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Nitric oxide produced in the lungs of mice immunized with the radiation-attenuated schistosome vaccine is not the major agent causing challenge parasite elimination

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SUMMARY

Mice vaccinated with radiation-attenuated cercariae of *Schistosoma mansoni* exhibit high levels of protection against a challenge with normal larvae. The immune effector mechanism, which operates against schistosomula in the lungs, requires $CD4^+$ T cells capable of producing interferon-γ (IFN-γ). This cytokine can stimulate production of nitric oxide (NO), via its ability to up-regulate inducible nitric oxide synthase (iNOS). We have therefore evaluated the potential role of NO in the effector mechanism operating in vaccinated mice. Evidence for the production of NO in the lungs of such animals was obtained from assays on antigen-stimulated airway cell cultures. Enhanced levels of NO, compared with those in cultures from control mice, were detected both after vaccination and after challenge; elevated levels of iNOS mRNA were also present in whole lung after challenge. However, administration of an iNOS inhibitor to vaccinated mice after percutaneous challenge did not significantly increase the worm burden. Furthermore, when mice with a disrupted iNOS gene were vaccinated they showed a highly significant level of protection. Although NO from activated macrophages can mediate cytotoxic killing of newly transformed schistosomula *in vitro*, we have demonstrated that the addition of erythrocytes to these larvicidal assays abolishes its effects. We interpret this to mean that once migrating schistosomula enter the bloodstream they will be protected against the cytotoxic actions of NO. Our data thus provide little evidence to implicate NO as a major component of the pulmonary effector response to *S. mansoni* in vaccinated mice.

INTRODUCTION

A single vaccination of mice with radiation-attenuated cercariae of *Schistosoma mansoni* confers a high level of protection against a challenge with normal larvae,[1] immune elimination occurring predominantly in the lungs[2] via a cell-mediated mechanism. This is clearly dependent upon the induction of a T helper type I (Th1) response since *in vivo* ablation of $CD4^+$ T cells[3,4] or neutralization of the Th1 cytokine interferon-γ (IFN-γ)[5,6] largely abrogates protection. Challenge schistosomula reaching the lungs of vaccinated mice provoke anamnestic inflammatory responses[7,8] which result in the development of cellular aggregates around individual larvae. Mononuclear cells,[9,10] including $CD4^+$ T lymphocytes,[11] feature prominently in these focal responses. Additionally, airway leucocytes recovered from vaccinated and challenged mice by bronchoalveolar lavage (BAL), produce abundant IFN-γ when stimulated *in vitro* with schistosome antigen.[8]

The precise mechanism of challenge parasite elimination in the lungs is unclear. It has been suggested that the crucial event is the physical blocking of parasite migration through the pulmonary vasculature by the effector focus.[12] This hypothesis is based on two observations. The first is that challenge larvae trapped within foci showed negligible evidence of cytotoxic damage at the ultrastructural level.[9] The second is that, in an assay of viability, most schistosomula recovered from the lungs of vaccinated mice 17 days after challenge were capable of maturing when transferred to the portal vein of naive recipients.[13] Furthermore, autoradiographic tracking studies revealed that if such larvae had remained *in situ* in the inflamed lung, they would not have matured.[2]

An alternative hypothesis is that lung schistosomula are eliminated by a cytotoxic killing mechanism.[14] Nitric oxide (NO) has been implicated as the cytotoxic agent, on the basis of its larvicidal properties *in vitro*.[15] Indirect evidence for its production in the lungs was found in a recent study where mRNA for inducible nitric oxide synthase (iNOS) was up-regulated at the time of challenge parasite elimination.[16] It is also notable that IFN-γ, the dominant cytokine in lung phase immunity,[6,8] can up-regulate iNOS,[17] to generate NO from L-arginine.[18] Thus, the appropriate conditions would appear to be fulfilled for the operation of NO-mediated killing

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of lung schistosomula *in vivo*. However, it must be borne in mind that much of the evidence for cytotoxic killing is derived from *in vitro* assays using peritoneal macrophages as effector cells and newly transformed (3 hr) schistosomula as the target, neither of which has direct relevance to the pulmonary immune mechanism operating *in vivo*. Indeed, it is paradoxical that 7–10-day-old lung stage schistosomula have proved refractory to killing in the larvicidal assays,[19,20] even when alveolar macrophages were used as the effector cells.[21]

In an attempt to resolve this paradox, we have sought to determine whether NO has an *in vivo* role in the pulmonary effector response in mice exposed to the radiation-attenuated vaccine. We have measured the production of NO by airway cells *ex vivo* after vaccination and challenge, and examined the induction of iNOS mRNA in whole lung tissue. We have determined the effect on protective immunity of administering an iNOS inhibitor after challenge, and have performed protection experiments in mice with a disrupted iNOS gene. Our data provide little evidence to implicate NO as an important component of the pulmonary effector response. The profound inhibitory effect of erythrocytes on the cytotoxic killing of larval parasites may provide a partial explanation for our observations.

MATERIALS AND METHODS

Animals and parasites

C57BL/6 and C3H.He mice, bred at the Universities of York and Aberdeen, respectively, were maintained with standard laboratory care. Specific pathogen-free C57BL/6 animals, purchased from B & K Universal (Grimston, Hull, UK) and kept under isolator conditions, were used in experiments involving mRNA analysis. MF1 × 129 mice with a homozygous disruption of the iNOS gene ($iNOS^{-/-}$;[22]) and heterozygous littermates ($iNOS^{+/-}$), were bred and maintained in the Joint Animal Facility, University of Glasgow; no difference in responses to *Leishmania* was found between $iNOS^{+/-}$ and wild-type mice in the earlier study.[22] A Puerto Rican isolate of *S. mansoni*, maintained by passage through MF1 mice and albino *Biomphalaria glabrata* snails, was used in all experiments. Newly transformed schistosomula for larvicidal assays were prepared by vortex shearing of cercarial tails,[23] fractionation over a 70% Percoll gradient,[24] and incubation in Dulbecco's modified minimal essential Eagle's medium (DMEM; ICN Biomedicals, Irvine, UK) containing 100 μg/ml penicillin/streptomycin (Gibco, Paisley, UK) for 3 hr at 37°, 5% CO_2/air.

Exposure regimes and experimental design

C57BL/6 mice were vaccinated via the shaved abdomen with 500 cercariae attenuated by exposure to 200 gy gamma irradiation (^{60}Co; Department of Radiobiology, Cookridge Hospital, Leeds, or Department of Veterinary Physiology, University of Glasgow Veterinary School, Glasgow, UK). These animals (VC), together with age-matched naive controls (CC) were challenged percutaneously 35 days later with 200 normal cercariae, either percutaneously via the tail or, in the case of the $iNOS^{-/-}$ and $iNOS^{+/-}$ mice, via the flank. The level of protection was calculated from the mean adult worm burdens obtained by portal perfusion at 35 days post-challenge, using the formula: $[(CC - VC)/CC] \times 100$. For the time–course of iNOS mRNA expression in the lungs, vaccinated mice were challenged intravenously with a synchronous pulse of 120 lung-stage schistosomula, injected via the femoral vein.[25,26] Airway leucocytes were recovered by BAL from groups of mice after vaccination, and after percutaneous or intravenous (i.v.) challenge, and cultured to assess their ability to produce NO *in vitro*. Whole lung tissue was harvested from similar groups of animals after i.v. challenge, for analysis of iNOS mRNA by reverse transcription–polymerase chain reaction (RT-PCR). To investigate the effects on vaccine-induced immunity of inhibiting or boosting production of NO, vaccinated mice were given 5 mg L-N^G-monomethyl arginine (L-NMMA) or L-arginine daily, via intraperitoneal (i.p.) injection, on days 6–13 post-percutaneous challenge, timed to coincide with parasite migration through the lungs;[2] challenge controls and a group of vaccinated mice received sterile phosphate-buffered saline (PBS) alone. In the repeat experiment, an additional group of vaccinated mice were given D-NMMA at the same dose.

RNA isolation and purification

Lung tissue was removed aseptically from individual mice, and frozen at −70° until use. Total RNA was isolated as previously described[27] by homogenization of lung tissue in guanidium thiocyanate solution, extraction in phenol, and precipitation in ethanol. The RNA yield was measured spectrophotometrically, and confirmed by 1% agarose gel electrophoresis in the presence of ethidium bromide. This latter technique also verified the intact nature of the RNA preparation.

RT-PCR detection of iNOS mRNA

RT-PCR analysis, on total RNA pooled from individual mice at each time-point, was carried out for iNOS, using the sense (GCAGCTCCTCACTGGGACAGCAC) and antisense (ATGAGGCAGGAGCTCCTCCAGAGG) primers,[28] and for the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) as previously described.[27] Semiquantitative analysis of cytokine message was performed using a modification of the method of Dallman *et al.*[29] described elsewhere.[25,27] Briefly, amplification was carried out, in duplicate, for three given cycle numbers (27, 30 and 33 for iNOS and 21, 24 and 27 for HPRT), using a temperature programme specific for each primer set. Positive and negative controls were included with each assay to ensure that all PCR reagents were free from contamination. PCR products were slot-blotted onto Zeta-Probe membrane (Bio-Rad, Hemel Hempstead, UK) and detected by hybridization with specific ^{32}P end-labelled oligonucleotide probes for iNOS (ACGGGTCGATGTCACATG[28] and HPRT[27] bound radioactivity was estimated using a Phosphorimager (Molecular Dynamics; Sunnyvale, CA). For each time-point post-challenge, a linear regression was performed on the counts obtained for the reaction product of iNOS at the different cycle numbers. A statistical comparison of the slopes of all regression lines obtained, for both VC and CC samples, revealed no significant differences, thereby confirming that the rates of PCR amplification were equal in all reactions. The values at 30 cycles were estimated and, after normalization using HPRT values, the levels of iNOS were compared across the time–course.

Pulmonary leucocytes

Pulmonary leucocytes recovered by BAL[7,8] were pooled, and brought to 2×10^6 cells/ml in Glasgow MEM supplemented with 10% fetal calf serum (FCS; Globefarm, Esher, Surrey, UK), 200 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical Co., St. Louis, MO) (GMEM+). Cells were incubated in flat-bottomed 96-well plates (Nunclon, Gibco) at 200 μl/well, ±50 μg/ml soluble 18 hr schistosomula antigen (SSP[30]) for 72 hr at 37°, 5% CO_2/air. Cultures relied upon the presence of abundant endogenous alveolar macrophages to act as antigen-presenting cells. Supernatants were harvested and stored at −70° until tested for the accumulation of nitrite.

Peritoneal cells

Resident peritoneal cells, recovered from naive C3H.He mice by lavage with DMEM containing 2 mM L-glutamine (Gibco) and antibiotics, were washed twice by centrifugation at 250 *g* for 10 min at 4°, and brought to 2×10^6 cells/ml in DMEM as above plus 10% FCS (DMEM+). Peritoneal macrophages were separated from the total leucocyte population using a simple adherence assay. Briefly, all cells were incubated in sterile 96-well plates (Nunclon, Gibco; 200 μl/well) for 2 hr at 37°, 5% CO_2, after which the non-adherent leucocytes were removed and medium was replaced with fresh DMEM+. Adherent cells were predominantly macrophages (>90%), as determined by non-specific esterase staining.[31]

Larvicidal assays

Larvicidal assays were performed in 96-well plates, using naive peritoneal macrophages which were activated *in vitro* for 24 hr (2×10^5 cells/well) with murine recombinant IFN-γ (100 U/ml; kind gift of G. Adolf, Ernst Boehringer Institute, Vienna, Austria) plus lipopolysaccharide (LPS; 100 ng/ml; 0.127:B8, derived from *Escherichia coli*, Sigma). Then, L-NMMA (0·5 mM) or whole mouse blood (derived from naive C3H.He mice; 1:200 final dilution) was added to parallel sets of cultures. Unstimulated cells were also included, as controls. All cultures were set up in four replicate wells. Following incubation, 100 μl of supernatant was aspirated, and frozen at −20° until tested for the accumulation of nitrite and nitrate. Schistosomula (3 hr) were added to macrophage cultures at an effector:target ratio of 2×10^4:1, for 48 hr at 37° and 5% CO_2/air, in DMEM+ supplemented with 2·5% glucose. Previous experiments established that these treatment regimes had no detrimental effects on either the macrophages or the schistosomula cultured alone (data not shown). After 48 hr, larval killing was determined by the criteria of internal granularity and loss of motility, and culture supernatant was harvested and frozen until tested for the accumulation of nitrite and nitrate.

NO assay

NO oxidizes stoichiometrically to nitrite and nitrate under aerobic conditions. Production of NO by airway cells was therefore quantified by measuring the accumulation of nitrite in the culture medium, using the Griess reaction.[32,33] Briefly, 50 μl of culture medium was incubated with an equal volume of Griess reagent (1% sulphanilamide/0·1% naphthylethylene diamine dihydrochloride/2·5% H_3PO_4) at room temperature for 10 min. The absorbance at 570 nm was determined, and values were quantified against a sodium nitrite standard curve, which was run on every plate. Nitrate was stoichiometrically reduced to nitrite, for measurement in the Griess assay, by incubation of sample aliquots (100 μl culture medium) for 1 hr at 37° in the presence of 0·1 U *Aspergillus* nitrate reductase/ml [NAD(P)H, nitrate oxidoreductase, EC 1.6.6.2; Sigma], 120 μM reduced NADPH and 5 μM flavine adenine dinucleotide. Subsequently, excess NADPH was oxidized with 10 U L-lactic hydrogenase/ml (EC 1.1.1.27; type XI, from rabbit muscle; Sigma), and 10 μM sodium pyruvate for 30 min at 37°. Sodium nitrate was used as a standard.[34]

Statistical analysis

Arithmetic means are shown ± SEM. All data comparisons were tested for significance using Student's *t*-test. Worm burden data from experiments involving iNOS$^{-/-}$ mice were also subjected to analysis of variance.

RESULTS

NO release by airway cells

Since the immune effector mechanism operates in the lungs of mice exposed to the radiation-attenuated vaccine, we first wished to establish that airway, as opposed to peritoneal, macrophages could produce NO *ex vivo*. Low levels of nitrite were detected in the culture supernatants of SSP-stimulated BAL cells from naive animals (Fig. 1) whereas significant production was observed at day 21 post-vaccination ($P<0·01$). Nitrite was also detected in culture supernatants from vaccinated mice at day 14 after percutaneous challenge, levels being fourfold greater than in challenge controls ($P<0·005$). Even in the absence of antigen, nitrite levels were significantly elevated in BAL culture supernatants from vaccinated compared to control mice ($P<0·002$).

In order to observe the kinetics of NO production after challenge, we administered a pulse of lung schistosomula to

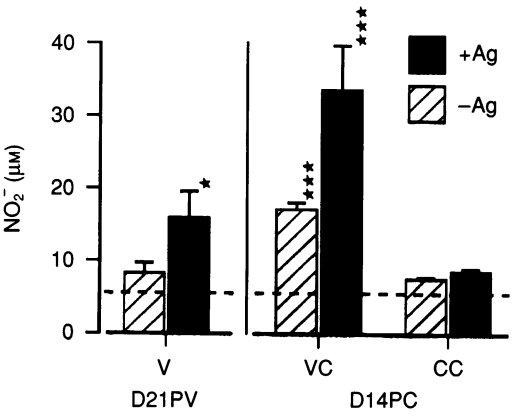

Figure 1. Comparison of nitrite production by airway leucocytes, recovered by BAL, from vaccinated mice at day 21 post-exposure to irradiated cercariae (D21PV), and from VC and CC animals at day 14 post-percutaneous challenge (D14PC). BAL cells from individual mice ($n=5$) were cultured for 72 hr with or without SSP antigen. Values are the mean ± SEM of nitrite levels estimated by the Griess reaction. The horizontal line represents the nitrite level detected in supernatant from 72 hr antigen-stimulated BAL cells recovered from naive mice ($n=5$). *$P<0·05$; ***$P<0·001$ relative to naive value. Results are representative of two experiments.

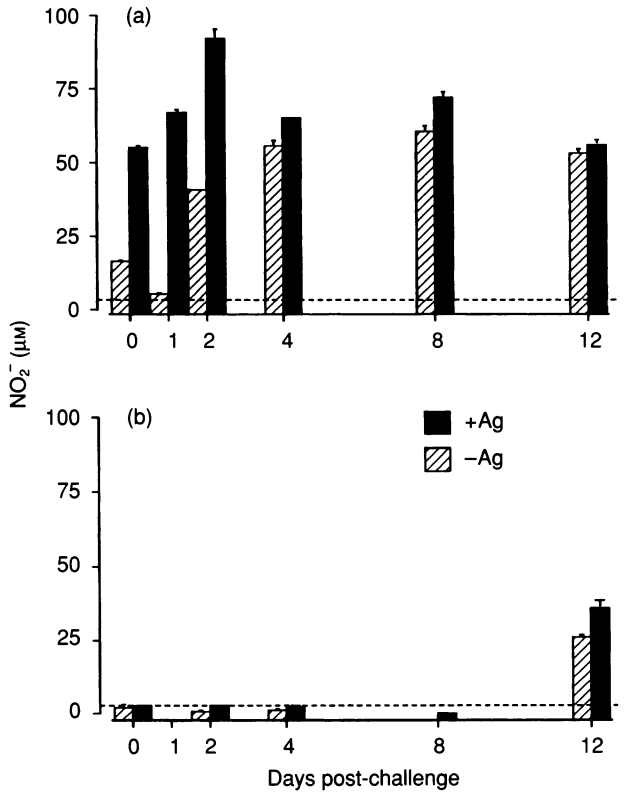

Figure 2. Nitrite production by airway leucocytes from VC (a) and CC (b) mice on days 0, 1, 2, 4, 8 and 12 post-synchronous i.v. challenge. Cells were cultured for 72 hr with or without SSP antigen. Values are the mean ± SEM of duplicate measurements from each of five mice. The horizontal line represents the nitrite level detected in supernatant from 72 hr antigen-stimulated BAL cells recovered from naive mice ($n = 5$). Similar results were obtained in a repeat experiment with time-points on days 0, 2, 4 and 12 post-challenge.

vaccinated and control mice, to synchronize inflammatory events in the lungs. Nitrite levels in BAL cultures from vaccinated mice were significantly elevated above naive values throughout the timecourse (Fig. 2a). Without stimulation, low to moderate nitrite accumulation was observed on days 0 and 1 post-challenge, levels increasing gradually thereafter to reach a maximum value at day 8. After antigen stimulation, strongly elevated nitrite levels were observed at all time-points, peaking at day 2. On days 0, 1 and 2 nitrite levels in these cultures were approximately twofold greater than in unstimulated cultures. After this time, high levels of nitrite were detected in supernatant from both culture regimes. When CC mice were challenged i.v. no accumulation of nitrite was observed in BAL culture supernatants, above that of naive levels, until day 12 when a moderate increase was detected even in the absence of antigen (Fig. 2b).

Pulmonary iNOS mRNA expression

Since activated macrophages (and other cells) release NO through the cytokine-induced action of NOS,[15] we assessed the level of iNOS mRNA expression in whole lung tissue of vaccinated and control mice on three occasions after i.v. challenge. The HPRT and iNOS PCR reactions both yielded single bands when run on a 2% agarose gel. Pulmonary iNOS mRNA levels were increased on day 4, and still elevated above naive levels at day 12 post-challenge (Fig. 3). In challenge control mice a small increase in iNOS message level was observed on day 2 only.

Treatment with L-NMMA does not abrogate immunity in vaccinated mice

To assess the role of NO in the pulmonary effector mechanism operating in this model, groups of vaccinated mice were given L-NMMA or L-arginine daily between days 6 and 13 post-percutaneous challenge, to block or enhance NO production, respectively, during lung-phase migration. The pattern of worm recovery at day 35 post-challenge was similar in both experiments undertaken (Fig. 4). Vaccinated mice which received PBS alone showed a highly significant reduction in worm burden compared to their controls ($P < 0{\cdot}001$), consistent with the usual level of immunity seen in this model. After treatment of vaccinated mice with L-arginine or L-NMMA changes in worm burden, compared to mice receiving PBS, or D-NMMA (second experiment only), were not statistically significant. The resistance of the vaccinated mice receiving L-NMMA was 22·2% and 14·3% lower than their PBS-treated counterparts in the first and second experiments, respectively.

iNOS$^{-/-}$ mice show no significant reduction in immunity

The derivation of mice homozygous for a disrupted iNOS gene has permitted direct evaluation of the involvement of NO in the protective response in this model. Vaccination of iNOS$^{+/-}$ mice resulted in a significant reduction in worm burden relative to control animals, as judged by Student's *t*-test ($P < 0{\cdot}001$ in experiments 1 and 2; Fig. 5). The vaccinated iNOS$^{-/-}$ mice were also immune, with significantly reduced worm numbers compared to those from their respective challenge controls ($P < 0{\cdot}001$). The level of protection displayed in the two experiments by the vaccinated iNOS$^{-/-}$ mice (46·4% and 48·8%) appeared somewhat lower than that of the heterozygous counterparts (69·5% and 67·0%), representing a 33% and 27% reduction, respectively. However, when the total data set was subjected to analysis of variance to test the effects of vaccination and of genotype on worm burden, the iNOS$^{-/-}$ mice proved to have significantly higher worm burdens than the heterozygotes, in both vaccinated ($P < 0{\cdot}001$) and control ($P < 0{\cdot}02$) groups. As a consequence, there was no significant interaction between the vaccine and the genotype ($P = 0{\cdot}202$), i.e. the vaccine influences worm burden by an equal amount in both genotypes.

Whole mouse blood quenches NO released from activated macrophages, and protects parasites

Since no substantial effect on immunity was apparent after *in vivo* inhibition of iNOS, or after vaccination of iNOS$^{-/-}$ mice, we examined further the cytotoxic killing of schistosomula by NO *in vitro*. We first determined that murine peritoneal macrophages, stimulated for 24 hr *in vitro* with IFN-γ and LPS, released reactive nitrogen intermediates, as judged by nitrite and nitrate accumulation in the medium. We then assessed the killing of newly transformed schistosomula

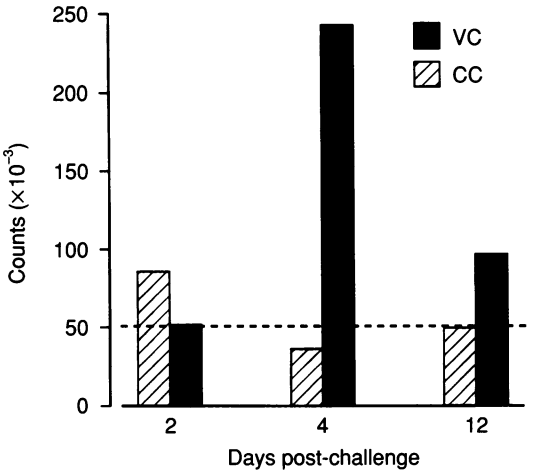

Figure 3. Pattern of iNOS mRNA expression in whole lung tissue extracts from VC and CC mice ($n=5$), harvested after synchronous i.v. challenge, and assayed using multiple cycle RT-PCR analysis. The cDNA product was estimated at 30 cycles from the linear regression performed on the counts obtained from different cycle numbers. The horizontal line is the iNOS mRNA level in the naive CC mice on the day of challenge, estimated in the same way.

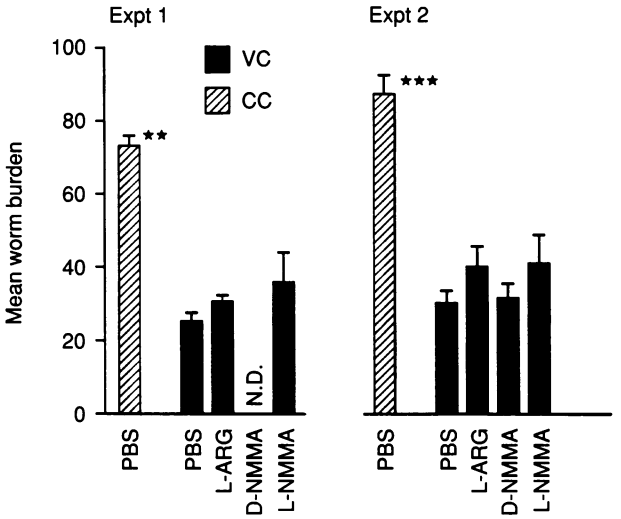

Figure 4. Mean worm burdens obtained by perfusion of groups of VC and CC mice ($n=5$ or $n=7$), following treatment with PBS alone, L-arginine (L-ARG), D-NMMA, and L-NMMA post-percutaneous challenge. $^{**}P<0{\cdot}01$; $^{***}P<0{\cdot}001$ relative to all VC worm burdens in each experiment.

co-cultured with such macrophages for the 48 hr following activation. Neither the addition of target schistosomula to macrophage cultures, nor the culture of parasites in the absence of macrophages, affected the accumulation of nitrite or nitrate (data not shown). In the absence of macrophage stimulation, both the levels of nitrite and nitrate found in 24 hr culture supernatants, and the numbers of parasites killed in these cultures, were low (Fig. 6). When IFN-γ and LPS were added to macrophage cultures, the accumulation of nitrite and nitrate, and the incidence of larvicidal killing, were sharply and significantly elevated ($P<0{\cdot}001$). These phenomena were clearly NO-dependent, since addition of L-NMMA to cultures prevented both the accumulation of nitrite and nitrate, and larvicidal killing.

As schistosome migration from the skin to the portal tract is entirely intravascular, larvae are in close proximity to erythrocytes throughout. Oxyhaemoglobin acts as a scavenger for NO, resulting in the formation of methaemoglobin and nitrate;[35] in such circumstances nitrite is not detectable. We therefore investigated the outcome of adding mouse erythrocytes to the macrophage cultures. This had a dramatic effect, reducing schistosomula killing to background levels. In parallel, the accumulation of nitrite in the culture medium was greatly diminished, whilst the level of nitrate actually increased.

DISCUSSION

There is a general concensus that the major part of challenge parasite elimination in mice exposed once to the radiation-attenuated schistosome vaccine occurs in the lungs.[13] The production of NO by pulmonary macrophages would provide a potential effector response to bring about this elimination.[16] Evidence for such a mechanism is based on previous *in vitro* studies using thioglycollate-elicited peritoneal macrophages[20,36] or activated murine endothelial cell lines,[36] to cause cytotoxic killing of newly transformed schistosomula and 18-day-old worms from the portal system. The purpose of our study was to explore whether NO is involved in lung-phase immunity, in view of the fact that schistosomula recovered from the lungs of normal mice 7–10 days after infection are refractory to killing in such *in vitro* assays.[20,21]

We have shown that airway leucocytes, recovered from vaccinated mice after challenge and cultured *in vitro*, secrete abundant NO. Production appears to be regulated at least in part by specific T cells since co-culture of BAL samples, from vaccinated but not naive mice, with schistosome antigen stimulates a marked increase in NO production. This observation implies a pivotal role in NO production for the $CD4^+$ effector/memory Th1 cells, via the cytokines they release. These cells are recruited to the lungs post-vaccination[37] and constitute a major component of the effector foci which develop around challenge schistosomula.[11] Additional evidence for T-cell involvement is provided by the rapid increase in NO production by airway cultures after i.v. challenge of vaccinated mice which coincides with a second influx of T lymphocytes into the airways.[25] This NO release, observed even in the absence of added antigen (day 2) or prior to a corresponding increase in IFN-γ levels *in vitro*, suggests that leucocytes may have already been activated *in vivo* through exposure to antigen from migrating larvae. Alternatively, other cytokines such as tumour necrosis factor-α (TNF-α) or interleukin-1β (IL-1β), known to up-regulate iNOS message,[36] might be involved in stimulating NO production. In contrast, the low level of NO released by BAL cultures from control animals probably reflects the scarcity of schistosome-specific T lymphocytes in airway populations recovered from such mice after i.v. challenge.[25]

The observation of elevated levels of iNOS mRNA in the lungs of vaccinated mice after i.v. challenge provides indirect evidence for NO production *in vivo*. Our data reveal that there was a sharp rise in iNOS mRNA between days 2 and 4, with levels still elevated above the naive background at day 12. Indeed, the pattern of iNOS mRNA expression closely matches

that for IFN-γ mRNA after i.v. challenge described in our earlier study;[25] it also matches the pattern of leucocyte accumulation around larvae embolized in the lungs. However, our results differ from those of Wynn *et al.*[16] who reported a sharp spike of iNOS mRNA in whole lung tissue at day 2 post-challenge, returning to background by day 4. This was accompanied by a similar spike of IFN-γ mRNA at day 1, returning virtually to background by day 2. We attribute the discrepancies between the two studies to differences in experimental approach. Wynn *et al.*[16] injected 500 mechanically transformed skin schistosomula whereas we used 120 lung-stage larvae extracted from donor mice. The fourfold greater parasite dose is clearly one factor which could potentially contribute to the early spikes of message described by these authors. The release of residual cercarial products from the newly transformed larvae, which would not be present in the lung-stage larvae, may also have triggered non-specific responses.

We have sought to establish the *in vivo* involvement of NO in challenge parasite elimination both by administration of an iNOS inhibitor to mice and by performing vaccination experiments in mice with a disrupted iNOS gene. We found that treatment with L-NMMA produced only 14–22% abrogation of protection. It could be argued that insufficient inhibition was achieved by the treatment, but the source and dose of L-NMMA were identical to those used to demonstrate a significant effect in *Leishmania*-infected mice.[38] Wynn *et al.*[16] reported a 32–33% increase in worm burden using aminoguanidine hemisulphate as an iNOS inhibitor. This is equivalent to 15% abrogation using our method of calculation in the one experiment where treatment groups were strictly comparable. We conclude that *in vivo* inhibition of iNOS does not provide strong evidence for the role of NO as the major mediator of the protective response against lung schistosomula.

Our experiments using mice with a disrupted iNOS gene further support the conclusion that NO does not play a dominant role in the immune effector mechanism. These animals are highly susceptible to *Leishmania major* infection, and their peritoneal macrophages produce only background levels of nitrite when stimulated *in vitro* with IFN-γ and LPS, implying an absence of inducible NO production.[22] When both heterozygous and iNOS$^{-/-}$ mice were vaccinated and challenged, they showed highly significant levels of protective immunity, independent of the challenge parasite dose. Interpretation of the results is complicated by the higher worm burdens in both vaccinated and control iNOS$^{-/-}$ mice relative to the heterozygous counterparts. Nevertheless, analysis of variance failed to reveal any effects on the level of immunity that could be attributed to disruption of the iNOS gene. The increased worm burdens (+16%) in gene-disrupted challenge controls (also observed by Wynn *et al.*[16] in mice receiving hemiguanidine sulphate) do suggest that maturation in naive mice is, to a small degree, influenced by NO production. However, this need not be the result of a cytotoxic mechanism. Parasites migrate from the skin to the hepatic portal system entirely within the circulation, the vascular beds of the lungs and systemic organs representing a major obstacle to larval progress. NO can function as a vascular relaxing factor,[39] and may thus influence the ability of parasites to negotiate the vascular beds by affecting smooth muscle tone and hence vessel diameter.

It is pertinent to ask why the *ex vivo* and *in vivo* observations reported here cannot be equated with the results of *in vitro* killing assays. Host–parasite associations where NO has been established as a cytotoxic agent involve intracellular protozoa such as *Toxoplasma* and *Leishmania*.[40,41] In the case of a large extracellular helminth, it is plausible that other factors could intervene to prevent the actions of NO. Schistosomes are surrounded by erythrocytes during their intravenous migration yet the larvicidal assays are routinely performed in the absence of these cells. We have shown that the addition of erythrocytes (at a 1/200 dilution of whole blood) to larvicidal assays abolishes all cytotoxic killing of newly transformed schistosomula. This parallels the observations of Mabbott *et al.* with *T. brucei*,[34] another extracellular parasite, where erythrocytes completely blocked the anti-proliferative effects of NO. Schistosome larvae at any stage of development, are thus unlikely to be exposed to cytotoxic concentrations of NO whilst resident in the host bloodstream. This is particularly relevant in the radiation-attenuated vaccine where challenge elimination occurs predominantly during migration through the pulmonary vasculature. The evidence

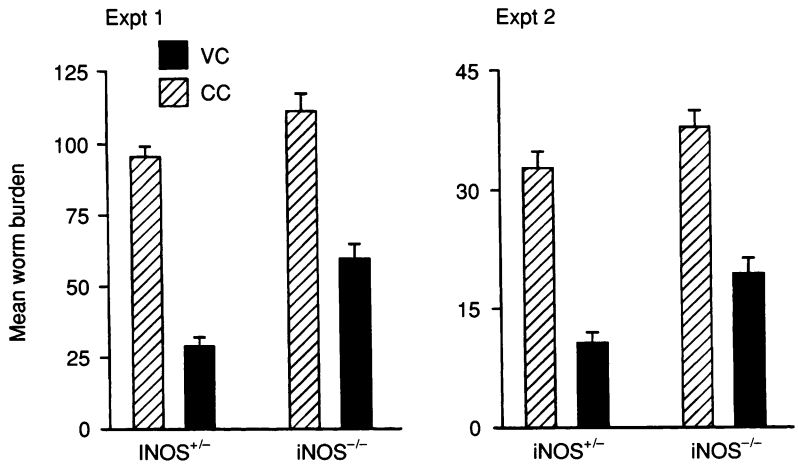

Figure 5. Mean worm burdens of groups ($n=7$) of VC and CC iNOS$^{-/-}$ and iNOS$^{+/-}$ mice. In Exp. 1, mice received a high dose cercarial challenge and in Exp. 2 a low dose.

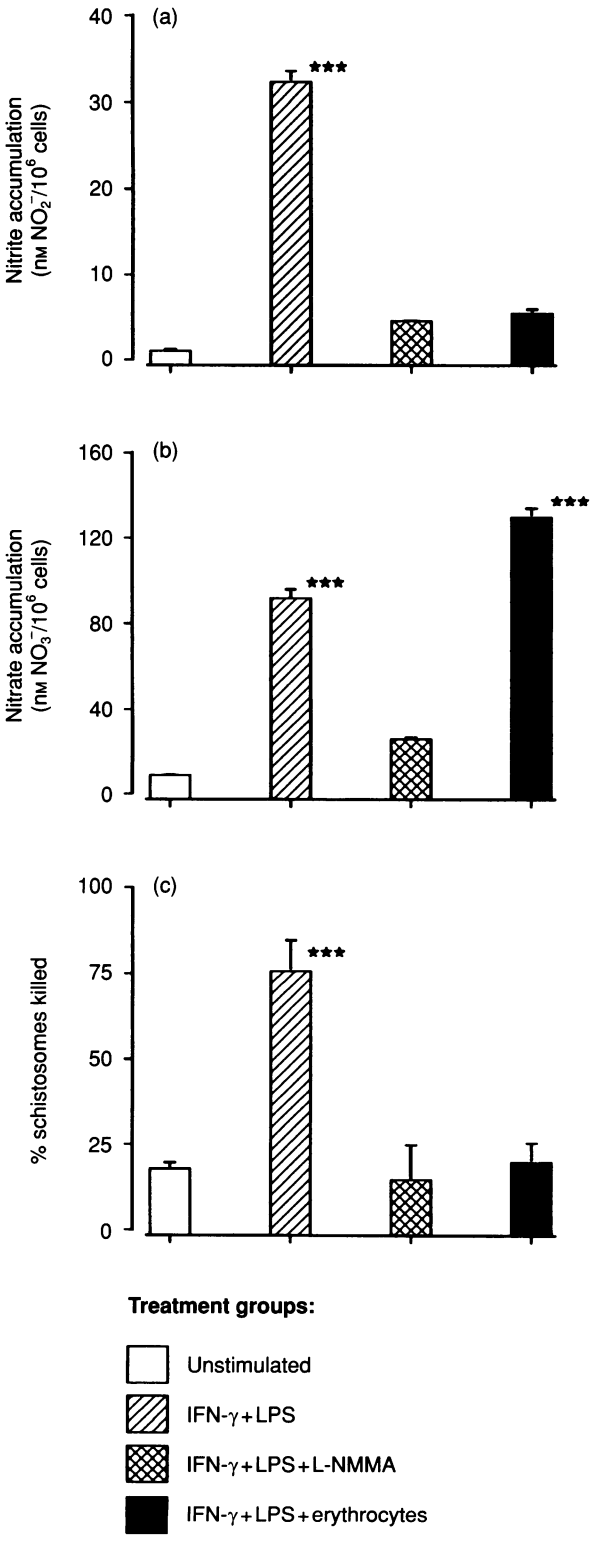

Figure 6. The accumulation of nitrite (a) and nitrate (b), measured in 24 hr culture supernatant of peritoneal macrophages, using the Griess reaction, and the percentage killing of schistosomula of *S. mansoni* cultured with peritoneal macrophages for 48 hr after activation (c). Cells were unstimulated, or incubated with 100 U/ml IFN-γ plus LPS (100 ng/ml) alone, or together with 0·5 mM L-NMMA or whole mouse blood at a 1:200 final dilution. ***$P<0{\cdot}001$ compared with unstimulated macrophage cultures. All cultures were set up in four replicate wells, and experiments were repeated at least three times.

that haemoglobin plays a scavenging role is provided by our observations that in stimulated cultures containing erythrocytes, the product of NO degradation was predominantly nitrate rather than nitrite, while more nitrite accumulated in cultures lacking erythrocytes.

It has recently been suggested that NO acts on pathogens to inactivate enzymes involved in oxidative energy metabolism.[19] However, 7-day-old lung schistosomula are not killed *in vitro* by inhibitors of aerobic metabolism, unlike day 18 lung schistosomula or liver worms.[19] These results accord with earlier observations made by Lawson & Wilson[42] that lung schistosomula are in a semi-quiescent state, relying on anaerobic pathways for energy metabolism during the period of intravascular migration. This situation changes when worms reach the portal tract where growth is initiated and oxygen consumption increases exponentially with time. Thus, the status of aerobic metabolism in the intravascular stage parasite correlates well with susceptibility to NO-mediated killing *in vitro*, but is unlikely to be relevant *in vivo* due to the overwhelming scavenging capacity of haemoglobin. We must assume that the *in vitro* susceptibility of day 18 lung schistosomula to killing is because they have metamorphosed to the 'liver stage' in an unsuitable location. In any case, it is unlikely that this late susceptibility is a factor determining the worm burden in vaccinated mice as migration to the portal tract is virtually complete at this time.[2]

On the basis of results presented here, the nature of the principal mechanism which brings about challenge parasite elimination in the radiation-attenuated vaccine model remains to be elucidated. The hypothesis that the focus which develops around an embolized parasite in the lungs acts by blocking further migration rather than by direct cytotoxic killing is still tenable. However, the parameters of focus formation have yet to be fully characterized. In mice with a disrupted IFN-γ receptor gene, where resistance is greatly diminished, cell aggregates are more extensive, looser and with an altered composition in which eosinophils predominate.[27] We infer from these observations that the type of cell which is attracted and the strength of intercellular adhesions are crucial to the success of the focus in trapping parasites. It is plausible that chemokines play a central role in the attraction process. The increased expression of RANTES mRNA in the lungs of vaccinated and challenged mice compared to challenge controls provides evidence for this last supposition.[43] We are currently investigating the relevance of these various parameters.

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