Immunology 1980 **41** 807

The isolation, long-term cultivation and characterization of bovine peripheral blood monocytes

J. R. BIRMINGHAM* & E. L. JESKA *Immunobiology Program, Veterinary Medical Research Institute, Iowa State University, Ames, Iowa, U.S.A.*

Accepted for publication 2 June 1980

Summary. Bovine peripheral blood monocytes were isolated from buffy coats of ACD-anticoagulated whole blood. Cells cultivated on plastic surfaces for 20 h were judged to be 95% monocytes based on non-specific esterase-1 staining, uptake of latex particles and surface receptor characteristics. Bovine monocytes were maintained up to 80 days *in vitro* in Dulbecco's modified Eagle medium with 15% horse serum and 15% foetal bovine serum. Several morphological and physiological features of bovine monocytes were examined during the course of culture. Cell size and cytoplasmic spreading, granulation and vacuolization increased progressively. Multinucleated giant cells predominated during the latter stages of *in vitro* culture. A high percentage of bovine monocytes possessed C3 and IgG Fc receptors, whereas IgM Fc and sheep erythrocyte receptors were not detected. Phagocytosis was mediated by the IgG receptor, but not by the C3 receptor. Peroxidase activity declined in a linear fashion, with cells essentially negative after 8 days of culture. Total cell protein and acid phosphatase increased during cultivation. Lysozyme activity was undetectable in both lysates and supernatants of bovine monocyte. These findings are consistent with the concept of maturation of mononuclear phagocytes. The procedures for isolation and cultivation described in this paper will provide methodology for detailed study of bovine mononuclear phagocytes.

Abbreviations: HBSS, Hanks's balanced salt solution; DMEM, Dulbecco's modified Eagle medium; M199, Medium 199; FBS, foetal bovine serum; HS, horse serum; LH, 0·2% lactalbumin hydrolysate; ACD, 2X acid-citrate-dextrose; PBS, phosphate-buffered saline; E, sheep erythrocytes; EA_{IgG}, IgG-coated erythrocytes; EA_{IgM}, IgM-coated erythrocytes; $EA_{IgM}C$, C3-coated erythrocytes.

* Present address and correspondence: Dr J. R. Birmingham, Department of Medicine, National Jewish Hospital and Research Center, 3800 E. Colfax, Denver, Colorado 80206, U.S.A.

0019-2805/80/1200-0807$02.00

INTRODUCTION

The role of macrophages as effector cells against microbial pathogens and tumour cells has been extensively investigated (reviews by North, 1978; Keller, 1977). In addition, macrophages are known to play a critical role in the induction of immune responses (Rosenthal, 1978; Rosenstreich & Mizel, 1978). Recently a large body of evidence has established the macrophage as a major secretory cell (reviewed by Davies & Bonney, 1979). The above mentioned studies have been conducted primarily with rodent models.

Peripheral blood monocytes are generally thought to be the immature precursors of tissue macrophages (Van Furth & Willemze, 1979). The differentiation of monocytes *in vitro* into macrophage-like cells appears to be related to maturational events that occur *in vivo* (Cohn & Benson, 1965). Examination of the maturation process *in vitro* has only been conducted with

peripheral blood monocytes from horses (Bennett & Cohn, 1966) and from humans (Johnson, Mei & Cohn, 1977; Zuckerman, Ackerman & Douglas, 1979).

Detailed study of bovine peripheral blood monocytes has been hindered by difficulties in obtaining and cultivating these cells. Bovine erythrocytes cannot be separated from leucocytic elements by sedimentation techniques standard to most animal species and humans (Carson, Sells & Ristic, 1975) and bovine monocytes survive poorly in culture after separation on sucrose or Ficoll–diatrizoate gradients (Rossi & Kiesel, 1977a). A recent paper described procedures for establishing continuous macrophage cell lines from peripheral blood monocytes of seven animal species, however, the authors indicated an inability to establish continuous lines using bovine cells (Wardley, Lawman & Hamilton, 1980). Thus, previous investigations have been limited to short-term studies on freshly isolated bovine cells or have utilized procedures requiring at least 6–12 days of culture in order to purify mononuclear phagocytes (Fitzgeorge, Solotorovsky & Smith, 1967; Rossi & Kiesel, 1977a). In this paper, a procedure is presented for isolating monocytes from whole blood and we describe optimal conditions for maintaining long-term primary cultures. In addition, a number of the morphological and physiological characteristics of bovine monocytes have been examined and the development of these cells followed during *in vitro* cultivation.

MATERIALS AND METHODS

Reagents

Hanks's balanced salt solution without Ca^{2+} and Mg^{2+} was obtained from Microbiological Associates (Walkersville, MD), Dulbecco's modified Eagle medium, Medium 199, RPMI-1640, Neuman–Tytell serumless medium, foetal bovine serum, and horse serum were from Grand Island Biological Co. (Grand Island, NY), gentamycin sulphate was from Schering Corp. (Bloomfield, NJ), α-naphthyl butyrate, *p*-nitrophenyl phosphate, 0·09 M citrate buffer, bovine serum albumin, and *Micrococcus lysodeikticus* were from Sigma Chemical Co. (St Louis, MO), Triton X-100 was from BioRad Laboratories (Richmond, CA), IgG anti-sheep erythrocyte and IgM anti-sheep erythrocyte antibody were from Cordis Corp. (Miami, FL) and 0·81 μm latex particles were from Difco (Detroit, MI).

Monocyte isolation

Normal 8–24 month old Hereford-Angus heifers were selected at random from the herds at the National Animal Disease Center, Ames, IA (provided by Dr B. L. Deyoe) and the Veterinary Medical Research Institute. Blood was collected by jugular venipuncture using 2X acid-citrate-dextrose (ACD) as anticoagulant (1 part 2X ACD: 10 parts whole blood). Mononuclear leucocytes were isolated by a modification of the procedure of Carlson & Kaneko (1973). Briefly, the anti-coagulated blood was centrifuged at 1000 ***g*** for 20 min and the buffy coat collected. Contaminating red cells were lysed by the addition of 2 volumes of cold phosphate-buffered (0·013 M) deionized H_2O and isotonicity restored with 1 volume of phosphate-buffered (0·013 M) 2·7% NaCl. The suspension was centrifuged (150 ***g***, 10 min) and washed 4× with Hanks's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. The washed leucocytes were resuspended in Dulbecco's modified Eagle medium (DMEM) supplemented with 50 μg/ml gentamycin sulphate, 15% foetal bovine serum (FBS) plus 15% horse serum (HS), and counted in a haemocytometer. All sera were previously heated at 56° for 30 min. Cell concentrations were adjusted to 10^7 cells/ml and 2 ml was seeded into 35 × 10 mm plastic tissue culture dishes (Falcon, Los Angeles, CA). The cell cultures were incubated at 37° in a humidified incubator with 5% CO_2 in air. Non-adherent cells were removed by washing with phosphate-buffered saline (PBS), pH 7·2, containing 5% FBS. Adherent cells were cultivated in 1·5 ml DMEM-15% FBS-15% HS. The culture medium was changed every 2 days. Viability was determined by trypan blue dye exclusion.

Phase-contrast morphological studies

For phase-contrast microscopy, adherent cells were washed, overlaid with a cover slip, and examined in the living state with a Zeiss microscope equipped with a Zeiss polaroid attachment.

Cytochemistry

Non-specific esterase-activity was determined by the method described by Li, Lam & Yam (1973), with α-naphthyl butyrate as substrate. Peroxidase activity was determined by the method described by Kaplow (1965). Activity was determined on a minimum of 200 cells per culture.

Latex phagocytosis

For phagocytosis of latex particles by cells in suspen-

sion, 2×10^7 buffy coat leucocytes were incubated in 9 ml of Medium 199 (M199) containing 20% FBS and 1 ml of a 1:50 dilution of 0·81 μm latex beads. The suspension was incubated for 1 h at 37° on a continuous rotator at 8 r.p.m. (Fisher Scientific Co., Itasca, IL). The cells were then centrifuged at 150 *g* for 10 min and washed 3 × with HBSS. Wet mounts were prepared and examined by phase contrast microscopy. For phagocytosis by adherent cells latex particles were added to cultures at a ratio of 40:1, incubated 1 h at 37°, washed 3 × with HBSS and examined under phase contrast microscopy (630 ×). Two hundred cells were examined per test.

Surface receptor enumeration and function

Sheep erythrocytes (E) were stored in Alsever's solution at 4° and used within 2 weeks of collection. The erythrocytes were centrifuged and washed 5 × with M199. A 0·5% concentration of sheep erythrocytes in M199 was used as the source of E. Equal volumes of 1% E and the highest subagglutinating dilution of either IgG anti-E (EA_{IgG}) or IgM anti-E (EA_{IgM}) in M199 were incubated at 37° for 30 min, followed by 30 min at 4°. The cells were centrifuged and resuspended to the original volume (0·5% concentration). Complement-coated erythrocytes ($EA_{IgM}C$) were prepared by incubating 0·2 ml of fresh serum with 3 ml of 0·5% EA_{IgM} for 30 min at 37°. C5-deficient DBA/2 mouse serum (preabsorbed with E) was used as the source of complement.

Adherent cells in plastic dishes were washed and covered with 1·5 ml of the appropriate indicator particle. The dishes were incubated 30 min at 37° and the monocytes carefully washed 4 × with PBS. The cells were fixed for 5 min with 2·5% glutaraldehyde in PBS and examined under an inverted microscope or stained with Wright's for high power examination (970 ×). A positive rosette was considered to be a cell with 4 or more erythrocytes attached to the surface. At least 10 replications were performed in testing for each receptor, with 200 cells examined in each test.

Receptor-mediated phagocytosis was determined by incubating adherent cells with either 1 ml of EA_{IgG} or $EA_{IgM}C$ for 45 min at 37°, followed by four washes with PBS. Erythrocytes attached to the surface of monocytes were lysed with distilled H_2O and the monocytes examined for internalized erythrocytes.

Lysozyme assay

Washed adherent cells were lysed by incubation for 30 min on ice with 1 ml of 0·2% Triton X-100 in PBS. Lysozyme activity was determined spectrophotometrically at 550 nm in cell lysates and supernatants according to the procedures described by Gordon, Todd & Cohn (1974).

Acid Phosphatase assay

Acid phosphatase was determined by the method described by Beck, Mahadevan, Brightwell, Dillard & Tappel (1968). Two hundred microlitres of cell lysate were incubated for 30 min at 37° with 200 μl of *p*-nitrophenyl phosphate in 0·09 M citrate buffer, pH 4·8. The reaction was stopped with 0·1 N NaOH and formation of *p*-nitrophenol was measured at 410 nm on a Gilford 250 spectrophotometer. Specific activity was expressed as nanomoles of *p*-nitrophenol liberated per h per mg cell protein.

Protein determination

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951) as modified by Wang & Smith (1975). Bovine serum albumin was used as a standard.

RESULTS

Monocyte isolation and cultivation

Centrifugation of ACD–anticoagulated bovine blood for 20 min at 1000 *g* resulted in a buffy coat containing an average of 2×10^8 leucocytes/100 ml blood (average of 30 determinations). Ninety-two per cent of the buffy coat cells were mononuclear based on Wright's stained smears. Approximately 30% of the buffy coat cells were non-specific esterase-1 positive and 33% phagocytosed latex particles. Viability was consistently 95–99% after four washes.

Monocytes were isolated by adherence to plastic dishes. Plastic surfaces resulted in more cells adherent to the substratum and better viability than glass surfaces. Non-specific esterase-1 reactivity and uptake of latex particles by adherent cells increased from 80–85% at 2 h to 95% after overnight incubation.

A variety of basal media and sources and concentrations of serum were examined in experiments designed to establish optimal cultural conditions for bovine monocytes. Basal media RPMI-1640, M199 and DMEM were all satisfactory for establishing and maintaining monocyte cultures. Serumless medium (Neuman-Tytell) did not support bovine monocytes adequately beyond 48 h. Foetal bovine serum and HS were determined to support growth better than swine

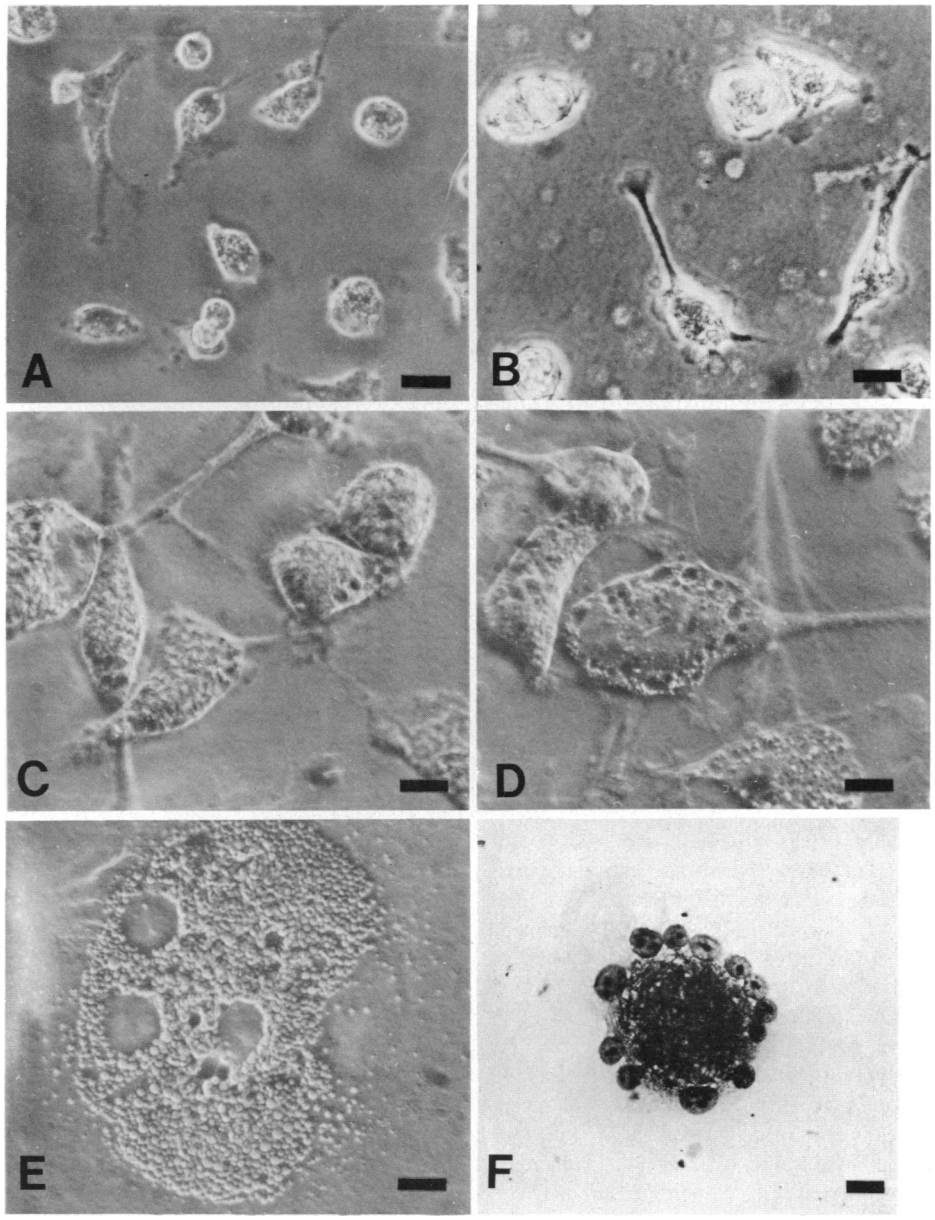

Figure 1. Morphology of bovine monocytes during cultivation. A-E: phase-contrast light microscopy; bar indicates 5 μm. Final magnification × 1260. (A) Day 3 culture; small monocytes showing some cytoplasmic spreading. (B) Day 6 culture; larger monocytes with long cytoplasmic extensions. (C) Day 16 culture; large cells with prominent granulation of cytoplasm and long cytoplasmic extensions. (D) Day 24 culture; large spread cells with peripheral vacuoles. (E) Day 47 culture; cell shown has diameter > 100 μm, numerous granules and vacuoles. (F) Day 47; Wright's stain, light microscopy. Multinucleate giant cell, prominent nucleoli; bar indicates 5 μm. Final magnification × 1000.

serum. Higher concentrations of FBS and HS resulted in greater cell growth (30 > 20 > 10%). The effects of variability in lots of HS and FBS could be reduced by combining 15% HS and 15% FBS. Autologous serum resulted in some clumping of cells and inconsistently supported monocytes. Acid-treatment of serum (2 h at pH 3·2) did not impair capacity to support monocyte cultures. Lactalbumin hydrolysate (LH, 0·2%) was unsatisfactory as a serum substitute for periods exceeding 2–3 days.

Culture characteristics and morphology

Cultivation of bovine monocytes in DMEM with 15% FBS and 15% HS as described above reproducibly resulted in cultures suitable for long-term growth. Over 200 cultures have been established (success rate >95%) in our laboratory for periods up to 80 days. Detachment of cells from the substratum was minimal after 2 days *in vitro* cultivation. Mitotic figures were not observed during the cultivation period under study. Incubation of cultures for periods up to 24 h in [^{3}H]-thymidine consistently resulted in less than 1% uptake.

The morphology of bovine monocytes during *in vitro* cultivation was observed by phase-contrast microscopy. Initially, the cells were small (7–9 μm in diameter), rounded and homogeneous in morphology. In 3 day cultures the cells appeared to be more heterogeneous with some cells appearing circular to oblong in shape and others displaying cytoplasmic spreading characteristic of mononuclear phagocytes (Fig. 1A). After 1 week of cultivation, the cells were approximately twice the size of freshly isolated monocytes. Fusiform shaped cells, often with long, slender cytoplasmic extensions were mixed with cells that retained an oval appearance (Fig. 1B). Sixteen-day-old cultures contained cells that showed further increases in mass and granulation of the cytoplasm (Fig. 1C). At 24 days, the cells had increased to an average of 50 μm in diameter and prominent peripheral vacuoles were evident (Fig. 1D). Multinucleated cells were infrequently seen at this time. Forty-seven day cultures were heterogeneous in size, some exceeding 100 μm in diameter (Fig. 1E). Staining revealed that nearly all cells were multinucleated at 47 days, some containing 10–20 nuclei (Fig. 1F).

Surface receptor studies

A high percentage of bovine monocytes were positive when examined for IgG Fc (98·7 ± 2·1%) and C3 (93·3 ± 6·3%) receptors. Receptors for the Fc portion of IgM and for E were not detected. Receptor-mediated internalization of erythrocytes was apparent with IgG-coated particles, but not with C3-coated particles. For the period studied (up to 14 days), no significant differences were noted for either the percentage of rosette-positive cells or for ingestion characteristics.

Peroxidase reactivity

Figure 2 illustrates granule-associated myeloperoxidase activity of bovine monocytes during maturation *in vitro*. Approximately 75% of newly isolated monocytes are positive for peroxidase. Activity declines in a linear manner, reaching nearly 0 after 8 days culture.

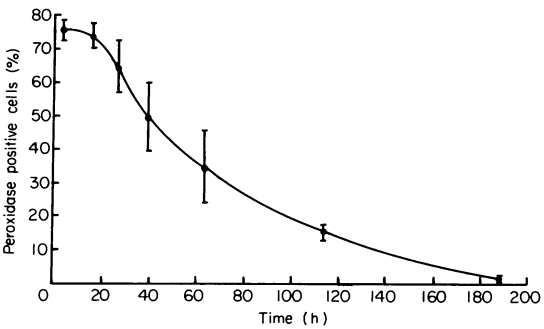

Figure 2. Granule-associated peroxidase activity of bovine monocytes during culture. Each point represents at least three determinations, ± one standard deviation.

Lysozyme activity

Newly established and long-term (up to 30 days) monocyte cultures were studied under a variety of conditions in attempts to detect lysozyme activity. The cells were cultivated in acid-treated serum, medium with no serum, in Neuman-Tytell medium, with 0·2% LH, and after phagocytosis of zymosan or latex particles. Lysozyme activity was not detectable in lysates of bovine monocytes, nor in their culture supernatants. Similarly maintained mouse peritoneal macrophages or porcine alveolar macrophages were positive when tested for lysozyme activity (data not shown).

Cell protein and acid phosphatase

Table 1 shows that cellular protein increased at a linear rate from day 5 to day 15 and then levelled off. Total

Table 1. Cell protein and acid phosphatase activity during cultivation*

	Days in culture			
	5	10	15	20
Cell protein/dish†	25·8 ± 7·4	53·3 ± 16·1	90 ± 10·1	95 ± 24·8
Total acid phosphatase/dish‡	362·5 ± 314·0	770 ± 175·0	660 ± 316·1	2408 ± 945·5
Acid phosphatase, specific activity§	15·5 ± 10·3	12·4 ± 7·0	7·1 ± 2·7	26·1 ± 7·8

* $n=6$ (day 5); $n=3$ (days 10–20) for all determinations.
† Mean μg/dish ± 1 SD.
‡ Mean nmol *p*-nitrophenol liberated/h/dish ± 1 SD.
§ Mean nmol *p*-nitrophenol liberated/h/μg cell protein ± 1 SD.

acid phosphatase activity increased early in culture, levelled off between days 10 and 15, and then increased sharply at 20 days' culture. The specific activity of acid phosphatase decreased from day 5 to day 15, followed by a sharp increase at 20 days of culture.

DISCUSSION

The present paper describes procedures that can be used for reproducible isolation of fresh bovine peripheral blood monocytes, and for cultivation of purified cells for periods up to 80 days. Centrifugation of ACD–anticoagulated blood yields a buffy coat, which, after washing, contains primarily mononuclear leucocytes. Washing the buffy coat cells 3–4 × is important because it removes platelets which cause cells other than monocytes to adhere to surfaces. Cultivation of the cells for approximately 20 h yields an adherent cell population which is 95% monocytes based on uptake of latex beads, non-specific esterase staining and surface receptor characteristics. Similar to human monocytes (Johnson *et al.*, 1977; Berman & Johnson, 1978), bovine monocyte yield and survival is better on plastic surfaces than on glass surfaces.

Dulbecco's modified Eagle medium plus 15% FBS and 15% HS was determined to be the optimal medium for long-term bovine monocyte cultivation. Foetal bovine serum and HS supported cell cultures equally well. Effects of variability in lots of these sera could be reduced by combining them in equal proportions as described by Zuckerman *et al.* (1979). Swine serum, autologous serum and LH were found to be unsatisfactory in their ability to support monocyte cultures. Several basal media are suitable for bovine monocyte culture, but we used DMEM because it provides better control of pH than M199 (Edelson & Cohn, 1976). Human monocytes have recently been successfully cultured for long periods in Neuman–Tytell serumless medium (Johnson *et al.*, 1977). Bovine monocytes contract and begin to detach from culture vessels after several days in this medium.

The procedures developed for isolation and long-term cultivation of freshly obtained bovine monocytes made it possible to study maturation and differentiation of these cells *in vitro*. A progressive enlargement of the monocyte occurs in culture with increasing granulation and vacuolization of the cytoplasm. Initially, the bovine monocytes are small (7–9 μm) and rounded with cytoplasmic spreading becoming apparent in some cells after 2 days. In contrast, Johnson *et al.* (1977), described 90–95% of human monocytes to be well spread with extensive ruffled plasma membranes and mean cell diameters of 30–40 μm after 30 min culture. These differences may be related to exposure to activated complement and coagulation factors (Bianco, Eden & Cohn, 1976) during isolation of human peripheral blood monocytes.

In the present study, multinucleated giant cells were observed to develop between 24 and 47 days' culture. Development of multinucleated giant cells, usually from long-term peripheral blood monocyte cultures, has been previously described (Lewis, 1925; Goldstein, 1954; Cohn, 1968). However, polynucleated cells have also been found as early as 2–3 days in human (Zuckerman *et al.*, 1979) and bovine (Rossi & Kiesel, 1977a) peripheral blood monocyte cultures. The factors influencing formation of multinucleated giant cells *in vitro* are poorly understood.

The presence of a surface receptor for the Fc portion of IgG has been previously described on 75–90% of bovine monocytes (Rossi & Kiesel, 1977b). Ninety-nine per cent of the bovine monocytes in this study were found to possess IgG Fc receptors. The use of

different procedures for demonstrating IgG receptors may account for this discrepancy. The presence of complement receptors has also been described on bovine monocytes, although the percentage of cells bearing the receptor were not reported (Rossi & Kiesel, 1977a). We found that 93% of bovine monocytes possessed these receptors. IgG receptors are believed to mediate both attachment and ingestion of particles and complement receptors are considered to mediate primarily attachment to normal mononuclear phagocytes (Bianco, Griffin & Silverstein, 1975). In this study, we have found similar functions for these receptors on bovine monocytes.

Peroxidase activity, associated with a population of cytoplasmic storage granules, has been demonstrated in human (Nichols & Bainton, 1973) and rat (Bentfeld, Nichols & Bainton, 1977) monocytes. Tissue macrophages, apparently derived from blood monocytes (Van Furth & Willemze, 1979), do not contain granule-associated peroxidase activity (Daems, Wisse, Brederoo & Emeis, 1975). It has been suggested that the loss of granule-associated peroxidase activity by monocytes in culture is related to maturation of these cells into macrophages (Bodel, Nichols & Bainton, 1977; Beelen, van't Veer, Fluitsma & Hoefsmit, 1978). We have examined the kinetics of peroxidase activity in bovine monocytes during cultivation. The progressive loss of granule-associated peroxidase activity by bovine monocytes resembles that reported for murine (Van Furth, Hirsch & Fedorko, 1970) and human monocytes (Van Furth, Raeburn and van Zwet, 1979).

Associated with a progressive enlargement in cell size bovine monocytes exhibited increases in total cell protein which levelled off beyond 15 days culture, although the cells continued to increase in size. Striking increases in acid phosphatase specific activity were noted in bovine monocytes from 15 to 20 days in culture, at a time when cell protein values were constant. These changes are comparable to 5–10 day human monocyte cultures as reported by Zuckerman *et al.* (1979).

In humans and mice, immature granulocytes, monocytes, and macrophages are the cells primarily responsible for production of lysozyme (McClelland & Van Furth, 1975). Secretion of lysozyme is a constitutive function of mononuclear phagocytes (Gordon *et al.*, 1974). In the present study, bovine mononuclear phagocytes neither secreted lysozyme nor contained it intracellularly. A previous investigation indicated the absence of lysozyme in bovine polymorphonuclear leucocytes, tears, saliva, and nasal exudates (Padgett & Hirsch, 1967). These findings, in conjunction with the present report, suggest an inability of cattle to produce lysozyme. The bactericidal role of lysozyme is well established (Glynn, 1968) and recent investigations have suggested that lysozyme plays a role in modulation of inflammation (Gordon, Douglas, Kay, Yamada, Osserman & Jacob, 1979). Lysozyme may also play a role in responses to tumours and can interact with mammalian cell membranes (Osserman, Klockars, Halper & Fischel, 1973). The identity of substances performing these functions in cattle remains largely unclear.

The techniques described in this paper should facilitate study of the physiology and differentiation of mononuclear phagocytes and provide an *in vitro* model for examination of the roles of these cells in inflammatory reactions.

ACKNOWLEDGMENTS

We wish to thank Drs B. L. Deyoe and M. L. Kaeberle for reading the manuscript and J. Lang for secretarial assistance.

The work was supported in part by co-operative agreement with the Science and Education Administration–Agricultural Research, United States Department of Agriculture.

REFERENCES

Beck C., Mahadevan S., Brightwell R., Dillard C.J. & Tappel A.L. (1968) Chromatography of lysosomal enzymes. *Arch. Biochem. Biophys.* **128,** 369.

Beelen R.H.J., van't Veer M.B., Fluitsma D.M. & Hoefsmit E.C.M. (1978) Identification of different peroxidatic patterns in human macrophages *in vivo* and *in vitro*. *J. reticuloendothel. Soc.* **24,** 351.

Bennett W.E. & Cohn Z.A. (1966) The isolation and selected properties of blood monocytes. *J. exp. Med.* **123,** 145.

Bentfeld M.E., Nichols B.A. & Bainton D.F. (1977) Ultrastructural localization of peroxidase in leukocytes of rat bone marrow and blood. *Anat. Rec.* **187,** 219.

Berman J.D. & Johnson W.D. (1978) Monocyte function in human neonates. *Infect. Immun.* **19,** 898.

Bianco C., Griffin F.M. & Silverstein S.C. (1975) Studies of the macrophage complement receptor. Alteration of receptor function upon macrophage activation. *J. exp. Med.* **141,** 1278.

Bianco C., Eden A. & Cohn Z.A. (1976) The induction of macrophage spreading: role of coagulation factors and the complement system. *J. exp. Med.* **144,** 1531.

Bodel P.T., Nichols B.A. & Bainton D.F. (1977) Appearance of peroxidase reactivity within the rough endoplas-

mic reticulum of blood monocytes after surface adherence. *J. exp. Med.* **145,** 264.

CARLSON G.P. & KANEKO J.J. (1973) Isolation of leukocytes from bovine peripheral blood. *Proc. Soc. exp. Biol Med.* **142,** 853.

CARSON C.A., SELLS D.M. & RISTIC M. (1975) A method for separation of bovine blood leukocytes for *in vitro* studies. *Am. J. vet. Res.* **36,** 1091.

COHN Z.A. & BENSON B. (1965) The differentiation of mononuclear phagocytes. Morphology, cytochemistry, and biochemistry. *J. exp. Med.* **121,** 153.

COHN Z.A. (1968) The structure and function of monocytes and macrophages. In: *Advances in Immunology* (Ed. by F. J. Dixon and H. G. Kunkel), p. 164. Academic Press, New York.

DAEMS W.T., WISSE E., BREDEROO P. & EMEIS J.J. (1975) Peroxidatic activity in monocytes and macrophages. In: *Mononuclear Phagocytes in Immunity, Infection, and Pathology* (ed. by R. Van Furth), p. 57. Blackwell Scientific Publications, Oxford.

DAVIES P. & BONNEY R.J. (1979) :Secretory products of mononuclear phagocytes: a brief review. *J. reticuloendothel. Soc.* **26,** 37.

EDELSON P.J. & COHN Z. A. (1976) Purification and cultivation of monocytes and macrophages. In: In Vitro *Methods in Cell-Mediated and Tumor Immunology* (Ed, by B. Bloom and J. R. David), p. 333. Academic Press, New York.

FITZGEORGE R.B., SOLOTOROVSKY M. & SMITH H. (1967) The behaviour of *Brucella abortus* within macrophages separated from the blood of normal and immune cattle by adherence to glass. *Br. J. exp. Pathol.* **48,** 522.

GLYNN A. A. (1968) Lysozyme: antigen, enzyme and antibacterial agent. *Sci. Basis med. Ann. Rev.* **31,** 31.

GOLDSTEIN M.N. (1954) Formation of giant cells from human monocytes cultivated on cellophane. *Anat. Rec.* **118,** 577.

GORDON L.I., DOUGLAS S.D., KAY N.E., YAMADA O., OSSERMAN E.F. & JACOB H.S. (1979) Modulation of neutrophil function by lysozyme. Potential negative feedback system of inflammation. *J. clin. Invest.* **64,** 226.

GORDON S., TODD J. & COHN Z.A. (1974) *In vitro* synthesis and secretion of lysozyme by mononuclear phagocytes. *J. exp. Med.* **139,** 1228.

JOHNSON W.D., MEI B. & COHN Z.A. (1977) The separation, long-term cultivation, and maturation of the human monocyte. *J. exp. Med.* **146,** 1613.

KAPLOW L.S. (1965) Simplified myeloperoxidase stain using benzidine dihydrochloride. *Blood,* **26,** 215.

KELLER R. (1977) Mononuclear phagocytes and antitumour resistance: a discussion. In: *The Macrophage and Cancer* (Ed. by K. James, W. J. McBride and A. Stewart), p. 31. Edinburgh University Medical School.

LEWIS M.R. (1925) The formation of macrophages, epithelioid cells and giant cells from leukocytes in incubated blood. *Am. J. Path.* **1,** 91.

LI C.Y., LAM K.W. & YAM L.T. (1973) Esterases in human leukocytes. *J. Histochem. Cytochem.* **21,** 1.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. & RANDALL R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265.

MCCLELLAND D.B.L. & VAN FURTH R. (1975) *In vitro* synthesis of lysozyme by human and mouse tissues and leucocytes. *Immunology,* **28,** 1099.

NICHOLS B.A. & BAINTON D.F. (1973) Differentiation of human monocytes in bone marrow and blood. Sequential formation of two granule populations. *Lab. Invest.* **29,** 27.

NORTH R.J. (1978) The concept of the activated macrophage. *J. Immunol.* **121,** 806.

OSSERMAN E.F., KLOCKARS M., HALPER J. & FISCHEL R.E. (1973) Effects of lysozyme on normal and transformed mammalian cells. *Nature (Lond.),* **243,** 331.

PADGETT G.A. & HIRSCH J.G. (1967) Lysozyme: its absence in tears and leucocytes of cattle. *Aust. J. exp. Biol. med. Sci.* **45,** 569.

ROSENSTREICH D.L. & MIZEL S.B. (1978) The participation of macrophages and macrophage cell lines in the activation of T lymphocytes by mitogens. *Immunol. Rev.* **40,** 102.

ROSENTHAL A.S. (1978) Determinant selection and macrophage function in genetic control of the immune response. *Immunol. Rev.* **40,** 136.

ROSSI C.R. & KIESEL G.K. (1977a) Bovine peripheral blood monocyte cultures: growth characteristics and cellular receptors for immunoglobulin G and complement. *Am. J. vet. Res.* **38,** 559.

ROSSI C.R. & KIESEL G.K. (1977b) Bovine immunoglobulin G subclass receptor sites on bovine macrophages. *Am. J. vet. Res.* **38,** 1023.

VAN FURTH R., HIRSCH J.G. & FEDORKO M.E. (1970) Morphology and peroxidase cytochemistry of mouse promonocytes, monocytes, and macrophages. *J. exp. Med.* **132,** 794.

VAN FURTH R. & WILLEMZE R.(1979) Phagocytic cells during an acute inflammatory reaction. In: *Current Topics in Pathology* (Ed. by H. Z. Movat), p. 179. Springer-Verlag, Berlin.

VAN FURTH R., RAEBURN J.A. & VAN ZWETT T.L. (1979) Characteristics of human mononuclear phagocytes. *Blood,* **54,** 485.

WANG C.-S. & SMITH R.L. (1975) Lowry determination of protein in the presence of Triton X-100. *Anal. Biochem.* **63,** 414.

WARDLEY R.C., LAWMAN M.J. & HAMILTON F. (1980) The establishment of continuous cell lines from peripheral blood monocytes. *Immunology,* **39,** 67.

ZUCKERMAN S.H., ACKERMAN S.K. & DOUGLAS S.D. (1979) Long-term human peripheral blood monocyte cultures: establishment, metabolism and morphology of primary human monocyte-macrophage cell cultures. *Immunology,* **38,** 401.