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Isolation and functional studies on feline bone marrow derived macrophages

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ABSTRACT

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In this report, we describe an *in vitro* culture method for feline bone marrow cells, which yields large numbers of quiescent macrophages after 14 days of culture. The bulk of the cultured cell population consists of macrophages as assessed by morphology, macrophage specific cytochemistry, and phagocytosis. The remaining cells were lymphocytes, bone marrow stromal cells, fibroblasts and occasional polymorphonuclear leukocytes. While resting cells produced no detectable interleukin 1, stimulation with lipopolysaccharide (LPS) induced the production of biologically active interleukin 1. After 6 h LPS stimulation, mRNA for tumor necrosis factor α and interleukin 1 β was detectable. The absence of mRNA in unstimulated cells indicates cultured macrophages were not activated until stimulated by LPS or plastic adherence. This approach provides a useful means to measure potential modulatory effects by virus infections or other agents upon feline macrophage gene expression.

ABBREVIATIONS

BM, bone marrow; FeLV, feline leukemia virus; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; IL-1, interleukin 1; LPS, lipopolysaccharide; PHA-P, phytohemagglutinin P; PMN, polymorphonuclear leukocytes; TNF, tumor necrosis factor.

INTRODUCTION

Viral-macrophage interactions may crucially influence the pathogenesis of infections, particularly retroviruses such as human immunodeficiency virus (HIV) (Narayan and Zink, 1988; Meltzer et al., 1990), feline leukemia virus (FeLV) (Hoover et al., 1981), and feline immunodeficiency virus (FIV)

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(Brunner and Pedersen, 1989). Given the interactions of HIV and macrophages (Narayan and Zink, 1988; Meltzer et al., 1990), it seems likely that infection of feline macrophages by agents such as FIV or FeLV may influence their function, but this point requires further investigation. A practical problem with studies on feline macrophages is that cell lines are lacking and obtaining adequate numbers of macrophages for study may at times be difficult.

In this report, we describe an approach which yields a large population of mature macrophages at the end of 14 days of *in vitro* culture. The technique involves aspiration of a small amount of bone marrow followed by *in vitro* suspension culture for a minimum of 14 days. Because of the small amount of bone marrow necessary for culture, bone marrow samples may be taken multiple times from the same cat by alternating bone marrow harvest sites. Results of morphological and functional studies on cultured cells are described.

MATERIALS AND METHODS

Animals

In all experiments, eight FeLV and FIV seronegative random-source adult cats were used as bone marrow donors. Animals were housed separately at the animal facilities of the University of Tennessee School of Veterinary Medicine, Knoxville, Tennessee (a fully accredited AALAC facility).

Cell lines and culturing conditions

P388D1 (TIB 63) and LBRM 33-1A5 (CRL 8079) cells were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Cell lines were cultured in accordance with ATCC recommendations. For production of murine interleukin 1 (IL-1), P388D1 cells (1×10^6 cells ml^{-1}) were stimulated in RPMI plus 5% heat-inactivated fetal bovine sera (HI-FBS; Hyclone, Logan, UT) with $10 \mu\text{g ml}^{-1}$ *Escherichia coli* serotype 0127:B8 lipopolysaccharide (LPS; Sigma Chemicals, St. Louis, MO) for 48 h (Gearing et al., 1985). Supernates were harvested and frozen at -70°C until assayed for IL-1 activity. LBRM 33-1A5 cells were used in an IL-1 conversion assay for determination of biologically active IL-1 in supernates (Gillis and Mizel, 1981).

CTLL-2 cells were obtained from ATCC and cultured according to recommendations, with the addition of IL-2 generated from phorbol myristic acetate stimulated EL4.IL-2 cells (Farrar et al., 1980).

Feline bone marrow isolation technique and in vitro culturing method

Bone marrow (BM) was obtained with cats under general ketamine hydrochloride (Ketalar^R; Parke Davis, Morris Plains, NJ) anesthesia (1 mg lb^{-1}

i.v. or 10 mg lb^{-1} i.m.). The sacrolumbar area was clipped, surgically prepped and draped. Approximately 0.5 ml of BM was aspirated by a BM needle from the femur directly into a 10 ml syringe containing 3 ml of Iscove's modified Dulbecco's medium (IMDMEM; GIBCO BRL, Grand Island, NY) with 100 units heparin sulfate ml^{-1} . Heparinized BM was mixed, placed into tubes containing heparinized IMDMEM and placed on ice until processed. Following anesthesia and BM sampling, cats were allowed to recover. BM samples from each cat were cultured separately.

For separation, heparinized BM samples were brought to 10 ml final volume with heparinized IMDMEM, vortexed for 20 s then left undisturbed for 5 min to allow bone chips and cartilage to settle out. The bulk of the supernate was removed and 5 ml aliquots layered over 3 ml of Ficoll-sodium diatrizoate (Histopaque 1.077; Sigma Chemicals) in 15 ml centrifuge tubes. Tubes were centrifuged at $400\times g$ for 25 min at 25°C . The cellular band was removed, washed twice with heparinized IMDMEM, followed by a final wash with non-heparinized IMDMEM. Cells were counted and viability assessed by 0.2% trypan blue dye exclusion. Viability averaged greater than 97% and never dropped below 95%. Cell numbers were adjusted to 2.0×10^6 cells ml^{-1} IMDMEM containing 15% HI-FBS (HyClone), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units penicillin ml^{-1} , 100 μg streptomycin ml^{-1} , 10 mM HEPES and sodium bicarbonate to pH of 7.3. No additional additives were necessary to provide feline specific colony stimulating factors.

Cell suspension (10 ml) was added to 55 cm^2 tissue culture dishes (Falcon, Baxter, Atlanta, GA) and cultured at 37°C in a humid 6% CO_2 chamber. After 7 days, cultures were refed with 10 ml of the above medium and incubation continued for a total of 14 days in vitro culture.

Macrophages assessed for IL-1 production or harvested for Northern blot analysis were pulsed with $10 \mu\text{g}$ LPS ml^{-1} by one of two methods.

Method 1. Medium was gently removed from cell cultures and the few non-adherent cells pelleted by centrifugation. Pelleted cells were resuspended in medium containing $10 \mu\text{g}$ LPS ml^{-1} . Then 20 ml of medium plus LPS were gently layered over the undisturbed macrophage monolayers. Incubation was continued at 37°C in a humid 6% CO_2 chamber for 6, 24, or 48 h, at which point supernates were harvested, frozen in small aliquots at -70°C for quantitation of IL-1 biological activity and cells lysed for RNA isolation.

Method 2. Loosely adherent macrophages were gently washed from culture dishes, while adherent cells were removed by incubating at room temperature with Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS; GIBCO BRL) plus 2.6 mM EDTA (Sigma Chemicals) followed by gentle pipetting. Cells were resuspended in complete medium at 1×10^6 cells ml^{-1} plus $10 \mu\text{g}$ LPS ml^{-1} . Cultures were incubated in a 37°C , humid 6% CO_2 incubator for

6, 24, or 48 h, at which point supernates were harvested, frozen in small aliquots for IL-1 testing, and cells lysed for RNA isolation.

For total RNA isolation, cell pellets were lysed in 4 M guanidinium isothiocyanate (Sigma Chemicals) containing 0.5% sodium lauryl sarcosine, 25 mM sodium citrate (pH 7.0), and 47 mM 2-mercaptoethanol. Samples were stored at -70°C until RNA separation.

Morphological and cytochemical characterization of population

Non-adherent cells were recovered by gentle pipetting, while adherent cells were removed by incubating with 5 ml of HBSS plus 2.6 mM EDTA. Adherent and non-adherent cells were mixed, concentrations determined, and cytopspins prepared (Cytospin; Shandon, Pittsburgh, PA) for differential and non-specific esterase stains. Cytopspins were air dried and stained with Diff-Quik stain (Harleco, Baxter, Atlanta, GA) for differentials. Non-specific esterase stains were performed by the method of Li et al. (1973). A minimum of 200 cells were counted for differential analysis and esterase determination. For phagocytosis assays, cells were plated at 2.0×10^5 cells ml^{-1} IMDMEM plus $10 \mu\text{g}$ LPS ml^{-1} in eight chamber Lab-Tek slides. In each well, $10 \mu\text{l}$ of a 1:100 dilution of $0.8 \mu\text{m}$ latex beads (Sigma Chemicals) were added. Cultures were incubated at 37°C in a humid 6% CO_2 atmosphere for 24 h; slides were then fixed, Diff-Quik stained and examined for phagocytosis of latex beads. A cell was deemed phagocytic if it engulfed a minimum of ten beads; however, the vast majority of cells contained massive numbers of beads which often obscured the nucleus.

IL-1 conversion assay for detection of biologically active IL-1

The IL-1 conversion assay was performed by the method of Gillis and Mizel (1981). Briefly, 1×10^5 washed LBRM 33-1A5 cells were added to sterile snap-capped tubes. Each tube contained the following: 25% putative IL-1 containing supernate plus $0.75 \mu\text{g}$ of phytohemagglutinin P (PHA-P; Sigma Chemicals) in a final volume of $300 \mu\text{l}$. Additionally, the following controls were run: LBRM cells plus medium alone, and LBRM cells plus 25% IL-1 containing supernate in the absence of PHA-P. Samples were run in triplicate and incubated at 37°C in 6% CO_2 for 24 h. Samples were centrifuged; supernates were harvested and frozen in small aliquots at -70°C until CTLL-2 assay for quantitation of IL-2.

In Step 2 of the conversion assay, two-fold serial dilutions of LBRM supernate for IL-2 quantitation were added to washed CTLL-2 cells plated at 5×10^3 cells per well of a 96 well round-bottomed microtiter plate. Human recombinant IL-2 (GIBCO BRL) was titered in each assay as a control for CTLL-2 proliferation and to provide a means to quantify test sample IL-2. Additionally, supernate harvested directly from stimulated macrophage cultures was

tested for the presence of IL-2, produced endogenously by residual lymphocytes. Plates were incubated 24 h at 37°C in a humid 6% CO₂ atmosphere. Wells were pulsed with 1 µCi of [³H]thymidine for 24 h then harvested and the radioactivity quantitated.

Total RNA isolation procedure and Northern blot analysis

Cells for total RNA isolation were lysed in 4 M guanidinium isothiocyanate lysing solution as previously described. Cell lysates were ultracentrifuged at 36 000 r.p.m. for 18 h through a 5.7 M cesium chloride cushion and the RNA pellets harvested (Chirgwin et al., 1979). All molecular reagents were prepared as described in standard protocol texts for molecular biology (Ausubel et al., 1989).

RNA was quantitated by spectrophotometry, and 10 µg per sample denatured for fractionation through a 1.0% agarose/1.1 M formaldehyde gel containing ethidium bromide for RNA visualization. The 10× running buffer consisted of 0.5 M boric acid, 50 mM sodium borate, 100 mM sodium sulfate and 10 mM EDTA. RNA samples were denatured by boiling for 10 min in 20 µl of the following buffer mix: 0.63 ml denatured formamide (GIBCO BRL), 0.20 ml 37% formaldehyde, 0.063 ml of 10× running buffer, 0.10 ml of 50% glycerol in diethylpyrocarbonate treated water, and 0.030 ml of a 0.6% solution of bromophenol blue/xylene cyanol. Gels were loaded with RNA samples and run at a constant current of 95 V until the bromophenol blue dye front had traveled approximately 14 cm. RNA was transferred from gels to nylon filters (Nytran; Schleicher and Schuell, Keene, NH) overnight by a sponge modification of the capillary transfer method (Fourney et al., 1988).

After capillary transfer, nylon filters were UV-crosslinked to stabilize RNA (Khandjian, 1986) (Stratalinker UV Crosslinker; Stratagene, La Jolla, CA) and filters prehybridized at 60°C for 1 h in 5× SSC (from 20× sodium chloride/sodium citrate stock), 0.05 M sodium phosphate buffer pH 6.5, 5× Denhardt's solution, 1% sodium dodecyl sulfate (SDS), with 200 µg ml⁻¹ denatured sperm DNA as a blocker. Following prehybridization, filters were hybridized overnight at 60°C in prehybridization mix plus the appropriate ³²P-labeled nick-translated (Amersham Kit N5500; Amersham International, Amersham, UK) cDNA probe. Nick-translated probes were labeled to a specific activity of 1 to 2×10⁷ counts min⁻¹ µg⁻¹. Post hybridization, filters were washed: (1) twice at room temperature in 2× SSC plus 0.1% SDS for 15 min each; (2) twice at room temperature for 15 min each in 0.2× SSC plus 0.1% SDS; (3) once for 20 min at 55°C with 0.2× SSC plus 0.1% SDS; (4) final wash, once for 30 min at 60°C with 0.1× SSC plus 0.1% SDS.

Following washing, blots were exposed for 70 h at -70°C to Kodak XAR 5 film in the presence of intensifying screens. The nylon filters were then stripped of radioactive cDNA probes by washing them in 50% formamide/

6× SSPE (from 20× sodium chloride/sodium phosphate stock solution) at 65°C for 30 min followed by rinsing briefly in 2× SSPE. Filters were re-probed with additional radiolabeled cDNA probes after stripping. Filters were first probed with tumor necrosis factor (TNF) α specific probes, blots were stripped followed by probing with IL-1 β specific probe, and blots were re-stripped then probed with an actin specific cDNA probe.

DNA probes for feline TNF α and IL-1 β were constructed in this laboratory (Daniel et al., 1992). The ³²P-labeled TNF α probe was prepared from the plasmid, pTNF α B9, which contains a 570 bp Eco R1, Hind III fragment of feline TNF α cDNA. The ³²P-labeled IL-1 β probe was prepared from the plasmid, pIL1#2, which contains a 750 bp, Eco R1, Hind III fragment of feline IL-1 β cDNA. The actin cDNA plasmid (pA1) was a gift from Dr. Janet Lathey (Scripps Institute) and contains a 750 bp Eco R1, Hind III cDNA fragment of chicken actin.

RESULTS

Morphological characterization of bone marrow derived population

Feline BM aspirates yielded an average of 1.5×10^8 nucleated cells (range 2.0×10^7 to 2.8×10^8). After 7 days of in vitro culture, the cell population was mixed and consisted of approximately equal numbers of mature polymorphonuclear leukocytes (PMNs) and immature cells of the myelomonocytic series (Table 1). However, after 14 days in culture, the average number of cells recovered was 2.3×10^7 (range 4.2×10^6 to 1.3×10^8) of which 90% were monocyte/macrophages and 10% were variously composed of lymphocytes, PMNs, immature myelomonocytic or erythroid progenitor cells and an occasional megakaryocyte (Table 1). After 14 days in culture, macrophages appeared round, elongated or stellate, differing significantly in the amount of cytoplasmic spreading and ruffling seen (Fig. 1(A)). Macrophage nuclei were kidney shaped or slightly ovoid and eccentrically located with small nucleoli and a large cytoplasm: nucleus ratio (Fig. 1(B)). The predominant cell types were macrophages but occasional lymphoid cells were present (Fig. 1(B)).

Fourteen-day-old BM cultures were examined for esterase and phagocytic activities (Table 1). The majority of cells (average 93%) were positive for butyrate esterase activity (Table 1; Fig. 1(C) and 1(D)). There was variability in the amount of enzyme activity with some cells staining intensely dark while others stained lightly for esterase activity. In Fig. 1(C), numerous darkly stained macrophages are present while esterase negative adherent bone marrow stromal cells and lymphocytes are evident in the background. Occasional esterase positive stellate or elongated cells could be seen, suggesting that these were of monocyte/macrophage lineage, but the predominant esterase negative cell was triangular with long cytoplasmic extensions suggestive of bone

TABLE 1

Characterization of cell population present following 7 and 14 days of in vitro culture of bone marrow cells

Cell type	Differential analysis ¹	
	7 day cultures	14 day cultures
Macrophages	(6–11%) 8.5%	(80–95%) 90%
Lymphocytes	(4–8%) 6.0%	(3–9%) 4%
PMN	(37–50%) 43.5%	(0–17%) 4%
Other ²	(35–49%) 42.0%	(0–2%) 1%
	Phagocytosis and cytochemical evaluation of 14 day cultures	
	Positive	Negative
α -Naphthol butyrate esterase ³	(79–99%) 93%	(1–12%) 7%
Latex bead phagocytosis ³	(78–97%) 89%	(3–22%) 11%

¹Differential analysis as described in Materials and Methods. Values within parentheses are ranges observed with the average percentage listed to the right of the parentheses. $N=8$ cats.

²Other cells: granulocyte-macrophage progenitor cells, erythroid precursors, megakaryocytes, bone marrow stromal cells.

³Assays described in Materials and Methods.

marrow stromal cells. In Fig. 1 (D) two esterase negative lymphocytes may be seen nestled against a macrophage.

Fourteen-day-old macrophages also displayed significant phagocytic capacity for latex beads (Table 1; Fig. 1 (E)). LPS activated macrophages engulfed significant numbers of latex beads, often leading to cellular distortion and obscuring of the nucleus. On the average, 89% of the population present on Day 14 phagocytized latex beads. Non-phagocytic cells were adherent, triangular shaped cells with long cytoplasmic extensions, having the appearance of bone marrow stromal cells, but occasional non-phagocytic lymphocytes were also present.

Presence of biologically active IL-1 in LPS stimulated bone marrow derived macrophage cultures

Culture fluids collected directly from 14 day cultured macrophages contained no detectable IL-1, but biologically active IL-1 was readily detectable following stimulation with LPS (Table 2).

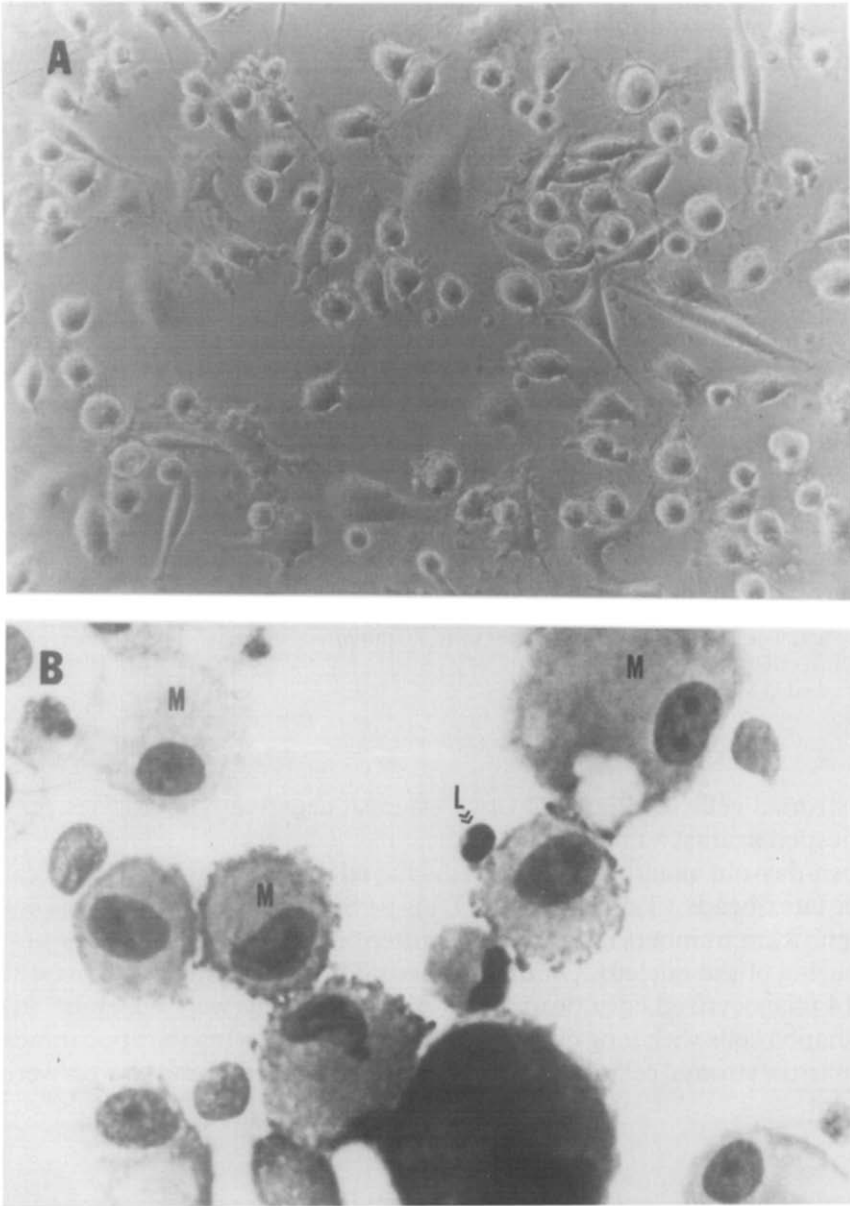


Fig. 1. Identification of BM derived cells as macrophages based upon morphology, cytochemistry, and phagocytosis assays. Cytospins of BM cells cultured in vitro for 14 days were made, stained by Diff-Quik (B) and evaluated morphologically. Lab-Tek chamber slide cultures of bone marrow cells were evaluated for α -naphthol butyrate esterase activity (C) and (D), while latex bead phagocytosis was evaluated following $10 \mu\text{g LPS ml}^{-1}$ stimulation (E). (A) is representative of the typical appearance of BM derived macrophages after in vitro culture for 14 days (magnification $\times 66$). (B) is a representative Diff-Quik stain of the cultured population (magnification $\times 400$). In (C) and (D), the darkly staining cells are positive for esterase activity (magnification $\times 100$ for (C) and $\times 400$ for (D)). In (E), the phagocytic macrophages have been stained with Diff-Quik allowing visualization of the nucleus. Extensive phagocytic activity is readily apparent, massive numbers of latex beads are visible within the cytoplasm of many cells. A non-phagocytic bone marrow stromal cell as well as a lymphocyte are visible in the center of the micrograph (magnification $\times 400$). M, macrophage; L, lymphocyte; BM, bone marrow stromal cell.

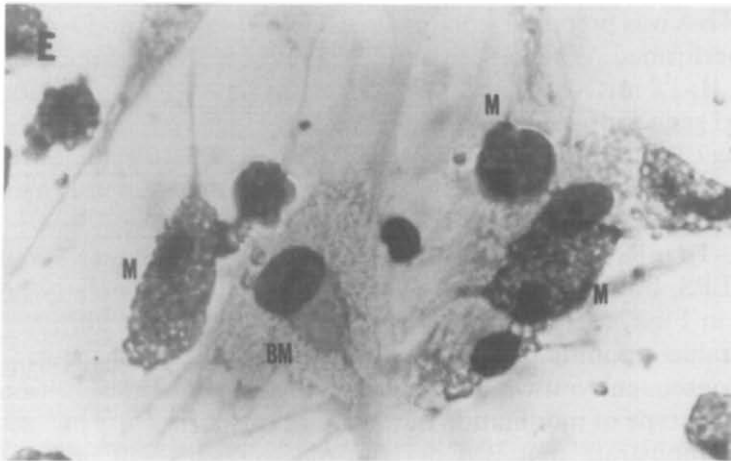
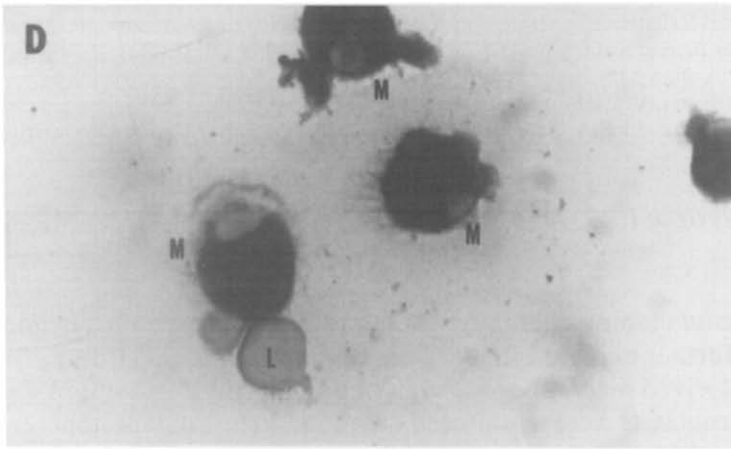
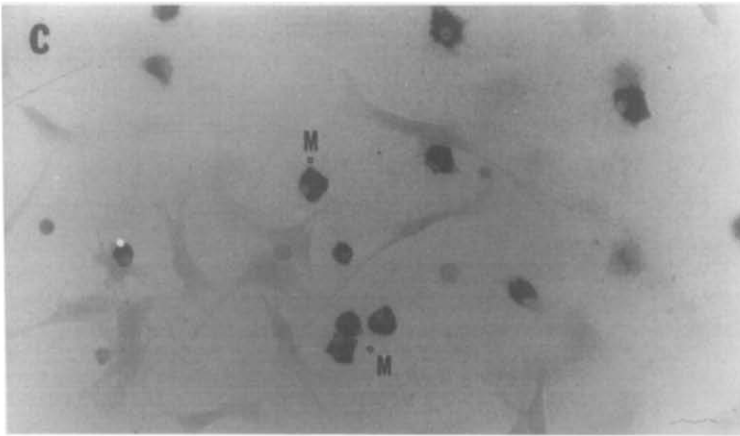


TABLE 2

BM macrophages produce biologically active IL-1: IL-2 production by IL-1 stimulated LBRM 33-1A5 cells

LBRM cells stimulated with		IL-2 present in 24 h supernate (units ml ⁻¹)
Test supernate	PHA	
Medium	None	0
Medium	+	0
P388D1	None	0
P388D1	+	40
Cat 1	None	0
Cat 1	+	40
Cat 2	None	0
Cat 2	+	50

IL-1 conversion assay and IL-2 detection are described in Materials and Methods. Units of IL-2 present in culture supernates determined by comparison with human recombinant IL-2 standard curve. Supernates taken directly from LPS stimulated macrophages were negative for endogenously produced IL-2. Supernates from LBRM 33-1A5 cells previously exposed to medium only, medium plus PHA-P, or test medium containing IL-1 without the addition of PHA-P were also negative for IL-2 production.

Northern blot analysis of LPS stimulated macrophages for IL-1 β and TNF α mRNA

With the successful cloning of cDNAs for feline IL-1 β and TNF α (Daniel et al., 1992), we further explored the kinetics of cytokine production by 14 day cultured BM derived macrophages. After 14 days in culture, medium was removed and macrophages were re-cultured (without disturbing the adherent monolayer—Method 1) in the presence or absence of 10 μ g LPS ml⁻¹ for 6, 24 or 48 h. Total RNA was prepared from macrophage cell lysates and Northern blot analysis performed. Whereas unstimulated cells failed to produce detectable TNF α or IL-1 β mRNA (Fig. 2(A) and 2(B), Lane 1), stimulation with LPS activated gene expression for both cytokines (Fig. 2(A) and 2(B), Lane 2). Following LPS stimulation, TNF α mRNA was readily detectable at 6 h post activation; however, TNF α mRNA was barely detectable or absent after 24 and 48 h (Fig. 2(A)).

In Fig. 2(B), IL-1 β mRNA was readily detectable following 6 and 48 h of stimulation with LPS, but at 24 h mRNA levels for IL-1 β were greatly reduced. As shown in Fig. 2(C), actin mRNA levels are similar in all lanes, indicating comparable amounts of total RNA were loaded per lane during electrophoresis. Consequently, it would appear that IL-1 β gene transcription was undergoing some type of modulation at the 24 h time point.

These studies demonstrate that BM derived macrophages will produce TNF α and IL-1 β after appropriate stimulation; however, their gene tran-

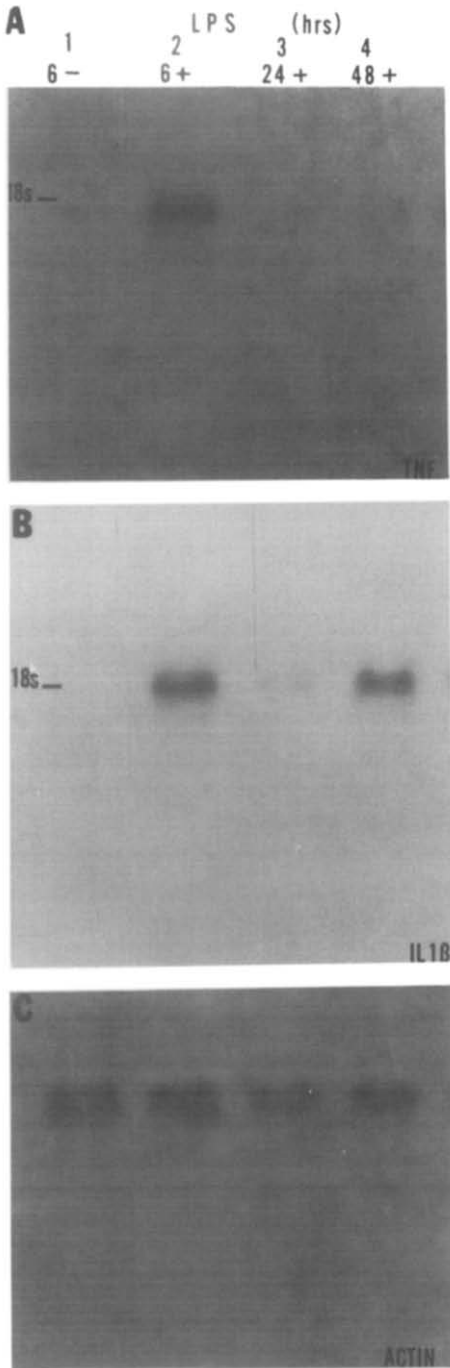


Fig. 2. Kinetic study: Northern blot analysis of LPS stimulated BM derived macrophages—bl Method 1. After 14 days in culture, growth medium was changed and LPS stimulation of cultures begun without disturbing the adherent macrophage cell monolayer. Procedure is fully described in Materials and Methods. Macrophages were stimulated for selected periods of time at which point total RNA was harvested and Northern blot analysis performed as outlined in Materials and Methods. Autoradiographs were exposed for 70 h at -70°C in the presence of an intensifying screen. Then $10\ \mu\text{g}$ of total RNA were added per lane for electrophoresis. Location of the 18S ribosomal band is marked. Blots were hybridized to ^{32}P -labeled cDNA probes for $\text{TNF}\alpha$ (A), $\text{IL-1}\beta$ (B), or actin (C) for 18 h followed by washing and autoradiography. Total RNA was derived from cells cultured in the presence of fresh growth medium in the absence of LPS for 6 h (Lane 1), and in the presence of LPS for 6, 24 or 48 h (Lanes 2, respectively).

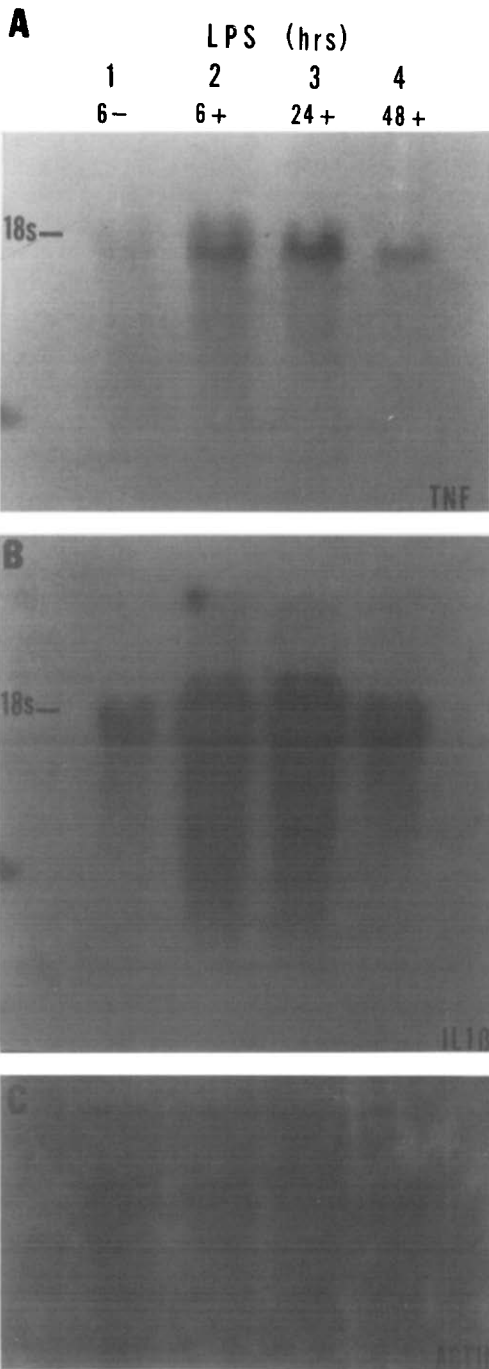


Fig. 3. Kinetic study: Northern blot analysis of LPS stimulated BM derived macrophages—b1Method 2. After 14 days in culture, adherent macrophages were removed from plastic, counted and replated for defined time periods in the presence or absence of $10 \mu\text{g LPS ml}^{-1}$. Procedure is fully described in Materials and Methods. Macrophages were stimulated for selected periods of time at which point total RNA was harvested and Northern blot analysis performed. Autoradiographs were exposed for 70 h at -70°C in the presence of an intensifying screen, for all blots. Filters were probed, stripped and reprobed in the following order: $\text{TNF}\alpha$, $\text{IL-1}\beta$, actin. Then $10 \mu\text{g}$ of total RNA were added per lane for electrophoresis. Location of the 18S ribosomal band is marked. Total RNA was hybridized to ^{32}P -labeled cDNA probes for $\text{TNF}\alpha$ (A), $\text{IL-1}\beta$ (B) and actin (C) for 18 h followed by washing. RNA was derived from cells cultured in the absence of LPS for 6 h (Lane 1), or in its presence for 6, 24 or 48 h (Lanes 2, 3 and 4, respectively).

scription appears to be tightly regulated, with $\text{TNF}\alpha$ mRNA expression declining rapidly after 6 h of LPS stimulation. $\text{IL-1}\beta$ mRNA shows a biphasic pattern with mRNA levels dropping significantly by 24 h of LPS stimulation followed by re-expression at 48 h. This pattern of $\text{TNF}\alpha$ and $\text{IL-1}\beta$ mRNA expression was reproducible as long as care was taken to prevent inadvertent activation of macrophages prior to LPS stimulation.

Interestingly, a separate pattern of cytokine mRNA induction was observed if cells were first harvested from tissue culture dishes, washed and replated onto tissue culture plastic in the presence or absence of LPS (compare Figs. 2(A) and (B) and 3(A) and (B)). Removal of macrophages with subsequent re-adherence to plastic for even a short time activates cytokine gene expression as demonstrated by the presence of $\text{IL-1}\beta$ mRNA in the absence of LPS stimulation (Fig. 3(B), Lane 1). Adherence to plastic may also have primed cells to produce increased amounts of $\text{TNF}\alpha$ mRNA following LPS stimulation since $\text{TNF}\alpha$ message is present in Lane 1 and mRNA levels do not begin to decrease until 48 h (Fig. 3(A), Lanes 2, 3 and 4). The protracted presence of $\text{TNF}\alpha$ mRNA in Fig. 3 (A), Lanes 2, 3 and 4) compared with Fig. 2 (A), Lanes 2, 3 and 4) may be attributed to LPS acting as a second signal for superinduction of $\text{TNF}\alpha$ message by previously 'primed' macrophages.

In the absence of LPS stimulation (Fig. 3(B), Lane 1) macrophages were producing a significant amount of $\text{IL-1}\beta$ mRNA which increased greatly following LPS stimulation (Fig. 3(B), Lanes 2, 3 and 4). Since comparable amounts of total RNA were loaded per lane (Fig. 3(C), all lanes for actin probe), LPS activation of an already 'primed' macrophage population (Lane 1, (B)) may have resulted in superinduction of gene transcription with enhanced $\text{IL-1}\beta$ mRNA production. Alternatively, normal degradation of $\text{IL-1}\beta$ mRNA could be blocked, extending the mRNA half-life with consequent increased expression of $\text{IL-1}\beta$ mRNA at a given point in time. Whatever the mechanism, it is apparent that even a slight modification in protocol (removal of cells prior to stimulation with LPS for determination of cell concentration versus a gentle medium change with no disturbance of the monolayer prior to LPS stimulation) may drastically alter cytokine induction patterns.

DISCUSSION

Large numbers of functional feline macrophages may be successfully obtained from bone marrow by culturing *in vitro* for 14 days. From an average input of 1.5×10^8 nucleated cells, an average of 2.3×10^7 cells was obtained at the end of 14 days of which approximately 90% were typical mature macrophages. Following culture for only 7 days, the cell population included many additional cell types such as PMNs and immature progenitor cells of varying lineage specificity. BM macrophage cultures could be successfully maintained

(to at least 21 days) without adding an exogenous source of feline colony stimulating factors. The probable source of endogenous macrophage colony stimulating factor or granulocyte-macrophage colony stimulating factor which is driving cellular development is the adherent bone marrow stromal cells (Dorshkind, 1990). These cells are known to be major producers of colony stimulating factors and to play a significant role in hematopoietic cell development (Metcalf, 1989; Dorshkind, 1990).

Because of the small sample size of BM which is required for *in vitro* culturing, this technique has the advantage that cats (after a short rest period) may serve as repeated BM donors, so large colonies of cats need not be maintained. Additionally, if cultures are properly maintained in LPS free medium, macrophages may be kept in a non-activated state as measured by a lack of detectable mRNA for TNF α or IL-1 β . However, when activated with LPS, biologically active IL-1 was detectable in cell culture supernatants as well as mRNA for IL-1 β and TNF α in cell lysates.

The isolation of large numbers of macrophages by peritoneal lavage with isotonic saline has also been reported (Stoddart and Scott, 1988). This technique shares the advantage that large numbers of macrophages may be obtained from one cat. However, there are several disadvantages to this technique, including the need to subject cats to two peritoneal lavages separated by 9–11 days for good macrophage recovery, the possibility of peritoneal adhesion development in cats, and the macrophage population which is obtained has gone through several potential activation steps, including elicited migration of cells into the peritoneal cavity and adherence for separation (Stoddart and Scott, 1988).

As a measure of macrophage function, the expression of certain cytokine genes was measured. It was important with such studies to stabilize culture conditions since procedures such as adherence or manipulation can change cytokine gene expression (Haskill et al., 1988). In our studies, unstimulated macrophages failed to express either IL-1 β or TNF α mRNA, but both genes were readily turned on following stimulation with LPS. Since the kinetics of expression for the two cytokines differed following activation, their transcription was assumed to be independently regulated with TNF α expression detectable at 6 h but essentially absent by 24 h. This is in agreement with similar studies on murine and human LPS stimulated monocyte-macrophages, where TNF α gene expression and mRNA levels are rapidly downregulated (Haas et al., 1990; Stein and Gordon, 1991; Takasuka et al., 1991). In contrast, gene transcription for IL-1 β underwent a different type of regulation with mRNA present at 6 h, decreased significantly by 24 h, but recovered by 48 h. Since actin mRNA levels during all time periods were similar, we can assume that comparable amounts of total RNA were loaded per lane. Our studies provided no explanation for the modulatory effects on IL-1 β expression, but this topic is under further investigation in our laboratory. We are considering the

possibilities of superinduction of gene expression or blockage of IL-1 β mRNA degradation.

The importance of the macrophage as a first-line host defense as well as its pivotal role in immunoregulation underscores the need for a system by which feline macrophages may be reproducibly generated for in vitro study. The system described herein fulfills this need and the numbers of macrophages routinely generated should permit in vitro studies into the interaction of macrophages with viral agents such as feline retroviruses and feline infectious peritonitis virus. Additionally, since these macrophages can be maintained in a quiescent state, macrophages cultured in this manner can be examined for modulation of monokine production following induction by specific activating agents.

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