Effect of increasing intravesicular pH on nitrite production and leishmanicidal activity of activated macrophages

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We examined the effect of bafilomycin A₁ (BAF), an inhibitor of vacuolar-type H⁺-ATPases, on macrophage activation (measured as increased nitrite production and leishmanicidal activity) induced by interferon γ alone or together with lipopolysaccharide or tumour necrosis factor α . BAF increased intravesicular pH and enhanced nitrite release by activated macrophages; however, the NO concentration necessary to kill parasites was higher in BAF-exposed than control macrophages, suggesting that microbicidal nitrogen derivatives were less active at alkaline pH.

Antibody to tumour necrosis factor α inhibited BAF-induced nitrite production in interferon-activated cultures. To determine if enhanced NO synthesis was related to vesicular alkalization, macrophages were incubated with the lysosomotropic bases NH₄Cl and methylamine. These agents also increased intravesicular pH and nitrite production. Nitrite production was correlated with enhanced NO synthase activity in cytosolic extracts of the activated cells.

INTRODUCTION

Acidification of intracellular organelles plays a critical role in various functions of the phagocyte. The ability of the cell to decrease phagosomal pH appears to be crucial to microbicidal activity. Indeed, the capacity of certain micro-organisms such as *Legionella pneumophila* and *Toxoplasma gondii* to block phagosome acidification may be critical to their intracellular survival and replication [1,2]. Moreover, it is known that an elevated proton concentration promotes the spontaneous dismutation of O₂^{•-} to H₂O₂ [3], facilitates the activity of lysosomal hydrolases [4] and is a prerequisite for phagosome-lysosome fusion [2]. The mechanisms underlying vesicle acidification are not yet completely resolved [5]. However, some studies [6] strongly suggest the involvement of a vacuolar-type H⁺-ATPase active in the endosomal membranes, which catalyses the electrogenic translocation of protons from the cytoplasm to the intraendosomal space. This process creates both a positive internal membrane potential and a low internal pH. The membrane potential is eventually dissipated by passive influx of permeant cytoplasmic anions or efflux of internal cations [5].

Bafilomycin A₁ (BAF) is the most potent and specific inhibitor of vacuolar-type H⁺-ATPases described [7,8]. This antibiotic has been reported to inhibit phagosomal acidification on phagocytosis of fluorescein isothiocyanate (FITC)-labelled bacteria [6] and to reduce cell-surface oxidized-low-density lipoprotein receptor activity and degradation of oxidized low-density lipoprotein by inhibiting ATP-dependent acidification of endosomes and lysosomes [9].

In the present study, the effect of BAF on nitrite production, leishmanicidal activity and intravesicular pH of activated macrophages was examined. In addition to increasing intravesicular pH, BAF induced nitrite production in macrophages stimulated with interferon γ (IFN- γ) alone and potentiated nitrite secretion by macrophages activated with suboptimal doses of IFN- γ plus lipopolysaccharide (LPS) or tumour necrosis factor α (TNF- α). Although leishmanicidal activity was enhanced by BAF in

suboptimally activated cultures, the absolute levels of NO production necessary to induce killing were increased. The lysosomotropic bases NH₄Cl and methylamine (MET) and the carboxylic ionophore nigericin also increased intravesicular pH and enhanced macrophage activation measured as increased nitrite secretion and leishmanicidal activity. The possible mechanisms by which an increase in intravesicular pH modulates macrophage function are discussed.

MATERIALS AND METHODS

Mice

CBA (male or female) mice were obtained from IFFA CREDO (Saint-Germain-sur-l'Arbresle, France) and were used between 8 and 18 weeks of age.

Reagents

BAF was provided by Professor K. Altendorf, Universität Osnabrück, D-4500 Osnabrück, Germany. IFN- γ (murine; lot no. 2309-24) produced by Genetech was kindly supplied by Boehringer Ingelheim, Vienna, Austria. FITC/dextran (FITC-D, FD-405), nigericin and MET were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Anti-fluorescein rabbit IgG (H+L) fraction was purchased from Molecular Probes Inc., Eugene, OR, U.S.A. Human recombinant TNF- α was a gift from BASF/Knoll (Ludwigshafen, Germany). Human and murine TNF- α display similar potencies for activation of macrophage nitrite production and leishmanicidal activity [10]. Rabbit anti-(murine TNF- α) polyclonal antibody was purchased from Genzyme (Luzern, Switzerland). Dulbecco's minimal essential medium (DMEM) with or without NaHCO₃ was purchased respectively from Seromed, Munich, Germany, and Flow AG, Allschwil, Switzerland; unless specifically indicated, DMEM with NaHCO₃ was used for macrophage activation. Na⁺ medium contained 127 mM NaCl, 1.2 mM KH₂PO₄, 5.4 mM KCl, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 5.6 mM glucose and 10 mM

Abbreviations used: BAF, bafilomycin A₁; DMEM, Dulbecco's minimal essential medium; FCS, fetal calf serum; FITC-D, fluorescein isothiocyanate/dextran; HBSS, Hanks' balanced salt solution; IFN- γ , interferon γ ; LPS, lipopolysaccharide; MET, methylamine; NGM, N^G-monomethyl-L-arginine; TNF- α , tumour necrosis factor α .

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Hepes; pH was adjusted to 7.4 at 37 °C. K⁺ medium contained 127 mM KCl, 1.2 mM NaH₂PO₄, 5.4 mM NaCl and was otherwise identical with Na⁺ medium [11]. Fetal calf serum (FCS) was purchased from Seromed. N^G-Monomethyl-L-arginine (NGM) was from Chemical Dynamics Corp. (South Plainfield, NJ, U.S.A.).

Macrophage cultures

Macrophages were obtained by *in vitro* differentiation of bone marrow cells [12]. They were then distributed in DMEM supplemented with 10% (v/v) FCS as follows: in 96-U-bottomed-well microculture plates (Costar 3799, Cambridge, MA, U.S.A.) for determination of nitrite secretion (8×10^4 cells/well) and in 96-flat-bottomed-well microculture plates (Costar 3596) for determination of leishmanicidal activity (8×10^4 cells/well) or for determination of intravesicular pH (10^5 cells/wells). For determination of leishmanicidal activity, macrophages were infected with promastigotes of *Leishmania enriettii* [13] at a ratio of 20 parasites per macrophage. The cultures were then incubated at 37 °C for 18–20 h to allow phagocytosis.

Macrophage activation

Infected and non-infected macrophages were washed twice with warm Hanks' balanced salt solution (HBSS) and reincubated in the absence of serum with the various modulators of pH for 30 min; for some experiments, anti-TNF- α or NGM was added together with the pH modulators; IFN- γ with or without LPS (from *Escherichia coli* 0:55-B.5, Difco Laboratories, Detroit, MI, U.S.A., 1–3 ng/ml) or TNF- α was then added to cultures in the presence of 10% FCS.

Nitrite determination

After 20–24 h of macrophage activation, 100 μ l of supernatant was taken for nitrite determination. Macrophage supernatants were mixed with an equal volume of Griess reagent and incubated for 10 min at room temperature [14]. The nitrite concentration was determined by measuring A_{550} in a micro-e.l.i.s.a. reader (Easy Reader EAR 340, Kontron Analytik) using a 690 nm reference filter. NaNO₂ was used as a standard.

Measurement of intracellular parasite killing

At 20 h after treatment of the infected cells with IFN- γ , LPS and the various pH modulators, macrophages were washed and lysed by exposure to 0.01% SDS as described previously [15]. The wells were then supplemented with Hosmen II medium [16] and parasite growth was recorded by measuring [³H]thymidine uptake [17].

Measurement of intravesicular pH

Intravesicular pH was measured by the method of Ohkuma and Poole [18], adapted to microtitre wells. Macrophages were incubated for 16 h at 37 °C with FITC-D (2 mg/ml) in DMEM supplemented with 10% FCS. Cells were then washed twice with HBSS, incubated for 1–2 h in DMEM at 37 °C, washed twice again, and reincubated for 30 min at 37 °C with IFN- γ + LPS in Na⁺ medium in the presence or absence of the various pH modulators. Leakage of fluorescence during the treatment was less than 10% of the total. Then 10 min before the end of the incubation, the remaining extracellular fluorescence was quenched by addition of 0.4 unit/ml anti-fluorescein-IgG. Flu-

orescence intensity was monitored with excitation at 485 nm and emission at 538 nm, using a microtitre plate fluorimeter (Fluoroskan II; Flow Laboratories). Calibration was performed by adding nigericin (10 μ M) to FITC-D-loaded cells in K⁺ media of various pH [19]. Fluorescence was determined as above and pH was monitored using a glass microelectrode (Ingold Messtechnik AB, Urdorf, Switzerland). Results are given as means of at least four replicates for which the S.D. $\leq$ 0.15 pH unit.

Preparation of macrophage cytosolic extracts [20,21]

Macrophages were incubated in 100 mm plastic Petri dishes (1.0×10^7 – 1.5×10^7 cells/dish) with IFN- γ (3–100 units/ml) with or without LPS (6 ng/ml), BAF (300 nM) or MET (10 mM) for 6 h at 37 °C. Cells were then washed, resuspended in PBS containing various protease inhibitors (0.1 mM phenylmethanesulphonyl fluoride, 10 μ g/ml soya-bean trypsin inhibitor, 5 μ g/ml each leupeptin, antipain and pepstatin) and sonicated. The extracts were then centrifuged at 11 000 g for 45 min at 4 °C. Supernatant fractions containing 0.1–0.6 mg/ml protein (as determined by the method of Baumgarten [22]) were tested for enzyme activity as described by Marletta et al. [20], in the presence or absence of NGM (0.5 mM), BAF (300 nM) or MET (10 mM). The incubation was carried out for 18 h at 25 °C.

RESULTS

Effect of BAF on macrophage activation

Maintenance of a low intravesicular pH by vacuolar-type H⁺-ATPases [7] appears to be relevant to many aspects of phagocyte function, including microbicidal activity. Moreover, in murine macrophages, leishmanicidal capacity is directly correlated with NO production measured as nitrite [13,23]. We therefore examined the effect of BAF, a specific inhibitor of vacuolar H⁺-ATPases, on killing of *Leishmania enriettii* and nitrite production by (IFN- γ + LPS)-activated bone-marrow-derived murine macrophages *in vitro*. Table 1 shows that BAF (300 nM) increased both nitrite production and, in several instances, intracellular parasite destruction in the activated cultures. The BAF-mediated increase in leishmanicidal activity was related to enhanced synthesis of NO, as treatment of the cultures with the NO synthase inhibitor, NGM, abrogated parasite killing in the presence or absence of BAF (Table 2). The lower number of parasites in BAF-pretreated control cultures (which secrete only low levels of nitrite) in the presence or absence of NGM can be attributed to a direct effect of the increase in vacuolar pH (see Table 4); indeed, several physiological activities of the amastigote form of *Leishmania* have been shown to proceed optimally at acid pH and to be markedly reduced at neutral pH [24]. Interestingly, induction of microbicidal activity in BAF-treated cultures appeared to require increased threshold levels of NO production. Thus for a given degree of nitrite secretion [for example 2.5 nmol/well (Table 1, Expt. II)], macrophages killed fewer parasites in the presence of BAF (16%) than in its absence (80%). Similar results were obtained whether activation was performed in DMEM with (Table 1, Expts. I and II) or without (Table 1, Expt. III) NaHCO₃, indicating that the bicarbonate-transport system reported as one of the regulation mechanisms of cytosolic pH [25] was not involved. Normally, low doses of IFN- γ alone induce neither high levels of nitrite release nor leishmanicidal activity in our system which depends on a second signal (LPS or TNF- α). It is interesting to note, however, that BAF increased nitrite release in cultures stimulated with a low dose of IFN- γ even if the level was too low itself to induce leishmanicidal activity (Table 1). Differences in the sensitivity of

Table 1 Effect of BAF on nitrite production and parasite killing by activated macrophages

Leishmania-infected macrophages were incubated for 30 min in the presence or absence of 300 nM BAF in 100 μ l of DMEM/NaHCO₃ (Expts. I and II) or DMEM without NaHCO₃ (Expt. III). The volume was adjusted to 200 μ l by adding IFN- γ (units/ml), LPS (ng/ml) or medium. After 24 h, supernatants were harvested to measure nitrite production (values represent the mean of three determinants for which the S.E.M. $\leq$ 0.15 nmol/well) and cells were lysed for determination of parasite survival (c.p.m.; means $\pm$ S.D.). Numbers in parentheses represent percentage of parasites killed.

	Expt. I		Expt. II		Expt. III	
	Parasite survival (c.p.m.)	Nitrite production (nmol/well)	Parasite survival (c.p.m.)	Nitrite production (nmol/well)	Parasite survival (c.p.m.)	Nitrite production (nmol/well)
IFN (1)/LPS (1)	36490 $\pm$ 1647	0.9	36481 $\pm$ 1657	0.8	46872 $\pm$ 1374	0.8
+ BAF	34069 $\pm$ 1978	1.7	25689 $\pm$ 2683 (16%)	2.5	29893 $\pm$ 1259	2.4
IFN (1)/LPS (3)	26042 $\pm$ 3363	1.9	6066 $\pm$ 6 (80%)	2.1	39854 $\pm$ 1356	1.8
+ BAF	20191 $\pm$ 5963 (46%)	3.1	2867 $\pm$ 703	3.9	20325 $\pm$ 357 (51%)	3.4
IFN (3)/LPS (1)	533 $\pm$ 100 (99%)	3.2	15066 $\pm$ 299	1.9	2621 $\pm$ 1299 (94%)	3.4
+ BAF	102 $\pm$ 7	6.0	375 $\pm$ 97	4.8	185 $\pm$ 49	6.0
IFN (3)	33145 $\pm$ 4191	0.1	32983 $\pm$ 2861	0.0	32962 $\pm$ 2540	0.0
+ BAF	34951 $\pm$ 1163	0.9	30050 $\pm$ 1797	1.0	28882 $\pm$ 676	0.6
LPS (3)	27905 $\pm$ 1046	1.2	36282 $\pm$ 3200	1.2	42690 $\pm$ 1311	0.9
+ BAF	28979 $\pm$ 3867	2.4	30703 $\pm$ 904 (6%)	2.2	36441 $\pm$ 1942	1.8
DMEM	37213 $\pm$ 3672 (0%)	0.1	32610 $\pm$ 3810 (0%)	0.1	41704 $\pm$ 338 (0%)	0.0

Table 2 Effect of an inhibitor of NO synthase on BAF-mediated increase in leishmanicidal activity and nitrite secretion by activated macrophages

Infected macrophages were preincubated with BAF (300 nM), NGM (0.3 mM) or DMEM for 30 min, then IFN- γ (3 units/ml) was added to the cultures in the presence or absence of LPS (1 ng/ml). After 24 h, supernatants were prepared for nitrite assay and cells were lysed for determination of parasite survival. For nitrite production, results are means of triplicates and are representative of three experiments; S.E.M. $\leq$ 0.1 for nitrite. For parasite survival, results are means $\pm$ S.D. of the same number of determinations and experiments.

	Parasite survival (c.p.m.)		Nitrite production (nmol/well)	
	— NGM	+ NGM	— NGM	+ NGM
IFN- γ /LPS	25216 $\pm$ 2500	39094 $\pm$ 3481	3.1	0.6
+ BAF	1672 $\pm$ 404	32432 $\pm$ 8242	6.6	1.0
IFN- γ	45512 $\pm$ 1472	40195 $\pm$ 5169	0.5	0.4
+ BAF	31239 $\pm$ 1172	32489 $\pm$ 5629	1.7	0.5
LPS	42870 $\pm$ 3378	41033 $\pm$ 5869	0.5	0.3
+ BAF	36257 $\pm$ 782	32240 $\pm$ 4291	0.7	0.4
DMEM	48104 $\pm$ 3694	37796 $\pm$ 6264	0.6	0.6
+ BAF	32363 $\pm$ 7376	27739 $\pm$ 4613	0.6	0.6

macrophages to IFN- γ and LPS from one experiment to another were presumably related to (as yet unexplained) variations in the culture conditions during the 10-day *in vitro* differentiation step from bone-marrow precursors to mature macrophages.

In order to ensure that the activity of BAF was not due to contamination by LPS, we examined its effect on macrophage activation by high doses of IFN- γ with or without added TNF- α , in the presence of polymyxin B (10 μ g/ml). As shown in Table 3 (Expt. I), BAF also potentiated nitrite production under these conditions. Similar results were obtained with LPS-low-responder C3H/HeJ macrophages (not shown). Surprisingly, BAF induced nitrite synthesis in macrophages incubated with a high dose of IFN- γ alone. This effect could be partially reversed by the addition of anti-TNF- α antibody (Table 3, Expt. II), suggesting that autocrine production of TNF- α in the IFN- γ -treated cultures was responsible in part for enhanced nitrite release. In contrast, anti-TNF- α antibody had no effect on macrophages activated by IFN- γ + LPS in the presence of BAF. These results,

Table 3 Effect of BAF on nitrite production by macrophages activated by IFN- γ with or without TNF- α in the presence of polymyxin B: effect of anti-TNF- α

Macrophages were preincubated with BAF (300 nM) or DMEM in the presence or absence of anti-TNF- α (diluted 1:500) for 30 min, then IFN- γ (3–100 units/ml) was added to the cultures in the presence or absence of LPS (1 ng/ml). Polymyxin B (10 μ g/ml) was present in the cultures without LPS. Nitrite production was determined after 24 h of incubation at 37 °C. Results are means $\pm$ S.E.M. of triplicates and are representative of three experiments. n.d., Not determined.

	Nitrite production (nmol/well)	
	— BAF	+ BAF
Expt. I		
IFN- γ (100 units/ml)	0	3.3 $\pm$ 0.09
+ TNF- α	1.2 $\pm$ 0.08	4.1 $\pm$ 0.09
Expt. II		
IFN- γ (50 units/ml)	0	5.6 $\pm$ 0.08
+ Anti-TNF- α	n.d.	2.2 $\pm$ 0.14
IFN- γ (3 units/ml) + LPS	3.2 $\pm$ 0.09	7.4 $\pm$ 0.17
+ Anti-TNF- α	n.d.	7.3 $\pm$ 0.12

together with the ability of BAF to enhance nitrite response to IFN- γ and high levels of exogenous TNF- α suggest that BAF modulates macrophage function by a process more complex than solely the induction of TNF- α secretion.

Effect of BAF, lysosomotropic bases and nigericin on intravesicular pH and activation of macrophages

As BAF is an inhibitor of the vacuolar H⁺-ATPases, we examined its effect on the intravesicular pH of activated macrophages. For this purpose, macrophages were labelled for 16 h with FITC-D, washed and reincubated for 1–2 h in DMEM before being tested in the presence of various pH modulators (see the Materials and methods section). Such pulse-chase treatment has been shown to label primarily lysosomes in mouse macrophages [26]. Table 4 shows that 300 nM BAF increased intravesicular pH by approximately 1.7 units when measured after 30 min of treatment.

Lysosomotropic weak bases are known to increase the pH

Table 4 Effect of BAF, lysosomotropic bases and nigericin on intravesicular pH of activated macrophages

FITC-D-loaded macrophages were incubated with IFN- γ (3 units/ml) and LPS (1–3 ng/ml) in the presence or absence of BAF (300 nM), NH_4Cl (10 mM), MET (10 mM) or nigericin (1 μM) in Na^+ medium containing L-arginine (0.4 mM) at 37 °C. After 30 min, the pH was determined as described in the Materials and methods section. Four experiments are shown. n.d., Not determined.

	pH				
	Control	+ BAF	+ NH_4Cl	+ MET	+ Nigericin
Expt. I	4.85	6.75	6.25	6.05	6.00
Expt. II	4.75	6.20	6.00	6.05	6.10
Expt. III	4.65	6.45	n.d.	n.d.	5.75
Expt. IV	4.60	n.d.	5.90	5.95	5.50

Table 5 Effect of lysosomotropic compounds on nitrite production and leishmanicidal activity of activated macrophages

Infected macrophages were preincubated with NH_4Cl (10 mM), MET (10 mM), BAF (300 nM) or DMEM for 30 min, then IFN- γ (1 unit/ml) was added to the cultures in the presence of LPS (1 ng/ml). After 24 h of incubation at 37 °C, supernatants were harvested for nitrite assay (nmol/well) and cells were lysed for determination of parasite survival (c.p.m.). Results are means $\pm$ S.D. of triplicates for parasite survival and means $\pm$ S.E.M. for nitrite production and are representative of three experiments. Values for parasite survival and nitrite production in non-activated cells were respectively $30\,811 \pm 2706$ c.p.m. and 0 nmol/well and were unaffected by the various lysosomotropic compounds.

	Parasite survival (c.p.m.)	Nitrite production (nmol/well)
DMEM	15786 $\pm$ 666	1.5 $\pm$ 0.03
NH_4Cl	10434 $\pm$ 992	2.0 $\pm$ 0.05
BAF	5047 $\pm$ 1264	2.7 $\pm$ 0.16
MET	1691 $\pm$ 258	2.9 $\pm$ 0.13

within acidic vacuoles of living cells [5,27]. The effect of two such bases, NH_4Cl and MET, on (IFN- γ +LPS)-activated macrophages was examined. These agents, at a concentration of 10 mM, increased intravesicular pH by approximately 1.3 units when measured after 30 min of exposure (Table 4). Under these conditions, increased nitrite production and leishmanicidal activity of (IFN- γ +LPS)-activated cells were observed (Table 5).

Carboxylic ionophores such as nigericin belong to another group of agents which can be used to increase vacuolar pH [5]. As shown in Table 4, 1 μM nigericin increased intravesicular pH by 1.1 units after 30 min of incubation. This agent also potentiated nitrite secretion by macrophages activated with IFN- γ +LPS (Table 6). The effect on leishmanicidal activity of activated macrophages could not be determined because nigericin itself was toxic to the parasites (results not shown).

Effect of an increase in intravesicular pH on cytosolic NO synthase activity

When macrophages were incubated for 6 h with 100 units/ml IFN- γ , low NO synthase activity was detectable in isolated macrophage cytosolic fractions (Table 7). Interestingly, the presence of BAF or MET led to significant enhancement of enzyme induction in macrophages activated by exposure to IFN- γ in the presence or absence of LPS. BAF or MET had no effect when added during the assay of NO synthase activity, and the

Table 6 Effect of nigericin on nitrite production of activated macrophages

Macrophages were preincubated for 30 min with nigericin before addition of IFN- γ (3 units/ml), LPS (1 ng/ml) or DMEM. Final concentration of nigericin was 1 μM . After 24 h at 37 °C, supernatants were harvested for nitrite determination. Results are means of triplicates; S.E.M. ≤ 0.06 . Three representative experiments are shown. n.d., Not determined.

	Nigericin	Nitrite (nmol/well)		
		Expt. I	Expt. II	Expt. III
IFN- γ + LPS	—	1.2	2.1	0.8
	+	2.1	3.0	2.5
IFN- γ	—	0	0.1	n.d.
	+	0.5	0.3	n.d.
LPS	—	0.1	0.3	0
	+	0.2	n.d.	0
DMEM	—	0	0.1	0
	+	n.d.	0.1	n.d.

Table 7 Effect of BAF and MET on cytosolic NO synthase activity

Cells were cultured for 6 h with IFN- γ (100 units/ml) in the absence of LPS and 3 units/ml in the presence of 6 ng/ml LPS) in the presence or absence of BAF (300 nM) or MET (10 mM). Polymyxin B (10 $\mu\text{g/ml}$) was present in the cultures incubated with IFN- γ in the absence of LPS. Enzyme activity was determined in the cytosolic fractions in the presence or absence of NGM (0.5 mM), BAF (300 nM) or MET (10 mM) as described in the Materials and methods section. Results are means $\pm$ S.D. of triplicates and are representative of four experiments. n.d., Not determined.

Cell culture	NO synthase activity (nmol of $\text{NO}_2^-/10^6$ cells)			
	+ PBS	+ NGM	+ BAF	+ MET
DMEM	0.10 $\pm$ 0.01	0	n.d.	n.d.
IFN- γ	1.40 $\pm$ 0.11	0.15 $\pm$ 0.06	1.30 $\pm$ 0.04	1.50 $\pm$ 0.16
IFN- γ + BAF	3.80 $\pm$ 0.12	0.80 $\pm$ 0.04	n.d.	n.d.
IFN- γ + MET	3.30 $\pm$ 0.16	0.60 $\pm$ 0.11	n.d.	n.d.
IFN- γ + LPS	6.40 $\pm$ 0.13	1.80 $\pm$ 0.11	6.40 $\pm$ 0.13	7.01 $\pm$ 0.22
IFN- γ + LPS + BAF	11.20 $\pm$ 0.53	3.01 $\pm$ 0.08	n.d.	n.d.
IFN- γ + LPS + MET	9.30 $\pm$ 0.65	2.80 $\pm$ 0.29	n.d.	n.d.

well-characterized NO synthase inhibitor NGM inhibited activity of the cytosolic enzyme.

DISCUSSION

Intravesicular acidification, which is regulated by vacuolar-type H^+ -ATPases, plays a critical role in maintaining the normal function of the endocytic and exocytic pathways of the phagocyte [5]. Acidic conditions have been reported to be necessary for recycling of receptors [5,28] and for the entry of certain viruses and bacterial toxins [5]. A low pH facilitates the fusion of phagosomes with lysosomes [2] and the activity of lysosomal enzymes [4] and promotes the spontaneous dismutation of $\text{O}_2^{\cdot-}$ to H_2O_2 [3]. The acidification accompanying phagocyte ingestion of micro-organisms is particularly important for the normal process of degradation of microbial molecules.

In the present report, we examined effects of the macrolide antibiotic BAF, a selective inhibitor of vacuolar H^+ -type ATPases [8], on leishmanicidal activity and nitrite production of activated macrophages. In parallel, we measured the effect of BAF on intravesicular pH of activated cells. BAF potentiated nitrite secretion by macrophages activated by IFN- γ +LPS or IFN- γ +TNF- α ; moreover, it induced nitrite synthesis in macrophages

incubated with a high dose of IFN- γ in the absence of added TNF- α or LPS. IFN- γ alone induced the production of negligible amounts of nitrite. The effect of BAF on macrophages treated with IFN- γ alone but not with IFN- γ + LPS could be inhibited by the addition of anti-TNF- α antibody. These results suggest that, although TNF- α may play a role in BAF-induced enhancement of nitrite secretion by cells stimulated with IFN- γ alone, additional mechanisms are involved in the BAF effect. Interestingly, increased TNF- α levels were not detected in supernatants of (IFN- γ + BAF)-treated cultures relative to BAF-free controls (results not shown). It is possible that BAF might stimulate expression of the membrane-bound rather than the soluble form of the molecule. Alternatively, low levels of soluble TNF- α secreted in response to IFN- γ alone [29] may be sufficient to activate macrophages in the presence of BAF.

pH measurement was performed using FITC-D as a fluorescent marker. FITC-D has been shown to accumulate in macrophage lysosomes and in parasitophorous vacuoles after lysosome-phagosome fusion [30]. BAF increased the intravesicular pH of (IFN- γ + LPS)-activated cells from approximately 4.7 to 6.5 after 30 min of incubation. The pH reached 7.3 when measured after 6 h of incubation with BAF (results not shown). Leishmanicidal activity of activated cells was also increased by BAF, although there was not a strict correlation between the amount of nitrite secreted and the number of parasites killed in BAF-treated cells. In fact, more NO was required to induce killing in cultures activated with suboptimal doses of IFN- γ + LPS in the presence of BAF than in cultures activated with an optimal dose of IFN- γ + LPS in the absence of BAF: in cultures in which 50% of parasites were killed, 1.3–1.8 times more NO derivatives were measured when BAF was present. This might be due to the fact that BAF increased intravesicular pH, an event that is unfavourable to microbicidal activity. Although BAF potentiated the production of L-arginine-derived toxic NO derivatives, these metabolites might, however, be less efficient at a higher pH. Indeed, we have previously shown that when free parasites were incubated with NaNO₂ at acid pH (5.0) (a pH at which nitrite gives rise to HNO₂ and NO [31]), the micro-organisms stopped multiplying and started to degenerate, whereas they remained unharmed when the incubation was performed at pH 7.2 [13]. It is likely that alkalization prevents the 'recycling' of nitrite (derived from NO) to NO, reducing the level of NO in the phagolysosome [31]. Thus in BAF-treated cultures, leishmanicidal activity might depend on the balance between increased intravacuolar pH and the amount of NO produced.

Treatment of macrophage cultures with the weak bases NH₄Cl and MET or the carboxylic ionophore nigericin increased intravesicular pH (as also shown by others [5,11,18]) and potentiated nitrite production by activated cells. Weak bases also induce a transient increase in cytosolic pH, whereas nigericin decreases it [5,11,26,32]. In contrast, BAF was shown to inhibit phagosomal acidification on phagocytosis of FITC-labelled bacteria [6] and had no effect on cytosolic pH around 7.35 [33]. As BAF shares with weak bases and nigericin the property of increasing intravesicular pH, it is conceivable that intravesicular alkalization was responsible for increased nitrite production.

Although other mechanisms cannot be ruled out, the stimulation of NO production by BAF and the lysosomotropic bases appears to involve an increase in NO synthase activity. Indeed, exposure of activated macrophages to BAF or MET induced higher levels of NO synthase activity in cytosolic extracts (Table

7), suggesting that an increase in vesicular pH might promote expression of the NO synthase gene. Moreover, preliminary results suggest that BAF enhances NO synthase mRNA levels in activated cells (Y. Buchmüller-Rouiller, unpublished work). It is particularly interesting that a change in intravesicular pH affected the activity or induction of a presumably cytosolic enzyme. However, it should be noted that, in addition to the macrophage cytosolic NO synthase, a membrane-bound enzyme has been described in BCG-activated rat macrophages [34]. As assays of NO synthase activity in our studies were performed with an 11000 g supernatant fraction of macrophage extracts, the presence of a membrane NO synthase cannot be excluded. It remains to be determined which form(s) of the enzyme is altered by a change in intravesicular pH.

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REFERENCES

- Sibley, L. D., Weidner, E. and Krahenbuhl, J. L. (1985) *Nature* (London) **315**, 416–419
- Horwitz, M. A. and Maxfield, F. R. (1984) *J. Cell Biol.* **99**, 1936–1943
- Fridovich, I. (1978) *Science* **201**, 875–880
- Coffey, J. W. and de Duve, C. (1968) *J. Biol. Chem.* **243**, 3255–3263
- Mellman, I., Fuchs, R. and Helenius, A. (1986) *Annu. Rev. Biochem.* **55**, 663–700
- Lukacs, G. L., Rotstein, O. D. and Grinstein, S. (1990) *J. Biol. Chem.* **265**, 21099–21107
- Werner, G. and Hagenmaier, H. (1984) *J. Antibiot.* **37**, 110–117
- Bowman, E. J., Siebers, A. and Altendorf, K. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7972–7976
- Naganuma, S., Kuzuya, N., Sakai, K., Hasumi, K. and Endo, A. (1992) *Biochim. Biophys. Acta* **1126**, 41–48
- Betz Corradin, S., Buchmüller-Rouiller, Y. and Mauel, J. (1991) *Eur. J. Immunol.* **21**, 2553–2558
- Tapper, H. and Sundler, R. (1990) *Biochem. J.* **272**, 407–414
- Kelso, A., Glasebrook, A. L., Kanagawa, O. and Brunner, K. T. (1982) *J. Immunol.* **129**, 550–556
- Mauel, J., Ransijn, A. and Buchmüller-Rouiller, Y. (1991) *J. Leuk. Biol.* **49**, 73–82
- Ding, A. H., Nathan, C. F. and Stuehr, D. J. (1988) *J. Immunol.* **141**, 2407–2412
- Mauel, J. (1984) *Mol. Biochem. Parasitol.* **13**, 83–96
- Berens, R. L. and Marr, J. J. (1978) *J. Parasitol.* **64**, 160
- Buchmüller-Rouiller, Y. and Mauel, J. (1986) *J. Immunol.* **136**, 3884–3890
- Ohkuma, S. and Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3327–3331
- Thomas, J. A., Buchsbaum, R. N., Zimniak, A. and Racker, E. (1979) *Biochem. J.* **18**, 2210–2218
- Marletta, M. A., Yoon, P. S., Iyengar, R., Leaf, C. D. and Wishnok, J. S. (1988) *Biochemistry* **27**, 8706–8711
- Buchmüller-Rouiller, Y., Schneider, P., Betz-Corradin, S., Smith, J. and Mauel, J. (1992) *Biochem. Biophys. Res. Commun.* **183**, 150–155
- Baumgarten, H. (1985) *J. Immunol. Methods* **82**, 25–37
- Green, S. J., Meltzer, M. S., Hibbs, J. B., Jr. and Nacy, C. A. (1990) *J. Immunol.* **144**, 278–283
- Mukkada, A. J., Meade, J. C. and Glaser, T. A. (1985) *Science* **229**, 1099–1101
- Frelin, C., Vigne, P., Ladoux, A. and Lazdunski, M. (1988) *Eur. J. Biochem.* **174**, 3–14
- Geisow, M. J., D'Arcy Hart, P. and Young, M. R. (1981) *J. Cell Biol.* **89**, 645–652
- Poole, B. and Ohkuma, S. (1981) *J. Cell Biol.* **90**, 665–669
- Woo, J. T., Shinohara, C., Sakai, K. and Endo, A. (1992) *Eur. J. Biochem.* **207**, 383–389
- Betz Corradin, S. and Mauel, J. (1991) *J. Immunol.* **146**, 279–285
- Chang, K. P. (1980) in *The Host-Invasor Interplay* (Van den Bossch, H., ed.), pp. 231–234, Elsevier, Amsterdam
- Stuehr, D. J. and Nathan, C. F. (1989) *J. Exp. Med.* **169**, 1543–1555
- Dean, R. T., Jessup, W. and Roberts, C. R. (1984) *Biochem. J.* **217**, 27–40
- Swallow, C. J., Grinstein, S., Sudsbury, R. A. and Rotstein, O. D. (1990) *Surgery* **108**, 363–369
- Hiki, K., Yui, Y., Hattori, R., Eizawa, H., Kosuga, K. and Kawai, C. (1991) *Jpn. J. Pharmacol.* **56**, 217–220