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Morphological Changes Caused by Experimental *Streptococcus uberis* Mastitis in Mice following Intramammary Infusion of Pokeweed Mitogen (42375)

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Abstract. Mice were used as models for bovine mastitis in an attempt to modify the susceptibility of mammary glands to *Streptococcus uberis* infection. Murine mammary glands were injected with pokeweed mitogen (PWM) prior to experimental bacterial challenge to accelerate involution and enhance antimicrobial mechanisms. PWM injection reduced the numbers of streptococci recovered when compared to controls. Histological examination of tissues from PWM-treated mice revealed a reduction in secretory activity and advanced involution. PWM-treated tissues had considerably more leukocytes infiltrating the epithelium, lumen, and underlying connective tissue. Bacteria were observed within the epithelium and alveolar lumen and internalized within neutrophils and macrophages in both PWM-injected and control tissue. Results of this study suggest PWM injection provided some protection against *S. uberis* mastitis by accelerating mammary involution, enhancing antimicrobial defenses, and facilitating a marked cellular response prior to bacterial challenge.

Streptococcus uberis is second only to *S. agalactiae* in the causation of bovine streptococcal mastitis. Recent reports indicate that 73% of British herds harbor at least one animal infected with this organism (1). *S. uberis* is the major cause of infection prior to calving in first calf heifers and is the most common pathogen during the dry period of cows (2, 3). Extensive epidemiological studies on *S. uberis* indicate a high percentage of isolates on udder surfaces, as well as on other sites on the cow and in the environment which may serve as reservoirs of infection (4). *S. uberis* is not an obligate pathogen and can survive on extramammary sites such as pastures and bedding (5). Because this is a widely disseminated pathogen, conventional control measures based on postmilking teat disinfection and antibiotic therapy are generally ineffective against *S. uberis* mastitis. Attempts have been made to enhance the natural resistance of the mammary gland to mastitis by increasing neutrophil populations to protective levels (6) and increasing nonspecific soluble components of lacteal secretions during early involution when new infections are prevalent (7). Plant lectins concanavalin A and phytohemagglutinin have been intramammarily injected in an attempt to stimulate local immune mechanisms and accelerate mammary involution (8). However, the possibility of modifying natural resistance to *S. uberis* through elevating leukocyte populations within mammary parenchyma has not previously been examined. Therefore, the purpose of this study was to characterize the pathogenicity and leukocytic response of the murine mammary gland to experimentally induced *S. uberis* mastitis following immunostimulation with the lectin pokeweed mitogen (PWM).

Materials and Methods. *Experimental animals.* Mice of the CD-1 strain were purchased from Charles River Breeding Laboratories, Inc., and bred at the Hill Farm Research Station. Preliminary attempts were made to use mammary glands lactating 7–10 days. However, morphometric analysis was impossible due to the large percentage of adipocytes (approximately 90%) occupying the total tissue area. For this reason, intramammary inoculations of PWM were performed on Day 2 of lactation. The offspring were removed 6 hr prior to initial treatment of their lactating parent.

Bacteriological media. Tryptose blood agar (Difco Laboratories, Detroit, Mich.) was prepared with 5% bovine calf blood and 0.1% esculin (Sigma Chemical Co., St. Louis, Mo.) and incubated for 24 hr at 37°C.

S. uberis preparation. A virulent strain of *S. uberis,* isolated from a clinical case of bovine mastitis, was used for experimental challenge.

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TABLE I. INFLUENCE OF PWM ON THE COLONIZATION OF *S. uberis* FOLLOWING EXPERIMENTAL CHALLENGE

Treatment	Animal no.	Bacterial isolation[a]
PWM	1	1.7×10^3
	2	3.5×10^3
	3	6.4×10^4
	4	1.0×10^4
	5	9.5×10^4
	6	1.3×10^4
Average		3.1×10^4
Control	7	7.3×10^5
	8	1.8×10^7
	9	5.2×10^6
	10	6.3×10^6
Average		7.6×10^6

[a] Mean number of organisms estimated from tissue homogenate by the plate count method.

The inocula were prepared from frozen cultures of the strain in Todd Hewitt broth and then incubated for 24 hr at 37°C. Subcultures were grown for 18 hr on tryptose blood agar, washed in physiological saline, and centrifuged at 10,000 rpm. The organisms were suspended in isotonic saline to a turbidity equivalent to a No. 1 McFarland ($BaSO_4$) standard (approximately 1×10^8 colony-forming units (CFU)/ml). A challenge suspension of approximately 1×10^5 CFU/ml was obtained through a series of 10-fold dilutions of the 1×10^8 CFU/ml suspension. Viable cell counts of the challenge inoculum were verified by inoculating 0.1-ml portions of 10-fold diluted *S. uberis* suspension onto the tryptose blood agar plates. Colonies appearing on the plates after 24 hr of incubation at 37°C were counted and the numbers of viable bacteria were estimated per milliliter of original *S. uberis* suspension.

Mitogen preparation. Pokeweed mitogen from *Phytolacca americana* (Sigma Chemical Co.) was the immunopotentiator used which was previously demonstrated to stimulate both T- and B-cell proliferation (9). PWM (25 μg/ml) was prepared in physiological saline and sterilized through a 0.45-μm Millipore filter.

Infusions. Intramammary injections were carried out on 10 mice lightly anesthetized with sodium pentobarbital. Abdominal mammary glands were swabbed with absolute alcohol to facilitate the visibility of the four posterior teats. Sterile 1-ml polypropylene syringes equipped with 26-gauge $\frac{3}{8}$-in. needles were used to deliver the inocula into abdominal teat ends as described by Chandler (10). PWM suspension (0.1 ml) was intramammarily injected into the right and left fourth abdominal gland of 6 mice before intramammary challenge with *S. uberis*. The remaining 4 mice received no PWM treatment and served as controls. *S. uberis* suspension (0.1 ml) was intramammarily injected into the 10 mice 48 hr following the PWM treatment. All animals were killed with an overdose of sodium pentobarbital 48 hr following *S. uberis* challenge.

Tissue preparation. Tissue samples for histological and cytological examination were obtained at necropsy and prepared for microscopy. From each mouse, the right injected gland was removed and fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.0 at 37°C) for 2 hr. Tissue was then postfixed in 0.1 *M* cacodylate-buffered osmium tetroxide (pH 7.0 at 5°C) for 1.5 hr, dehydrated in a graded series of ethanol, and embedded in epoxy resins. Thick sections (0.5–1 μm) were obtained on a Porter Blum MT-5000 microtome and stained with toluidine blue for light microscopy. Ultrathin sections approximately 60 nm thick were stained with 5.0% uranyl acetate in 50% methanol for 20 min followed by 0.4% lead citrate for 20 min and examined using a Phillips EM 300 electron microscope at 60 kV.

Bacteriological procedures. Left injected glands of all mice were aseptically dissected and transferred to tubes containing 10 ml sterile 0.1 *M* phosphate-buffered saline (PBS) solution. Each gland was completely homogenized through a tissue grinder in 1.0 ml PBS.

TABLE II. HISTOLOGICAL ANALYSIS[a] OF MURINE MAMMARY TISSUE TAKEN FROM PWM-INFUSED AND CONTROL GLANDS

Tissue classification	Treatment groups	
	PWM	Control
Epithelium	40.06 ± 2.2^b	40.73 ± 3.8^b
Lumen	18.83 ± 4.8^b	30.85 ± 4.4^c
Stroma	41.10 ± 3.8^b	28.45 ± 3.6^c

[a] Data are expressed as mean percent of tissue area ± SD.
[b,c] Means between treatment groups lacking identical superscript differ significantly ($P \leq 0.005$).

The CFU of *S. uberis* were estimated from homogenates by the plate count method.

Morphometric analysis. Quantitative morphologic analysis was used to determine percentage mammary tissue area composed of interalveolar connective tissue, epithelia, and alveolar lumen. For each tissue sample, 10 replications of 100 contact points were counted per slide at a magnification of 1000×. A reference grid in the microscope ocular provided fixed points used in the counting process. Alveolar epithelium was further characterized as (1) nonactive, (2) moderately active, or (3) fully active. For each tissue sample, cells were classified in alveolar cross-sections of 10 different fields at 1000×.

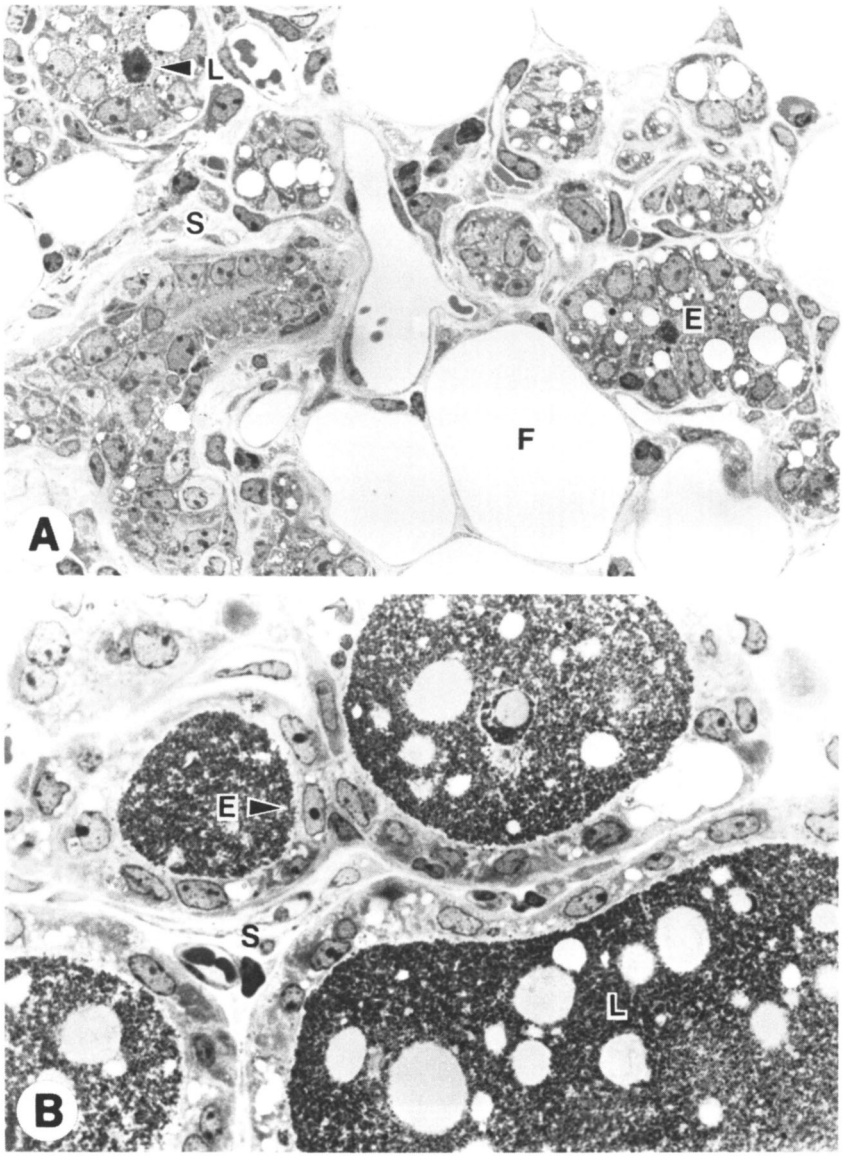

FIG. 1. Murine mammary tissue obtained 48 hr following experimental *S. uberis* challenge. (A) PWM-infused tissue demonstrated reduced luminal area and increased interalveolar stroma when compared with (B) control tissue (×800). E, alveolar epithelium; F, interalveolar fat; L, alveolar lumen; S, interalveolar stroma.

TABLE III. CYTOLOGICAL ANALYSIS[a] OF EPITHELIUM FROM PWM-INFUSED AND CONTROL GLANDS

Epithelial classification	Treatment groups	
	PWM	Control
Nonactive	38.33 ± 13.3[b]	15.00 ± 10.0[c]
Moderately active	33.33 ± 8.2[b]	30.00 ± 8.2[b]
Fully active	28.33 ± 17.2[b]	55.00 ± 12.9[c]

[a] Data are expressed as mean percentage of tissue area ± SD.

[b,c] Means between treatment groups lacking identical superscript differ significantly ($P \leq 0.005$).

Tissue specimens of mammary parenchyma were also examined for the presence of neutrophils, lymphocytes, macrophages, mast cells, and plasma cells. Prevalence of these cell populations was quantitated morphometrically at 1000× in 10 randomly selected microscopic fields per sample.

Ultrastructural examination. Based on light microscopic observations, tissue areas were selected to evaluate secretory cell activity and cytological integrity. Epithelial cells were examined for the presence and development of organelles including Golgi components, rough endoplasmic reticulum (RER), mitochondria, and nuclei. Cytoplasmic areas were also examined for presence of lysosomes, degenerating junctional complexes, microvilli at apical surfaces, and accumulation of milk stasis vacuoles, secretory vesicles, and fat droplets.

Pathological changes of infected glands were also examined. Parameters examined included the presence of bacteria within the secretory epithelium and alveolar lumen, degree of epithelial cell degeneration, and influx of leukocytes within the alveolar epithelium.

Results. Infusion of PWM into murine mammary glands 48 hr prior to challenge reduced the CFU recovered when compared to control glands. Numbers of streptococci recovered from injected mammary glands 48 hr after challenge are presented in Table I. Although *S. uberis* was not eliminated from glands receiving the immunopotentiating agent, growth of the organisms was inhibited.

Histological analysis of tissues from PWM-injected mice revealed a reduction in secretory activity when compared to control mice (Table II). PWM-treated tissue exhibited more interalveolar stroma (+12.7) and less luminal area (−12.0), indicating a progression toward involution (Figs. 1A, 1B). Both treatment groups had similar percentages of total alveolar epithelium (approximately 40%). However, cy-

TABLE IV. CYTOLOGICAL COMPARISON[a] OF INFILTRATING CELLS IN MAMMARY PARENCHYMAL TISSUE FROM PWM-INFUSED AND CONTROL GLANDS

Cell types	Treatment groups		Difference between groups	
	PWM	Control	No. of cells	% Difference
Epithelial lining				
Mononuclear	15.3	3.0	−12.3	−80.4
Neutrophils	12.7	6.8	−5.9	−46.6
Total	27.9	9.8	−18.2	−65.2
Lumen				
Mononuclear	34.5	10.3	−24.2	−70.1
Neutrophils	4.5	4.0	−0.5	−11.1
Total	38.5	14.3	−24.7	−64.2
Stroma				
Mast cells	9.7	2.0	−7.7	−79.4
Plasma cells	21.3	13.8	−7.5	−35.2
Mononuclear	34.0	13.0	−21.0	−61.8
Neutrophils	11.0	8.5	−2.5	−22.7
Total	76.0	37.3	−38.7	−50.9

[a] Data are expressed as mean number of cells/6 × 10^5 μm^2 of mammary parenchyma.

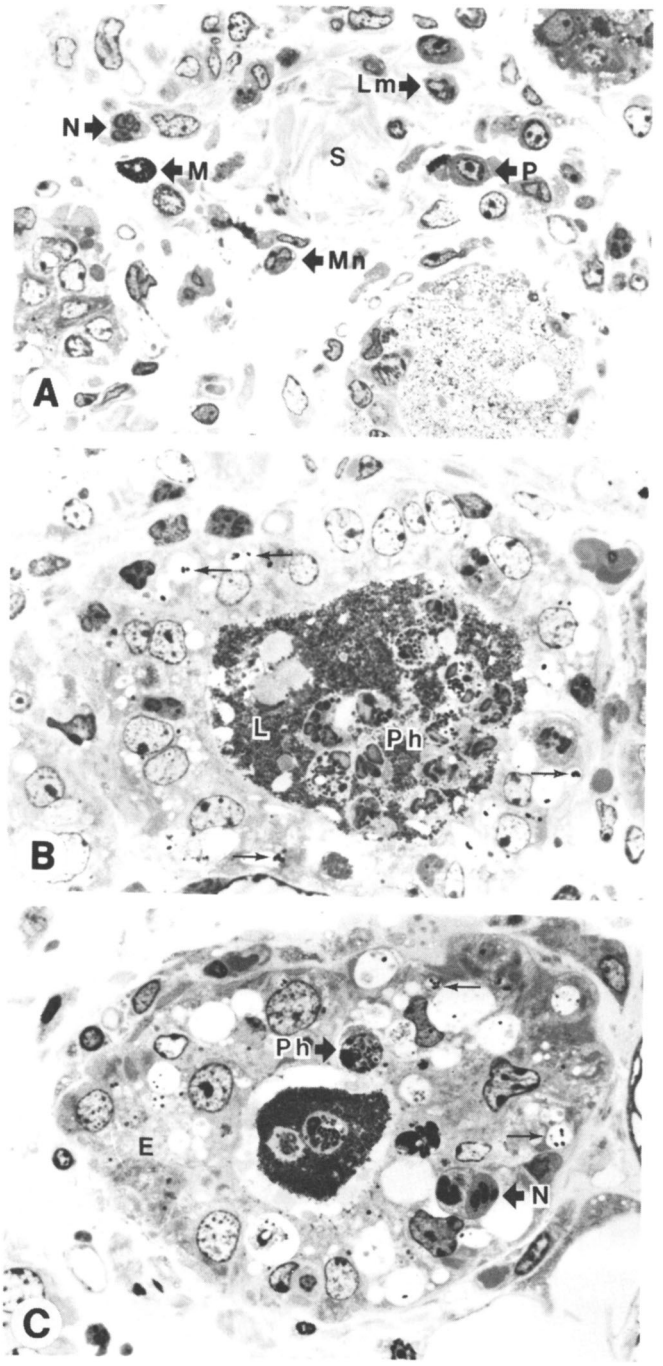

FIG. 2. (A) Subepithelial stromal area representative of both PWM-infused and control tissue demonstrating the high degree of leukocyte infiltration. (B) Alveolar lumenal area representative of PWM-infused tissue exhibiting numerous monocytes and neutrophils. (C) Alveolar lining representative of both PWM-infused and control tissues demonstrating leukocyte penetration of the epithelium (×800). Arrows, cytoplasmic vacuoles containing bacteria; L, alveolar lumen; Lm, lymphocyte; M, mast cell; Mn, monocyte; N, neutrophil; P, plasma cell; Ph, phagocyte; S, interalveolar stroma.

tological analysis of epithelia demonstrated a marked increase in nonactive cells and a reduction in fully active cells in PWM-injected tissue when compared with control tissue (Table III).

Quantitative microscopic analysis of PWM-injected glands demonstrated an intense cellular reaction to the immunopotentiating agent. Although a moderate leukocyte response was evident in control tissues, immunostimulated tissues had considerably more cells infiltrating underlying connective, lumen, and epithelial tissue (Table IV).

Subepithelial stromal areas of both PWM-injected and control tissues were the most common sites of leukocyte infiltration with a total mean number of 76.0 and 37.3 cells enumerated per area of parenchyma, respectively. Comparison of total cell numbers found in the subepithelial stroma of PWM-injected and control glands demonstrated a 50.9% reduction of all cell types in the control tissue. Mononuclear cells (lymphocytes and macrophages) were the most prevalent cell types in the subepithelial connective tissue of PWM-injected glands followed by plasma cells, neutrophils, and mast cells. Both mononuclear cells and plasma cells were the most prevalent cell types in the subepithelial connective tissue of control glands followed by neutrophils and mast cells (Fig. 2A).

Quantitation of leukocytes within the alveolar lumen of PWM-injected and control glands demonstrated a 64.2% reduction of all cell types in control tissue. Mononuclear cells were the most prevalent cell types in both PWM-injected and control tissue with a mean of 34.5 and 10.3 cells per area of parenchyma, respectively. Neutrophils were observed less frequently within the alveolar lumen with approximately four cells per area of parenchyma in each treatment group (Fig. 2B).

The epithelial lining of PWM-injected and control tissues was the least common site of

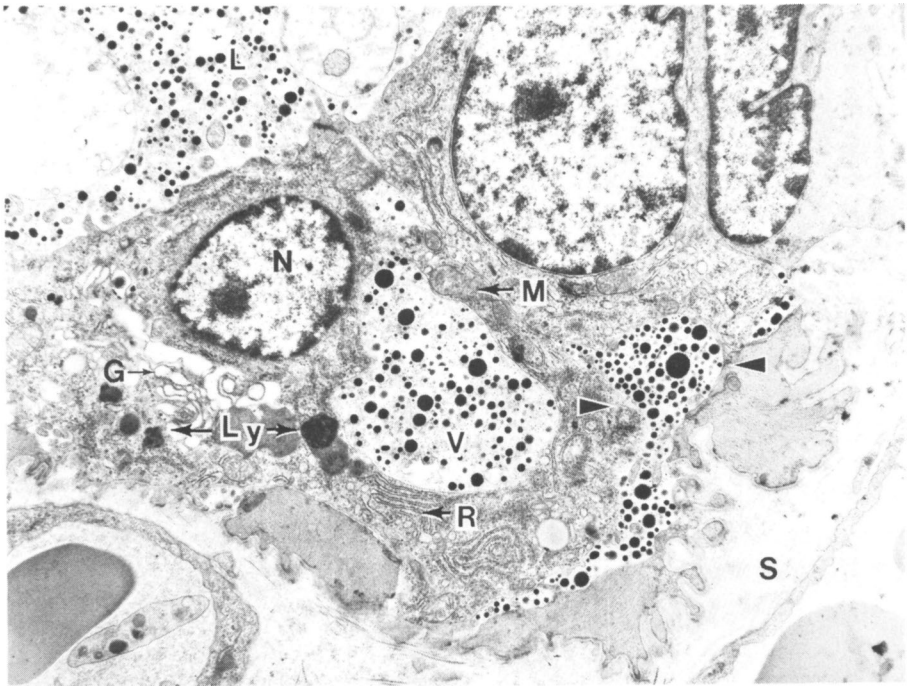

FIG. 3. Involuted mammary cell typical of those found in PWM-infused gland 48 hr following experimental *S. uberis* challenge. Golgi components consist only of dictyosome elements, mitochondria are few and appear swollen, and RER is discontinuous with only a few scattered fragments. Cytoplasmic area is occupied by lysosomes and milk stasis vacuoles. Presence of milk components is observed in the alveolar lumen as well as between the basal plasma membrane and the underlying connective tissue (arrow heads) (×6340). G, Golgi dictyosomes; Ly, lysosomes; M, mitochondria; R, rough endoplasmic reticulum; S, interalveolar stroma.

leukocyte infiltration with a total mean number of 27.9 and 9.8 cells enumerated per area of parenchyma, respectively. Comparison of total cell numbers found within the epithelial lining of PWM-injected and control glands demonstrated a 65.2% reduction of all cell types in the control tissue. Mononuclear cells were the most prevalent cell type of the epithelium of PWM-injected glands with a mean number of 15.3 cells per area of mammary parenchyma. Neutrophils were less numerous with a mean number of 12.7 cells. Conversely, neutrophils were the most prevalent cell type of the epithelial lining of control glands, and mononuclear cells were observed less frequently (Fig. 2C).

Ultrastructural examination of epithelial cells demonstrated a decrease in secretory activity and advanced involution in tissue from PWM-injected glands when compared with tissue from control glands. Cells from PWM-injected glands exhibited minimal cytoplasmic area, lack of cellular polarity, pyknotic and medially-located nuclei, and abundant fat droplets scattered about the cytoplasm. The cytoplasmic area of cells from PWM-injected glands was characterized by a lack of organelle development. Golgi dictyosomes were few and indistinct, RER was observed as scattered fragments, mitochondria were infrequently observed and only appearing as swollen masses, and microtubules were absent. The cytoplasm also contained lysosomes and milk stasis vacuoles. Plasma membranes of PWM-injected cells were indistinct with little to no apparent apical microvilli or lateral junctional complexes (Fig. 3).

Ultrastructural examination of alveolar epithelial cells from control tissues indicated increased secretory activity when compared with PWM-injected cells. Cells were more polarized with often rounded medially to basally located nuclei and a larger cytoplasmic-to-nuclear ratio. The cytoplasm of control cells exhibited more organelle development with some RER located in the basal and lateral cytoplasm and a distinct supranuclear Golgi apparatus.

Pathological changes resulting from *S. uberis* challenge were frequently observed in tissue obtained from control glands. Infected epithelial cells were identified by their flattened appearance (Fig. 4). The cytoplasm of these

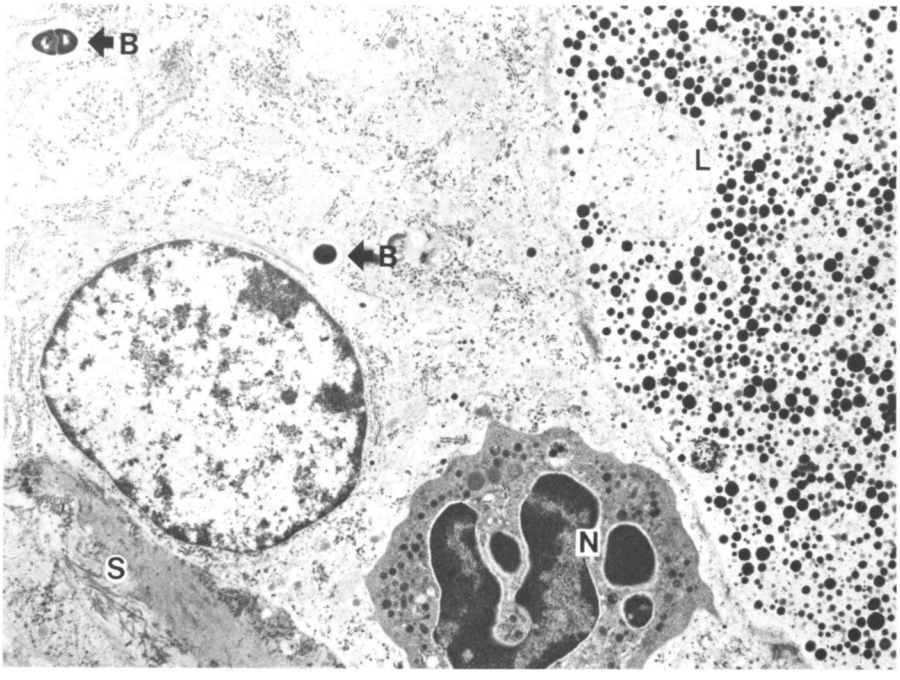

FIG. 4. Infected secretory cells observed in tissue from control glands 48 hr following *S. uberis* challenge appeared flattened. The cytoplasm is occupied by a disruptive RER network, dividing bacterial cells, and penetrating neutrophils (×6340). B, bacteria; L, alveolar lumen; N, neutrophil; S, interalveolar stroma.

degenerating cells demonstrated disruptive RER cisternae, swollen mitochondria, and internalized cocci. Necrotic epithelial cells of infected control tissue were devoid of any organelle organization and often contained lysed nuclear membranes (Fig. 5). The cytoplasm was largely occupied by cellular debris and frequently contained bacterial cells, neutrophils, and degenerative phagocytes with internalized cocci.

Discussion. Although considerable information is available regarding natural defense systems of the mammary gland (11, 12), a practical method of enhancing resistance to mastitis has not been elucidated. The importance of neutrophils in containing bacteria and suppressing their growth has been identified, and several techniques for stimulating rapid infiltration into mammary tissue are currently being developed (6). However, relatively little research has been directed toward the defense potential of the entire cell-mediated immune (CMI) response of the mammary gland mastitis. This study examined leukocyte infiltration following PWM injection, and the effect of these cell populations on *S. uberis* mastitis.

Bacteriological examination of murine mammary glands 48 hr following *S. uberis* inoculation demonstrated a reduction in the numbers of streptococci recovered from PWM-treated tissues. Fewer bacterial isolates can, in part, be attributed to the presence of elevated leukocyte populations in the mammary gland at the time of streptococcal challenge. Previous studies indicated that high phagocytic cell populations protect the nonlactating gland from intramammary infection (IMI) (13, 14). Results of this study suggest a substantial percentage increase in both neutrophils (+11.1%) and mononuclear cell (+70.1%) populations within the alveolar lumens of PWM-stimulated tissue when compared to controls. Microscopic examination revealed the ability of these phagocytic cells to ingest and internalize the streptococci.

Histological analysis of tissues from PWM-injected mice revealed a reduction in secretory activity and advanced involution when com-

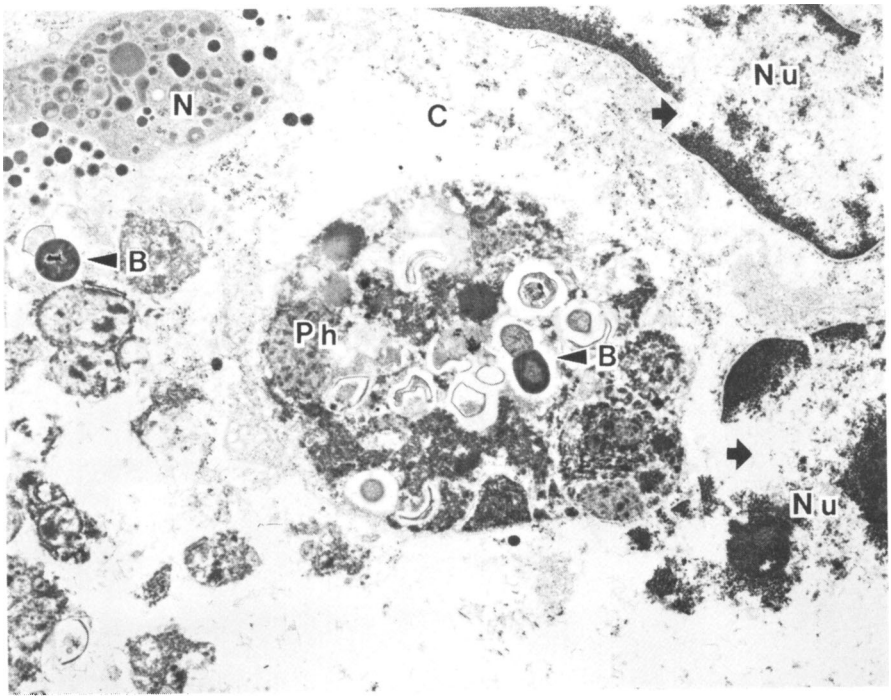

FIG. 5. Damaged mammary cells observed in control tissues often contained bacteria within the cytoplasm; cocci were also internalized within degenerative phagocytes. The nuclear membranes are perforated (arrows) and the cytoplasm is occupied by cellular debris (×10,275). B, bacteria; C, cytoplasmic area; N, neutrophil; Nu, nucleus; Ph, phagocyte.

pared to control mice. Previous studies found involuted glands to have an increased resistance to intramammary infection (7). Mammary secretion from nonlactating cows has a distinct composition which is unique from either milk or colostrum. Secretion from involuted mammary glands contains elevated natural protective factors including phagocytes, lymphocytes, immunoglobulins, and bacteriostatic proteins such as lactoferrin (15, 16). Mammary secretion from dry cows also contains lower concentrations of casein, lactose, and citrate which can be utilized by invading bacteria for growth and colonization (15, 16). Intramammary injection of plant lectins (concanavalin A and phytohemagglutinin) and endotoxin during early involution have been shown to accelerate the involution process, increase levels of natural protective factors, and inhibit bacterial growth (7, 8). In this study, intramammary injection of PWM 48 hr prior to *S. uberis* challenge expedited the involutionary process which would normally occur gradually over several days. PWM-injected tissue exhibited 12.7% more interalveolar stroma and 12.0% less luminal area when compared to controls. Cytological analysis of PWM-injected epithelia demonstrated a marked increase in nonactive cells and a reduction in fully active cells. Moreover, ultrastructural examination of secretory epithelium exhibited minimal cytoplasmic area, lack of cellular polarity, and accumulation of large fat droplets within the cytoplasm. During involution, lysosome formation was triggered, and a large influx of phagocytic cells caused heterophagocytic degeneration of secretory tissue. These observations are consistent with previous studies which suggest degenerative alterations of epithelial cells during involution result from both lysosomal digestion and phagocytosis by macrophages (17).

The pathogensis of *S. uberis* mastitis was effectively modified by intramammary PWM injected prior to experimental challenge. Elevating leukocyte numbers may have played a role in preventing rapid streptococcal colonization and the subsequent production of pathogenic toxins.

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