Macrophage activation for intracellular killing as induced by a Ca^{2+} ionophore

Dependence on L-arginine-derived nitrogen oxidation products

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Mouse macrophages activated by interferon- γ kill intracellular *Leishmania* by a process that depends on the generation of L-arginine-derived nitrogen oxidation products. Interferon-induced intracellular killing can be mimicked by exposure of macrophages to the Ca^{2+} ionophore A23187 in the presence of lipopolysaccharide. The mechanisms of this effect were therefore investigated. Destruction of the parasite was accompanied by accumulation of nitrite in the macrophage culture fluids. Leishmanicidal activity and nitrite production in cultures stimulated with ionophore A23187 and lipopolysaccharide were abrogated when cells were activated in medium containing arginase or the L-arginine analogues L-canavanine, guanidine or N^G -monomethyl-L-arginine. L-Arginine was required during the lipopolysaccharide-induced triggering phase only. Indeed, macrophage priming with ionophore A23187 in L-arginine-depleted medium led to full microbicidal activity and nitrite generation provided that L-arginine was present during subsequent triggering by lipopolysaccharide. Addition of N^G -monomethyl-L-arginine to ionophore-activated macrophages increased O_2^- production on phorbol myristate stimulation, while inhibiting glucose oxidation through the hexose monophosphate shunt pathway. Leishmanicidal activity and nitrite production were also inhibited when ionophore-treated cultures were incubated with excess iron, implying a role for iron as a defence mechanism against the toxicity of nitrogen derivatives. These results indicate that the ionophore-induced leishmanicidal activity occurs through a process similar to that evoked by interferon- γ , i.e. the production of L-arginine-derived nitrogen oxidation products.

INTRODUCTION

On activation with lymphokines or recombinant interferon- γ (IFN- γ), mouse macrophages acquire the capacity to kill *Leishmania* parasites (Buchmüller & Mauël, 1979; Nacy *et al.*, 1988). It has been recently reported by this and other laboratories that leishmanicidal activity in IFN- γ -activated macrophages is dependent on the generation of L-arginine-derived nitrogen oxidation products (Green *et al.*, 1990; Liew *et al.*, 1990; Mauël *et al.*, 1991). These metabolites (particularly NO^*) appear to be responsible for killing tumour cells, and possibly protozoan and metazoan parasites, through inhibition of mitochondrial respiration and DNA synthesis in the targets (Hibbs *et al.*, 1988; Stuehr & Nathan, 1989). IFN-mediated intracellular parasite killing can be mimicked by exposure of macrophages to the Ca^{2+} ionophore A23187 in the presence of lipopolysaccharide (LPS) (Buchmüller-Rouiller & Mauël, 1991). The present report deals with the mechanisms of this microbicidal effect, and more precisely addresses the question of whether the leishmanicidal activity of ionophore-treated macrophages is also mediated by L-arginine-dependent effector mechanisms.

MATERIALS AND METHODS

Mice

CBA and C57BL/6 mice were obtained from Iffa Credo (Saint-Germain-sur-l'Arbresle, France) and were used between 8 and 18 weeks of age. These two strains were used interchangeably, as their macrophages have been found to respond similarly to Ca^{2+} ionophore (Buchmüller-Rouiller & Mauël, 1991).

Reagents

L-Arginine hydrochloride, L-canavanine sulphate salt, guani-

dinium chloride, arginase from bovine liver (40–60 units/mg of protein) and Ca^{2+} ionophore A23187 (calcium salt) were purchased from Sigma GmbH (Munich, Germany). N^G -Monomethyl-L-arginine (N^G -MeArg) was purchased from Chemical Dynamics Corporation (South Plainfield, NJ, U.S.A.).

Macrophage cultures

Bone marrow-derived macrophages were prepared as described previously (Kelso *et al.*, 1982). Cells were suspended in Dulbecco's medium supplemented with 10% (v/v) fetal bovine serum (FBS; Seromed, Munich, Germany) and distributed as follows: in 96-U-bottomed-well microculture plates (Costar 3799, Cambridge, MA, U.S.A.; 6×10^4 – 7×10^4 cells/well) for hexose monophosphate shunt (HMPS) activity, O_2^- secretion and protein determinations; in 96-flat-bottomed-well microculture plates (Costar 3596; 7×10^4 – 8×10^4 cells/well) for determination of leishmanicidal activity and NO_2^- secretion; flat-bottomed wells gave more consistent results than U-bottomed wells in the leishmanicidal assay, probably because of a more uniform phagocytosis of the parasites by cells cultured on a flat surface.

Infection of macrophage cultures

Macrophages were infected with *Leishmania enriettii* promastigotes (Mauël *et al.*, 1989) at a ratio of 20 parasites per cell and were incubated for 18–20 h at 37 °C to allow for phagocytosis of the parasite.

Macrophage activation

Infected and non-infected macrophages were washed twice with warm Hanks' balanced salt solution and reincubated in the absence of serum with ionophore A23187 with or without added lipopolysaccharide (from *Escherichia coli* 0:55-B.5; Difco Labor-

Abbreviations used: IFN- γ , interferon- γ ; LPS, lipopolysaccharide; N^G -MeArg, N^G -monomethyl-L-arginine; FBS, fetal bovine serum; HMPS, hexose monophosphate shunt; SOD, superoxide dismutase; PMA, phorbol myristate acetate.

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Table 1. Reversal of the L-arginine analogue-induced inhibition of A23187-dependent activation by addition of excess L-arginine

Infected C57BL bone-marrow macrophages were incubated in L-arginine-containing (0.4 mM) medium with ionophore A23187 (0.5 μ M) and LPS (10 ng/ml) in the presence or absence of added L-arginine analogues (experiment I: guanidine, 3.5 mM; L-canavanine, 4 mM; experiment II: *N*^G-MeArg, 0.2 mM) and excess L-arginine (4 mM). After 24 h, supernatants were harvested for NO₂⁻ measurement and cells were lysed for determination of parasite survival. Results are means of quadruplicates $\pm$ S.D. Two representative experiments are shown.

		Inhibitor	L-Arginine	Parasite survival (c.p.m.) (mean $\pm$ S.D.)	NO ₂ ⁻ (nmol/well) (mean $\pm$ S.D.)
Experiment I					
	A23187 + LPS	—	—	918 $\pm$ 489	1.52 $\pm$ 0.11
	A23187 + LPS	—	+	621 $\pm$ 293	2.35 $\pm$ 0.12
	A23187 + LPS	Guanidine	—	7303 $\pm$ 1163	0.79 $\pm$ 0.05
	A23187 + LPS	Guanidine	+	732 $\pm$ 226	1.80 $\pm$ 0.05
	A23187 + LPS	L-Canavanine	—	7177 $\pm$ 590	0.97 $\pm$ 0.05
	A23187 + LPS	L-Canavanine	+	590 $\pm$ 106	2.02 $\pm$ 0.10
	Control	—	—	13046 $\pm$ 1712	0.13 $\pm$ 0.01
Experiment II					
	A23187 + LPS	—	—	2201 $\pm$ 246	1.08 $\pm$ 0.07
	A23187 + LPS	<i>N</i> ^G -MeArg	—	12476 $\pm$ 1472	0.22 $\pm$ 0.01
	A23187 + LPS	<i>N</i> ^G -MeArg	+	5116 $\pm$ 1067	1.01 $\pm$ 0.04
	Control	—	—	14760 $\pm$ 2109	0.04 $\pm$ 0.01

atories, Detroit, MI, U.S.A.; 1–10 ng/ml) in the presence or absence of various L-arginine analogues or arginase. After 4 h, the microwell cultures were supplemented with FBS up to a final concentration of 10%; serum was not added at the beginning of ionophore treatment, because it is known to antagonize the cellular response to ionophore A23187 because of the presence of serum proteins which can bind the small hydrophilic ionophore molecule (Drummond *et al.*, 1987). When ionophore treatment was used as a priming step, cells were incubated in serum-free medium for 4 h with the drug in the presence or absence of arginase, then washed twice with Hanks' balanced salt solution and reincubated with Dulbecco's medium supplemented with 10% FBS in the presence or absence of LPS and arginase.

NO₂⁻ determinations

After 20 h of activation, 100 μ l portions of macrophage supernatants were harvested and tested for the presence of NO₂⁻ by the Griess reaction, as previously described (Ding *et al.*, 1988). Briefly, samples were allowed to react with a similar volume of the Griess reagent (1% sulphanilamide/0.1% naphthylethylenediamine dihydrochloride/2.5% H₃PO₄), and the NO₂⁻ concentration was determined by measuring the absorbance at 550 nm in a micro-e.l.i.s.a. reader (Easy Reader EAR 340, Kontron Analytik, Zürich, Switzerland) using a 690 nm reference filter. NaNO₂ was used as a standard.

Measurement of HMPS activity

HMPS activity was determined as previously described (Mauël *et al.*, 1984; Buchmüller-Rouiller & Mauël, 1991) from the amount of ¹⁴CO₂ evolved by macrophages from D-[1-¹⁴C]glucose (3.94 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.).

Cytochrome c reduction test

O₂⁻ production was assayed by the superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c, using a micro-assay as previously described (Buchmüller-Rouiller & Mauël, 1987). Phorbol myristate acetate (PMA) at 500 ng/ml was used as the triggering agent. Cytochrome c reduction was measured at 550 nm with a 492 nm reference filter. Results are expressed as the differences in absorbance/mg of cell protein (ΔA /mg of protein) between cultures incubated in the absence (–SOD) and

in the presence (+SOD) of SOD at each time point (*t*_i) according to the formula:

$$\frac{\Delta A_{t_i-t_0}[-SOD] - \Delta A_{t_i-t_0}[+SOD]}{\text{mg of protein}}$$

Determination of cellular protein

Cellular protein in macrophage cultures was determined by the Coomassie Blue dye-binding assay as previously described by Baumgarten (1985).

Measurement of intracellular parasite killing

At 24 h after treatment of the infected cells with ionophore, LPS, the various L-arginine analogues or arginase, macrophages were washed and lysed by exposure to 0.01% SDS as described previously (Mauël, 1984). The wells were then supplemented with Hošmem II medium (Berens & Marr, 1978), and parasite growth was recorded by measuring [³H]thymidine uptake (Buchmüller-Rouiller & Mauël, 1986).

RESULTS

Requirement for L-arginine in the ionophore-dependent induction of macrophage leishmanicidal activity and NO₂⁻ production

We have previously shown that bone marrow-derived mouse macrophages exposed for 24 h to the ionophore A23187 together with LPS were induced to secrete NO₂⁻ and acquired the ability to kill *Leishmania enriettii* (Buchmüller-Rouiller & Mauël, 1991). The presence of both the ionophore and LPS was required for activation, as either factor alone failed to induce macrophages to kill the parasite or to release appreciable amounts of NO₂⁻. Moreover, leishmanicidal activity and NO₂⁻ secretion were presumably correlated, since intracellular killing was never observed in the absence of NO₂⁻ production and, conversely, release of this metabolite always signalled parasite destruction.

Nitrites and nitrates are end products of the L-arginine-dependent pathway through which activated mouse macrophages become cytotoxic for certain micro-organisms and tumour cells (Hibbs *et al.*, 1988; Stuehr *et al.*, 1989; Green *et al.*, 1990). We thus tested the L-arginine dependence of ionophore-induced leishmanicidal activity and NO₂⁻ secretion by adding L-arginine

Table 2. Requirement for L-arginine during the triggering phase of macrophage activation

CBA bone-marrow macrophages were incubated for 4 h with ionophore A23187 (0.1 μ M) in the presence or absence of 5 units of arginase/ml. Cells were then washed twice and reincubated with LPS (experiment I: 3 ng/ml; experiment II: 10 ng/ml) with or without arginase (5 units/ml). After 20 h, NO₂⁻ production was determined in the supernatants and cells were lysed by SDS for determination of parasite survival. Results are means $\pm$ S.D. Controls corresponded to 8235 $\pm$ 1851 c.p.m. and 0.26 $\pm$ 0.09 nmol of NO₂⁻ for experiment I and 18482 $\pm$ 2262 c.p.m. and 0.44 $\pm$ 0.07 nmol of NO₂⁻ for experiment II. Two experiments representative of eight experiments are shown.

1st incubation	2nd incubation	Experiment I		Experiment II	
		Parasite survival (c.p.m.)	NO ₂ ⁻ (nmol)	Parasite survival (c.p.m.)	NO ₂ ⁻ (nmol)
A23187	LPS	1703 $\pm$ 471	0.97 $\pm$ 0.06	1362 $\pm$ 561	3.37 $\pm$ 0.13
A23187 + arginase	LPS	773 $\pm$ 391	1.50 $\pm$ 0.09	557 $\pm$ 49	5.76 $\pm$ 0.12
A23187	LPS + arginase	10259 $\pm$ 2236	0.11 $\pm$ 0.01	25838 $\pm$ 3823	0.33 $\pm$ 0.13
A23187 + arginase	LPS + arginase	8113 $\pm$ 340	0.15 $\pm$ 0.00	25932 $\pm$ 3348	0.44 $\pm$ 0.07

Table 3. Effect of N^G-MeArg on the activation of macrophage HMPS by ionophore A23187 and LPS

CBA bone-marrow-derived macrophages were incubated with ionophore A23187 (0.5 μ M) in the presence or absence of LPS (10 ng/ml), N^G-MeArg (0.2 mM) and L-arginine (4 mM). After 24 h, supernatants were harvested and tested for the presence of NO₂⁻, and cells were washed and assayed for HMPS activity. Protein content was determined in parallel cultures treated similarly. Two experiments representative of four are shown. Results are means of four determinations $\pm$ S.D. In parentheses, percentage inhibition of HMPS activity in the presence of the L-arginine analogue, N^G-MeArg.

	Experiment I		Experiment II	
	HMPS (c.p.m./ μ g of protein)	NO ₂ ⁻ (pmol/ μ g of protein)	HMPS (c.p.m./ μ g of protein)	NO ₂ ⁻ (pmol/ μ g of protein)
Medium	99 $\pm$ 40	21 $\pm$ 3	72 $\pm$ 11	27 $\pm$ 4
A23187	159 $\pm$ 20	49 $\pm$ 9	67 $\pm$ 10	45 $\pm$ 10
LPS	132 $\pm$ 41	88 $\pm$ 13	98 $\pm$ 25	139 $\pm$ 16
A23187 + LPS	536 $\pm$ 49	241 $\pm$ 19	261 $\pm$ 30	248 $\pm$ 31
A23187 + LPS + N ^G -MeArg	248 $\pm$ 68 (54 %)	106 $\pm$ 9	132 $\pm$ 8 (49 %)	57 $\pm$ 7
A23187 + LPS + N ^G -MeArg + L-arginine	491 $\pm$ 76	232 $\pm$ 37	270 $\pm$ 30	189 $\pm$ 17
A23187 + LPS + L-arginine	525 $\pm$ 78	329 $\pm$ 21	315 $\pm$ 36	292 $\pm$ 55

analogues (N^G-MeArg, canavanine, guanidine) to the cultures. Table 1 shows that these analogues, which block the activity of enzymes that utilize L-arginine, were very efficient at preventing the development of leishmanicidal activity and NO₂⁻ release when added to culture medium at concentrations close to that of L-arginine. That these agents acted as competitive inhibitors was shown by the fact that the above activities were partially or completely restored by the addition of excess L-arginine (4 mM) in the incubation media. The inhibitors were without any effect on parasite survival in control cultures (Mauël *et al.*, 1991).

Requirement for L-arginine in the triggering phase of ionophore-dependent macrophage activation

Ionophore-induced activation of macrophages for intracellular killing is a two-signal process, the ionophore acting as the first (priming) and LPS as the second (triggering) signal (Buchmüller-Rouiller & Mauël, 1991). In order to determine if the presence of L-arginine was necessary during both phases of activation, infected macrophages were incubated for 4 h with ionophore A23187 in the presence or absence of arginase, then washed and exposed to LPS for 20 h with or without arginase. Table 2 shows that removal of L-arginine by arginase during the ionophore-dependent priming phase of activation did not impair the subsequent development of leishmanicidal properties and NO₂⁻

secretion on triggering by LPS in L-arginine-containing medium. In contrast, the enzyme inhibited both effects when present during the LPS-induced triggering phase. These observations are consistent with L-arginine being required as a late effector molecule responsible for the complete functional expression of the activated state.

Role of L-arginine in the development of a metabolic burst in ionophore-activated macrophages

On activation by lymphokines, macrophages undergo a metabolic burst which is characterized by increased HMPS activity and the production of oxygen metabolites as triggered by membrane-active agents or phagocytosis (Murray, 1984; Buchmüller-Rouiller & Mauël, 1986). We have shown that bone marrow-derived macrophages stimulated with ionophore A23187 and LPS exhibited a severalfold increase in their HMPS activity relative to control macrophages. When such cells were exposed to ionophore and LPS in the presence of the L-arginine analogue N^G-MeArg, HMPS activity was decreased by approx. 50 % (Table 3). This inhibitory effect was reversed by the addition of excess L-arginine during activation, providing evidence that L-arginine is required for full development of the ionophore-dependent HMPS burst. The effect of N^G-MeArg was tested next on PMA-triggered superoxide (O₂⁻) production. As shown in

Fig. 1. Effect of N^G -MeArg on O_2^- secretion by macrophages stimulated by ionophore A23187 + LPS

C57BL/6 bone-marrow derived macrophages were incubated in L-arginine-containing Dulbecco's medium with ionophore A23187 and LPS (3 ng/ml), in the presence (●, ▲) or absence (○, △) of N^G -MeArg (0.2 mM). After 24 h, supernatants were harvested and tested for NO_2^- production (stippled line). Cells were then washed and O_2^- production (solid line) was determined using the cytochrome *c* reduction assay. Parallel cultures treated similarly were used for protein determination. Results are means of four determinations $\pm$ S.D. and are representative of three experiments.

Fig. 1, exposure of macrophages to ionophore plus LPS (in the presence of L-arginine) led to decreased O_2^- release when compared with LPS alone. This effect was reversed by N^G -MeArg, which restored O_2^- concentrations to those seen in macrophages treated with LPS alone. As expected, NO_2^- generation was, however, markedly inhibited by the non-metabolizable L-arginine analogue. Thus, N^G -MeArg appears to have different effects on HMPS activity and O_2^- production.

Effect of Fe on A23187-induced leishmanicidal activity of macrophages

It has been suggested that activated macrophages exercise their cytotoxic effects through NO -mediated Fe efflux leading to inhibition of Fe-dependent enzymes in the target cells (Drapier & Hibbs, 1986, 1988; Hibbs *et al.*, 1988). In order to study the dependence of ionophore-induced macrophage leishmanicidal activity on Fe, infected macrophages were activated with ionophore A23187 plus LPS in the presence of $FeSO_4$. As shown in Fig. 2, Fe inhibited macrophage leishmanicidal activity. Moreover, NO_2^- concentrations in macrophage supernatants were strikingly decreased. This decrease was not due to cell death (less than 2% dead cells in $FeSO_4$ -treated cultures, as determined by the Trypan Blue-exclusion test). The observation that a decrease in NO_2^- was accompanied by a similar decrease in NO_3^- [measured by the Griess reaction after reduction of NO_3^- by nitrate reductase (Beutler & Wurst, 1986) (results not shown)], was consistent with impairment of NO_2^- secretion in the presence of $FeSO_4$ via decreased NO synthesis rather than increased NO_3^- versus NO_2^- production. This suggests that in addition to its capacity to prevent inactivation of target enzymes, Fe might interfere with the production of oxidized nitrogen derivatives.

Fig. 2. Inhibition of A23187-induced macrophage activation by $FeSO_4$

Infected CBA bone-marrow-derived macrophages were incubated with increasing concentrations of ionophore A23187 and 1 ng of LPS/ml in the presence or absence of 50 μM - $FeSO_4$. After 24 h, supernatants were tested for the presence of NO_2^- and cells were lysed for determination of parasite survival. Results are means of four determinations $\pm$ S.D. and are representative of three experiments.

DISCUSSION

The present report shows that macrophage activation for intracellular killing as induced by ionophore A23187 is strictly dependent on L-arginine-derived nitrogen oxidation products, as previously reported for lymphokine-induced tumoricidal and microbicidal activities (Drapier *et al.*, 1988; Granger *et al.*, 1988; James & Glaven, 1989; Green *et al.*, 1990; Mauël *et al.*, 1991). Removal of L-arginine from culture media by arginase (which converts arginine into ornithine and urea), or addition of the L-arginine analogues, guanidine, L-canavanine or N^G -monomethyl-L-arginine [which inhibit NO formation from L-arginine in mammalian cells (Hibbs *et al.*, 1987; Marletta, 1989)], depressed both parasite killing and NO_2^- production, which could be restored to their normal levels by addition of excess L-arginine.

Macrophage activation for intracellular or extracellular killing appears to proceed in a sequence of two reactions, i.e. a priming step (as induced by exposure to crude lymphokines, to recombinant IFN- γ or, as shown here, to a Ca^{2+} ionophore), followed by a triggering signal required for the development of full functional activity (Pace *et al.*, 1983; Nacy *et al.*, 1988; Buchmüller-Rouiller & Mauël, 1991). L-Arginine was clearly required during the LPS-induced triggering phase only. Indeed, incubation of macrophages in L-arginine-depleted medium during ionophore-induced priming resulted in the capacity to develop

full microbicidal activity and to generate NO₂⁻ provided that L-arginine was present during subsequent triggering by LPS. These results are consistent with a role for this amino acid as a substrate for generation of the final effector nitrogen oxidation products. Hibbs *et al.* (1987) have reported a similar L-arginine requirement for LPS to be effective as a second signal for the expression of the tumoricidal activity of macrophages activated as a result of BCG infection.

Macrophage activation is correlated with the stimulation of a metabolic burst characterized by enhanced HMPS activity and the capacity to release increased amounts of oxygen metabolites on proper triggering (Buchmüller-Rouiller & Mauël, 1986). A similar metabolic stimulation is observed in macrophages exposed to ionophore A23187 and LPS (Buchmüller-Rouiller & Mauël, 1991). Interestingly, the L-arginine analogue N^G-MeArg was found to exercise different effects on HMPS activity and PMA-triggered O₂⁻ production, i.e. the compound down-regulated HMPS stimulation but increased O₂⁻ release. Since treatment of ionophore-stimulated macrophages with N^G-MeArg blocks NO₂⁻ production, which itself appears to require NADPH (Marletta *et al.*, 1988; Stuehr *et al.*, 1989), the depressed HMPS activity might reflect the accumulation of NADPH resulting from inhibition of the conversion of L-arginine into NO₂⁻/NO₃⁻. This explanation is consistent with the observation that addition of excess L-arginine, which allowed NO₂⁻ production to resume, also restored HMPS activity to that observed in control activated macrophages. The apparent increase in O₂⁻ production by macrophages stimulated with ionophore A23187 in the presence of N^G-MeArg might be due to higher accumulation of this metabolite in the absence of NO[•] (which may react with O₂⁻ to produce NO₃⁻; Beckman *et al.*, 1990), or to increased production resulting from the availability of excess NADPH in the absence of NO₂⁻ formation. A similar increase in O₂⁻ production by macrophages activated with IFN-γ and LPS in the presence of N^G-MeArg, as well as in resident and *Corynebacterium parvum*-activated rat macrophages, has been reported by others (Ding *et al.*, 1988; Albina *et al.*, 1989).

The possible role of oxygen metabolites in the leishmanicidal activity of ionophore A23187 plus LPS-activated mouse macrophages remains to be evaluated. No significant killing was observed during the first 8 h of activation by ionophore plus LPS (results not shown), similar to results obtained with interferon-γ plus LPS-activated macrophages (Mauël *et al.*, 1991). These observations correlate with the time-course of NO₂⁻ production: NO₂⁻ released by macrophages became measurable only after 8–10 h of activation. Furthermore, no O₂⁻ secretion was detected when ionophore A23187 and LPS were used as triggering agents (in place of PMA), even in the presence of N^G-MeArg (results not shown). These results, together with the strict L-arginine dependence of ionophore A23187 plus LPS-induced leishmanicidal activity, strongly suggest that putative production of oxygen metabolites during an early macrophage response to activating factors is not responsible for our observations. We do not, however, exclude a possible role for O₂⁻ in *Leishmania* killing by activated macrophages. Indeed, it is generally considered that O₂⁻ itself has little microbicidal activity (Badwey & Karnovsky, 1980); it may, however, interact with NO[•] within the acidic phagosome to generate new derivatives such as peroxy-nitrite (Beckman *et al.*, 1990) that would be more microbicidal than the initial reactants.

Recent studies on the mechanisms of macrophage activation have focused on the importance of iron in the survival of target micro-organisms and cells (Hibbs *et al.*, 1984; Byrd & Horwitz, 1989). L-Arginine-derived NO[•] synthesized by activated macrophages has been reported to form complexes with iron from FeS proteins of target cells, leading to iron loss and subsequent

inactivation of iron-requiring enzymes active in mitochondrial respiration (Drapier & Hibbs, 1986, 1988; Hibbs *et al.*, 1988), the citric acid cycle (Drapier & Hibbs, 1986; Hibbs *et al.*, 1988) and DNA replication (Hibbs *et al.*, 1988). FeSO₄ has been previously reported to abolish the cytotoxic effect of lymphokine-activated macrophages against *Leishmania* (Mauël *et al.*, 1991) and *Schistosoma mansoni* parasites (James & Glaven, 1989). Similarly, the addition of FeSO₄ to ionophore A23187-activated macrophages inhibited intracellular parasite killing and led to decreased NO₂⁻ concentrations in macrophage supernatants. This decrease was not due to toxicity of FeSO₄ for the cells nor to preferential accumulation of NO₃⁻ relative to NO₂⁻ in iron-treated cultures. These results suggest that in addition to a possible protective action through reconstitution of the activity of target iron-containing enzymes as postulated in other systems (Drapier & Hibbs, 1986; James & Glaven, 1989), the metal also interfered with the release of toxic nitrogen oxidation products.

In conclusion, these studies indicate that the Ca²⁺ ionophore A23187 (together with LPS) does induce in macrophage microbicidal mechanisms that are similar to those evoked by IFN-γ (plus LPS), i.e. the generation of L-arginine-derived nitrogen oxidation products.

The question whether a rise in intracellular Ca²⁺ is a prerequisite for nitric oxide production remains open. A Ca²⁺-dependence for the cellular regulation of NO[•] synthesis has been shown in several systems including the vascular endothelium (Bredt & Snyder, 1990), brain (Knowles *et al.*, 1989) and adrenal gland (Palacios *et al.*, 1989). The enzyme generating NO[•] in macrophages appears, however, to be Ca²⁺-independent (Marletta *et al.*, 1988; Hauschildt *et al.*, 1990). Our previous results suggested a role for Ca²⁺ in the molecular events leading to NO₂⁻ secretion and microbicidal activity in ionophore A23187 plus LPS- or IFN-γ plus LPS-activated macrophages, because both effects could be inhibited by EGTA and reversed by an excess of Ca²⁺ (Buchmüller-Rouiller & Mauël, 1991). Hauschildt *et al.* (1990) showed that induction and activity of NO[•] synthase in macrophages stimulated by a unique signal (LPS at a high concentration, lipopeptides) was Ca²⁺-independent. Conceivably, stimulation by high doses of LPS might bypass the step that is Ca²⁺-dependent in our two-signal system where activation is induced by ionophore and low doses of LPS. Finally, although extracellular Ca²⁺ appears to be essential for macrophage priming by ionophore A23187, it cannot be excluded that the ionophore might stimulate cells by means other than Ca²⁺ influx. Indeed, as shown by other authors (Gemsä *et al.*, 1979), macrophage stimulation could be triggered by ionophore A23187 even in the absence of Ca²⁺ influx, although the presence of extracellular Ca²⁺ was required for induction of a biological response. Which step in the activation sequence would require extracellular Ca²⁺ under conditions preventing Ca²⁺ influx still remains to be determined.

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