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Effect of interferon on chemiluminescence and hydroxyl radical production in murine macrophages stimulated by PMA

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Summary. Considerably augmented chemiluminescence (CL) occurred when murine peritoneal resident macrophages (MPs), pretreated with murine interferon (MuIFN)-α within 24 hr, were stimulated by 4-β-phorbol, 12-β-myristate, 13-β-acetate (PMA). Augmentation of CL generation ceased when incubation in the presence of MuIFN was continued for 48 hr.

As 12 hr preincubation with MuIFN procured optimal CL generation, the various reactive oxygen species (OH, $O_2^{\bar{\bullet}}$, H_2O_2) were measured at that point. The hydroxyl radical (OH·) level in MuIFN-treated MPs was 19·44 times higher than in MuIFN-untreated MPs. However, the levels of $O_2^{\bar{\bullet}}$ and H_2O_2 generation were the same in both MuIFN-treated and untreated MPs. Moreover, by using the inhibitors lipoxygenase and cyclo-oxygenase, we established clearly that CL and OH· generation in MuIFN-treated MPs is due to the lipoxygenase pathway of arachidonic acid metabolism.

INTRODUCTION

It is well known that macrophages (Mps) can produce reactive oxygen species, such as superoxide anion ($O_2^{\bar{\bullet}}$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radical (OH·) (Klebanoff, 1980; Nathan, Murray & Cohn, 1980). These reactive oxygen species are toxic for both intra- and extracellular targets (either prokaryotic or eukaryotic). When MPs are activated *in vitro* with lymphokine-rich supernatants, the generation of reactive oxygen species increases (Murray & Cohn, 1980).

It is also known that MPs treated with homologus interferon (IFN) have an augmented ability to kill microorganisms and to inhibit tumour cell growth (Schultz, Papamatheakis & Chirigos, 1977). Nathan *et al.* (1983) have recently shown that human IFN-γ induced H_2O_2 generation in human macrophages after more than 3 days' incubation. Meanwhile, Boraschi *et al.* (1982, 1983) reported that murine IFN-β inhibits the $O_2^{\bar{\bullet}}$ and H_2O_2 releasing capacity of murine MPs after 20 hr incubation, but increases tumour cytotoxicity. However, they did not measure hydroxyl radical (OH·), a highly reactive microbicidal and tumoricidal agent, in IFN-treated MPs. We have, therefore, examined whether MuIFN affects the generation of reactive oxygen species ($O_2^{\bar{\bullet}}$, H_2O_2, OH·) in resident murine MPs.

We report here that considerably augmented chemiluminescence (CL) (an indicator of the presence of reactive oxygen species) and OH· production occur when murine MPs, stimulated by 4-β-phorbol, 12-β-myristate, 13-β-acetate (PMA), are pretreated with MuIFN-α under certain conditions. Our results also suggest that hydroxyl radical produced under these

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conditions can be attributed to the lipoxygenase pathway of arachidonic acid metabolism.

MATERIALS AND METHODS

MPs

MPs were obtained from female C57BL/6J mice (6–8 weeks old, from Jackson Laboratories, Bar Harbor, ME) injected intraperitoneally with 5 ml Hanks' balanced salt solution without phenol red (HBSS, Gibco, Grand Island, NY) containing two units of sodium heparin, 100 units of penicillin and 100 μg streptomycin per ml. Cells from peritoneal exudates were washed once in HBSS and resuspended in RPMI-1640 (Gibco) containing 15% heat-inactivated fetal calf serum (FCS, Gibco), 100 units penicillin and 100 μg streptomycin per ml (hereafter referred to as culture medium). Following 2 hr incubation at 37° and washing three times with serum-free medium, the adherent cells constituted the MP population. Culture medium was used in all our experiments, and all cultures were incubated at 37° in a humidified atmosphere containing 5% CO_2.

Reagents

4-β-phorbol, 12-β-myristate, 13-β-acetate (PMA), 2-keto-4-thiomethylbutyric acid (KMB), dimethylsulphoxide (DMSO), 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol), sodium azide, nordihydroguaiaretic acid (NDGA) and indomethacin were obtained from Sigma Chemical Co. (St Louis, MO). 5,8,11,14-eicosatetraynoic acid (ETYA) was received from Dr James G. Hamilton (Hoffmann LaRoche, Nutley, NJ).

Interferon

Purified murine interferon (MuIFN)-α ($1{\cdot}0 \times 10^8$ units/mg protein) was received from Dr Y. Kawade (Institute for Virus Research, Kyoto University). The purification method has been described previously (Iwakura Yonehara & Kawade, 1978). Human leucocyte interferon (HuIFN-α, 3×10^6 units/mg) was partially purified by Dr A. Walz at the Theodor Kocher Institute in Berne, Switzerland, following a method developed and previously described by Dr K. Cantell (Cantell & Hirvonen, 1978). The titres of both interferon preparations are expressed in International Reference Units. These materials were reconstituted with RPMI-1640, divided into aliquots and stored at −80°. Before use, MuIFN-α and HuIFN-α were titrated to determine their protective effects on L-929 cells or on human embryonic fibroblasts challenged with vesicular stomatitis virus, respectively.

Chemiluminescence test

Adherent cells from peritoneal exudates (2 ml, 1×10^6 cells/ml) were isolated under standard conditions in sterilized glass scintillation vials (Packard, Downers Grove, IL), and culture medium containing various concentrations of MuIFN or HuIFN was added to each vial. The cells were further incubated for various amounts of time in the presence of interferon, then washed three times with HBSS. HBSS, 2·2 ml, 0·1 ml of luminol in DMSA (for final concentrations of $2{\cdot}26 \times 10^{-5}$ M luminol and 0·2% DMSO), 0·1 ml of a solution of one of various chemical reagents used, and 0·1 ml of a solution of 25 μg/ml of PMA (stimulation of MPs by PMA 1 μg/ml is necessary to elicit CL) were added to each vial for a final volume of 2·5 ml.

Cultures were treated for 20 min at $25 \pm 1{\cdot}0°$ with ETYA, NDGA or indomethacin in the presence of a final concentration of 0·2% DMSO. Each vial was immediately capped and placed in a Beckman LS-355 Liquid Scintillation Counter (Fullerton, CA) out of phase, with one photomultiplier tube disconnected. For each test condition, duplicate vials were counted at 5-min intervals for 30 min at $25 \pm 1{\cdot}0°$. Results were recorded as mean counts per minute. The final values were calculated by subtracting background counts which averaged 1×10^4 c.p.m. (with peak control values of 7×10^4), while experimental values were up to 50-fold higher.

Assay for protein content

MPs cultured in glass scintillation vials were washed three times with HBSS, lysed with 1·0 ml 0·5 N NaOH, and their protein content determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard. Such determinations were performed in triplicate for each time point. Amounts of protein per vial for cultures with and without MuIFN were, respectively, 35 $\mu g \pm 2{\cdot}0$ (SD) and 34 $\mu g \pm 5{\cdot}6$ (SD) at 12 hr; 33·0 $\mu g \pm 2{\cdot}2$ (SD) and 31·2 $\mu g \pm 5{\cdot}1$ (SD) at 24 hr; 29·2 $\mu g \pm 4{\cdot}3$ (SD) and 29·5 $\mu g \pm 3{\cdot}1$ (SD) at 48 hr. The differences in protein content were not significant.

$O_2^{\cdot}$ and H_2O_2 assays

Aliquots of 2-ml MPs suspensions (1×10^6 cells/ml) in the culture medium were added to 6-well culture plates (Falcon no. 3046, Oxnard, CA). After 2 hr incubation, adherent cells were washed three times in HBSS, and 2

ml of culture medium were added. MP monolayers were cultured for 12 hr with MuIFN or without IFN, and washed three times with HBSS.

$O_2^{\bullet}$. MPs were incubated with 2 ml HBSS containing 80 μM ferricytochrome *c* (Type III, Sigma) and PMA at a final concentration of 1 μg/ml. At various times after incubation at 37° in humidified air with 5% CO_2, supernatants were harvested and centrifuged at 250 ***g*** for 10 min. The concentration of reduced ferricytochrome *c* was measured from spectrophotometric determinations of supernatant optical densities according to the equation:

$$\Delta \text{ E } 550 \text{ nm} = 2{\cdot}1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}.$$

Control wells included (i) MP monolayers incubated without PMA; (ii) MP monolayers incubated with cytochrome *c* and PMA, and with bovine erythrocyte superoxide dismutase (SOD, from bovine erythrocytes, 3000 units/mg, Sigma) at the final concentration of 150 units/ml; (iii) cell-free wells incubated with cytochrome *c* and PMA (blank).

H_2O_2 assay. MPs were covered with 2 ml HBSS containing PMA (1 μg/mg) horseradish peroxidase (8·5 units/ml, type VI, Sigma) and 7·8, nmoles scopoletin (Sigma). Incubation was carried out for various time intervals at 37° in humidified air with 5% CO_2. At the end of the incubation period, supernatants were harvested and centrifuged at 250 ***g*** for 10 min. Fluorescence was measured by spectrophotofluorometry (excitation 350 nm, emission 460 nm). A standard curve of H_2O_2 was built by adding known amounts of H_2O_2 to MP-free wells containing only the reaction mixture. In this assay, 6 nmoles H_2O_2 typically decreased fluorescence by 50%. Control wells included (i) MP monolayers incubated without PMA; (ii) MP monolayers incubated with PMA and catalase (from bovine liver, 15,500 units/mg, Sigma) at the final concentration of 400 units/ml; (iii) cell-free wellls incubated with PMA (blank).

Prostaglandin (PG) assay

PGE_2 and 6-keto-$PGF_{1\alpha}$ were measured by specific radioimmunoassay as previously described (Karmali *et al.*, 1982).

Assay of lipoxygenase products

The lipoxygenase products (5- and 15-HETEs) were measured by using high pressure liquid chromatography (HPLC, Japan Spectroscopic Co. (Jasco), Tokyo, Japan). MPs (1×10^6 cell) were incubated at 37° for 3 hr with 5 ml of RPMI-1640 medium devoid of FCS containing 15 μCi of [5,6,8,11,12,14,15- ^{3}H] arachidonic acid (100 Ci/mmol; New England Nuclear, Boston, MA). At the end of the incubation, the medium was discarded and the MPs were washed twice with 5 ml of serum-free medium. The cells were then incubated at 37° for 12 hr with the culture medium containing 10^3 units of MuIFN-α. Afterwards, the MPs were washed three times with HBSS and cultured with 2 ml of HBSS containing ETYA, NDGA and indomethacin at a final concentration of 5×10^{-5} M at 37°. After 20 min, PMA (at a final concentration of 1 μg/ml) was added to the MPs and the MPs were subsequently cultured for 1 hr at 37°. The supernatants were removed and acidified to pH 3–3·5 with 2 M citric acid and added to six volumes of diethylether and four volumes of distilled water. The ether layer was removed and the supernatant extracted again with the same volume of diethylether. Both ether layers were pooled and evaporated until dry under a nitogen stream. The dry residue was dissolved in 100 μl of HPLC eluent (acetonitrile/water/acetic acid = 65:35:0·1) and injected directly into the chromatograph (BIP-1, Jasco) equipped with a Finpak SIL C18 S column (5 μm, Jasco) and a u.v. detector (UVIDEC-100-V, Jasco). The sample was eluted at a solvent flow of 1 ml/min, and fractions were collected at 1-min intervals. The elution of hydroxyacids was monitored by measurement of u.v. absorbance at 235 nm. Authentic 5- and 15-HETE were added to samples before the purification procedure as internal standards. [^{3}H] radioactivity of fraction was measured in a liquid scintillation counter.

OH· assay

For a determination of OH· generation in IFN-treated and untreated MPs, the ethylene generation method was used. Resident peritoneal cells (in 5 ml culture medium, 1×10^6 cells/ml) were incubated for 2 hr in a plastic tissue culture flask (Falcon no. 3010) to allow cells to become attached. The cells were then washed three times with serum-free medium, and 9 ml of culture medium were added, as well as either 1 ml serum-free medium alone, or 1 ml serum-free medium containing 10^3 units of MuIFN or HuIFN. In several experiments, MuIFN (10^4 units/ml) was preincubated for 1 hr at 37° with 1/50 anti-MuIFN antibody (5×10^5 units/ml neutralizing antibody titre). The attached cells were incubated for 12 hr and then washed three times with HBSS. In order to determine the effect of

various agents on ethylene production, cells were then treated with such agents for 20 min at 37°. Since ETYA, NDGA and indomethacin were used at a final concentration of 0·2% DMSO in culture medium, 0·2% DMSO controls were included.

C_2H_4 production was determined by incubating the adherent cells at 37° in HBSS containing KMB (1 mM) in a final volume of 7 ml (Klebanoff & Rosen, 1978). The reaction took place in the plastic flasks sealed with rubber stoppers with or without PMA added at a final concentration of 1 μg/ml. Reactions were terminated after 60 min by rapid cooling in an ice-bath and adding N-ethyl-maleimide to a final concentration of 1 mM, injected through the rubber stopper. Reduction of temperature and addition of N-ethyl-maleimide completely arrested C_2H_4 generation. Portions of the vapour phase (1 ml) were analysed on a gas chromatograph (Finnigan 950, Finnigan Corp., Sunnyvale, CA). The chromatograph was equipped with a 120 cm × 3 mm stainless steel column packed with Porapak 289-100. Gas flow rates were 300 ml/min air, 25 ml/min nitrogen with the injector, detector and column at 160°, 300° and 101°, respectively. Standardization and quantification of C_2H_4 with this system have been described previously (Cunningham & Stahy, 1975).

Preparation and opsonization of zymosan

Zymosan (Sigma) was suspended in HBSS to a concentration of 10 mg/ml and opsonized by incubat-

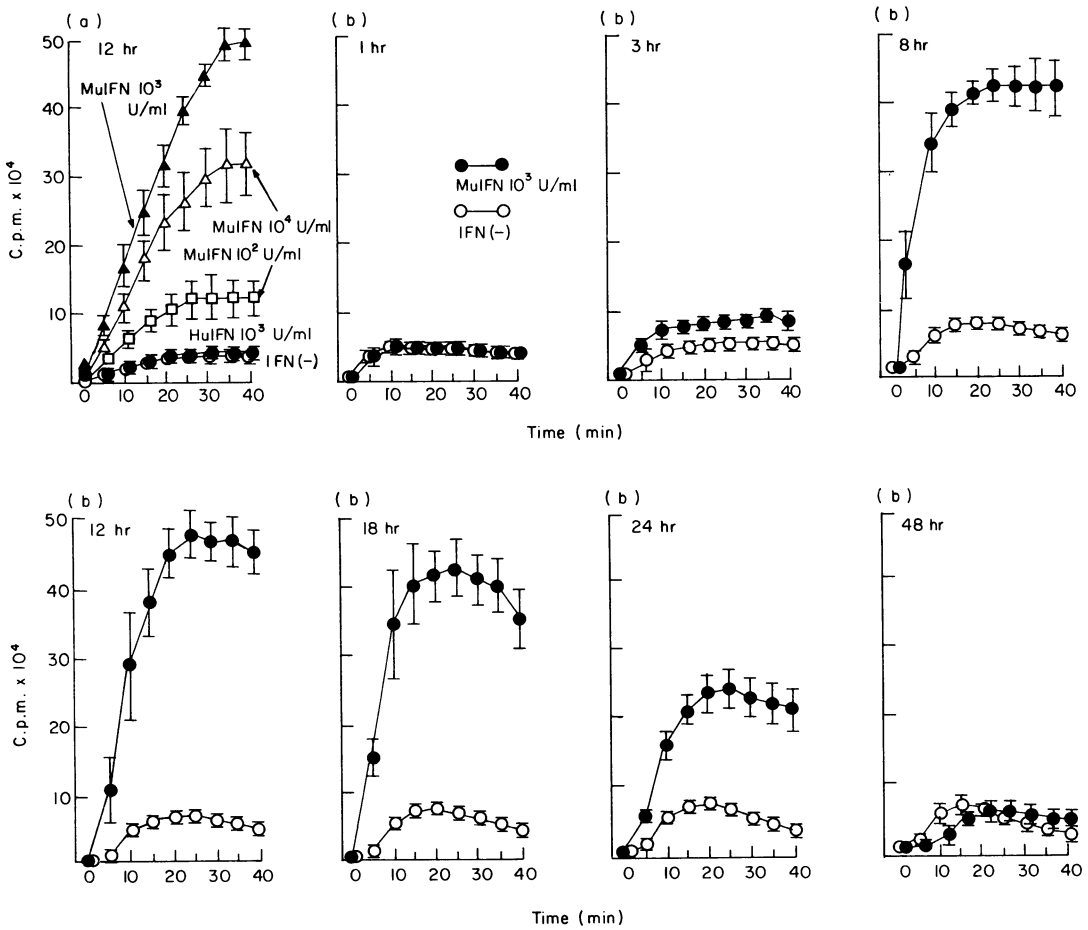

Figure 1. Chemiluminescence (CL) in murine peritoneal macrophages (MPs) treated with IFN-α. (a) Dose-dependent effect of MuIFN on CL. (b) CL in MPs pretreated with MuIFN (10^3U/ml) for 1, 3, 8, 12, 24 or 48 hr. Results are the mean ± SE of three to five experiments each one performed in duplicate (see 'Materials and Methods' for details).

ing one volume of zymosan (10 mg/ml) with one volume of fresh murine serum for 30 min at 37°. The suspension was centrifuged at 3000 *g* for 10 min before washing the pellet, and resuspended to a final concentration of 10 mg/ml in HBSS.

Phagocytosis assay

IFN-treated and untreated MPs were washed with HBSS and suspended in 2 ml HBSS. The cells were cultured with ETYA and NDGA for 30 min at 37°, and incubated for 15 min with 1 mg/ml opsonized zymosan. The MPs were washed three times with cold HBSS, stained with periodic acid-Schiff (PAS) reagent, and examined under light microscopy.

RESULTS

Chemiluminescence (CL)

Dose- and time-dependent effects of MuIFN on CL in MPs were observed.

We first examined the effects of various doses of MuIFN on the generation of CL in PMA-stimulated MPs, following 12 hr culture of MPs in the presence of MuIFN-α. With 10^3 units/ml of MuIFN, CL generation was increased 12·25-fold over the control value, and with 10^2 and 10^4 units/ml MuIFN values were only about 7·9 and 3-fold higher, respectively. Human interferon (HuIFN-α (Le), 10^3 units/ml) did not augment CL in murine MPs (Fig. 1a). There was no significant increase in CL generation in the absence of PMA stimulation.

We then examined the kinetics of CL generation in PMA-treated MPs cultured in the presence of 10^3 units/ml MuIFN, at which concentration maximum CL generation had been obtained. Following 1 hr incubation with MuIFN, no effect on CL could be observed. However, after 3 hr incubation with MuIFN, CL was slightly augmented, and with longer incubation times in the presence of this MuIFN concentration (i.e. 8, 12 and 18 hr) more than 6-fold increases were observed. CL augmentation decreased to 3-fold at 24 hr, and it ceased if incubation in the presence of MuIFN lasted as long as 48 hr (Fig. 1b). Subsequent experiments were done with MPs incubated for 12 hr in the presence of 10^3 units/ml MuIFN.

CL augmentation reflects an increase in the production of various reactive oxygen species (Hatch, Gardner & Menzel, 1978; Johnston, Lehmeyer & Guthrie, 1976; Stevens, Winston & Van Dyke, 1978). Thus, we attempted to determine what kind of reactive oxygen species contribute to this augmentation of CL

Table 1. Effects of azide, SOD, catalase and inhibitors of arachidonic acid metabolism on chemiluminescence in MuIFN-treated MPs

	MuIFN (−) % inhibition	MuIFN (+) % inhibition
Azide (3)		
0·2 mM	−18·2	−12·5
SOD (3)		
Heat-inactivated SOD (200 μg/ml)	28·0	12·0
SOD (200 μg/ml)	11·5	13·2
SOD (100 μg/ml)	2·5	3·8
Catalase (3)		
Heat-inactivated catalase (400 units/ml)	10·5	11·0
Catalase (400 units/ml)	13·0	9·2
Catalase (400 units/nml)+SOD (200 μg/ml)	10·8	13·6
ETYA (5×10^{-5} M) (3)	30·6	63·5
NDGA (5×10^{-5} M) (3)	20·3	40·0
Indomethacin (5×10^{-5} M) (3)	10·2	13·5

MPs were pretreated with MuIFN (10^3 U/ml) for 12 hr. After washing, various materials were added to the MPs. The MPs were stimulated with PMA (1 μg/ml) and CLs were measured. Peak activity in the presence of the added material was compared to peak activity obtained with buffer in matched experiments. Values are expressed as percentage inhibition of control. The number of experiments is given in parentheses (see 'Materials and Methods' for details).

with the use of various reactive oxygen species scavengers. Sodium azide (myeloperoxidese inhibitor) was used as a CL inhibitor of PMNs but not of MPs (Rosen & Klebanoff, 1976). Table 1 shows that 0·2 mM azide did not suppress CL in MuIFN-treated and untreated MPs. This confirms an earlier report stating that sodium azide does not suppress CL in the murine MP system (Hatch *et al.*, 1978). Superoxide dismutase (SOD), a superoxide anion ($O_2^{\bullet}$) scavenger and a strong inhibitor of CL in neutrophils and monocytes (Stevens *et al.*, 1978; Johnson *et al.*, 1976), did not suppress CL in MuIFN-treated or untreated MPs at doses of 100–200 μg/ml.

Catalase, a hydrogen peroxide (H_2O_2) scavenger, alone or with SOD, did not suppress the CL. These results suggest that the CL observed in MPs is not $O_2^{\bullet}$- or H_2O_2-dependent. On the other hand, ETYA (5×10^{-5}M) and NDGA (5×10^{-5} M), inhibitors of arachidonic acid cyclo-oxygenase and lipoxygenase, did suppress MuIFN-augmented CL in MPs, while indomethacin (5×10^{-5} M) (a cyclo-oxygenase inhibitor) did not (Smolen & Weissman, 1980). These results suggest that MuIFN-augmented CL may be lipoxygenase dependent.

$O_2^{\bullet}$, H_2O_2 and OH· generation in IFN-treated MPs

It has been shown, by using various scavengers, that CL generation does not reflect an increased production of $O_2^{\bullet}$ and H_2O_2. Therefore, we measured reactive oxygen species ($O_2^{\bullet}$ and H_2O_2 and OH·) generation in MPs treated with IFN for 12 hr, which is optimal preincubation time for CL augmentation (Fig. 1b). $O_2^{\bullet}$ production by PMA was 45·83 ± 9·10 (SD) nmol/mg protein/60 min or 44·40 ± 7·46 (SD) nmol/mg protein/60 min in IFN-treated or untreated MPs, respectively. These levels were diminished by one-tenth by the addition of SOD (150 units/ml). In the absence of PMA, the cells did not produce any detectable $O_2^{\bullet}$, which agrees with previously published observations (Johnston, Godzik & Cohn, 1978).

H_2O_2 releases by PMA were 17·54 ± 2·57 (SD) nmol/mg protein/60 min in untreated MPs, and 19·03 ± 2·75 (SD) nmol/mg protein 60/min in IFN-treated MPs. However, H_2O_2 values were less 1·7 nmol/mg protein/60 in unstimulated MPs or in PMA-stimulated MPs with catalase (400 units/ml).

Hydroxyl radical production was measured by using a method based on the oxidation of 2-keto-4-thiomethylbutyric acid (KMB) by OH·, with the generation of ethylene gas (C_2H_4) as an end product (Klebanoff & Rosen, 1978). The MPs treated with MuIFN (10^3 units/ml) produced 19·44 times more C_2H_4 than untreated cells. However, when mouse MPs were treated with heterologous interferon [i.e. HuIFN-α (10^3 units/ml)] or with MuIFN pretreated with anti-MuIFN antibody, C_2H_4 production was not augmented significantly (Table 2). This supports the view that an enhancement of C_2H_4 production is mediated by the mouse interferon itself.

ETYA and NDGA suppressed OH· production, while indomethacin did not (Table 3). There was no difference in uptake of opsonized zymosan by ETYA- and NDGA-treated and untreated MPs, which suggests that, at this concentration, ETYA and NDGA did not interfere with MP function (data not shown).

Indomethacin was employed in a concentration (5×10^{-5} M) at which PGE_2 and 6-keto-$PGE_{1\alpha}$ were

Table 2. Effect of MuIFN on OH· production by mouse peritoneal MPs

		10^{-11} moles/5×10^6 cells
	No. of assays	Mean ± SD
Cell	3	1·95 ± 0·49
Cell + PMA	4	3·78 ± 0·89
Cell + MuIFN	3	10·42 ± 2·20
Cell + MuIFN + PMA	5	73·47 ± 15·53
Cell + HuIFN + PMA	3	6·18 ± 0·59
Cell + MuIFN + anti-MuIFN + PMA	3	13·22 ± 3·47

MPs were pretreated with MuIFN (10^3 U/ml) or HuIFN (10^3 U/ml) for 12 hr. After washing, the MPs were stimulated with PMA (1 μg/ml) and OH· was measured (see 'Materials and Methods' for details).

Table 3. Effects on inhibitors of arachidonic acid metabolism on OH· production by MuIFN-treated MPs

		10^{-11} moles/5×10^6 cells
	No. of assays	Mean ± SD
Cell	3	1·34 ± 0·39
Cell + PMA	3	3·66 ± 0·94
Cell + MuIFN	3	9·86 ± 3·17
Cell + MuIFN + PMA	3	81·09 ± 16·10
Cell + MuIFN + PMA + 5×10^{-5} M, ETYA	3	13·19 ± 5·56
Cell + MuIFN + PMA + 5×10^{-5} M, NDGA	3	14·17 ± 7·51
Cell + MuIFN + PMA + 5×10^{-5} M, indomethacin	3	75·74 ± 11·44

For experimental details, see 'Materials and Methods'.

inhibited in MuIFN-treated MPs up to 90%. ETYA (5×10^{-5} M) and NDGA (5×10^{-5} M) diminished the lipoxygenase products (5- and 15-HETE) up to 90% compared with the products of the MPs untreated with the inhibitors. However, indomethacin did not affect lipoxygenase production.

DISCUSSION

We have demonstrated that MuIFN significantly augments CL and OH· production in resident murine peritoneal macrophages stimulated by PMA. Maximal effects were obtained with a MuIFN dose of 10^3 units/ml, and lesser effects with a higher dose such as 10^4 units/ml. The enhancing effect of MuIFN was optimal following 12 hr treatment of MPs with MuIFN, and it could not be observed after a 48-hr treatment.

Why the higher concentration of MuIFN could not enhance the CL maximally, and why a 48-hr incubation of MPs with MuIFN did not result in any effect on CL is not clear. CL is believed to result from a respiratory burst which produces a group of highly reactive microbicidal and tumoricidal agents through the partial reduction of oxygen, to forms of $O_2^{\bullet}$, H_2O_2, $^1O_2$ and OH·. $^1O_2$ and OH· are believed to be particularly potent in their ability to mediate the killing of bacteria and tumour cells by MPs (Klebanoff, 1980; Nathan *et al.*, 1980).

The augmentation of CL by MuIFN treatment was not suppressed by $O_2^{\bullet}$ and/or H_2O_2 scavengers. This suggested that MuIFN-treated PMA-stimulated MPs have an increased $^1O_2$ and/or OH· production. Moreover, we obtained evidence to suggest that only OH· among $O_2^{\bullet}$, H_2O_2 and OH· was increased in IFN-treated MPs.

Two metabolic pathways which mediate an increased $^1O_2$ and OH· production are the NADPH oxidase system (Klebanoff, 1980) and the arachidonic acid metabolic pathway (Kuehl *et al.*, 1979).

$O_2^{\bullet}$ and H_2O_2 are generated by the NADPH oxidase system, and OH· and $^1O_2$ are produced from $O_2^{\bullet}$ and H_2O_2 with metals (for example Fe^{2+}) or myeloperoxidase-halides in neutrophils and monocytes (Klebanoff, 1980).

SOD almost totally abolishes OH· and CL generation in these cells (Johnston *et al.*, 1976; Weiss, Rustagi & LoBuglio; 1978). In our experiments, SOD did not suppress either CL or production of OH· augmented by MuIFN, and no differences were observed between the PMA-stimulated generation of $O_2^{\bullet}$ and H_2O_2 in MuIFN-treated and untreated MPs after 12 hr IFN-treatment.

Therefore, it is unlikely that the NADPH oxidase system did participate in the elevated OH· and CL generation in IFN-treated MPs. Meanwhile, it is known that highly reactive oxygens are derived from the unsaturated fatty acid cascades, and the oxygens are responsible for some of the cellular damage that characterizes inflammatory lesions (Kuehl & Egan, 1980). Another report has shown that the CL generation in resident MPs relates to the lipoxygenase pathway of arachidonic acid, an unsaturated fatty acid abundantly present in many phospholipids of the cell membrane (Smith & Weidemann, 1980).

In our experiments with unsaturated fatty acid oxygenase inhibitors, ETYA and NDGA (inhibitors of both cyclo-oxygenase and lipoxygenase) did suppress CL and production of OH· augmented by

MuIFN, but indomethacin (a cyclo-oxygenase inhibitor) did not. This result shows that the CL and OH· production enhanced by MuIFN in the Mps are due to the lipoxygenase pathway and not to the cyclo-oxygenase pathway. Suthanthiran *et al.* (1984) showed that the tumoricidal activity in natural killer (NK) cells was inhibited by various OH· scavengers, but not by SOD and catalase. Moreover, NK activity was also inhibited by lipoxygenase inhibitors. Thus, they speculated that OH·, possibly generated via the lipoxygenase pathway of arachidonic acid metabolism, is critical for the NK cell cytotoxicity.

Although OH· can be directly produced from lipoxygenase pathway (i.e. arachidonic acid + O_2 → hydroperoxy-eicosatetraenoic acid (HPETE) → hydroxy-eicosatetraenoic acid (HETE) + OH·), it is not clear in our experiment whether the reactive oxygen augmented by MuIFN is a product derived from the cascade on this pathway.

Boraschi *et al.* (1982, 1983) showed that IFN-β inhibited the $O_2^{\bullet}$ and H_2O_2 releasing capacity of murine MPs for 20 hr incubation. In contrast, Nathan *et al.* (1983) reported that IFN-γ induced activity of H_2O_2 generation in human macrophages for more than 3 days' incubation. In our experiment, murine IFN-α generated only CL and OH· production, but did not change $O_2^{\bullet}$ and H_2O_2 levels. Although these discrepancies among Nathan, Boraschi and us will be explained as being due to different kinds of MPs, IFNs and stimulants, further studies will be required to resolve them. It is known that immune interferon (IFN-γ) induces cellular resistance to virus replication more slowly that does virus-induced interferon (IFN-α, -β) (Dianzani *et al.*, 1978). Thus, it would be of interest to know whether these IFNs activate MPs by different mechanisms.

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