Immunofluorescent Studies of the Local Immune Response in the Mammary Glands of Rats

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Two irritants, phosphate-buffered saline and alcohol, and antigens including killed Brucella abortus, live B. abortus, Staphylococcus aureus, and rat parvovirus were separately infused into rat mammary glands during pregnancy, and by using immunofluorescent techniques, the numbers of immunoglobulin-containing cells in glands during lactation and involution were determined. The study provided basic information on the local immune response of the mammary gland to antigens of various types. In all experiments, the number of immunoglobulin M (IgM) cells present was small and no trends were apparent. IgA cells were always more prevalent than IgG cells. Fewer IgA cells were in the glands of rats infused with phosphate-buffered saline and alcohol than in normal rats. IgA cell prevalence was greatest in response to infusion of live B. abortus. Responses to live S. aureus and parvovirus were less pronounced, and infusion of killed B. abortus did not induce an elevation in IgA cell prevalence. IgG cell prevalence was greatest in response to infusion of live B. abortus or S. aureus and was decreasingly less pronounced in response to killed B. abortus and rat parvovirus. With the exception of parvovirus infusion, in regard to IgA cells, all glands locally infused with antigen had elevated IgA and IgG cell numbers when compared with noninfused glands in the same animal.

The presence of specific antibody in milk after the infusion of bacterial antigens into the mammary gland has been recognized for some time (16, 26, 30); in particular, the inoculation of these antigens into glands during pregnancy resulted in a persistent local production of antibody (17, 18).

Apart from bacteria, viruses represent another type of agent which may cause mastitis. Natural and experimental viral infections of the mammary gland have been examined in cows (9, 10), sows (3, 4), and goats (24, 27, 28). Immunohistochemical studies concerning the appearance of immunocytes in the mammary gland after virus-induced mastitis appear to be lacking.

Although antibody-containing cells in normal mammary glands have been studied sequentially (19) and local immune responses to bacterial antigens have been reported (11, 20), little information is available on immunocyte populations in the mammary gland during lactation and involution of glands stimulated by antigens of different types. In the present studies, two irritants (phosphate-buffered saline [PBS] and alcohol) and a variety of antigens including killed Brucella abortus, live B. abortus, Staphylococcus aureus, and rat parvovirus were infused into rat mammary glands during pregnancy, and the numbers of specific immunoglobulin-containing cells were determined during lactation and involution.

MATERIALS AND METHODS

Animals. Virgin female albino rats were mated at 12 to 15 weeks of age. Pregnancy was determined by observation of spermatozoa in vaginal smears; the day after the appearance of spermatozoa was counted as day 0 of gestation. Average gestation length was 22 days, and rat pups were weaned at 20 days of age. A total of 141 rats were used; included were 25 normal, uninfused rats and 116 rats randomly allocated to six treatment groups as indicated below, each group containing 16 to 33 rats.

Antigens and infusion procedure. Three right-hand side teats, one abdominal and two inguinal, were used for intramammary infusion. The inocula were infused into the mammary glands through the teat canal at day 15 of gestation. The procedure was similar to the method described previously for mice (7). Each gland received 0.1 ml of inoculum.

Materials infused were PBS (pH 7.2), ethyl alcohol, 1% (vol/vol) in PBS, and killed B. abortus suspension (Commonwealth Serum Laboratories, Melbourne) washed three times in PBS and resuspended to contain

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approximately 10^7 organisms per ml in PBS.

Additionally, live B. abortus (strain 19) (Commonwealth Serum Laboratories, Melbourne) was infused after dilution in PBS to contain approximately 10^7 organisms per ml. A coagulase-positive strain of S. aureus was isolated from a case of acute clinical mastitis in a dairy cow and prepared so that the inoculum contained approximately 10^7 organisms per ml in PBS. The number of staphylococci cultured in nutrient broth for 24 h was determined as described (13), and the organism displayed strong double-zone hemolysis on sheep blood agar. Rat parvovirus was prepared to contain 40 hemagglutination units in 3 ml of PBS. The virus was kindly supplied by R. H. Johnson (Department of Tropical Veterinary Science, James Cook University).

For infusion, rats were anesthetized with ether; they were killed by exsanguination at 0, 4, 8, 12, and 16 days after parturition and 1, 5, and 10 days after weaning. Sampling procedures for the mammary gland have been reported previously (19).

**Immunofluorescent and histological procedures.** For immunohistological studies, single portions of mammary tissue (2 mm thick) were obtained from two right-side glands of each animal; inguinal glands of rats stimulated locally were infused with the above-mentioned materials whereas the thoracic glands of all animals were noninfused. Paraffin blocks were prepared according to the technique of Sainte-Marie (34), and sections cut for immunofluorescent studies were 6 μm thick.

Fluorescein-labeled rabbit anti-rat immunoglobulin G (IgG; Wellcome, England) was diluted 1:15 (vol/vol) in PBS, pH 7.2, before use in the assays. Rabbit anti-rat IgM and goat anti-rat IgA, lyophilized (Miles Laboratories, Inc., Elkhart, Indiana), were conjugated with fluorescein isothiocyanate (Sigma) as described (19). For specificity control IgG fractionated from normal rabbit and goat sera by ion-exchange chromatography was conjugated with fluorescein isothiocyanate. Staining procedures, specificity control, and microscopy for immunofluorescence have been described (19). Counts were made on stained cells present in 20 high-power fields (HPF) selected at random in each section, using a ×40, 0.65-numerical aperture objective and a X8 eyepiece. The cells counted were those which showed clearly defined cytoplasmic staining.

Duplicate portions of mammary tissue sampled for immunofluorescent studies from rats infused with visible antigens were fixed in 10% neutral buffered formalin and embedded in paraffin, and sections cut at 6 μm were stained with hematoxylin and eosin, and, where appropriate, with a modified Ziehl-Neelsen (31) or Gram stain, for the demonstration of bacteria.

**Serology.** The direct agglutination test was carried out on the sera of rats infused with B. abortus as described (2). Staphylococcal agglutination assays were performed as described (23). The hemagglutination inhibition technique was used for antiparvovirus antibody assays.

**Statistical analysis.** Because of the unequal number of animals in each treatment, results were analyzed by a split-plot analysis of variance (36) from which the standard errors quoted in Tables 1 and 2 were derived.

**RESULTS**

**Clinical and pathological observations.** Clinical signs were not observed after infusion of irritants or antigens. Gross pathological changes were seen only in the staphylococcus-infused group, which within 48 h had abscesses at infusion sites. The size of abscesses (8 mm in diameter) diminished after 3 days. At postmortem the abscesses were seen to contain pus until lactational day 16, and at postweaning day 10 only remnants of abscesses (white foci less than 2 mm in diameter) remained. In some adults, abscesses blocked the teat duct, but usually lactation was apparently not impeded.

S. aureus was isolated from the pus in the abscesses until lactational day 16; histological examination until this interval showed focal accumulations of plasma cells in the connective tissue adjacent to the abscesses. Only a few alveoli adjacent to the abscesses contained neutrophils, and inflammation was not observed in the mammary tissues at a distance from the abscesses. S. aureus organisms were demonstrated in sections stained by the Gram method. In rats infected with live B. abortus, histological examination revealed only occasional slight mononuclear cell infiltrations and accumulations of plasma cells in the interstitial tissue. Neither bacterial isolation nor the demonstration of the organisms in sections stained by a modified Ziehl-Neelsen method was achieved at any interval. Histological examination of mammary tissue infected with live rat parvovirus did not reveal inflammation at any stage.

**Immunofluorescent studies.** The prevalence of IgA- and IgG-containing cells in the mammary glands, during lactation and involution, or rats infused intramammarily with irritants and dead and live antigens is presented in Tables 1 and 2, respectively. Although rats were killed at eight separate lactation and involution intervals, no clear pattern of change in immunoglobulin containing cell prevalence was related to these specific intervals so that results for lactating rats were pooled, as were results for rats examined during involution. In all studies, the number of IgM cells present was small (0.3 to 6.2 cells per 20 HPF), and no trends were apparent.

Except in the case of rat parvovirus, the prevalence of both IgA and IgG cells was greater during lactation than during involution. The differences, however, were not significant when compared with the variation between days during lactation and involution. IgA cells were always more prevalent than IgG cells; the maximum number of IgG cells observed (25 per 20
HPF, after infusion of live *B. abortus* being comparable to the minimal number of IgA cells (20 per 20 HPF in involuting, alcohol-infused glands).

**IgA cells.** After the infusion of alcohol or PBS there was no obvious difference between the infused and noninfused glands in regard to IgA cell prevalence. However, the number of IgA cells in the mammary glands of these rats was less than in the mammary glands of normal (uninfused) rats (*P* < 0.05 and *P* < 0.01 for alcohol and PBS, respectively).

With the exception of parvovirus-infused mammary glands, all mammary glands locally infected with antigen had elevated IgA cell numbers when compared with noninfused glands in the same animal. Parvovirus infusion resulted in a reversal of this trend during involution, when more IgA cells (71 per 20 HPF) were found in the noninfused glands than in the infused glands (50 per 20 HPF).

The magnitude of IgA cell response to infused antigen was greatest with live *B. abortus* (84 cells per 20 HPF) and less pronounced with live *S. aureus* and parvovirus (51 cells each). Infusion of killed *B. abortus* did not induce an elevation of IgA cell prevalence above that observed in normal glands; more IgA cells were, however, found in glands with killed *B. abortus* than in noninfused glands from the same animal (*P* < 0.01).

The pattern of IgA cell distribution in the mammary tissue infused with live brucellae and staphylococci was irregular as compared with the distribution of these cells in the mammary tissue of normal rats. Focal accumulations of IgA cells were seen in the glands infused with live brucellae (Fig. 1), and although IgA cells were prevalent around abscesses in the glands infected with staphylococci, their distribution in other areas (away from abscesses) was the same as in normal glands.

**IgG cells.** As with the IgA cell response to most antigens, there were more IgG cells in antigen-infused than in noninfused glands (*P* < 0.01). The IgG cell response to parvovirus, however, followed a pattern similar to that found with other antigens rather than a contrasting pattern, as was the case with IgA.

Also, unlike findings in regard to IgA cells in alcohol- and PBS-infused glands, the prevalence of IgG cells in such glands was not suppressed below the IgG cell prevalence observed in normal (uninfused) glands.

As with the IgA cell response to antigens, glands infused with live *B. abortus* contained the greatest prevalence of IgG cells (18 cells per 20 HPF), but this was not significantly different.
from the IgG cell response to infusion of live *S. aureus* (17 cells per 20HPF). As was found with IgA cells after *S. aureus* infusion, IgG cells were numerous in the connective tissue around abscesses (Fig. 2).

The IgG cell response to killed *B. abortus* was also different from the IgA cell response to the same antigen, being significantly greater than the response to parovirus.

**SEROLOGY.** Anti-staphylococcal agglutinins were not detected in the sera of rats at any interval examined. Anti-brucella antibody titers in the sera of rats during lactation and involution are presented in Fig. 3; the titers were higher during early lactation, and thereafter they remained relatively constant.

Hemagglutination inhibition titers in the sera of rats infected with virus are also shown in Fig. 3; there was a continuous increase in these with advancing lactation and involution, and the variation between animals at each interval was minimal.

**DISCUSSION**

Previous studies have concerned the effect of local antigenic stimulation by bacteria (14, 22, 40) and viruses (3, 4) on immunoglobulin secretion in the udder, but these investigations have relied upon either the measurement of antibody titers as determined by serological tests or the detection of changes in absolute concentration of immunoglobulin. Many antibody assay methods do not distinguish between classes of immunoglobulin (25), and a problem in interpreting results in these studies is defining the extent of immunoglobulin contributed by the mammary gland as opposed to that derived from the serum. By quantitating and classifying the actual immunoglobulin-containing cells as in the present studies, we have obtained information which helps clarify the nature of the local immune response in the mammary gland.

Failure of an immunological response in rats infused with PBS and alcohol was expected because these irritants are nonantigenic (38). Since PBS was the standard diluent for antigens used in subsequent experiments, however, examination of its effect on immunoglobulin-containing cell populations was a desirable preliminary experiment which added interpretation of subsequent studies.

Depression of IgA cell prevalence in PBS- and alcohol-treated rats is of interest and may be related to stress factors rather than the injected substances per se. The effect appears to be systemic rather than local, since both infused and noninfused glands were so affected. It is recognized that corticosteroid release in response to stress is immunosuppressant (35); the mere handling and bleeding or parenteral injection of

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**Fig. 1.** IgA cells in the mammary gland of a rat infused with live *B. abortus*. Cells are aggregated in certain areas of the gland; 21 days postpartum. x225.
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Fig. 2. IgG cells in the mammary gland of a rat infused with S. aureus. The cells are most numerous in the connective tissue around an abscess; 12 days postpartum. ×225.

Fig. 3. Antibody titers during lactation and involution in sera collected from rats infused intramammarily with live B. abortus and rat parvovirus 7 days before parturition.

saline or alcohol into rabbits produced lymphopenia (37). It is also possible that decreased IgA cell numbers during involution was due to the stress of weaning. The fact that this decrease was seen in the treated, but not in the normal, rats may possibly be the result of repeated handling; whereas the normal rats were not handled before weaning, those in the treated group had previously been anesthetized and infused so that greater apprehension and stress may well have occurred with this second handling at the time of weaning.

Of particular interest in the present studies was the greater prevalence of both IgA and IgG cells in antigen-infused glands than in noninfused glands of the same animal. The single exception involved the parvovirus group, in which the noninfused gland contained more IgA cells than the infused one. This finding of more immunoglobulin-containing cells in infused glands probably indicates that they attracted more immunoblasts than the noninfused ones. It was suggested (11) that the tissues that receive the major share of the available immunoblasts are those most exposed to challenge from the environment. Subsequent studies have supported this hypothesis (21, 32), and it is now generally accepted that blast lymphocytes extravasate randomly but remain in tissues containing antigen (39).
A slight difference in the number of immunoglobulin-containing cells between the infused and noninfused glands in this study as opposed to a striking appearance of these cells in ewes after antigenic stimulation (21) indicates the species difference; whereas normal mammary glands of ewes have few immunoglobulin-containing cells, they are constantly resident in the rat mammary gland during the period from late pregnancy through parturition and lactation to involution (19).

Whereas histological changes in brucella-infected glands were minimal, abscess formation in S. aureus-infused glands was prominent and was typical of staphylococcal mastitis in other animals (1, 29). The finding in this experiment that generalized mastitis did not occur supports previous observations that the resting mammary gland is more resistant to staphylococci and that the gland has to be in a state of lactation to be most susceptible to the staphylococcal infection (1). The two live antigens chosen in the present experiment appear to represent a paradox with respect to inflammatory as opposed to immune responses; B. abortus caused little inflammation but an appreciable increase in immunoglobulin-containing cells, whereas the reverse was the case with S. aureus. Such results may be explained as follows. B. abortus organisms are taken up by phagocytic cells in which some survive and multiply and are transported to lymphoid tissue where they may persist as a chronic infection (5); hence, they can induce prolonged stimulation of immunocompetent cells. The response to S. aureus, on the other hand, is more intensive at the injection sites (29), and inflammatory mechanisms (especially neutrophilic infiltration) are relatively more important than local and/or systemic immunity against these organisms.

Of particular interest is the finding that live brucellae were far superior to live staphylococci in inducing both systemic and local immunoglobulin responses; no anti-staphylococcal antibody was detected in the sera of rats immunized with S. aureus, and in the infused glands, IgA cells were more prevalent in the brucella group than in the staphylococcal group.

In the present experiment the infusion of S. aureus into the preparturient mammary gland of rats resulted in a significant increase in immunoglobulin-containing (predominantly IgA) cells as early as 7 days postinfusion. In comparison, it has been confirmed that the bovine mammary gland has moderate deficiency in its ability to synthesize IgA both in vitro (6) and in vivo (25).

The response to parvovirus in this experiment probably illustrates more effectively than responses to the other antigens studied the difference in locally produced, as opposed to systemically produced (but perhaps locally secreted), antibody in the mammary gland. Although intramammary parvovirus infusion induced a significant ($P < 0.05$) elevation of IgA or IgG cells in the mammary gland, it was not striking and contrasted with a marked increase in serum antibodies as indicated by the hemagglutination inhibition test. The different pattern of response to parvovirus as compared with other antigens might be due to rapid transmission of the virus from the mammary gland to other body sites so that the mammary gland may serve simply as a "portal-of-entry." Probably other infused antigens remained more localized, thereby inducing a greater local response.

Questions concerning the apparent elevation of IgA cell numbers in the noninfected involuting glands and of IgG cells in the injected glands in the virus group essentially remain unanswered. Although the parvovirus selectively attacks rapidly dividing cells (15), it is possible that it may persist in other cells, e.g., macrophages, and could result in continued antigenic stimulation or could induce selective localization of immunocytes in various locations.

With regard to our finding of a greater number of IgG cells in the infused glands of the virus group, it is of interest that Bohl et al. (3, 4) also vaccinated pregnant sows intramammarily with live (attenuated transmissible gastroenteritis) virus and found that antibodies in milk from these animals were primarily of the IgG class. Such results indicate that the route of infection or vaccination with virus influences the immunoglobulin class of antibodies in secretion, since after natural infection or experimental oral infection with transmissible gastroenteritis virus, the antibody in mammary secretion was mainly of the IgA class (33).

LITERATURE CITED


5. Burrin, D. H., J. Kepple, and H. Smith. 1966. The isolation of phagocytes and lymphocytes from bovine blood and the effect of their extracts on the growth of
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