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KINETICS OF ENDOTOXIN-INDUCED INFLAMMATION IN OVINE MAMMARY GLAND

IAN G. COLDITZ[1] and B. L. PRESSON[2]

[1]*CSIRO Division of Animal Health*
Armidale NSW, Australia, 2350

[2]*Department of Animal Science, University of New England*
Armidale NSW, Australia, 2351

Abstract—The inflammatory response to stimulation with endotoxin of lactating and nonlactating mammary glands of sheep was examined. Similar numbers of neutrophils and mononuclear cells were recovered from nonlactating glands sampled every 2 h for 8 h and in glands sampled once at 8 h. Thus the inflammatory response was initiated by 2 h and repeated sampling did not modify the time course of the response. In contrast, in lactating ewes, fewer cells were recovered from glands sampled every 2 h than from glands sampled once at 8 h. Fewer neutrophils were also recovered when glands were serially sampled from 4 h to 8 h. Thus, removal of milk and inflammatory exudate modified the time course of the leukocyte influx into lactating glands. Significant accumulation of neutrophils occurred by 2 h in dose–response experiments in nonlactating glands. Peak accumulation of neutrophils occurred between 2 and 6 h, and a marked decline occurred after 8 h. In lactating glands, a slower onset and longer duration of neutrophil accumulation occurred. Twenty- to 30-fold more neutrophils were recovered by 8 h in lactating than nonlactating glands. This difference was not due to a lower threshold of sensitivity to endotoxin. Infusion of milk into nonlactating glands did not modify the intensity or time course of the inflammatory response to endotoxin. Thus, the physiological state of resident cells within the lactating gland, rather than the interaction of inflammatory exudate with milk, can account for the different reaction pattern in lactating glands. Inflammation in the nonlactating gland closely resembles inflammatory rsponses in skin and provides a convenient model for investigating the initiation and regulation of inflammatory processes.

INTRODUCTION

Many models have been developed for studying the pathobiology of inflammation. These models fall into two classes: those which permit harvesting the inflammatory exudate from body cavities and those which permit the observa-

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tion (by microscopy, spectroscopy, or gross tissue swelling) of the inflammatory infiltrate in solid tissues. The recovery of the inflammatory exudate from a body cavity allows analysis of cell populations and humoral products accumulating during the inflammatory response without requiring the death of experimental animals. The mammary glands of sheep provide an easily accessible cavity from which inflammatory exudates can be readily collected. In the present experiments, the characteristics of neutrophil accumulation in the ovine mammary gland have been investigated to determine the suitability of this model for further analysis of inflammatory processes.

The kinetics of the accumulation of radioisotope-labeled leukocytes in inflammatory lesions has been studied extensively in the skin of rabbits by Movat and his colleagues (1–4). It was found that the accumulation of neutrophils induced by a wide range of inflammatory agents peaked in lesions 1–3 h old. Very few neutrophils entered older lesions. Recently these observations have been repeated in skin of sheep, and a similar pattern of neutrophil accumulation was found (5). The large skin area of rabbits and sheep enables the placement of multiple lesions on individual animals for the study of the kinetics of the inflammatory response. The kinetics of inflammatory in body cavities has been less thoroughly studied because of the presence of just one or a few experimental sites per animal. This limitation could be avoided if serial sampling did not modify the progression of the inflammatory response in a body cavity. In the present experiments, the effect of repeated sampling on the kinetics of the inflammatory response in individual mammary glands has been investigated.

MATERIALS AND METHODS

Animals. Experiments were done in multiparous lactating and nonlactating Merino ewes kept at pasture. Lactating ewes were in the second or third month of lactation.

Reagents. Endotoxin (*Escherichia coli* serotype 055:B5, Sigma Chemical Company, St. Louis, Missouri) was dissolved in pyrogen-free saline (PFS, Travenol Laboratories, Toongabbie, Australia). A stock solution of 1 mg/ml was prepared and test concentrations of 5 μg/ml to 5 ng/ml were made by dilution in PFS. Oxytocin (Intervet Internation B.V., Boxmeer, Holland) was diluted to 50 mU/ml in sterile water for injection (Travenol).

Experimental Procedure. Intramammary infusions were made through sterile 18G cannulae via the teat canal. Mammary secretions were collected into silicone-coated flasks. In nonlactating ewes, mammary secretions were collected by infusing 20 ml PFS, gently massaging the gland and expressing the washings into flasks. In lactating ewes, milk was collected after intraveneous injection of 100 mU oxytocin. Lambs were withheld from ewes for the duration of each experiment.

Analysis of Secretions. Total cell counts were performed in a hemocytometer. Cells in mammary washings from nonlactating ewes were counted following appropriate dilution in phosphate-buffered saline (PBS). Cells in milk were first sedimented by centrifugation for 10 min at 160*g* and resuspended in PBS for hemocytometer counts. Air-dried smears were stained in Diff

Quick (Lab-Aids, Narrabeen, Australia) and 200 cells per smear were classified as mononuclear or neutrophil leukocytes.

Statistical Methods. Comparisons between treatments in paired glands were made by paired *t* tests on log-transformed data.

RESULTS

Cell Content of Unstimulated Mammary Secretions. The total cell concentration in washings from involuted glands was $3.9 \pm 0.7 \times 10^5$ cells/ml (mean ± standard error of mean of 26 glands). Neutrophils comprised 1.3 ± 1.0% of these cells. Milk contained $9.1 \pm 1.1 \times 10^4$ cells/ml of which 1.9 ± 0.4% were neutrophils (28 glands). One eosinophil was seen in 5200 cells from quiescent involuted glands, and two eosinophils were seen in 5600 cells examined in normal milk. No eosinophils were observed in inflammatory exudates at any time.

Effect of Repeated Sampling on Accumulation of Leukocytes in Inflammatory Exudates. The effect on the total leukocyte influx of withdrawing the inflammatory exudate at regular intervals was examined. After washing out glands at 0 h, nonlactating ewes received 500 ng endotoxin in each gland, then right glands were sampled every 2 h. After 8 h, the left glands were washed out for the first time, and total numbers of neutrophils and mononuclear leukocytes collected from each gland were compared (Table 1). A similar number of neutrophils and mononuclear cells were collected from both glands. This result indicates that withdrawing the inflammatory exudate at regular intervals did not modify the progression of the inflammatory response. In lactating ewes, significantly fewer cells were harvested from glands sampled every 2 h than from opposite glands sampled for the first time at 8 h. When the inflammatory response was allowed to proceed for 4 h before the first sample was collected from repeatedly sampled glands a difference in the yield of neutrophils from the paired glands was still apparent at 8 h. Similar numbers of mononuclear cells, however, were recovered from both glands in this experiment (Table 1). Thus the early and repeated removal of the inflammatory exudate in milk diminished the intensity of the inflammatory response in these glands. The total number of neutrophils collected from lactating glands by 8 h was 20–30 times that recovered from nonlactating glands.

Kinetics of Neutrophil Accumulation in Endotoxin-Stimulated Nonlactating Glands. The time course of neutrophil accumulation in glands receiving 500 ng endotoxin was calculated from the data in the previous experiment and is shown in Figure 1. Most neutrophils entered mammary secretions between 2 and 4 h. The kinetics of the inflammatory response to infusion of 5 μg and 50

Table 1. Total Number of Neutrophils Harvested from Mammary Glands Stimulated with 500 ng Endotoxin

Ewes	*N*	Gland	Sampling times (h)	Number of neutrophils ($\times 10^7$)	Probability left vs. right	Ratio R/L (probability)	Number of mononuclears ($\times 10^7$)	Probability left vs. right
Exp. 1. Nonlactating	5	Left	8	29.19 ± 6.10	$P > 0.2$	1.55 ± 0.43	1.87 ± 0.70	$P > 0.2$
		Right	2, 4, 6, 8	37.55 ± 4.53		($P > 0.4$)	1.37 ± 0.48	
Exp. 2. Lactating	4	Left	8	1173.6 ± 63.2	$P < 0.01$	0.24 ± 0.04	63.2 ± 25.3	$P < 0.05$
		Right	2, 4, 6, 8	2566.6 ± 15.3		($P < 0.001$)	15.3 ± 1.8	
Exp. 3. Lactating	4	Left	8	572.1 ± 11.22	$P < 0.05$	0.51 ± 0.13	13.9 ± 9.9	$P > 0.4$
		Right	4, 6, 8	251.0 ± 3.7		($P < 0.05$)	12.5 ± 3.0	

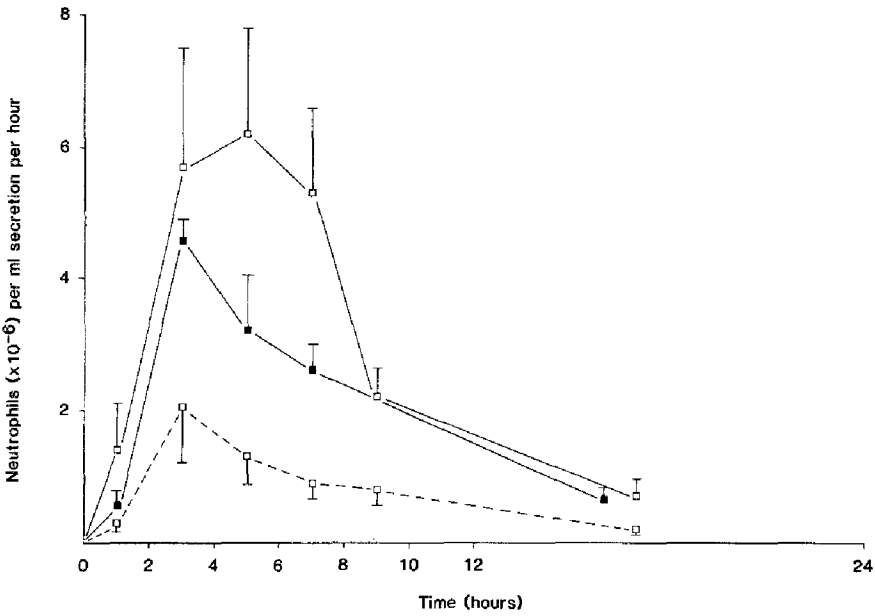

Fig. 1. Kinetics of the accumulation of neutrophils in nonlactating mammary glands. Glands were stimulated with 50 ng (□----□), 500 ng (■——■), or 5 μg (□——□) endotoxin at time 0 and the accumulation of cells in intervals up to 24 h was determined. Data points are means ± SEM of results in four or five ewes.

ng endotoxin was investigated in opposite glands of four nonlactating ewes. The peak neutrophil influx also occurred in the interval between 2 and 4 h in glands receiving 50 ng endotoxin. In contrast, the peak neutrophil influx occurred 2 h later in glands receiving 5 μg endotoxin. Thus, at these doses the responses in contralateral glands progressed independently. A marked decline in the number of neutrophils entering secretions occurred after 8 h and less than 1×10^6 neutrophils/ml/h entered secretions between 10 and 24 h (Figure 1).

Kinetics of Neutrophil Accumulation in Endotoxin-Stimulated Lactating Glands. The initial experiment indicated that regular removal of milk from inflammed glands modified the intensity of the inflammatory response in lactating glands. With this limitation in mind, the kinetics of neutrophil accumulation in repeatedly sampled lactating glands was investigated. From the earlier data the kinetics of the response to 500 ng endotoxin was calculated (Figure 2). In contrast to nonlactating glands, very few neutrophils entered secretions during the first 2 h. Subsequently, neutrophils continued to enter milk in increasing numbers up to 8 h when sampling ceased. As neutrophil concentrations in milk

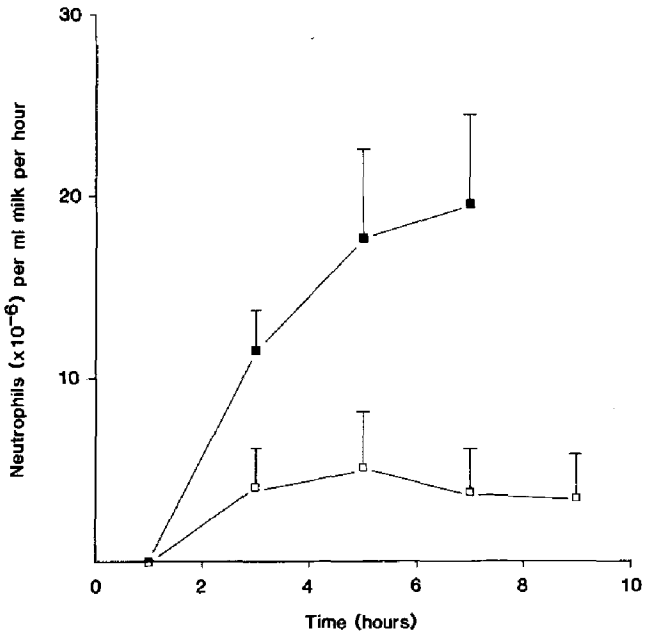

Fig. 2. Kinetics of the accumulation of neutrophils in lactating mammary glands following stimulation with 50 ng (□——□) or (500 ng) (■——■) endotoxin. Data points are means ± SEM of four animals.

from glands receiving 500 ng endotoxin were about three times greater than in washings from nonlactating glands receiving 5 μg endotoxin, the response in lactating glands to two lower doses, 50 ng and 5 ng, was examined. No response to 5 ng endotoxin was observed. (In a separate experiment, 5 ng endotoxin induced significant accumulation of neutrophils in nonlactating glands by 6 h.) A delay in the onset of neutrophil accumulation was again observed with 50 ng, and no distinct peak in the neutrophil influx was observed by 10 h (Figure 2). These results suggested that a more protracted influx of neutrophils occurred in lactating glands stimulated with endotoxin. To further investigate this point, the response to stimulation of lactating glands with 500 ng endotoxin was examined up to 32 h. Milk was removed at 4-h intervals up to 12 h; then ewes were returned to their lambs for the night. From 8 to 12 h, large numbers of neutrophils entered milk, whereas in nonlactating glands the number of neutrophils entering secretions declined markedly at this time. At 24 h, ewes were again milked out and fresh neutrophils accumulating in the subsequent two 4-h intervals up to 32 h were measured. Around 3×10^6 neutrophils/ml/h continued to enter milk up to 32 h (Figure 3).

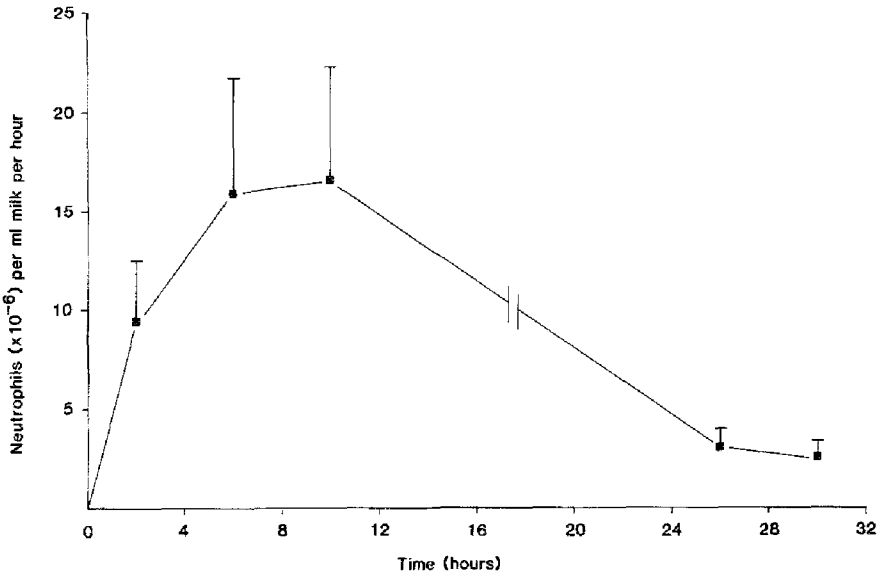

Fig. 3. Kinetics of the accumulation of neutrophils in lactating glands receiving 500 ng endotoxin. The inflammatory exudate was harvested at 4-h intervals up to 12 h and again from 24 to 32 h. Data points are means ± SEM of four animals.

Role of Milk in Modulating Neutrophil Influx in Lactating Glands. The delay in onset, the greater intensity, and the protracted time course of the neutrophil influx in lactating glands could be a consequence of the interaction of the inflammatory exudate with milk or may reflect an intrinsic difference in the response of the tissues of lactating and nonlactating glands to stimulation by endotoxin. The capacity of milk to modify the pattern of neutrophil accumulation was determined by infusing milk into nonlactating glands. Four nonlactating ewes received 500 ng endotoxin in 20 ml PFS in one gland and 500 ng endotoxin in 20 ml pooled milk in the contralateral glands. At 2-h intervals the PFS and milk were recovered for analysis of the inflammatory response, and a further 20 ml PFS or milk (without endotoxin) was infused into each gland. The pooled milk was collected aseptically from four ewes and held overnight at 4°C before infusion into nonlactating glands. The pooled milk contained 3.8×10^3 cells/ml of which 3% were neutrophils. In two control glands, a single infusion of pooled milk (without endotoxin) induced the infiltration of 4.6 and 1.6×10^5 neutrophils by 8 h. Figure 4 shows the kinetics of neutrophil accumulation in glands receiving endotoxin in PFS and in milk. No significant differences in concentrations of neutrophils in secretions from each gland was observed. The peak accumulation of neutrophils in both treatments occurred

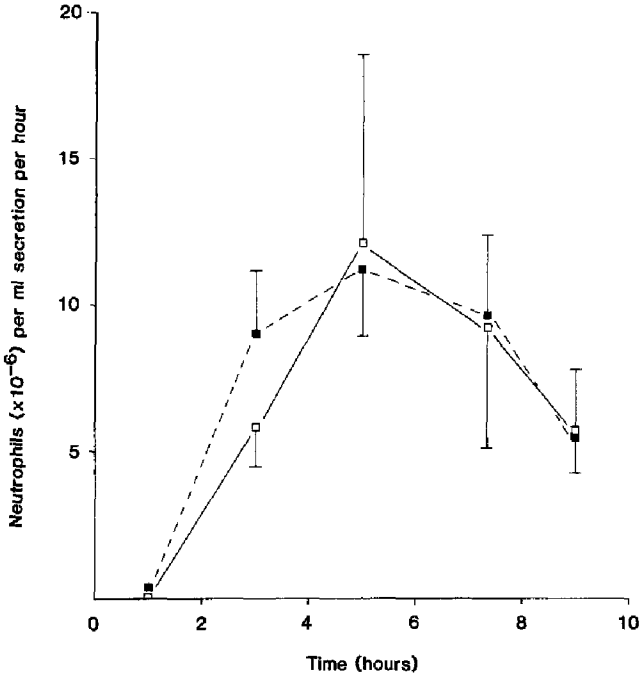

Fig. 4. Kinetics of the accumulation of neutrophils in nonlactating mammary glands receiving 500 ng endotoxin in 20 ml pyrogen-free saline (□——□) or 20 ml pooled milk (■----■). Milk and PFS were recovered after 2 h and replaced with 20 ml fresh milk or PFS for the subsequent 2 h. Data points are means ± SEM of results from four animals.

from 4 to 6 h, in contrast to the earlier peak recorded in nonlactating glands receiving 500 ng endotoxin shown in Figure 1.

DISCUSSION

The nonlactating mammary gland of multiparous Merino ewes has a potential volume of around 20 ml. It is easily accessible for a traumatic infusion of inflammatory agents and repeated sampling of inflammatory exudates. In these experiments, it was found that repeated sampling of the nonlactating gland did not modify the total leukocyte influx into the gland over an 8-h period. In contrast, removal of milk and inflammatory cells from the lactating gland diminished the total leukocyte influx even when glands were left 4 h before the first sample was withdrawn. Lactating glands yielded 20- to 30-fold more cells than did nonlactating glands by 8 h when stimulated with 500 ng endotoxin. This

was not due to a lower threshold of sensitivity of lactating glands as 5 ng endotoxin provoked a response in nonlactating but not in lactating glands. The accumulation of neutrophils in inflammatory lesions is correlated with blood flow to the site (6). The lactating gland is highly vascular, and this alone may account for the greater intensity of the neutrophil influx in lactating glands. Alternatively, amplification of the inflammatory signal or intrinsic regulation via negative feedback of the inflammatory response may be altered in lactating glands. Neutrophils rapidly phagocytose casein micelles (7) and milk fat globules (8), which could lead to the generation of secondary inflammatory signals in lactating glands. The transfer of milk to nonlactating glands failed, however, to increase the intensity of the inflammatory response. Similarly, this experiment indicates that putative negative feedback mechanisms were not modified by the presence of milk in the inflammatory exudate. Thus it seems likely that the greater intensity of inflammation in the lactating gland is a consequence of its physiological state rather than an effect of milk on the progression of the inflammatory response.

The kinetics of the inflammatory response in rabbit skin have been found to be independent of the concentration of chemotactic factor (formyl-methionyl-leucyl-phenylalanine) used to initiate the response (2). In contrast, the kinetics of the inflammatory response in nonlactating glands altered with the highest dose of endotoxin used here. The accumulation of neutrophils in inflammatory lesions induced in sheep skin by 100 ng endotoxin peaks in lesions during the second hour and declines to a very low level in the fourth hour (5). A similar pattern is induced by injection of casein and zymosan-activated plasma. The response in the nonlactating gland exhibited a longer delay and a more protracted time course for the neutrophil influx than occurs in skin. An abrupt decline in the leukocyte influx was observed after 8 h in glands receiving 5 μg endotoxin: this pattern is a common finding in inflammatory responses in skin. In lactating glands the onset and time course of the neutrophil influx was further drawn out, and an abrupt decline was not observed. Similar results have been recorded in the lactating bovine mammary gland by Jain et al. (9), who found little change in endotoxin-stimulated glands at 4 h and greatest neutrophil concentrations in milk at 8–24 h. Cell counts returned to normal at around 48–72 h. In nonlactating glands stimulated with endotoxin in milk, a marked decline in neutrophil numbers was evident by 10 h, again suggesting that the physiological state of resident cells in the lactating gland, rather than the presence of milk in the lumen of the gland, was responsible for the altered kinetics of the inflammatory response. Interestingly, the neutrophil influx in the nonlactating glands in which 20 ml milk or PFS was left in the lumen during each 2-h interval peaked later than in nonlactating glands washed out with 20 ml PFS at each 2-h time point and left empty during the intervening intervals. This suggests that dispersion of the inflammatory exudate in liquid in the lumen of the gland may

modify the time course of the cellular influx. This factor may contribute to the longer time course of the inflammatory response in lactating glands.

Endotoxin is not chemotactic for ovine neutrophils (10) and must initiate the release of an endogenous mediator to provoke inflammation. Recent studies suggest that interleulin 1 is the primary inflammatory mediator induced by endotoxin (11). Interleukin 1 is produced by many cell types, although the monocyte–macrophage series is the predominant source (12). In the mammary gland, interleukin 1 may be produced by intraepithelial leukocytes or by the epithelium itself. Removal of the inflammatory exudate and residual endotoxin at 2 h in nonlactating glands did not diminish the intensity of the inflammatory response, indicating that the induction of the response was presumably complete at this time. The slower onset of inflammation in lactating glands suggests that removal of milk and residual endotoxin at 2 h may have interfered with the induction of the response, although this cannot be distinguished in the present experiments from the effect of removal of milk and inflammatory cells on putative negative feedback control of the response.

Inflammatory lesions in the skin of sheep and rabbits show a high degree of stimulus-specific desensitization to the inflammatory effect of restimulation (5, 11, 13, 14). We have recently found that desensitization develops also in the lactating and nonlactating mammary gland (15). Thus, inflammation in the mammary gland shares many important characteristics with other models of inflammation. The ease of harvesting large numbers of inflammatory cells enables analysis of the structure and function of these cells (16, 17). The presence of paired glands in sheep should permit the transfer of autologous cells and exudate between glands to investigate factors contributing to the initiation and intrinsic regulation of inflammatory responses.

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