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IMMUNOLOGY

Calcium Dependency of Interleukin-1 Secretion by a Chicken Macrophage Cell Line

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ABSTRACT The role of calcium in transducing the signal for interleukin-1 (IL-1) secretion was examined in the MQ-NCSU chicken macrophage cell line. Cells were maintained in RPMI 1640 medium supplemented with 5% chicken serum and antibiotic-antimycotic solution at 40°C and 5% CO2. The effects of stimulation with lipopolysaccharide (LPS), calcium ionophore A23187, or a combination of both on IL-1 secretion were examined. Calcium ionophore A23187 did not replace LPS in MQ-NCSU stimulation but the LPS + A23187 combination stimulated more IL-1 than ionophore alone in these cells. The combination of LPS and ionophore did not increase IL-1 secretion above the levels observed with LPS alone. No synergistic effects between LPS and A23187 were evident. In order to demonstrate that IL-1 secretion by the MQ-NCSU cells is a calcium-dependent process, ethylene glycol bis(β-aminoethyl ether)N,N',N'-tetraacetic acid (EGTA) was used to chelate free calcium in the cultures. Incorporation of 5 mM EGTA in the cultures lowered IL-1 stimulated by LPS + A23187 to control levels. Addition of 5 mM CaCl2 to EGTA-suppressed cultures restored IL-1 secretion. These results indicate that the calcium ionophore, A23187, augments IL-1 secretion by LPS-stimulated MQ-NCSU macrophages and that IL-1 secretion by these cells is a calcium-dependent process.

(Key words: calcium, macrophage, interleukin-1, cytokine, ionophore)

INTRODUCTION Cytokines, soluble hormone-like molecules, exert multiple biological effects through intercellular communication. Interleukin-1 (IL-1), a polypeptide product of monocytes, macrophages, and various other cells, is one of the key mediators of the host’s response to immunological reactions, tissue injury, microbial invasion, and inflammation (Kampschmidt, 1984; Dinarello and Savage, 1989). The diverse biological effects of IL-1 include co-stimulation of T lymphocyte proliferation (Mizel, 1982), B cell differentiation into plasma cells (Wood and Cameron, 1976), acute phase protein production by the liver (Dinarello, 1984a), muscle proteolysis, fever, and secondary mediator production (Rae et al., 1992). These effects, originally attributed to individual factors, were eventually assigned to a single substance designated IL-1 (Dinarello, 1984a,b).

Interleukin-1 release from chicken macrophages has been examined by several investigators. Hayari and coworkers (1982) described IL-1 production by adherent spleen cells treated with lipopolysaccharide (LPS). Klasing and Peng (1987) characterized chicken IL-1 using different cell sources, stimulating agents, and incubation temperatures. Their results indicated that the chicken macrophage cell line, HD11, released more IL-1 than the other cell sources studied. In addition, LPS and heat-killed Staphylococcus aureus were effective stimulants of IL-1 in these cells. Bombara and Taylor (1991) found that extracellular IL-1 released from HD11 macrophages significantly increased when the cells were stimulated with LPS and silica, but not by a superinduction protocol with mezerein.

The role of calcium in cytokine production has been examined in other systems. The murine macrophage cell line, P388D1, released IL-1 following stimulation with LPS or calcium ionophore A23187. Combination of the stimulants increased IL-1 to levels higher than when either stimulant was used alone (Simon, 1984).
The current study investigated the role of calcium in IL-1 secretion by the MQ-NCSU chicken macrophage cell line. Growth patterns of the MQ-NCSU cell line were compared using various levels of serum supplementation in RPMI 1640 medium. Next, the stimulatory effects of LPS and the calcium ionophore A23187 on IL-1 secretion were examined. Finally, the calcium dependence of IL-1 secretion by this cell line was determined via the use of ethylene glycol bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) and CaCl₂ replacement.

MATERIALS AND METHODS

Macrophage Cell Line

MQ-NCSU, a chicken macrophage cell line established from splenic cells of a broiler chicken experimentally challenged with the JM/102W strain of Marek’s disease virus, was used (Qureshi et al., 1990). Cells were grown in RPMI 1640 medium with 1,000 U penicillin/mL, 1.0 mg streptomycin/mL, and 2.5 μg amphotericin B/mL added. This solution is referred to as culture medium. Chicken serum was supplemented at 5%. The same batch of chicken serum was used in all tests. Cells were grown in tissue culture tubes. One milliliter of the appropriate test serum: 2.5, 5, 7.5, and 10%. MQ-NCSU cells were seeded at a density of 1 to 3 × 10⁶ cells/mL per tube in side-flattened 24-well plates. The cells were allowed to attach for 4 h. Triplicate wells were used for each of eight treatments: unstimulated control, LPS from Escherichia coli 011:B4 (2.5 μg/mL), calcium ionophore A23187 alone at 5 × 10⁻⁷, 2.5 × 10⁻⁷, and 1.2 × 10⁻⁷ M, and LPS plus A23187 at each of the previously listed concentrations. Since the ionophore required reconstitution in dimethyl sulfoxide (DMSO) prior to dilution in RPMI 1640 medium, all experimental wells not containing A23187 had DMSO (3.3 μL) added to them. The cells were incubated at 40 C in RPMI 1640 medium containing 5% chicken serum. Cells were collected with a cell scraper and seeded at a density of 1 to 3 × 10⁶ cells/mL in 24-well plates. The cells were allowed to attach for 4 h.

Multiplication rate (r) = \frac{3.32(\log N - \log N_0)}{t_2 - t_0}

where N = total cell population; N₀ = initial cell population; t₂ = time when final population is calculated; and t₀ = time at inoculum.

Treatment of Cells with LPS and Calcium Ionophore

Using information obtained on serum levels and cell growth, MQ-NCSU cells were cultured in T-75 flasks in culture medium containing 5% chicken serum. Cells were collected with a cell scraper because trypsin-EDTA treatment can greatly affect cell viability due to cellular damage. The medium containing the detached cells was aspirated several times to remove clumps and then dispensed into new flasks at the appropriate density for continued growth.

Calcium Dependence of IL-1 Secretion

The calcium dependence of the A23187/LPS-induced IL-1 secretion was demonstrated by using 5 mM ethylene glycol bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (EGTA) to chelate free calcium in the cultures. The effects of the EGTA on IL-1 secretion were to be reversed by the addition of 5 mM CaCl₂ to the cultures. MQ-NCSU cells were seeded at a density of 1 to 3 × 10⁶ cells/mL in 24-well plates using culture medium. The cells were allowed to attach for 4 h. Quadruplicate wells were used for each of six treatments: unstimulated control, LPS (2.5 μg/mL), A23187 (2.5 × 10⁻⁷ M), A23187 + LPS, A23187 + LPS + 5 mM EGTA, and A23187 + LPS + EGTA + 5 mM CaCl₂. Cells were incubated for 24 h at 40 C, after which time the supernatants were collected for the thymocyte co-mitogen assay. Supernatants were dialyzed as described above and then stored at −20 C until IL-1 assays were performed.

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8Sigma Chemical Co., St. Louis, MO 63178-9916.
9JRH Biosciences, Lenexa, KS 66215.
8Corning Glass Works, Corning, NY 14831.
1MUNC Inc., Naperville, IL 60563.
9Micron Separations Inc., Westboro, MA 01581.
**IL-1 Assay**

Chicken thymocyte co-mitogenic proliferation (Klasing and Peng, 1987) was used to assay for the level of IL-1 in the supernatants. Thymic lobes were aseptically removed from four Line 105 B24B24 chickens and transported in culture medium. Thymic lobes were ground through a sterile wire mesh with the rubber end of a syringe plunger. The cell suspension from each thymic sample was then collected and washed three times by centrifugation for 10 min at 600 \( \times g \). Thymus cells were checked for viability and seeded at a density of 2 \( \times 10^6 \) cells/100 \( \mu L \) in each well of a 96-well plate. Sixty microliters of culture supernatants, 50 \( \mu L \) phytohemagglutinin (PHA)-P (4 \( \mu g/mL \) final concentration), and 50 \( \mu L \) of culture medium were added to each well to give a final volume of 250 \( \mu L \). Thymic cells were incubated at 40 \( ^\circ C \) for 72 h, the last 18 h in the presence of 1 \( \mu Ci \) per well \( {}^3H \)-thymidine, specific activity 6.7 Ci:mmol. Cells were collected on glass fiber filter mats using a cell harvester. Scintillation counting was used to determine the level of \( {}^3H \)-thymidine incorporation into cellular DNA. Each supernatant was assayed three times for each thymic sample (12 times total) in a 96-well plate. Data were expressed as stimulation indices (SI) using the following formula: (SI = counts per minute of thymocytes + PHA + supernatant)/(counts per minute thymocytes + PHA).

**Statistical Analysis**

Data from each experiment were analyzed by analysis of variance. Significant means were partitioned using Fisher's protected LSD (\( P \leq 0.05 \)).

**RESULTS**

The growth characteristics and multiplication rates of MQ-NCSU cells under the described culture conditions are shown in Figure 1. MQ-NCSU cells had a generation time of 26.7 \( \pm 0.9 \) h in RPMI 1640 medium supplemented with 10% chicken serum, but this value did not differ significantly from the 28.3 \( \pm 0.8 \) h generation time found with both 5 and 7.5% serum levels. Macrophages grown in RPMI 1640 supplemented with 2.5% chicken serum grew the slowest, with a generation time of 30.0 \( \pm 0.7 \) h, which was significantly lower than all other values. For subsequent experiments, MQ-NCSU macrophage cells were maintained in RPMI 1640 medium supplemented with 5% chicken serum at 40 \( ^\circ C \) and 5% \( CO_2 \). This serum concentration was chosen because it was the smallest amount that could be used without significantly affecting either cell generation times or the IL-1 assays. MQ-NCSU cells were cultured for 24 h in the presence of one of three concentrations of calcium ionophore, A23187, with or without LPS. The results shown in Figure 2 demonstrate that 2.5 \( \mu g/mL \) LPS and 2.5 \( \mu g/mL \) LPS + 1.2 \( \times 10^{-7} \) M A23187 stimulate greater IL-1 secretion than either of the two higher concentrations (5 \( \times 10^{-7} \) M, 2.5 \( \times 10^{-7} \) M) of ionophore alone. However, 2.5 \( \mu g/mL \) LPS + 1.2 \( \times 10^{-7} \) M A23187 did not increase IL-1 secretion above the levels observed with LPS alone, indicating no synergistic effects between LPS and A23187.

The Ca\(^{2+} \) dependence of the LPS/A23187-induced secretion of IL-1 by MQ-NCSU cells was demonstrated by adding 5 mM EGTA to chelate free calcium in the cultures. Figure 3 demonstrates that the addition of EGTA to the cultures resulted in IL-1 secretion, which was not statistically different from the unstimulated control. However, when calcium, in the form of CaCl\(_2\), was added to EGTA-suppressed cultures, IL-1 secretion was restored.

**DISCUSSION**

Growth data indicated that MQ-NCSU cells grew at similar rates in RPMI 1640 medium supplemented with 10, 7.5, or 5% chicken serum (Figure 1). When serum levels were decreased below 2.5%, cell growth was not supported for more than 72 h (unpublished data). The time necessary for the number of cells to double is affected by factors such as media composition, temperature, and level of serum. Transformed cells have a decreased serum dependence compared to normal cells, possibly because such cells may secrete their own growth factors (Freshney, 1987). The MQ-NCSU cells have been maintained in Leibovitz-McCoy’s complete medium containing 8% fetal bovine serum and 10% chicken serum in other laboratories. However, these

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conditions represented a much higher serum concentration than was desired for these experiments.

Several studies have demonstrated that avian and murine macrophages had increased levels of IL-1 upon stimulation. Adherent spleen cells produced IL-1 after LPS stimulation (Hayari et al., 1982). Klasing and Peng (1987) stimulated HD11 chicken macrophages with LPS, S. aureus, and a superinduction protocol that used 1 x 10^{-8} M mezerien. All three stimulating agents produced significantly higher IL-1 levels. Bombara and Taylor (1991) used LPS, silica, and the superinduction protocol as stimulating agents for the HD11 cells. In their experiments, both LPS and silica stimulation significantly increased extracellular IL-1 levels, whereas the superinduction protocol did not significantly increase IL-1. Cellular damage from the superinduction method may have been a reason for the discrepancy. Intracellular IL-1 levels were significantly increased by all three of the stimulating agents.

A murine macrophage cell line, P388D1, released IL-1 following stimulation with LPS, calcium ionophore A23187 or a combination of the two. The combination of both stimulants resulted in the production of more IL-1 than with either used alone (Simon, 1984). The results of the current study (Figure 2) demonstrate that LPS and A23187 can stimulate IL-1 in MQ-NCSU cells. In addition, the combination of 2.5 \( \mu g/mL \) LPS + 1.2 x 10^{-7} M A23187 stimulated more IL-1 secretion than two concentrations of ionophore alone. No synergistic effects between LPS and A23187 were noted because the combination of LPS + A23187 did not increase IL-1 secretion above the levels observed with LPS alone. Calcium ionophore A23187 slightly enhanced IL-1 production by LPS-stimulated macrophages, in contrast to the significant augmentation found by Simon (1984).

In addition to stimulation by different agents, macrophage IL-1 can be induced through adherence. When mouse peritoneal exudate macrophages adhere to polystyrene plates, increases in IL-1α and IL-1β mRNA can be detected (Fuhlbrigge et al., 1987). The MQ-NCSU culture procedure involves the removal of the cells from a T-75 culture flask and replating in a 24-well plate. Perhaps this change in environment and the reattachment process stimulated IL-1 secretion resulting in higher values for the unstimulated control.

Inhibitors of lymphocyte mitogenesis have been detected in supernatants of chicken macrophages (Schaefer et al., 1985). Therefore, the possibility exists that the amount of IL-1 released, as determined by the thymocyte co-mitogenesis assay, may be affected by the macrophage release of these inhibitors in response to various stimuli. To avoid this potential problem, inhibitory substances must be removed from culture supernatants prior to assaying for IL-1 (Klasing and Peng, 1987). This inhibition can be achieved by either dialysis, as was done in the present work, or Sephadex chromatography.

Besides the above mentioned agents, the addition of EGTA to stimulated cultures can completely eliminate cytokine production or secretion. Simon (1984) demonstrated that IL-2 production was abolished when EGTA was added to EL-4, a T lymphoma cell line, cultures stimulated with A23187 and IL-1. However, the addition of CaCl_2 to the EGTA-suppressed cultures completely restored the response. In the present study, MQ-NCSU cells stimulated with A23187 and LPS were shown to secrete increased levels of IL-1 (Figure 3). The addition of EGTA to cultures stimulated with LPS + A23187 suppressed IL-1 secretion below the level seen in the controls. When CaCl_2 was included in the EGTA-
suppressed cultures, IL-1 secretion was restored to levels similar to those seen with LPS and LPS plus ionophore stimulation.

These results indicate that the calcium ionophore A23187 augments IL-1 secretion by LPS-stimulated MQ-NCSU macrophages. In addition, IL-1 secretion by this cell line is a calcium-dependent process. Combination of the calcium-mediated event with another signal from a cytokine or soluble mediator, such as LPS, constitute a cellular immune response activation mechanism.

REFERENCES


