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Signal Transduction Events in Chicken Interleukin-1 Production

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ABSTRACT The HD11 chicken macrophage cell line was studied for growth characteristics using different culture media, serum concentrations, and incubation temperatures. Growth curves at 37°C revealed that the cells grew best in RPMI 1640 medium supplemented with 10% colostrum-free bovine serum (CPBS). Growth was not supported by RPMI 1640 medium supplemented with the serum replacement Nu Serum nor by PC-1 culture medium alone. However, coating culture plates with either chicken serum or Nu Serum enhanced HD11 cell growth in the PC-1 medium. Growth at 40°C was equivalent to that obtained at 37°C when RPMI 1640 medium with 10% CPBS or PC-1 medium in plates coated with chicken serum were used.

Several stimuli were tested for their ability to induce interleukin-1 (IL-1) in HD11 macrophages under serum-free conditions. Lipopolysaccharide (2.5 μg/mL) or silica (50 μg/mL), increased extracellular IL-1 significantly after a 24-h treatment. In contrast, a superinduction protocol using phorbol 12-myristate 13-acetate, cycloheximide, butyrate, and actinomycin D did not increase extracellular IL-1 significantly. All three stimulants significantly elevated intracellular IL-1 after treatment. Protein kinase C inhibitors (H7 and retinol) as well as calmodulin-dependent kinase inhibitors (W7 and TFP) significantly diminished IL-1 production in the intracellular and extracellular compartments. Elevated cyclic adenosine 5’-monophosphate (cAMP) actuated by dibutyryl cAMP also increased IL-1 significantly. The data indicate that both protein kinase C and calmodulin-dependent kinase mechanisms are important in signal transduction leading to IL-1 production.

(Key words: protein kinase C, calmodulin-dependent kinase, cyclic adenosine 5’-monophosphate, interleukin-1, macrophage)

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INTRODUCTION

Many cell types produce hormone-like factors called cytokines. These substances exert multiple biological and regulatory effects by providing intercellular communication. Interleukin-1 (IL-1), which exhibits a broad spectrum of activities, is a low molecular weight protein product of macrophages (Dinarello, 1984a,b; Dinarello and Savage, 1989). Tissue macrophages and other cells such as keratinocytes also produce IL-1 (Kampschmidt, 1984). Interleukin-1 has a pivotal role in the pathogenesis of many diseases and in the mediation of a host’s response to infection through inflammatory and immunological events (Dinarello, 1984a,b). In immune reactions, IL-1 supports activation of T-lymphocytes (Mizel, 1982) as well as B-lymphocyte differentiation into antibody-producing cells (Wood and Cameron, 1976).

Lipopolysaccharide (LPS)-stimulated adherent spleen cells from chickens produced IL-1 activity (Hayari et al., 1982). Klasing and Peng (1987) characterized IL-1 release from chicken macrophages using different cell sources, stimulating agents, and incubation temperatures. The HD11 chicken macrophage cell line produced greater amounts of IL-1 compared with adherent chicken cells from the peripheral blood, spleen, or peritoneal cavity. Lipopolysaccharide and heat-killed Staphylococcus aureus stimulated higher levels of IL-1 than did latex particles or a superinduction protocol with mezerien. Cross-reactivity studies revealed that chicken thymocyte comitogenesis was stimulated by chicken IL-1 but not by human and murine IL-1 (Klasing and Peng, 1987).

Second messenger pathways have an important role in IL-1 release. Kovacs et al. (1988), found that inhibitors of protein kinase C and calmodulin-dependent kinase blocked IL-1 mRNA expression and protein production in murine macrophages. Klasing and Peng (1987) used indomethacin to demonstrate that prostaglandins did not mediate IL-1 release from chicken macrophages. Little additional information is available on the mechanisms of chicken IL-1 synthesis and release.
The current study was undertaken to meet two objectives. First, cell growth and IL-1 production parameters under serum-free conditions were established for HD11 macrophages. Second, the role of second messenger pathways in chicken IL-1 release was evaluated using these optimum culture conditions.

MATERIALS AND METHODS

Cell Culture

A chicken macrophage cell line (HD11) transformed by MC29 retrovirus containing a myc-oncogene, was kindly provided by R. R. Dietert, Cornell University, Ithaca, NY 14853, and K. C. Klasing, University of California, Davis, CA 95616, with permission of Thomas Graff, European Molecular Biology Laboratory, Heidelberg, Germany. Initially, the cells were maintained in RPMI 1640 medium, supplemented with 10% colostrum-free bovine serum (CFBS), penicillin (100 U/mL), and streptomycin (100 µg/mL). These anchorage-dependent cells were grown in either T-25 or T-75 tissue culture flasks at 40°C, 5% CO2. Culture medium was replaced approximately every 2 days. Upon reaching confluency, cells were removed from the flasks with a rubber policeman, as trypsin-EDTA treatment greatly reduced cell viability. Medium containing the cells was aspirated several times to remove clumps and dispensed in new flasks at the appropriate density for continued growth.

Experiment 1—HD11 Growth Characteristics

Growth experiments were performed to determine the optimum culture conditions for the HD11 cells. The RPMI 1640 or PC-1 culture media were used with the following supplements: CFBS, Nu Serum, or chicken serum added in various percentages. The HD11 cells were seeded at a density of 1.25 x 10⁵ cells per tube in side-flattened tissue culture tubes. Two milliliters of the appropriate test medium were added to each tube and were not replaced throughout the course of the 7-day experiment. Triplicate cultures were incubated at either 37°C or 40°C, both at 5% CO2. In some experiments, culture tubes were coated by adding 1 mL of the appropriate serum to each tube, incubating for approximately 30 min, and then washing with fresh medium.

Cells were enumerated every 24 h for 7 days. On each day, cells were resuspended by gentle scraping with a rubber policeman in fresh medium. Viable cell counts were made using the trypan blue exclusion method. Generation times of the cells were determined using the following equation (Paul, 1975):

\[
multiplication\ rate\ (r) = \frac{3.32(\log X_t - \log X_0)}{t_2 - t_0}
\]

where

- \(N\) = total cell population,
- \(X_0\) = initial cell population,
- \(t_2\) = time when final population is calculated, and
- \(t_0\) = time at inoculum.

Experiment 2—Interleukin-1 Production From HD11 Macrophages

Using information obtained on growth, HD11 cells were cultured at 40°C in PC-1 medium in T-75 flasks that had been coated with chicken serum. The HD11 macrophages were grown to confluency in T-75 flasks. Cells were collected with a cell scraper and seeded at a density of 1 to 3 x 10⁷ cells per well in 24-well plates that had been coated with chicken serum. Cells were allowed to attach for 4 h.

Three sets of triplicate wells were used for each of the four treatments: unstimulated control, LPS from Escherichia coli 011:B4 (2.5 µg/mL), silica (50 µg/mL), and a superinduction protocol. Concentrations of LPS and silica were chosen on the basis of preliminary experiments. The cells were incubated in the presence of LPS or silica for 24 h after which the culture supernatant was harvested for the treatments plus the unstimulated control. After the 24-h treatment, HD11 cells from each treatment and the control were then subjected to three cycles of freezing and thawing in fresh medium to release intracellular IL-1. All super-
natants were stored at −20°C until assays for IL-1 were performed.

For the superinduction protocol (Mizel and Mizel, 1981), HD11 cells were exposed to medium containing 10 μg/mL phorbol 12-myristate 13-acetate (PMA),2 10 μg/mL actinomycin D,2 and 2 mM butyrate for 4 h. Thymic lobes were ground through a wire mesh with the rubber end of a syringe plunger. This cell suspension was then collected and washed three times to collect any intracellular IL-1. Cells were then incubated at 40°C for 24 h. The culture supernatant was harvested after 24 h. These cells were subjected to a series of freezing and thawing to collect intracellular IL-1 as in the above procedure. Supernatants from each set of three wells were pooled. The entire series of stimulation protocols was repeated four times.

Chicken thymocyte comitogenic proliferation (Klasing and Peng, 1987) was used to assay for the level of IL-1 in the supernatants. Extracellular and intracellular IL-1 were assayed separately with their respective controls. Thymic lobes were removed aseptically from Line UNH 105 B24/B24 chickens and transported to HD11 cell cultures for 6 h in presence of 2.5 μg/mL LPS or 50 μg/mL silica, using the contrast procedure.

Supernatants were then tested in the thymocyte comitogenic assay.

The effects of four second-messenger pathway inhibitors upon IL-1 production were examined. The inhibitors used were of two types. The protein kinase C inhibitors were 50 μM retinal2 and 20 μM 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7). The calmodulin-dependent kinase inhibitors were 20 μM N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7)2 and 20 μM trifluoperazine dichloride (TFP). These concentrations demonstrated inhibition in preliminary experiments.

The HD11 macrophages were seeded at a density of 2 × 10⁷ cells per well using PC-1 medium in 24-well plates coated with chicken serum. The cells were allowed to attach for 4 h. Cells were then incubated for 6 h at 40°C in the presence or absence of LPS (2.5 μg/mL) and silica (50 μg/mL), with or without a single second messenger pathway inhibitor W7, TFP, retinal, and H7. Cells were then washed and new PC-1 medium added. Cells were incubated for 24 h after which time the culture medium was collected. Cells were frozen and thawed three times to collect any intracellular IL-1. Supernatants were dialyzed for 24 h against culture medium to remove any residual inhibitors. The dialyzed supernatants were then tested in the thymocyte comitogenesis assay.

A second messenger modulator, dibutyryl cAMP (dbcAMP)2 was used to determine whether IL-1 production could be augmented by cAMP. The dbcAMP (50 or 100 μM) was added to HD11 cell cultures for 6 h in presence of 2.5 μg/mL LPS or 50 μg/mL silica. Cells were washed and fresh PC-1 medium was added. Cultures were allowed to incubate for 24 h at 40°C. Supernatants were collected and the cells were frozen and thawed three times to collect any intracellular IL-1. All supernatants were dialyzed against media for 24 h at 4°C. Supernatants were then tested in the thymocyte comitogenesis assay.

**Statistical Analysis**

Stimulation indices were evaluated by analysis of variance using a completely randomized design. Means for each treatment were tested against the control via contrasts (Steel and Torrie, 1980). The dbcAMP treatments were also tested against the stimulant, either LPS or silica, using the contrast procedure.
TABLE 1. Generation times for HD11 cells grown in various media supplements and temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Medium</th>
<th>Supplements</th>
<th>Generation time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>37</td>
<td>RPMI 1640</td>
<td>10% CFBS&lt;sup&gt;1&lt;/sup&gt;</td>
<td>20</td>
</tr>
<tr>
<td>37</td>
<td>RPMI 1640</td>
<td>10% Nu Serum</td>
<td>NA&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>37</td>
<td>RPMI 1640</td>
<td>5% Nu Serum</td>
<td>NA</td>
</tr>
<tr>
<td>37</td>
<td>RPMI 1640</td>
<td>2.5% Nu Serum</td>
<td>NA</td>
</tr>
<tr>
<td>37</td>
<td>RPMI 1640</td>
<td>2.5% CFBS + 2.5% Nu Serum</td>
<td>100</td>
</tr>
<tr>
<td>37</td>
<td>PC-1</td>
<td>None</td>
<td>33</td>
</tr>
<tr>
<td>37</td>
<td>PC-1</td>
<td>Chicken serum-coated</td>
<td>20</td>
</tr>
<tr>
<td>37</td>
<td>PC-1</td>
<td>Nu Serum-coated</td>
<td>25</td>
</tr>
<tr>
<td>37</td>
<td>PC-1</td>
<td>10% CFBS</td>
<td>20</td>
</tr>
<tr>
<td>40</td>
<td>RPMI 1640</td>
<td>5% Nu Serum</td>
<td>NA</td>
</tr>
<tr>
<td>40</td>
<td>PC-1</td>
<td>None</td>
<td>34</td>
</tr>
<tr>
<td>40</td>
<td>PC-1</td>
<td>Chicken serum-coated</td>
<td>20</td>
</tr>
</tbody>
</table>

<sup>1</sup>CFBS = colostrum-free bovine serum.

<sup>2</sup>NA = never achieved.

RESULTS

Because the time required for cells to double is influenced by a number of factors including media composition, temperature, and serum level, growth experiments were necessary to determine the optimum conditions for HD11 propagation. Table 1 shows the generation times for different media and incubation temperatures. The HD11 macrophages grew to the highest density and at the fastest rate in RPMI 1640 medium supplemented with 10% CFBS with a 20 h generation time. Experiments indicated that HD11 macrophage growth could not be supported in RPMI 1640 medium supplemented with any tested level of Nu Serum; an alternative to fetal bovine serum customarily used in the preparation of cell culture media. The combination of 2.5% CFBS and 2.5% Nu Serum allowed only very slow cell growth, as indicated by the 100 h generation time.

Cell growth was not well supported by PC-1 medium alone (Table 1). This is a low-protein, serum-free, modified DME:F12 base medium intended for the culture of primary cells and anchorage-dependent cell lines. However, pretreating plates with either chicken serum or Nu Serum facilitated HD11 cell growth in PC-1. Cell doubling time was 20 h in plates coated with chicken serum and 25 h in plates coated with Nu Serum. These results approximated the growth obtained in RPMI 1640 medium supplemented with 10% CFBS. Coating with Nu Serum and culturing in PC-1 medium eliminated problems associated with the Nu Serum-supplemented medium because plates were washed after coating.

To compare the effect of temperature upon HD11 cell growth, an experiment (Table 1) was executed at 40 °C rather than 37 °C. Generation times at 40 °C were identical to those obtained at the lower temperature for both RPMI 1640 medium containing 10% CFBS (20 h) and the PC-1 medium in plates coated with chicken serum (20 h). Cells in PC-1 medium alone and in medium supplemented with Nu Serum did not grow as well if at all.

For subsequent experiments, HD11 cells were maintained in PC-1 medium using tissue culture flasks coated with chicken serum. This medium was chosen because it provided defined components for optimizing cell growth while maintaining a low protein content. A low protein content in culture supernatants containing IL-1 was deemed appropriate to minimize interference from other proteins in the IL-1 assays.

The HD11 macrophages were stimulated for production of IL-1 using LPS and silica for 24 h, and a superinduction protocol. The LPS for 24 h stimulated extracellular IL-1 production that was significantly higher than the control (Figure 1A). Similar significant stimulations were found using silica for 24 h. The superinduction protocol produced a higher stimulation index that was not significantly different from the control.

Intracellular IL-1 was assayed following 24-h treatment with LPS, silica, or the superinduction protocol. Stimulation indices obtained in this assay were lower than those
FIGURE 1. A) Extracellular interleukin-1 (IL-1) produced by HD11 macrophages after 24-h treatment with different stimulants. B) Intracellular IL-1 produced by HD11 macrophages after 24-h treatment with different stimulants. Stimulation index = \[\text{counts per minute of } ^3\text{H} \text{ incorporation by thymocytes plus phytohemagglutinin (PHA) plus culture supernatants}] + (\text{counts per minute of } ^3\text{H} \text{ incorporation by thymocytes plus PHA})\). Asterisk indicates significant difference from control \((P<.05)\). Horizontal lines are SE. LPS = lipopolysaccharide.

obtained for extracellular IL-1 (Figure 1B). However, all three treatments significantly increased intracellular IL-1 above the control values. Superinduction elevated intracellular IL-1, unlike the result obtained for extracellular IL-1, which may suggest a slower release of the cytokine into the external environment.

Inhibitors of second messenger pathways were used to study their effect on IL-1 production. Both the H7 and retinal inhibit protein kinase C, and W7 and TFP inhibit calmodulin-dependent protein kinase. Extracellular IL-1 yield as stimulated by LPS was affected by all four second messenger inhibitors (Figure 2). The significantly lower stimulation indices for LPS plus each inhibitor compared with the stimulation index of LPS alone indicated that activation of protein kinase C and calmodulin-dependent protein kinase were involved in IL-1 gene expression, which leads to IL-1 production (Figure 2A). The same significant inhibitions were observed for intracellular IL-1 after LPS treatment (Figure 2B).

The IL-1 produced after silica treatment was also reduced significantly by all of the second messenger inhibitors. Extracellular IL-1 (Figure 3A) and intracellular IL-1 (Figure 3B) were both lower as observed in the LPS treatment. The percentage IL-1 reduction produced following silica stimulation plus inhibitors was similar to that found with LPS.

The role of cAMP in the IL-1 production response of HD11 cells was tested by adding the cAMP activator, dbcAMP to the cultures.
FIGURE 3. A) Extracellular interleukin-1 (IL-1) produced by HD11 macrophages after treatment with silica (50 μg/mL) or silica plus inhibitors. B) Intracellular IL-1 produced by HD11 macrophages after treatment with silica (50 μg/mL) or silica plus inhibitors. Stimulation index = [counts per minute of 3H incorporation by thymocytes plus phytohemagglutinin (PHA) plus culture supernatants] / (counts per minute of 3H incorporation by thymocytes plus PHA). Asterisk indicates significant difference from control (P<0.05). Horizontal lines are SE. W7 = N-(6-amino-hexyl)-5-chloro-l-napthalenesulfonamide; H7 = 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; RA = retinal, TFP = trifluoperazine dichloride.

Either 50 or 100 μM dbcAMP significantly increased extracellular (Figure 4A) and intracellular (Figure 4B) IL-1 above the level found with LPS stimulation alone. Silica stimulated IL-1 production was also significantly increased in the extracellular (Figure 5A) and intracellular compartments (Figure 5B). The addition of dbcAMP to either LPS or silica treatments gave the highest stimulation indices found in all assays.

DISCUSSION

The HD11 cells grew best in RPMI 1640 medium supplemented with 10% CFBS as shown in Table 1. The HD11 macrophages, although transformed and thus continuous, must attach to a substrate such as a plastic tissue culture flask in order to survive and replicate. The presence of serum allowed the cells to propagate at the minimum generation time of 20 h. The PC-1 medium without serum, which lacked necessary attachment factors, did not support growth for more than 72 h.

Attempts to replace CFBS with Nu Serum were unsatisfactory as no level of that supplement supported cell growth. Possibly, this serum replacement was deficient in one or more essential factors necessary for macrophage replication. It is also plausible that substances inhibitory for HD11 cells may be contained in the Nu Serum. On the other hand, coating flasks with either chicken serum or Nu
Serum promoted growth of HD11 cells. Culture flasks were washed after coating to leave only the attachment factors and remove any substances in Nu Serum that were inhibitory to macrophage growth. The temperature test showed that growth at 40 °C was equivalent to growth at 37 °C in RPMI 1640 medium with 10% CFBS and in PC-1 medium with flasks coated with chicken serum.

Klasing and Peng (1987) found that HD11 macrophages released significantly higher levels of IL-1 compared with nontransformed chicken macrophages. In their work LPS, *Staphylococcus aureus*, and a superinduction protocol using $1 \times 10^{-8} M$ mezerien all significantly increased IL-1. However, the former two stimulants produced greater amounts of IL-1. The present study showed that LPS or silica stimulation for 24 h significantly increased extracellular IL-1. The superinduction protocol used in the present study failed to produce a significant IL-1 increase. Cellular damage, including loss of viability, may have been a factor, as cultures stimulated in this manner had increased cellular debris in the supernatants.

Intracellular IL-1 production was significantly increased by all stimulating agents including the superinduction protocol. The discrepancy of an increased intracellular IL-1 without a significant increase in extracellular IL-1 following superinduction may indicate IL-1 secretion is delayed. Furthermore, action of serine proteases, which are important in processing IL-1, may be inhibited by the compounds used for superinduction.

Experiments by Fuhlbrigge et al., (1987) showed that IL-1 can be induced through adherence of mice peritoneal exudate macrophages to polystyrene plates. The HD11 culture procedure used in the present study called for cells to be removed from T-75 flasks and replated in 24-well plates. This change in environment and reattachment may have triggered production of IL-1, which resulted in control stimulation indices of almost 5. It is also possible that HD11 cells have a high basal level of IL-1 synthesis and release.

The H7 and retinal, protein kinase C inhibitors, (Taffet et al., 1983; Kawamoto and Hidaka, 1984) as well as W7 and TFP, calmodulin-dependent protein kinase inhibitors, all suppressed IL-1 production in the current study. Their inhibition was equally effective in LPS or silica stimulations. These results confirm the experiments of Kovacs et al., (1988) showing that inhibitors of protein kinase C and calmodulin-dependent protein kinase block the IL-1 mRNA expression and IL-1 production stimulated by LPS. Their experiments pretreated murine macrophages with the stimulant (LPS or silica) prior to the addition of the inhibitor. This allowed an appropriate time interval for IL-1 gene activation because these genes are not expressed constitutively. Once the inhibitors were added, gene expression was deactivated. The current results, coupled with those of Kovacs et al. (1988), suggest that control of IL-1 by these inhibitors may occur both at the level of signal transduction and posttranscription.
Inhibitors of protein kinase C and calmodulin-dependent protein kinase reduced IL-1, which demonstrates that both enzyme systems are important in the generation of the cytokine. However, other biochemical pathways may be involved in the effects on IL-1 seen with these inhibitors. At the 20 \( \mu M \) dose tested, H7 blocked protein kinase C and cAMP but retinal was selective for protein kinase C. The inhibition of IL-1 by H7 and retinal suggested the importance of cAMP as well as protein kinase C. Both TFP and W7 lowered IL-1 levels, indicating a role for calmodulin-dependent enzymes as well. The TFP inhibits calmodulin-dependent enzymes in vitro but other pathways involving calmodulin-independent enzymes may be affected. Luthra (1982) found that TFP inhibits calmodulin-activated calcium dependent adenosine triphosphatase (ATPase), but the exact mechanism of its action is unknown. The TFP may inhibit calmodulin binding to calmodulin-dependent second messenger intermediates.

The effect of elevated cAMP levels was also tested. Addition of 50 or 100 \( \mu M \) dbcAMP significantly increased the amounts of extracellular and intracellular IL-1. The synergistic action of either LPS or silica with dbcAMP was shown by the highest stimulation indices found in any protocol utilized. The present result differs from prior studies, showing that inhibitors of cAMP did not affect LPS induced IL-1 (Kovacs et al., 1988). Elevation of cAMP by dbcAMP may have influenced the level of expression of other genes subject to control from cAMP-dependent second messenger pathways in addition to those involved in IL-1 output. On the other hand, cAMP serves as a negative regulator in many cellular systems, so the IL-1 increase may result from an effect on an inhibitor within the cells.

In B-cell cultures, cAMP-raising agents, dbcAMP, and isobutylmethylxanthine (IBMX) enhanced entry into S phase in some mitogenic stimulation regimens but inhibited the process in others. B-cell stimulation by ionomycin, a calcium ionophore, plus PMA was enhanced, but stimulation induced by anti-immunoglobulin antibodies was inhibited (Cohen and Rothstein, 1989). More important, enhancement was more sensitive to dbcAMP and inhibition was more sensitive to IBMX. The authors suggested that the differential effect resulted from the kinetics of increased cAMP brought about by dbcAMP and IBMX. Enhancement was promoted by sustained levels of cAMP but inhibition required only a transitory cAMP change. The increased IL-1 following addition of dbcAMP in the present study may have been due to a sustained level of intracellular cAMP.

In summary, the results of the present study indicate that LPS and silica are equally effective in stimulating intracellular and extracellular IL-1 from HD11 macrophages. Signal transduction from these stimuli leading to IL-1 production occurs through mechanisms dependent on both protein kinase C and calmodulin-dependent protein kinase. Moreover, prolonged levels of cAMP resulting from dbcAMP also increase cytokine release.

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REFERENCES


