INFECTION AND IMMUNITY, July 1997, p. 2732–2739 Vol. 65, No. 7
0019-9567/97/$04.00+0

Cytostatic and Cytotoxic Effects of Activated Macrophages and Nitric Oxide Donors on *Brugia malayi*

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Received 18 February 1997/Returned for modification 28 March 1997/Accepted 24 April 1997

The susceptibility of *Brugia malayi* microfilariae and adults to injury by the murine macrophage cell line J774 activated with gamma interferon and bacterial lipopolysaccharide has been examined in vitro. Parasites of both stages showed a decline in viability over 48 h of coculture with activated macrophages, assessed by their capacity to reduce the tetrazolium salt 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), although adult parasites were more resistant than microfilariae. Removal of parasites to cell-free medium following exposure to activated macrophages for up to 48 h resulted in partial recovery of their capacity to reduce MTT, suggesting that the effects were primarily cytostatic. However, prolonged exposure to activated J774 cells for 72 h resulted in parasite death. Addition of the nitric oxide synthase inhibitor L-NMMA (N^G-monomethyl-L-arginine monoacetate) indicated that nitric oxide derivatives were responsible for cytostasis and ultimate toxicity. The toxicity of nitric oxide derivatives was confirmed by coincubation of parasites with chemical donors, although far higher concentrations were required than those generated by activated J774 cells, implying additional complexity in macrophage-mediated cytotoxicity. These experiments further suggested that peroxynitrite or its by-products were more potently damaging to filariae than nitric oxide per se. Examination of ultrastructural changes on exposure of parasites to activated macrophages or donors of nitric oxide indicated that hypodermal mitochondria were highly vacuolated, with less prominent cristae. The data are discussed with reference to immunity to lymphatic filariae and their mechanisms of energy generation.

Filarial nematodes are noted for their longevity in the mammalian host, implying a high degree of sustained resistance to or subversion of host immunity (26). Nevertheless, studies in animal models have unequivocally demonstrated the acquisition of immunity to lymphatic filariae (12), and this status may be mirrored in amicrofilaremic or endemic normal individuals in human populations continuously exposed to challenge (32). Despite these observations, little is known of the immunological mechanisms that lead to rejection of lymphatic filariae. Studies of mice point to the essential participation of T cells (45, 50) and more specifically $CD4^+$ cells (5), although the precise contribution of different T-helper (Th) cell subsets and the role of specific cytokines produced by these cells in antifilarial immunity have not been resolved (24). Work on alternative animal models has suggested a potentially protective role for immunoglobulin E (IgE) (4, 18), and a high ratio of parasite-specific IgE/IgG4 is a characteristic feature of amicrofilaremic individuals exposed to filarial infection (22). In addition, these individuals also show a cytokine profile indicative of intact Th1-like responses to parasite antigen (14, 21, 27), although it cannot be definitively concluded that these mechanisms promote clearance of parasites, since T-cell proliferation, gamma interferon (IFN-γ) release, and the IgE/IgG4 ratio are all enhanced by chemotherapy of microfilaremic patients (23, 36, 42).

Nematode parasites present a formidable challenge to the immune system in that they are bound by a tough elastic extracellular matrix (the cuticle), which in the case of adult *Brugia malayi* is approximately 2 μm in diameter (51). Possible immune effector mechanisms operative against tissue-dwelling nematodes are currently thought to be afforded by (i) antibody-dependent cellular cytotoxicity (ADCC), elaborated by a repertoire of antibody classes and myeloid cells, resulting in release of toxic granule proteins and activation of reactive oxygen intermediates, and (ii) generation of nitric oxide derivatives by macrophages activated by lymphokines such as IFN-γ, with possible alternative cellular sources of nitric oxide or appropriate stimuli in humans (8, 53). In vitro experiments have demonstrated that the former mechanism, targeted by antibodies to the surface of the parasite, can effectively kill larval (L1 and L3) stages of filariae (26), although no studies have yet reported that adult parasites are susceptible to this mode of attack. This may be due in part to the expression of extracellular antioxidant enzymes (11, 33), although the existence of relatively saturated fatty acids and the deployment of lipid-soluble antioxidants in the cuticle of adult *B. malayi* probably contribute equally to the observed resistance of this stage to oxidative stress (34, 43, 44).

Given the apparent resistance of adult *B. malayi* to ADCC and reactive oxygen metabolites in vitro, we were interested in investigating their susceptibility to activated macrophages and reactive nitrogen intermediates. Results of recent studies suggest that nitric oxide or its by-products may play a role in limiting the development of *B. malayi* in vivo (40) and adversely affect the viability of microfilariae in vitro (48). The current data investigate the relative susceptibility of adult and larval *B. malayi* to damage by activated murine macrophages and address the nature of potentially toxic species via the use of chemical donors of nitric oxide.

MATERIALS AND METHODS

Isolation and culture of parasites and macrophages. *B. malayi* parasites were recovered from the peritoneal cavity of Mongolian jirds (*Meriones unguiculatus*) infected more than 3 months earlier and obtained from TRS Labs, Athens, Ga. Microfilariae were separated from host cells by passage through Sephadex G-10 columns equilibrated with RPMI 1640 preheated to 37°C. Parasites were washed extensively and maintained in vitro in a mixture containing phenol red-free

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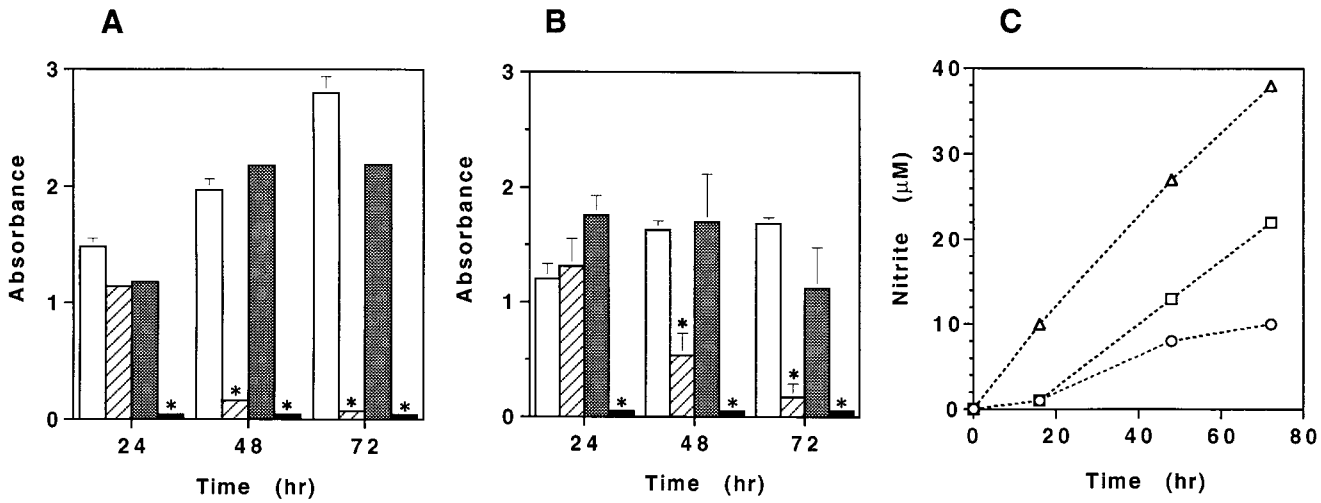

FIG. 1. (A and B) Viability of microfilariae (A) and adult female *B. malayi* (B) exposed to activated macrophages. Parasites were cultured in the presence of 5×10^5 J774 cells for up to 72 h, and viability was assessed by the reduction of MTT. All assays were carried out in triplicate. The mean absorbance at 510 nm plus 1 standard deviation is shown. Symbols: □, parasites coincubated with unstimulated cells; ▨, parasites coincubated with cells stimulated with 40 U of IFN-γ ml^{-1} and 10 ng of LPS ml^{-1}; ▩, parasites coincubated with cells stimulated with 40 U of IFN-γ ml^{-1} and 10 ng of LPS ml^{-1} in the presence of 500 μM L-NMMA; ■, heat-killed parasites. *, $P < 0.05$ versus parasites coincubated with unstimulated cells. (C) Time course of nitrite release from J774 cells. Symbols: ○, unstimulated; △, stimulated with IFN-γ and LPS; □, stimulated with IFN-γ and LPS plus L-NMMA (concentrations as described for panels A and B).

RPMI 1640, 2 mM glutamine, 1% glucose, 100 U of penicillin ml^{-1}, and 100 mg of streptomycin ml^{-1} at 37°C and 5% CO_2. The murine monocyte-macrophage tumor cell line J774 was grown under the same conditions but with the addition of 10% fetal calf serum. Cells were allowed to adhere to tissue culture dishes overnight, and fresh medium was added before stimulation. Optimum stimulation of nitric oxide production was achieved with a combination of 40 U of recombinant mouse IFN-γ ml^{-1} and 10 ng of lipopolysaccharide (LPS) ml^{-1} from *Escherichia coli* on cells plated at a density of 5×10^5 ml^{-1}. Supernatants were assayed at various time points following stimulation for nitrite accumulation as detailed below, in the presence or absence of 500 μM N^G-monomethyl-L-arginine monoacetate (L-NMMA), an inhibitor of nitric oxide synthase.

Nitric oxide donors and measurement of nitrite. Nitric oxide was generated by *S*-nitroso-*N*-acetyl-penicillamine (SNAP) and 3-morpholino-sydnonimine hydrochloride (SIN-1). Titration curves for the production of nitrite were established with a range of concentrations of each donor. In solution, SNAP releases nitric oxide and penicillamine, whereas SIN-1 auto-oxidizes to form an intermediate which reduces oxygen to superoxide. The radical intermediate then releases nitric oxide, which combines with superoxide to form peroxynitrite (19). *S*-Acetyl penicillamine (SAP), which does not release nitric oxide, was used as a control in these experiments.

Determination of the stable oxidation product nitrite was used as an indicator of nitric oxide production. Nitrite was measured with Greiss reagent (1% aminobenzenesulfonamide, 0.1% naphthylethylene diamine dihydrochloride in 2.5% phosphoric acid). An aliquot of test medium or culture supernatant was incubated with an equal volume of Greiss reagent for 10 min at room temperature with shaking, and the absorbance was determined at 550 nm. Nitrite concentrations were determined via a standard curve established with 1 to 100 μM sodium nitrite prepared in phenol red-free RPMI 1640 reacted with Greiss reagent under the same conditions.

Cytotoxicity of activated macrophages and nitric oxide donors. J774 cells were plated at a density of 5×10^5 cells in 1 ml, the culture medium was changed 24 h later, and cells were stimulated with IFN-γ and LPS as detailed above. At this point, either 2×10^4 microfilariae or two size-matched adult female *B. malayi* parasites were added to the cultures. Parasites were removed at different time points for up to 72 h of coculture, and viability was assessed. The cytotoxic effect of SNAP and SIN-1 was determined by incubating the same numbers of parasites for 1 to 48 h in the presence of a range of concentrations of SNAP or SIN-1 before assay of parasite viability. All assays were performed in triplicate.

The ability of parasites to recover from exposure to activated macrophages was assessed by incubation as detailed above for periods up to 72 h. Parasites were then removed, washed three times in RPMI 1640, and returned to culture medium for a further 24 h prior to assessment of viability. Controls consisted of parasites continuously exposed to activated macrophages or maintained in culture medium alone for the duration of the experiment. Assays were again performed in triplicate.

Assay of viability. Uptake and reduction of the tetrazolium salt 3-[4,5-diethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was utilized as a measure of parasite viability (10, 31). Either 2×10^4 microfilariae or two size-matched adult females were incubated in 0.5 mg of MTT ml^{-1} in phenol red-free RPMI 1640 at 37°C for 1 h. Resulting formazan crystals formed within the parasites were solubilized by transferring parasites to 200 μl of dimethyl sulfoxide and incubating at room temperature for 1 h with shaking. Parasites were removed, and the absorbance of the supernatant was determined at 510 nm with dimethyl sulfoxide as a blank. Background levels were established by performing the assay on parasites killed by heating to 65°C for 10 min. All assays were carried out in triplicate. Statistical analysis of data was performed by Student's *t* test.

Electron microscopy. Gross alterations in the morphology of parasites exposed to activated macrophages or nitric oxide donors were assessed by transmission electron microscopy of thin sections. Adult female parasites were fixed for 2 h in 3% glutaraldehyde–75 mM sodium cacodylate buffer (pH 7.4) at 4°C. Parasites were then washed overnight at 4°C in 75 mM sodium cacodylate buffer–200 mM sucrose. Thin sections were cut, processed for electron microscopy, and stained with uranyl acetate and Reynold's lead citrate.

RESULTS

Effects of activated macrophages. Murine macrophages of the J774 cell line were stimulated with 10 ng of LPS ml^{-1} and a range of concentrations of IFN-γ between 0 and 200 U ml^{-1} to optimize the activation of inducible nitric oxide synthase. Increasing the concentration of IFN-γ above 80 U ml^{-1} had little effect on the production of nitrite, and a combination of 10 ng of LPS ml^{-1} and 40 U of IFN-γ ml^{-1} was chosen for all experiments. Under these conditions, the concentration of nitrite in the culture medium rose steadily over 72 h, reaching 10, 27, and 38 mM at 16, 48, and 72 h, respectively (Fig. 1C). Addition of 500 μM L-NMMA at the same time as IFN-γ and LPS considerably reduced the amount of nitrite produced. In the absence of stimulation, this cell line constitutively produced a low level of nitric oxide, detectable as 9 μM nitrite after 72 h of culture (Fig. 1C).

The susceptibility of *B. malayi* to damage induced by activated J774 cells was determined by an assay of MTT reduction as a measure of parasite viability. Both microfilariae and adult worms were incubated with cells stimulated with IFN-γ and LPS, and viability was monitored at several time points over a 72-h period. After 24 h, there was little difference in the viability of microfilariae cocultured with activated or control macrophages (Fig. 1A), although motility was considerably reduced in the former group. Extended culture resulted in a dramatic decline in the viability of microfilariae incubated with

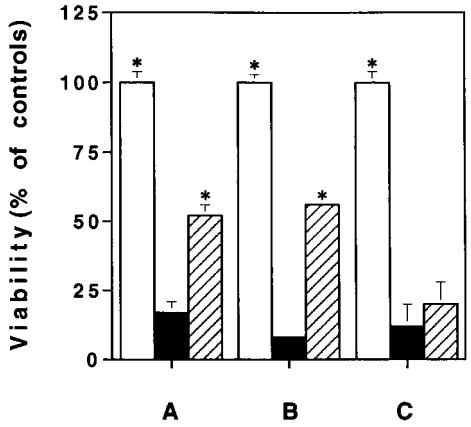

FIG. 2. Recovery of parasite viability after exposure to activated macrophages. Adult *B. malayi* parasites were incubated with activated J774 cells for 24 h (A), 48 h (B), and 72 h (C) before removal to cell-free culture medium for 24 h. Following the recovery period, parasite viability was assessed by MTT reduction. Assays were performed in triplicate, and data are expressed as mean percent viability + 1 standard deviation compared to control samples. Symbols: □, parasites cultured for the duration of the experiment in the absence of cells; ■, parasites incubated in the presence of activated macrophages for the duration of the experiment; ▨, parasites incubated with activated macrophages and removed to cell-free medium for 24 h. *, $P < 0.05$ versus parasites incubated in the presence of activated macrophages for the duration of the experiment.

activated cells, so that by 48 h, reduction of MTT in this group was measured at a level of 8% that of controls, and by 72 h, this had diminished to a value of only 2.5% that of controls, similar to the baseline absorbance determined for heat-killed parasites. Microfilariae cocultured for 48 and 72 h with activated cells were rendered completely immotile. Addition of the nitric oxide synthase inhibitor L-NMMA to the cultures blocked the apparent cytotoxic effect of activated cells on parasites and restored motility (Fig. 1A).

When these experiments were repeated with adult female *B. malayi*, activated J774 cells again reduced parasite viability after 48 and 72 h of coculture but to a lesser extent than that observed with microfilariae (Fig. 1B). The variability in MTT reduction between replicates was higher, despite size-matching of adult parasites. Addition of L-NMMA again restored parasite motility and MTT reduction to near-control levels.

The permanence of these effects was assessed by challenging parasites with activated macrophages for 24, 48, or 72 h and then removing them to a cell-free culture for an additional 24 h prior to assessment of viability. Figure 2 demonstrates that although the viability of adult worms was severely impaired in these experiments, removal from activated macrophages even after 48 h of coculture resulted in significant restoration of their ability to reduce MTT to approximately 50% of control values, with a concomitant increase in motility. Recovery was ablated by extended exposure of parasites to activated macrophages for 72 h prior to removal to a cell-free culture. Microfilariae showed a similar pattern of recovery after short-term exposure but were also killed by activated J774 cells after 72 h of coincubation (data not shown). Thus, although the primary effects of activated macrophages on *B. malayi* could be most accurately described as cytostatic, prolonged exposure was cytotoxic under the conditions examined.

Toxicity of nitric oxide donors. The ability of L-NMMA to block impairment of parasite viability implicated nitric oxide or subsequent by-products in the cytotoxic properties of activated J774 cells. The sensitivity of *B. malayi* to nitric oxide was therefore tested by challenge of microfilariae and adult worms with the chemical donor SNAP. SNAP, at a range of concentrations, was dissolved in phenol red-free RPMI 1640 and incubated at 37°C with 5% CO_2 in a humidified incubator. Considerable levels of nitrite were detected after 4 h at each concentration of SNAP and reached a plateau at 24 h (Fig. 3C). The control compound SAP did not release detectable levels of nitrite (data not shown). The viability of both microfilariae and adult *B. malayi* was progressively impaired by increasing concentrations of SNAP, but again differences were observed in the susceptibility of these two parasite stages. After 24 h of incubation in the presence of 0.2 or 0.5 mM SNAP, the viability of adult worms was reduced to 75 and 58% that of controls, respectively (Fig. 3B), whereas the viability of microfilariae was reduced to 43 and 16% under the same conditions (Fig. 3A). The motility of microfilariae was severely impaired by incubation for 24 h in 0.2 mM SNAP and had ceased altogether at the higher concentration, whereas incubation of

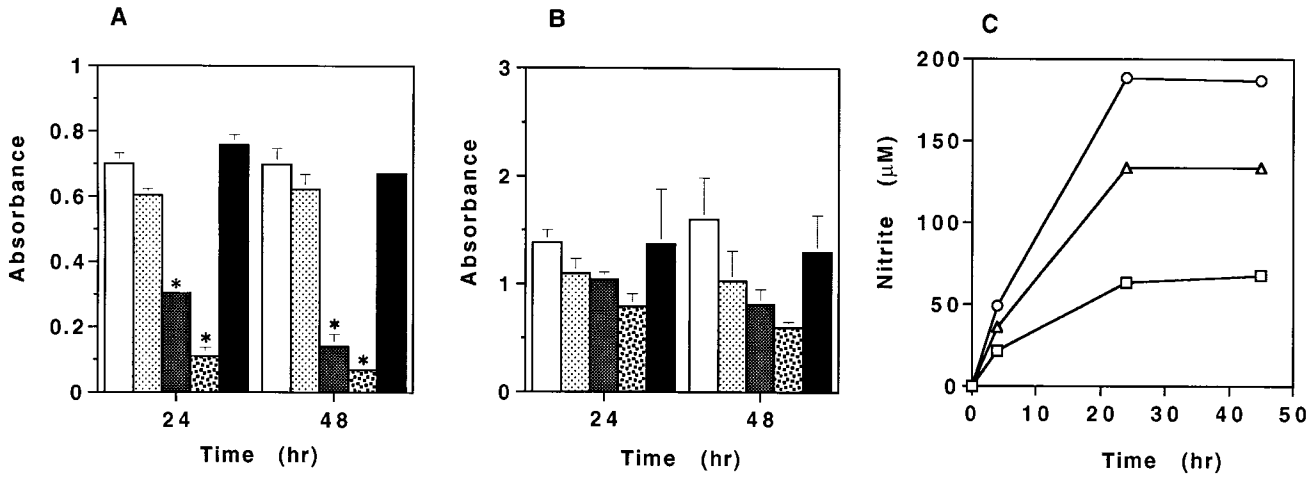

FIG. 3. (A and B) Susceptibility of *B. malayi* microfilariae (A) and adults (B) to nitric oxide generated by SNAP. Parasites were exposed to SNAP for 24 or 48 h, and viability was assessed by the MTT assay. Results are expressed as the mean + 1 standard deviation of triplicate assays. Parasites were cultured alone (□) and with 0.1 mM SNAP (▤), 0.2 mM SNAP (▨), 0.5 mM SNAP (▩), and 0.5 mM SAP (■). *, $P < 0.05$ versus parasites cultured alone. (C) Time course of nitrite release from SNAP in RPMI 1640 incubated at 37°C. Symbols: □, 0.1 mM SNAP; △, 0.2 mM SNAP; ○, 0.5 mM SNAP.

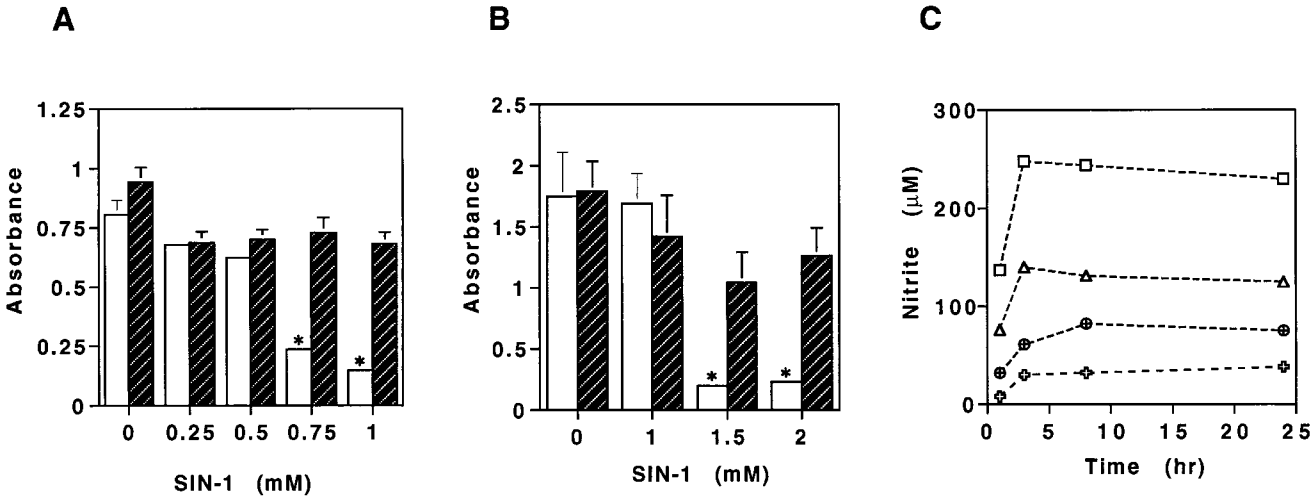

FIG. 4. (A and B) Susceptibility of *B. malayi* to nitric oxide derivatives released by SIN-1. Microfilariae (A) or adult parasites (B) were exposed to various concentrations of SIN-1 in RPMI 1640 for 24 h in the presence (▨) and absence (□) of 50 mU of SOD ml^{-1} and 50 U of catalase ml^{-1}. Viability was assessed by the MTT assay, and data are expressed as the mean absorbance + 1 standard deviation of triplicate assays. *, $P < 0.05$ versus parasites cultured in the absence of SIN-1. (C) Time course of nitrite release from SIN-1 in RPMI 1640. Symbols: ⌖, 0.25 mM SIN-1; ⊕, 0.5 mM SIN-1; △, 1 mM SIN-1; □, 2 mM SIN-1.

adult parasites in 0.5 mM SNAP for the same period of time resulted in only a partial reduction in motility. Incubation of 0.2 and 0.5 mM SNAP for 24 h in the absence of parasites generated 130 and 190 mM nitrite, respectively (Fig. 3C). Extending the incubation period to 48 h resulted in greater reductions in parasite viability but did not significantly alter the pattern of results. Adult worms retained a degree of mobility even at the highest concentration (0.5 mM) of SNAP used, and their values for reduction of MTT were not statistically different from that of untreated parasites ($P < 0.05$). Higher concentrations of SNAP were clearly cytotoxic to both stages, but the levels of nitric oxide generated far exceeded physiological conditions (data not shown). Incubation in the presence of 0.5 mM SAP for up to 48 h had no effect on parasites of either stage (Fig. 3A and B).

SIN-1 auto-oxidizes in solution to yield superoxide anion and nitric oxide, which react to form peroxynitrite and subsequent by-products (19). The levels and kinetics of nitrite produced by a range of concentrations of SIN-1 dissolved in RPMI 1640 were established as described for SNAP. At each concentration used, the levels of nitrite in solution peaked between 3 and 8 h (Fig. 4C). Parasites were incubated in a range of concentrations of SIN-1 over a 24-h period, and viability was assessed as described previously. Microfilariae were adversely affected by increasing amounts of SIN-1, and at concentrations of 0.5 mM and 0.75 mM, their capacity to reduce MTT was restricted to 77 and 29%, respectively, that of controls (Fig. 4A). Adult parasites were unaffected by concentrations up to 1 mM, but thereafter showed rapidly impaired viability (Fig. 4B). Superoxide dismutase (SOD) was added to the system to remove superoxide and thus limit the formation of peroxynitrite, and catalase was included to catabolize hydrogen peroxide formed by dismutation of the superoxide radical. Addition of these enzymes at concentrations of 50 U/ml inhibited SIN-1-mediated toxicity to parasites (Fig. 4A and B), suggesting that peroxynitrite or subsequent by-products are more potently toxic to *B. malayi* than nitric oxide per se.

Ultrastructural changes. The effect of exposure to nitric oxide derivatives on parasite ultrastructure was next examined by incubating adult female worms for 24 h in vitro in the presence of 0.5 mM SNAP prior to preparation for thin sections and examination by electron microscopy. Figure 5A and B illustrate the ultrastructure of adult female parasites cultured in the absence of SNAP. Figure 5A demonstrates that the morphology of the cuticle, hypodermis, and underlying muscle appeared normal and intact, and Fig. 5B shows that the mitochondria immediately subjacent to the inner projections of the hypodermal membrane retained their characteristic cristate appearance. Figure 5C and D illustrate representative sections of parasites following exposure to SNAP. The gross architecture of the body wall appeared unchanged, but the mitochondria in the hypodermis were typically highly vacuolated. This is illustrated at a higher power in Fig. 5D. The outer membrane of mitochondria remained intact, but the matrix appeared vacuolated, and the inner membrane frequently showed a degenerate appearance, with less-prominent cristae. Analogous alterations in mitochondrial morphology were observed when parasites were incubated with macrophages activated with IFN-γ and LPS under the conditions described earlier (Fig. 5E and F).

DISCUSSION

These experiments demonstrate that nitric oxide derivatives generated from activated macrophages are cytostatic for both larval and adult *B. malayi* and may ultimately effect cytotoxicity on prolonged challenge. Analogous experiments with chemical donors suggested that peroxynitrite or its subsequent by-products were more potently toxic than nitric oxide to parasites. However, to adversely affect filarial viability, it was necessary to use concentrations of chemical donors which generated very high concentrations of nitrite (100 to 200 μM), far higher than that reported to be released by activated human neutrophils (8) or rat macrophages (20). In our current experiments, J774 cells stimulated with IFN-γ and LPS were ultimately cytotoxic to parasites after 72 h of coincubation, although culture of these cells for the same time period in the absence of parasites yielded an extracellular concentration of only 37 μM nitrite, suggesting that other factors in activated macrophages may contribute to or synergize with nitric oxide derivatives to mediate cytostasis and toxicity.

Congenors of nitrogen monoxide (NO) exist in a variety of

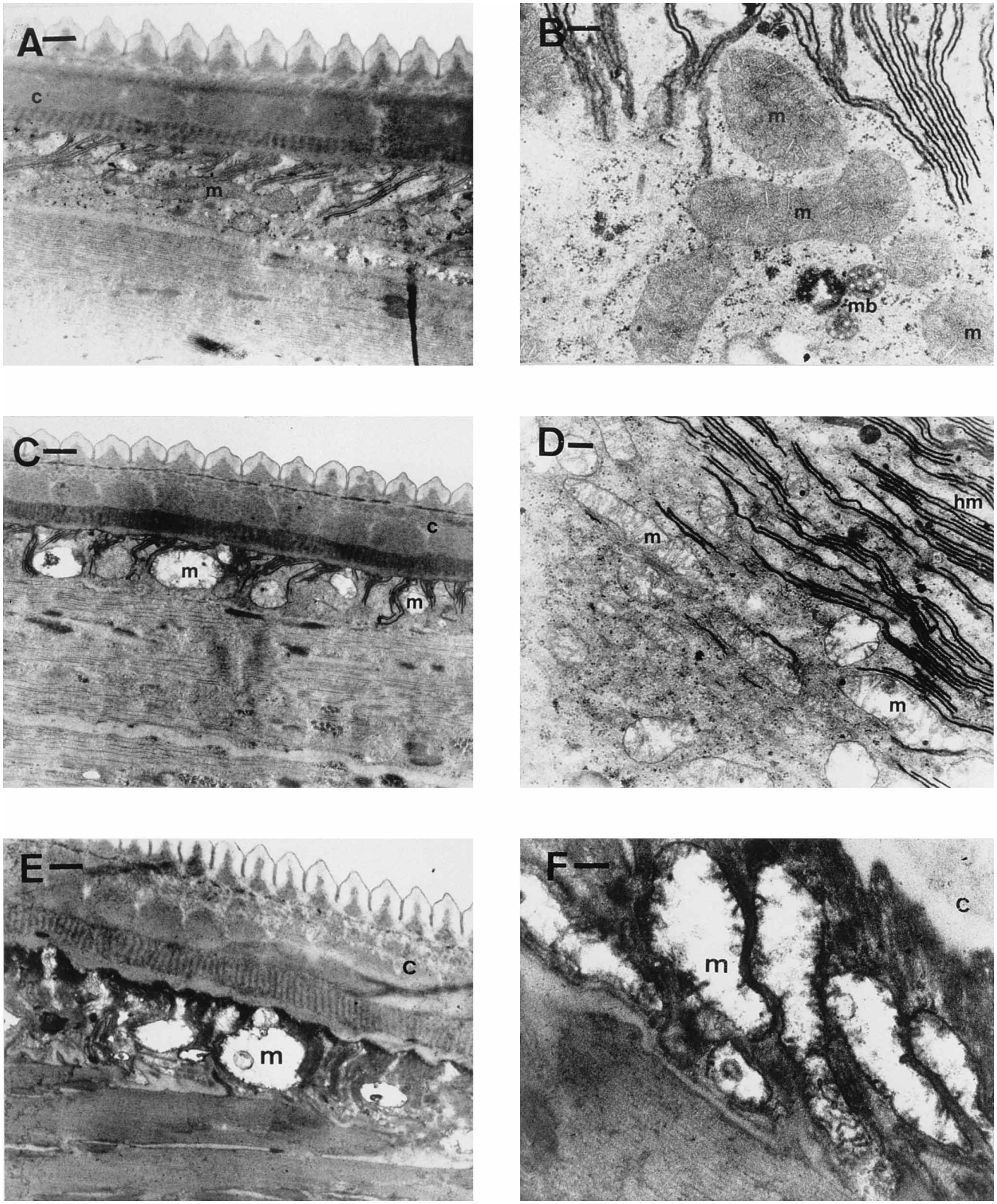

FIG. 5. Electron micrographs showing morphological changes in adult *B. malayi* on exposure to activated J774 cells or SNAP. (A and B) Parasites cultured in RPMI 1640 for 24 h; (C and D) parasites cultured in RPMI 1640 containing 0.5 mM SNAP for 24 h; (E and F) parasites cultured for 48 h with J774 cells stimulated with IFN-γ and LPS. Abbreviations: m, mitochondrion; c, cuticle; hm, hypodermal membrane; mb, multivesicular body. Bars, 500 (A, C, and E) and 200 (B, D, and F) nm.

alternative redox states, such as nitric oxide (NO·), nitroxyl anion (NO^-), and nitrosonium cation (NO^+), which have distinct properties and reactivities which have clouded the issue of whether nitric oxide itself is cytotoxic. Nitric oxide reacts with oxygen to form nitrogen dioxide radical (NO_2·) and also reacts with superoxide to generate peroxynitrite anion ($ONOO^-$). The protonated form of peroxynitrite rapidly decays to form nitrogen dioxide and hydroxyl radical (6, 19). Peroxynitrite itself is a potent oxidant, capable of oxidizing protein and nonprotein sulfydryls (37) and initiating lipid peroxidation (38). Lipton et al. (25) reported that nitric oxide-mediated neurotoxicity is mediated in part by reaction with superoxide to generate peroxynitrite and not by nitric oxide alone. Moreover, recent data indicate that biologically relevant concentrations of peroxynitrite inhibit mitochondrial respiration via inactivation of electron transport components complex I and II (39). Peroxynitrite appears to be a more potent suppressant of mitochondrial respiration than nitric oxide (47) and has also been implicated as a more potent inhibitor of aconitase (9).

Collectively, these data imply that the cytotoxic capacity of activated macrophages may be due in part to generation of peroxynitrite rather than nitric oxide per se. A number of studies have verified the preferential toxicity of peroxynitrite over nitric oxide towards microorganisms as diverse as *E. coli* (7), *Trypanosoma cruzi* (13), and *Candida albicans* (49), although the opposite effect was observed for *Leishmania major* (3). Coactivation of the respiratory burst and nitric oxide synthesis in macrophages or granulocytes could result in peroxynitrite formation (8, 20), and studies utilizing intact mitochondria suggest that peroxynitrite can cross membranes to reach and inactivate target enzymes (39). The protective effect of SOD against SIN-1-mediated toxicity (Fig. 4) suggested that secretion of this enzyme by adult *B. malayi* (33) might be responsible for enhanced resistance of this stage to activated J774 cells, since LPS elicits superoxide production in this cell line (2). However, addition of exogenous SOD and catalase to microfilariae cocultured with J774 cells did not protect parasites from toxicity and neither did addition of the membrane-permeant SOD mimetic Mn(III)tetrakis (4-benzoic acid) porphyrin (46; data not shown). Previous data suggest that exogenous SOD is an inefficient inhibitor of peroxynitrite formation in macrophages (20), and thus it is unlikely that parasite-derived SOD confers any direct resistance to nitric oxide derivatives from activated macrophages.

The susceptibility of adult and microfilarial stages of *B. malayi* to the toxic effects of activated macrophages is of particular interest given the nature of energy metabolism in these parasites and the identification of mitochondrial respiratory enzymes as specific targets of inhibition by nitric oxide or its by-products (16, 39). Adult *Brugia* spp. have been described as homolactate fermenters (52). Data acquired from *Brugia pahangi* microfilariae suggest that, in contrast, this stage has a significant aerobic component of glucose metabolism, although this was interpreted as resulting from the single-step oxidative decarboxylation of pyruvate to acetate and CO_2 (41). Interestingly, when microfilariae were cultured under anaerobic conditions, the parasites showed a decreased utilization of glucose coupled with a shift to homolactate fermentation and a loss of motility (41). This was not fatal to the parasites, however, since after extended (7 days) maintenance under anaerobic conditions, reintroduction of air led to a resumption of motility. It was therefore concluded that microfilariae have an aerobic requirement for motility but not survival (41). Rapid cessation of motility in microfilariae cocultured with activated macrophages is consistent with the effects of anaerobiosis, which suggests that microfilariae are more dependent than adults on respiration for energy generation required to sustain motility in vitro (41, 52). These experiments illustrate several important points, namely, that (i) motility cannot be utilized as an accurate measure of viability for filarial parasites and (ii) one would expect both microfilariae and adult worms to exhibit considerable resistance to the toxic effects of nitric oxide derivatives targeted to mitochondrial respiration but still remain susceptible to alternative targets and processes such as DNA synthesis (17). Consistent with this interpretation, a recent study implies that resistance of lung-stage larval *Schistosoma mansoni* to nitric oxide derivatives is correlated with a shift from aerobic to anaerobic metabolism (1).

The existence of high numbers of cristate mitochondria in adult stages of *Brugia* spp. is inconsistent with their assignation as homolactate fermenters. Middleton and Saz (30) assayed and detected six tricarboxylic acid cycle enzymes in a particulate (mitochondrial) fraction of adult *B. pahangi* but considered energy generation via the tricarboxylic acid cycle to be of doubtful significance. More recently, Mendis and Townson (29) demonstrated that oxygen uptake by adult male and female *B. pahangi* and *Acanthocheilonema viteae* was inhibited by a blockade of respiratory electron transport with rotenone (inhibitor of complex I) and antimycin A (inhibitor of complex III) and inferred the existence of a classical respiratory electron transport sequence in addition to a pathway bifurcating on the substrate side of complex III. The existence of a respiratory electron transport pathway in adult *B. malayi* would be consistent with the damage to mitochondria elicited in the present study by both activated macrophages and nitric oxide donors (Fig. 5). Degeneration appeared to be limited to the inner mitochondrial membrane, on which enzymes of the electron transport chain are localized. Previous studies in which schistosomula of *S. mansoni* were cultured with activated macrophages indicated that the earliest morphologically identifiable lesions occurred in muscle cell mitochondria, characterized by swelling and disorganization of cristae similar to that observed in our current study (28, 35).

The reversibility of cytostasis effected by activated macrophages illustrated in Fig. 2 suggests that *Brugia* spp. are capable of recovery from this form of immune attack unless it is sustained over a prolonged period of time. This is analogous to the recovery of the capacity for cell division of tumor cells exposed to activated macrophages, which correlates with the resumption of mitochondrial respiration (15). It is interesting to compare these in vitro observations with those engendered by in vivo treatment of immunodeficient mice with a nitric oxide-releasing compound (40). Intraperitoneal administration of sodium (*N*,*N*-diamino)diazen-1-ium-1,2-diolate (DEA/NO) blocked the development of *B. malayi* from infective larvae to adult worms, but daily treatment for 5 days did not result in the direct death of mature adult parasites when examined 2 days later. Adult worms removed from the peritoneum of DEA/NO-treated SCID mice showed greatly reduced motility which recovered following incubation in vitro (40), consistent with our current observations.

It is still unclear whether nitric oxide metabolites contribute to elimination of lymphatic filariae from humans, in particular, given the apparent difference in cellular sources and/or elicitors in comparison with rodents. Nevertheless, the current data indicate the potential of this system for cytostasis and cytotoxicity of both microfilariae and adult worms, the latter of which show marked resistance to alternative known immune effector mechanisms.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council through a studentship to Ruth Thomas.

We thank Will Reece for the kind provision of the J774 cell line.

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Editor: J. M. Mansfield