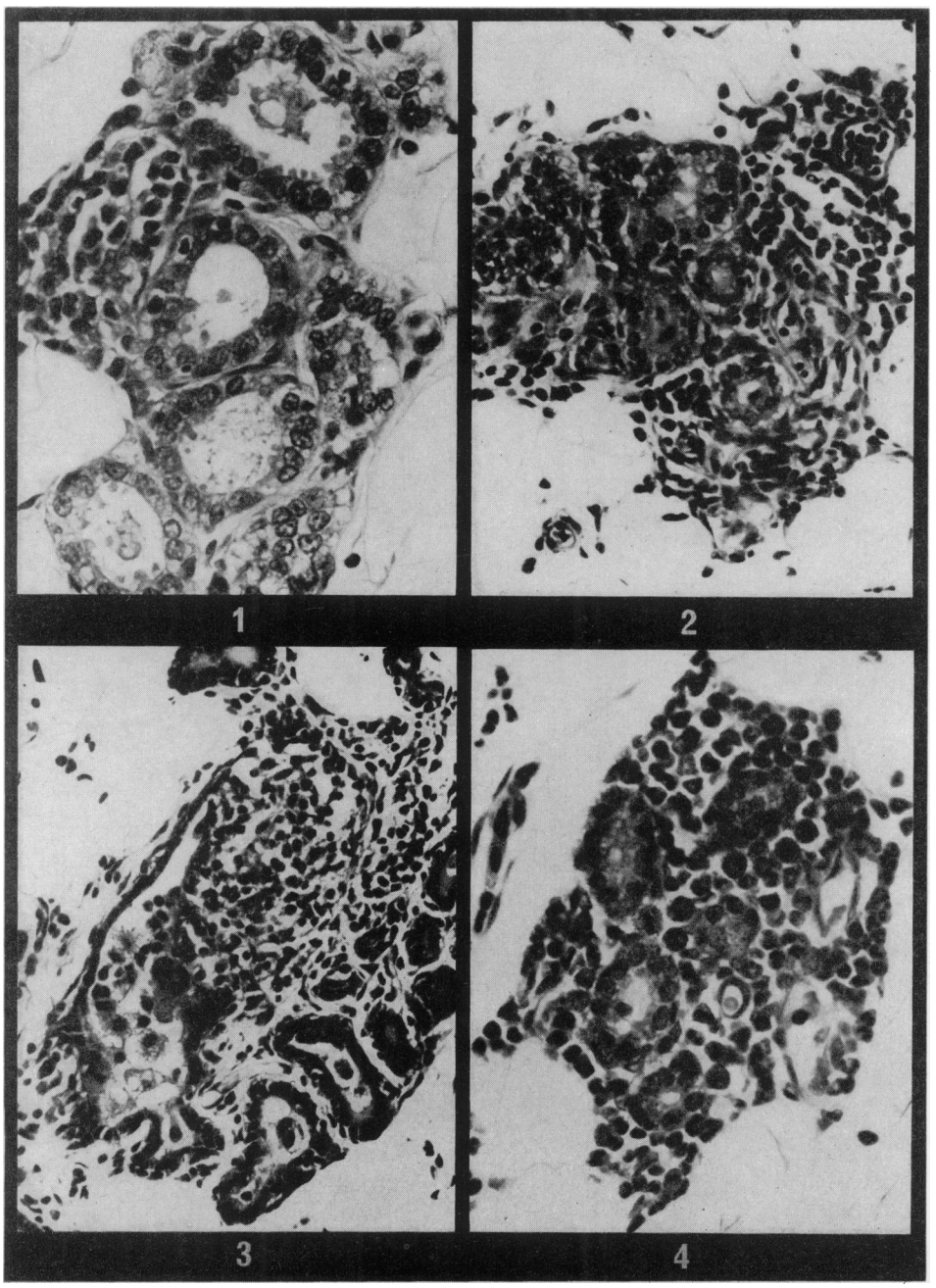
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TABLE III.—*Numbers of Leucocytic Foci in Hind Mammae of Oestrogenized Female Rats Inoculated FCA-mamma at Base of Tail*

Time (from inoculation)	No. mammae examined	No. mammae with specified no. foci							
		0	1	2	3	4	5	6	7+
3 days	28	18	7	1	1	1	0	0	0
7 days	12	9	1	2	0	0	0	0	0
14 days	48	17	17	6	3	2	0	0	3
21 days	75	7	15	12	9	7	5	4	16
28 days	40	5	5	6	4	5	3	2	10
35 days	14	2	1	3	2	4	1	0	1
56 days	22	9	7	5	2	0	0	1	0

mammae occasionally contained 7 foci at 21 and 35 days, 8 or more foci were limited to the inoculated animals at 14, 21 and 28 days after inoculation. In inoculated animals 7 or more foci were present in 6·3% of hind mammae at 14 days, 21·3% of hind mammae at 21 days, 25·0% of hind mammae at 28 days, and in 7·1% of hind mammae at 35 days.

Macroscopically, the only change attributable to inoculation was purple discolouration of some mammae. This first appeared 21 days after inoculation, and was associated with increased vascularity and leucocytic infiltration in the mammae. Brown discolouration was unrelated to inoculation or to the inflammatory reaction.

Microscopically, the first changes associated with inoculation of Freund's complete adjuvant and mammary homogenate occurred at 14 days when tight perivenular exudates of mononuclear cells appeared close to small ducts and lobules (Fig. 1). These cells were small round cells, morphologically either lymphoid or macrophages; granulocytes were scanty. At 21 days the exudates were larger (Fig. 2); many of the component cells possessed pyroninophilic cytoplasm, some early epithelial permeation was visible, and only a few granulocytes were present. The parenchyma was often condensed where the cellular infiltration had occurred. At 28 days the leucocytic foci had further increased in size, the proportion of histiocytes became larger and focal destruction of the parenchyma appeared (Fig. 3). Thereafter the size and numbers of the foci declined, though some focal fibrosis was seen.

Leucocytic foci in the control animals differed from the lesions described above in that they were looser knit, and showed more prominent granulocytes, particularly eosinophils, and histiocytes with conspicuously foamy cytoplasm.

Effect of site of inoculation

Inoculation of mammary homogenate and Freund's complete adjuvant did not evoke significantly different proportions of 7 or more leucocytic foci in the hind mammae when the sites on intradermal inoculation were altered. Seven or more foci were found in 21·3% of 75 hind mammae after inoculation at the base of the tail, 16·7% of 12 hind mammae after inoculation in the ipsilateral hind foot-pad, and 15·4% of 26 hind mammae after inoculation in the ipsilateral front foot-pad. Whilst the thoracic mammae, partly owing to interdigitation with local skeletal muscle, were too variable in their cross-sectional area for quantitation of leucocytic responses, their reactions resembled those in the hind mammae after inoculations at the base of the tail.

Effect of pertussis vaccine

Inoculation of pertussis vaccine alone, or in combination with an injection of Freund's complete adjuvant emulsified with saline, did not evoke an increased incidence of 7 or more leucocytic foci in the hind mammae for only 1 mamma of 48 contained 7 foci 21 days after such inoculations (Table IV). The prevalence of lesions in mammae 21 days after inoculation of pertussis vaccine combined with Freund's complete adjuvant emulsified with mammary homogenate did not differ significantly from that found in animals given only Freund's complete adjuvant and mammary homogenate (Table IV).

TABLE IV.—*Effect of Pertussis Vaccine on Numbers of Leucocytic Foci in Hind Mammae of Oestrogenized Female Rats at 3 Weeks*

Inoculum (base of tail, front or hind foot pads)	No. mammae examined	No. mammae with specified no. foci							
		0	1	2	3	4	5	6	7+
Pertussis alone	12	4	2	3	3	0	0	0	0
Pertussis + FCA-saline	36	8	9	7	4	4	2	1	1
Pertussis + FCA-mamma	51	15	6	7	6	0	5	2	10

Effect of antilymphocyte serum

The mean lymphocyte count of the post-mortem venous blood of the animals administered antilymphocyte serum was reduced from 18,800 mm^{-3} to 9280 mm^{-3}, whereas the erythrocyte, neutrophil and monocyte parameters were unchanged. The intramammary lymph nodes of the antilymphocyte serum treated rats exhibited variable suppression of the paracortical nodules, and expansion and plasmacytosis of the medullary cords together with enhanced germinal centre formation in the cortical follicles. All sera from the rats given antilymphocyte serum produced precipitin arcs in the agar gel opposite wells containing rabbit serum.

The administration of antilymphocyte serum concurrently with the inoculation of lactating mammary homogenate and Freund's complete adjuvant reduced the proportion of hind mammae containing 7 or more leucocytic foci at 21 days from 21·4% to 5·5% (Table V). Furthermore, the composition of the foci in the

TABLE V.—*Effect of Antilymphocyte Serum on Numbers of Leucocytic Foci in Hind Mammae of Oestrogenized Rats Inoculated with FCA-mamma at Base of Tail 3 Weeks Previously*

Inoculum	No. mammae examined	No. mammae with specified no. foci							
		0	1	2	3	4	5	6	7+
FCA-mamma alone	75	7	15	12	9	7	5	4	16
FCA-mamma + antilymphocyte serum	18	7	3	2	1	3	0	1	1

mammae of the animals administered antilymphocyte serum differed strikingly from that seen after inoculation of mammary homogenate and Freund's complete adjuvant alone. The mononuclear component of the exudate was suppressed and replaced by eosinophil and neutrophil granulocytes (Fig. 4).

DISCUSSION

The character and prevalence of the leucocytic foci in the hind mammae after inoculation of an emulsion of mammary homogenate and Freund's complete adjuvant differed from those seen in control animals administered oestrone alone. Advantage was taken in these experiments of the sparse leucocytic infiltration in the early stages of mammary response to oestrogens, for longer periods of oestrogenization are associated with pronounced spontaneous inflammatory infiltration in the mammae of mice (Burrows, 1935–1936; Bonser, 1936) and rats (Eisen, 1942; Geschickter, 1945). The sites at which the intradermal inoculations were made did not have significant effect upon the numbers of leucocytic foci observed 21 days afterwards. This was confirmed by the similarity of the mammary reactions in thoracic and abdominal regions after inocula at the base of the tail. The only differences in the mammae that did appear related to the site of inoculation were perilymphangitic lesions. These were, however, excluded by definition in the assessment of the intramammary foci.

Of the various inoculations, that comprising mammary homogenate and Freund's complete adjuvant evoked most foci of leucocytes in the mammae. Other combinations of saline, hepatic or mammary homogenates emulsified with Freund's complete or incomplete adjuvants did not yield significantly different proportions of mammae containing 7 or more foci to that observed after oestrogenization alone. No lesion in other organs appeared attributable specifically to inoculation of mammary homogenate and Freund's complete adjuvant. Such findings resemble those in other experimental organ-specific lesions induced in the brain (Freund, Stern and Pisani, 1947), testis (Freund, Lipton and Thompson, 1953), and thyroid (Terplan *et al.*, 1960). The low incidence and mildness of the arthropathies in all groups may be related to oestrogenization (Kappas, Jones and Roitt, 1963; Mueller and Kappas, 1964). However, the interaction between oestrogenization and the leucocytic reactions to inoculation was not tested here, for all inoculated animals were also oestrogenized.

The chronological development and morphology of the mammary lesions are also similar to many experimental allergic organ-specific diseases. The interval of 14 days between inoculation and the appearance of the first leucocytic exudates, the gradually increasing size of the foci with parenchymal disruption, and the subsequent resolution of the lesions, which thus lack progressive character, resemble other experimental allergic models. The rat has shown a pronounced regression of experimental allergic lesions of the thyroid after the fourth and fifth weeks (Jones and Roitt, 1961; Willoughby and Coote, 1966) but a similar, if later, regression was also noted in the thyroid lesions of guinea-pigs (Flax, 1963; Lerner, McMaster and Exum, 1964).

Though the mammary lesions remained strictly focal in oestrogenized animals, the microscopic morphology also resembles other models of experimental organ-specific allergic disease. Mononuclear predominance in the inflammatory exudate, which has been stressed by Waksman (1959) as a hall-mark of experimental autoallergic diseases, and focal destructive disruption of the parenchyma are consistent with cell-mediated immunological reactions to elements of the mammary parenchyma.

The augmentation of experimental autoallergic lesions by pertussis vaccine (Levine and Wenk, 1964, 1965; Paterson and Drobish, 1968) was not convincingly

reproducible here. Perhaps the variation in effectiveness of different pertussis vaccines noted by Paterson *et al.* (1970) was responsible.

The potency of the antilymphocyte serum was shown by the differential reduction of the peripheral blood lymphoid cell count by about 50% and by the suppression of the paracortical nodules in the intramammary lymph nodes. Though it apparently evoked an antibody response, as was shown by the plasma cell-rich expansion of the medullary cords of the lymph nodes and the appearance of a precipitating antibody to rabbit serum proteins in the sera of the treated rats, the stability of the red blood cell and granulocyte counts in the treated animals would indicate that it was not toxic. These effects and the suppression of the mononuclear infiltrates in the mammae of rats inoculated with mammary homogenate and Freund's complete adjuvant resemble the effects of antilymphocyte serum in experimental allergic thyroiditis in the rat (Kalden *et al.*, 1968; MacSween *et al.*, 1970). The appearance of granulocytes around mammary lobules in the mammae in some of the animals administered antilymphocyte serum raises the possibility of a local Arthus-type reaction in the mammae to xenogeneic immunoglobulin, which is known to be excreted into the mammary secretions (Ehrlich, 1892; Dixon, Weigle and Vazquez, 1961).

Though Freund (1957) suggested that the mammae might provide a suitable model whereby to study experimental allergic reactions, few accounts of such investigations have been published. Sell and Weigle (1961) were unable to show significant inflammatory lesions in mammary biopsies from *post-partum* does after repeated immunization with skim milk or casein, though all the rabbits administered allogeneic milk produced iso-antibodies to casein and other milk proteins. However Shinohara (1968) evoked inflammatory infiltration and fibrosis in the mammae of oestrogenized rabbits by inoculations of saline extracts of oestrogen-stimulated mammae.

The present study has shown in about one-quarter of the oestrogenized rats a focal, predominantly mononuclear, inflammatory infiltration of the mammae after inoculation of an emulsion comprising allogeneic lactating mammae and Freund's complete adjuvant. The histological appearances of the lesions, their chronological development, lack of dependence upon the site of inoculation, and suppression by antilymphocyte serum support the hypothesis that they represent an allergic reaction which may be directed against an unidentified component of the mammary gland.

There are resemblances between the experimental lesions in the rat and those of human periductal mastitis. The predominance of mononuclear cells, comprising lymphoid cells, histiocytes and plasma cells, together with focal ulceration and a periparenchymal distribution of the leucocytes are common to the 2 conditions. Granulocytes are usually in a minority in both lesions, though occasionally they figure more prominently in human periductal mastitides. The major difference between the two species is the largely lobular distribution of the lesions in the rat, and the predominantly ductal involvement in human periductal mastitis. Possibly this difference relates to microanatomical differences in the mammae of the two species, but it may be that the early lesions of the human disease are overshadowed by the more marked periductal distribution of the inflammatory infiltrate as the disease becomes advanced.

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