Clin. exp. Immunol. (1986) **66**, 615–624

Locally dividing macrophages in normal and inflamed mammary glands

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(Accepted for publication 17 July 1986)

SUMMARY

Goat mammary macrophage division *in vivo* was assessed by detection of mitotic figures, by autoradiographic measurement of the uptake of ^{3}H thymidine, and by a 96-well proliferation assay. Autoradiography revealed that $3{\cdot}74 \pm 0{\cdot}77\%$ of nonstimulated mammary macrophages were actively synthesizing DNA. Eight days of sterile inflammation, induced by lipopolysaccharide or thioglycollate, increased mammary macrophage division ($10{\cdot}9 \pm 2{\cdot}1\%$). The division increased within 2 h after inducing inflammation with thioglycollate. After 1 day, the rate of division decreased, and another increase occurred 3–4 days later. The high rate of division was maintained for >60 days after the induction of sterile inflammation. Division was further shown to occur by injecting ^{3}H-thymidine directly into the mammary gland, harvesting the macrophages 1·5 h later, and determining incorporation by autoradiography. The results of all assays of division were in agreement, suggesting they reflected the same event. The dividing cells were nonspecific esterase-positive, adherent, motile, phagocytic, and had morphological characteristics of macrophages.

Keywords division macrophages mammary gland

INTRODUCTION

The origin of tissue macrophages is controversial. Since van Furth's studies (van Furth & Cohn, 1968; Thompson & van Furth, 1969; van Furth, Diesselhoff-den Dulk & Mattie, 1973), tissue macrophages were thought to be direct lineal descendants of bone-marrow-derived circulating monocytes. Resident macrophages were considered to be 'dead-end' cells that were incapable of self-renewal. This hypothesis was seriously doubted, however, when it was observed that 1–5% of the macrophages in the liver (Volkman, 1977), spleen (van Furth & Diesselhoff-den Dulk, 1984), peritoneum (Shands & Axelrod, 1977; Sawyer, Strausbauch & Volkman, 1982), lungs (Sawyer *et al.*, 1982; Coggle & Tarling, 1984), and brain (Raedler & Raedler, 1984) were dividing. Recent reports indicate that macrophage proliferation *in situ* can significantly contribute to the maintenance of lung alveolar (Sawyer *et al.*, 1982) and splenic (van Furth & Diesselhoff-den Dulk, 1984) macrophage populations. Studies have not been conducted to determine the capability of mammary macrophages to divide.

Mammary macrophages are the predominant cell type in the fluid of the noninflamed mammary gland of many species of animals (Jensen & Eberhart, 1975; Wardley, Rouse & Babiuk, 1976; Lee & Outteridge, 1976; Desiderio & Campbell, 1980; Paape *et al.*, 1980; Anderson & Banks, 1980) including humans (Cole *et al.*, 1981; Hanson, 1982; Cole *et al.*, 1985). Studies have been conducted

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of the morphology (Jensen & Eberhart, 1975; Lee & Outteridge, 1976; Desiderio & Campbell, 1980; Anderson & Banks, 1980), phagocytic capability (Paape *et al.*, 1980; Ward & Sebunya, 1980; Tashjian & Campbell, 1983; Craven, 1983), and complement-producing capability (Cole *et al.*, 1981; 1985) of mammary macrophages. Commonly, 10–100 ml of mammary gland fluid can be obtained from cows, goats, horses, or humans. This fluid contains approximately 1 million cells/ml, of which > 80% are macrophages. The ease of obtaining this fluid provides a unique opportunity for extensive study of a resident macrophage population. The study of mammary macrophages may contribute to the understanding of macrophages and macrophage function in general, as well as the physiology of the mammary gland itself, which is important in both human and veterinary medicine. For example, the mammary gland is normally a highly proliferative organ that has a greater frequency of neoplastic diseases, such as human breast cancer (MacMahon, Cole & Brown, 1973; Dulbecco, Henahan & Armstrong, 1982). Inflammatory reactions resulting in disease are also common in the mammary gland (Smith & Roguinsky, 1977; Blood, Radostits & Henderson, 1983). Finally, these proliferative and inflammatory events can lead to secretion of inflammatory mediators and growth factors that could have significant implications if they enter milk or milk products.

In this study, we determined if caprine mammary macrophages divide and, if so, how this division is affected by the induction of sterile inflammation.

METHODS AND METHODS

Animals

Female goats of the Saanen breed were used in the experiments. All goats had completed at least one lactation period and had developed mammary tissue but did not lactate during the study. The goats did not have clinically evident mastitis, as indicated by low numbers of neutrophils in the mammary gland.

Collection, identification and culture of monocytes and mammary gland cells

Goat monocytes were collected from heparinized blood by isopyknic centrifugation using Ficoll-Hypaque (Banks & Greenlee, 1982) and subsequent adherence on plastic Lab-tek slides (Lab-Tek Miles Scientific, Naperville, IL, USA) during autoradiography (described below).

Cells from both nonmanipulated and inflamed mammary glands were studied. Inflammation was induced by the infusion of 10 ml of sterile thioglycollate broth (Difco Laboratories, Detroit, MI, USA or 25 μg of *E. coli* lipopolysaccharide (0111, B4) (LPS; RIBI ImmunoChem Research, Inc., Hamilton, MT, USA). A catheter was inserted into a teat orifice that had been bathed with ethanol, and 5–10 ml of mammary fluid was aspirated into a 10 ml syringe. The fluid was diluted 1 : 3 in calcium-magnesium free Hanks's balanced salt solution (CMF-Hanks's), and the cells pelleted by centrifugation at 400 ***g***. The cell pellet was resuspended in CMF-Hanks's, and cells counted and cell types determined from cytospin preparations by staining with Wright's and staining for esterase content (Müller *et al.*, 1981). Phagocytosis of yeast cells or latex beads was evaluated before the preparation of the cytospins. On other occasions phagocytosis was evaluated by allowing 100,000 macrophages in 0·4 ml of tissue culture medium to adhere to plastic slides to which yeast or latex beads were then added. After 1–2 h of incubation, the adherent monolayers were washed in phosphate buffered saline (PBS), dried, and stained with Wright's. Cells containing more than three particles were considered phagocytic.

In fluids obtained from nonstimulated caprine mammary glands, macrophages normally accounted for > 85% of the cells, and neutrophils generally were the second major cell population. Lymphocytes were occasionally seen, as were fibroblasts and epithelial cells, but their combined percentages were normally < 5%. Total cell number ranged from $5–9 \times 10^5$/ml. After a sterile irritant was injected into the mammary gland, neutrophils initially became the major cell type, but macrophages predominated by 4 day after injection. Inflammatory macrophage suspensions for proliferation assays were obtained 8 days after the injection of the sterile irritants, when macrophages usually accounted for > 70% of the inflammatory cell population.

RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Flow Laboratories, Inglewood, CA, USA) and antibiotics (cRPMI) or Iscove's serumless (IS) medium (Gibco) were used to culture the monocytes and mammary macrophages. The IS medium was prepared as described by Iscove & Melchers (1978), except that it was supplemented with 1 μg/ml human transferrin (Sigma), 5 μg/ml insulin (Sigma), 900 μg/ml BSA (Sigma), 80 μg/ml sonicated phosphatidylcholine (Sigma), and 20 μg/ml cholesterol (Sigma). Antibiotics were added when necessary.

Assays of macrophage division

Mitotic index. Cytospin preparations of cells, incubated with or without yeast, directly obtained from the mammary gland were stained by Wright's and stained for esterase content, and were examined for mitotic figures. The percentage of mitotic figures in 10,000 macrophages was determined and recorded as the mitotic index.

Autoradiography. Autoradiography was done to determine the percentage of macrophages synthesizing DNA after tritiated thymidine labelling either *in vitro* or *in vivo*. The labelling assays *in vitro* were done using 100,000 macrophages in 0·4 ml of IS medium, culturing the cells with 0·25 μCi tritiated thymidine (6·7 Ci/mmole; New England Nuclear, Boston, MA, USA) and yeast cells in the wells of plastic Lab-Tek slides for 4 h. The slides were then vigorously washed in PBS, allowed to dry, dipped in Kodak NTB 2 liquid emulsion (Eastman Kodak Company, Rochester, NY, USA), exposed for 3 days at 4°C, developed in Kodak D-19 developer for 3 min, fixed (Kodak fixer), and stained by Wright's or stained for esterase content. Dividing macrophages were identified as those with 12 or more silver grains over their nuclei. The percentage of dividing macrophages was determined by counting a total of 500 cells with characteristics of macrophages. Motility was assessed by allowing macrophages to adhere to plastic slides that were tilted, washing the adherent monolayer, and reculturing the horizontal slides for autoradiography under the conditions described above. Motility was detected by counting the cells with 12 or more silver grains in the area of the slide that previously did not contain cells. For detection of labelled DNA-synthesizing cells *in vivo*, 100 μCi of tritiated thymidine was injected into the mammary gland, and 5 ml of fluid was collected 1·5 h later. Macrophages were harvested and incubated with yeast in plastic Labtek slides for 3–4 h to allow for adherence and phagocytosis, and autoradiography was performed.

96-Well macrophage division assay. The division of macrophages in 96-well plates was determined using a modification of a method used by Neumann & Sorg (1983). The mammary macrophages used in this assay were adherent cells that previously had been incubated for 2–3 h on plastic Petri dishes (60 × 15 mm) at 37°C under 6·5% CO_2. In this latter procedure the nonadherent cells were washed away, the adherent macrophages were removed from the plastic by cold shock, incubation in 0·01% EDTA, and gentle scraping with a rubber policeman. The resulting suspensions that contained < 5% lymphocytes were adjusted to 0·7–2·0 × 10^6 macrophages/ml in cRPMI or IS medium. The cells were split into two groups, and autoradiography was performed on the first group. The cells of the second group were seeded into flat-bottom 96-well microtitre plates (Corning Glass Works, Corning, NY, USA) in aliquots of 0·1 ml and cultured for 2–10 days. These cultures were pulsed with 1 μCi of tritiated thymidine for the final 36 h. After cold shock and incubation in 0·02% EDTA plus 0·02% lidocaine (Burns Veterinary Supply, Oakland, CA, USA), to remove adherent macrophages, cells were harvested mechanically onto filter disks with a semi-automated cell harvester (Brandell, Rockville, MD, USA), the disks were immersed in 1 ml of PPO-POPOP scintillation cocktail, and tritiated thymidine incorporation was measured by liquid scintillation spectrometry (Beckman Instruments, Fullerton, CA, USA).

RESULTS

Characteristics of the dividing mammary fluid cells. The cells synthesizing DNA in the autoradiography assays, described below, contained large, cytoplasmic granules that were positive for nonspecific esterase. The cells were adherent to plastic culture chambers but were motile, moving from one area of the chamber to another. The cells phagocytozed over three yeast particles

and latex beads. The cells synthesizing DNA had morphological characteristics of macrophages, such as cytoplasmic vacuoles, eccentric nucleus, high cytoplasm/nucleus ratio, and a diameter of 15–60 μm.

Macrophage division in unstimulated mammary glands. Based on the uptake *in vitro* of tritiated thymidine and subsequent autoradiography, 2–6% of resident macrophages from nonstimulated goat mammary glands from different animals were actively synthesizing DNA (Fig. 1). In contrast, 0·1–0·2% of blood monocytes were synthesizing DNA. As indicated in Fig. 1, there was variation among the goats tested. If the gland remained nonstimulated, however, significant day-to-day variation in the same goat did not occur. To control for pinocytosis of fluid containing radioactive thymidine, which may contribute to positive results in the autoradiographs, autoradiography was done using tritiated H_2O mixed with the mammary macrophages. There was no labelling of the cells when they were incubated with tritiated H_2O (data not shown).

Effect of inflammation within the mammary gland on macrophage division. When inflammation was initiated in the mammary gland by the injection of thioglycollate or LPS, the cells harvested 8 days later generally had a greater percentage of macrophages synthesizing DNA than did cells from nonstimulated glands (Fig. 1). The glands were examined at various times after being injected with thioglycollate (Fig. 2); the percentage of DNA-synthesizing macrophages increased 1 day after the

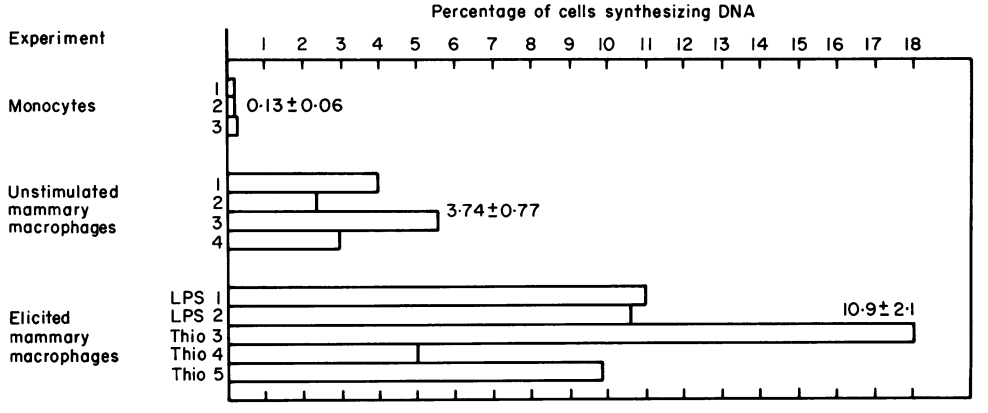

Fig. 1. DNA synthesis by monocytes and mammary macrophages detected by tritiated thymidine incorporation and subsequent autoradiography. All differences of means are significant at $P<0·05$.

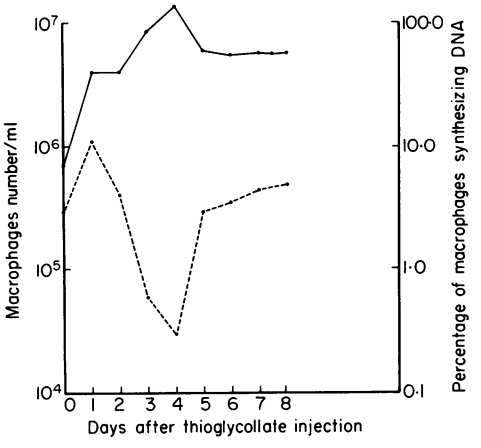

Fig. 2. Daily change in mammary macrophage DNA synthesis measured by autoradiography, and change in macrophage numbers/ml after the injection of thioglycollate into the mammary gland. ——=number of macrophages;- - -=percentage of DNA-synthesizing macrophages.

injection, correlating with the expected increase in macrophage number. After Day 1 and up to Day 4, however, macrophage numbers continued to increase, while the percentage of DNA-synthesizing macrophages dropped to < 1%. Finally, on Days 6, 7 and 8, the percentage of DNA-synthesizing macrophages increased to 3·5, 4·6 and 4·9% respectively. These values were slightly above those observed before the injection of thioglycollate. The contralateral gland, which did not receive thioglycollate, remained unchanged until Day 7, when the number of labelled cells increased to 7·5%. The percentage on all previous days had been 4·5% (data not shown). In other goats, experiments demonstrated that the initial increase in the percentage of DNA-synthesizing macrophages began within 2 h after the injection of thioglycollate (Table 1). When LPS was used as an inflammatory stimulant, mammary macrophage DNA synthesis increased by Day 8 (fig. 1), but the early (< 1 day) increase that was observed with thioglycollate did not occur with LPS (Table 1).

In four glands examined approximately 60 days after they were injected with a sterile irritant (LPS or thioglycollate), the percentage of DNA-synthesizing macrophages was maintained at 11–18%, compared with 2–6% in nonstimulated glands (Table 2). The concentration of cells was 5-fold to 10-fold higher than in nonstimulated glands and was mainly mononuclear cells of which normally > 85% were macrophages. Using these cells, we determined the influence of increasing the time that the tritiated thymidine was incubated with mammary macrophages on the number of detectable dividing cells. We varied the incubation time from 1–24 h and found a marked rise in the percentage of tritiated-thymidine-positive macrophages as incubation time was increased. When macrophages were labelled for 1·0 h, washed, and recultured without tritiated thymidine for 23 h (Table 3) (which controlled for the increase in the number of labelled cells due solely to the division of the 1 h labelled macrophages) an increased occurred, but not to the same extent. The greater increase detected with the cells continually labelled with tritiated thymidine compared with the cells that had been labelled for 1 h only indicates that there were macrophages that did not divide for the initial 1 h but entered S phase later in the assay.

Labelling of dividing macrophages in vivo. Experiments were done to confirm DNA synthesis *in vivo* by injecting tritiated thymidine into a stimulated goat mammary gland and measuring the incorporation by cells harvested 1·5 h later. To compare labelling *in vivo* and *in vitro*, macrophages were collected before tritiated thymidine was injected into the mammary gland, and the adherent

Table 1. Kinetic studies on the early effects of inflammation on mammary macrophage DNA synthesis

Inflammatory stimulant (h†)	No. of macrophages ($\times 10^6$)		%*	
	Exp 1	Exp 2	Exp 1	Exp 2
Thio (0)	0·9	1·0	8·7 ± 0·3‡	14·5 ± 0·4
Thio (1)	1·1	0·8	11·4 ± 0·6§	18·8 ± 0·4§
Thio (24)	1·8	7·0	12·3 ± 0·7	19·7 ± 0·5
LPS (0)	2·0	2·7	9·0 ± 0·1	14·6 ± 0·55
LPS (1)	2·0	2·2	7·8 ± 0·2	12·4 ± 0·5
LPS (24)	12·0	6·7	< 0·1	< 0·1

* Percentage of DNA-synthesizing macrophages based on autoradiography.

† Hours after thioglycollate (thio) or LPS injection.

‡ Standard error of duplicate values of 1000 total cells examined.

§ Difference of means between 0 and 1 h significant at $P < 0{\cdot}01$.

Table 2. Increased macrophage DNA synthesis 60 days after the induction of inflammation

Inflammatory stimulant	No. of macrophages/ml ($\times 10^6$)	%*
Thio	5·0	11·5
Thio	6·0	17·3
LPS	5·0	15·3
LPS	3·0	18·0
Mean ± s.e.m.	4·80 ± 0·63	15·50 ± 2·90
None		
Range	0·50–0·70	2·00–5·70
Mean ± s.e.m.	0·58 ± 0·048	3·74 ± 0·77

* Percentage of DNA-synthesizing cells based on autoradiography.

Table 3. Effect of incubation time on the percentage of macrophages synthesizing DNA in the autoradiography assay

	Time of incubation			
Experiment	1 h*	4 h*	24 h*	24 h†
1‡	14·8 ± 1·25§	20·8 ± 2·75	46·5 ± 1·50	27·3 ± 1·25
2	18·9 ± 1·60	22·1 ± 1·2	30·8 ± 1·2	22·2 ± 2·2
3	19·0 ± 1·41	21·4 ± 1·0	42·5 ± 1·44	ND

* Hours of ^{3}H-thymidine labelling.

† One hour ^{3}H-thymidine labelling followed by 23 h culturing in the absence of ^{3}H-thymidine .

‡ All macrophages in each experiment were from goat mammary glands that had been injected with thio or LPS at least 60 days before.

§ Standard error of the mean of 1000 macrophages counted in duplicate.

cells were labelled *in vitro* for 1·5 h. The labelling *in vitro* and *in vivo* was 21·2% and 23·4% DNA-synthesizing cells, respectively.

Association of mammary macrophage DNA synthesis with cell division and proliferation in vitro. Direct evidence for division was provided by the presence of mitotic figures in cells with the same characteristics described above, except that motility was not assessed. Prophase, metaphase and cytokinesis of cell division were all detected. The percentage of dividing macrophages determined by autoradiography was plotted against the percentage of mitotic figures of the same macrophage population, and a linear regression correlation coefficient of 0·96 was obtained (Fig. 3). Thus, DNA synthesis was associated with actual cell division.

As an additional assay to confirm macrophage division, we compared our autoradiography technique with the proliferation of macrophages cultured in 96-well microtitre plates. We found optimal conditions to be 70,000 macrophages/well in Iscove's modified serum-free medium (see Materials and Methods). When serum was used as a protein supplement, variation among replicates occurred. In some cases FBS stimulated division, whereas in other cases it inhibited division. When

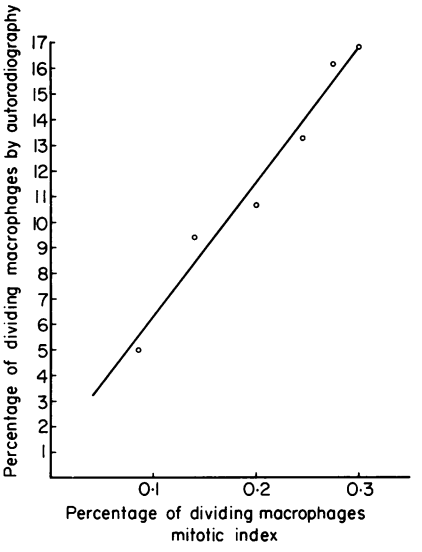

Fig. 3. Comparison of mammary macrophage division determined by autoradiography of tritiated thymidine labelled cells and division determined by the percentage of mitotic figures (mitotic index) of the same population. Values from six goats with varying degrees of inflammation within the mammary gland. ($r = 0{\cdot}96$ significant at a P value of less than 0·005 based on Student's t-test).

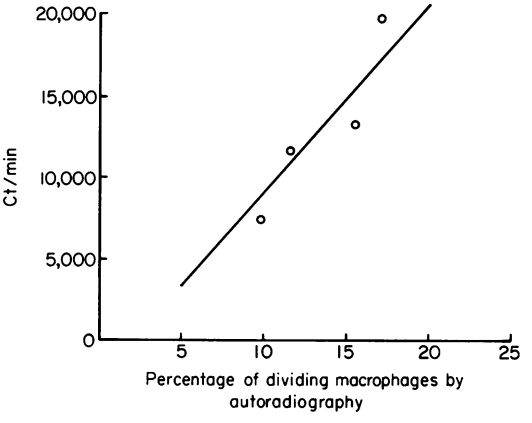

Fig. 4. Mammary macrophage poulations from four separate goats were cultured with tritiated thymidine for 4 h and the percentage of dividing cells determined by autoradiography. Macrophages from the same goats were also cultured for 3 days in the 96-well proliferation assay (see Materials and Methods). Autoradiography values of each mammary macrophage population was compared to the proliferation of each population in the 3 day assay. (ct/min were the average of quadruplicate wells).

RPMI plus FBS was used, there was greater variation among assays of cells obtained from the same animal on different days than when IS medium was used. The relatively low number of cells was necessary for maximal division. If macrophage numbers were increased to over 100,000 cells/well, as in lymphocyte proliferation assays, division decreased. The optimal proliferative conditions were found to be 37·5°C under 6·5% CO_2 and a culture period of 3–4 days (data not shown). We obtained values that ranged from 1376 ± 107 ct/min to $29{,}425 \pm 2100$ ct/min. Inflammatory macrophages

incorporated more tritiated thymidine, indicated by higher ct/min values, than did resident macrophages. The results of the 96-well proliferation assay were comparable with those of autoradiography. Again, when percentage of thymidine incorporation of a mammary macrophage sample determined by autoradiography was plotted against ct/min based on the 96-well proliferation assay, a high correlation coefficient was obtained ($r=0{\cdot}92$) (Fig. 4). Significant division by inflammatory macrophages could be detected up to 6–7 days in culture, but generally by 8–10 days proliferation was very low or nonexistent. Tritiated H_2O was again used as a control for pinocytosis, and there was never a significant uptake.

DISCUSSION

We conclude that mammary macrophages can divide *in vivo*. Mammary macrophage DNA synthesis was detected by labelling cells *in vivo* or *in vitro* with tritiated thymidine followed by autoradiography. Direct evidence for division was shown by the detection of mitotic figures in macrophages obtained directly from mammary glands. Mammary macrophage division was also shown to occur by using a 3-day 96-well proliferation assay. In comparison studies, all division assays correlated well with each other, indicating that each was a reflection of the other two. This was particularly important in assessing the validity of the various assays in measuring the same event. The 2–6% division noted for resident mammary macrophages agrees with reports of resident macrophage proliferation in other animals (Volkman, 1977; Shands & Axelrod, 1977; Sawyer *et al.*, 1982; Coggle & Tarling, 1984; van Furth & Diesselhoff-den Dulk, 1984) and at other locations. The dividing cells detected in this study were esterase-positive, adherent, mobile, could phagocytize yeast and latex beads, and had morphological characteristics of macrophages. These criteria preclude the possibility that we measured division by large lymphocytes or epithelial cells. We have not directly shown the origin of the dividing macrophages, nor have we shown that the dividing macrophages account for the increase in cell number within the gland, but, as shown below, these can be inferred from our data.

There are different views of the origin of resident macrophages. In one, resident macrophages are the direct lineal descendants of bone-marrow-derived blood monocytes (van Furth & Cohn, 1968; Thompson & van Furth, 1969; van Furth *et al.*, 1973; van Furth, 1985). This hypothesis interprets the 1–5% proliferating resident macrophages to be dividing monoblasts that have recently arrived from the bone marrow. This conclusion was difficult to reconcile, however, with the failure to find these dividing cells in the peripheral blood (van Furth & Cohn, 1968; Thompson & van Furth, 1969; van Furth *et al.*, 1973; Volkman, 1976). Recently, it has been proposed that nondividing bone-marrow-derived mononuclear phagocytes infiltrate resident macrophage sites and start to divide once in tissue, thus explaining the 1–5% division (Blusse van Oud Albas, Mattie & van Furth, 1983; van Furth & Diesselhoff-den Dulk, 1984; van Furth, 1985). However, when monocytopenia is induced in laboratory animals by treatments that do not affect alveolar or peritoneal macrophages, tissue macrophage division and numbers remain constant (Sawyer *et al.*, 1982). These criticisms of the first view have lead to the hypothesis that some tissue macrophages, possibly a distinct subgroup, divide and replenish resident populations (Volkman, 1976; 1977; Shands & Axelrod, 1977; Sawyer *et al.*, 1982; Coggle & Tarling, 1984).

It is apparent, as discussed in recent reviews (Bennet & Kumar, 1983; Stewart, 1984), that the issue of tissue macrophage proliferation is still a controversial one. The present study has shown that caprine mammary macrophages can divide. Although we frequently observed an increase in the number of macrophages when there was an increase in the rate of division, these two observations are consistent with, but do not prove, that mammary macrophages are proliferating *in vivo*. To prove that proliferation is occurring *in vivo* would require the elimination of all possible contributions of infiltrating blood cells and subsequent maintenance of the resident cell population. We can, however, estimate what the rate of division observed could contribute to the maintenance of mammary macrophage populations. When we assume that the generation time of mammary macrophages is like that of other resident macrophages (11–16 h) (Coggle & Tarling, 1984; Stewart, 1984; van Furth, 1985), 500,000 mammary cells/ml with a 4% division rate could contribute 1300–

1800 cells/ml/h. These numbers are consistent with the percentage of division we observed and the number of macrophages in the caprine mammary gland, suggesting that division alone could result in a turnover of the resident macrophage population of the mammary gland fluid in approximately 11–17 days. Certainly monocyte influx will alter these values and conclusions cannot be drawn until this contribution is determined; however, it is evident that macrophage division could have a significant impact on mammary macrophage homeostasis.

As described in other reports (Forbes, 1965; van Furth *et al.*, 1973; Spector, 1974; Adolphe *et al.*, 1976; Bennett & Kumar, 1983; Nagao & Tanaka, 1983; Stewart, 1984) and shown here, inflammatory macrophages had a significantly higher rate of division than resident, nonstimulated mammary macrophages. Our studies of the kinetics of the change in division during a thioglycollate-induced inflammatory event revealed two separate increases in macrophage division. There was an initial increase in division within 2 h. After 1 day the percentage of dividing macrophages decreased dramatically, although the number of macrophages in the gland continued to increase. These nondividing infiltrating cells were most likely from the blood. By Days 4 and 5 the percentage of dividing macrophages increased to levels higher than seen before the induction of inflammation. The initial enhanced division was probably by resident macrophages, because it occurred before we detected infiltrating blood cells, such as neutrophils. Also, it has been shown that 1–2 h is too short a time for contribution of bone-marrow-derived proliferating monocytes (van Furth *et al.*, 1973). These observations support the proposal by Adolphe *et al.* (1976) that resident macrophages proliferate during inflammation. Other studies of the kinetics of the change in macrophage division during inflammation (van Furth & Cohn, 1968; van Furth *et al.*, 1973; van Furth, 1985; Forbes, 1965) do not show increases at 1–2 h, but do show later increases. This may be a reflection of the times tested, the tissue macrophage site examined, or, since some inflammatory agents are toxic for cells, the agent used to elicit the inflammatory response. We found that LPS, an inflammatory agent toxic for macrophages (Patierno *et al.*, 1983), decreased division early in the inflammatory reaction, but by Day 8 the macrophage division exceeded the preinjection rate. This finding is similar to that of Forbes (1965).

Sixty days after the induction of inflammation, the concentration of mammary macrophages was 5-fold higher than in nonstimulated mammary glands, and macrophage division was increased 2-fold to 9-fold. These populations included cells that were dividing when they were removed from the gland and others that began to divide after being in culture for various lengths of time. The latter cells were presumably in G1 phase when removed and entered S phase during the 24 h of labelling with tritiated thymidine (Table 3). This suggested that the dividing cells were not solely newly arrived dividing monoblasts but were resident cells that entered division after being stimulated and then removed from the gland. This increased division probably contributed to the chronic mammary macrophage accumulation, an hypothesis originally proposed for chronic macrophage proliferation in granulomas induced by carrageenan (Spector, 1974). If we assume 16% proliferating cells and the generation time used above, this mammary macrophage population could double in approximately 2–4 days. This rather formidable proliferative activity underscores the significance of a question recently raised by Stewart in a review on macrophage proliferation (Stewart, 1984) concerning 'where the locally produced cells go and what they do.'

This study was supported by the Washington State University Agricultural and Home Economics Research Center (Project 0600, Scientific number 7559), the Washington State Dairy Products Commission, and Public Health Service grant AM27680 from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases. The authors thank Jan Carlson for technical assistance and Kjerstie Guenther for help in preparation of the manuscript.

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