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Induction of Alpha/Beta Interferon and Dependent Nitric Oxide Synthesis during *Chlamydia trachomatis* Infection of McCoy Cells in the Absence of Exogenous Cytokine

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The propensity of two *Chlamydia trachomatis* strains (L2/434/Bu [biovar LGV] and E/DK20/ON [biovar trachoma]) to induce putative host defense responses upon infection of McCoy (mouse) cell cultures was examined. Both strains induced production of alpha/beta interferon and nitric oxide (NO) by McCoy cells. NO synthesis was mediated by the inducible isoform of NO synthase as indicated by the ability of cycloheximide or the arginine analog N^G-monomethyl-L-arginine to abolish NO production; the extent of the response was dependent upon the dose of chlamydiae applied. Incubation of McCoy cells with chloramphenicol prior to infection reduced NO production by strain 434 but not by DK20, suggesting that initial chlamydial metabolism was essential to induction by the LGV strain. Antibody inhibition studies indicated that NO synthesis was dependent upon production of alpha/beta interferon and induction via lipopolysaccharide. Overall, our findings show that chlamydiae are capable of the induction of interferon and NO in murine fibroblasts in the absence of exogenous cytokines. However, the role of NO as an antichlamydial effector could not be clearly demonstrated since treatment with an arginine analog, while suppressing NO production, gave no consistent enhancement of infected cell numbers.

The genus *Chlamydia* consists of obligate intracellular parasitic bacteria that infect an extensive range of hosts, causing widespread disease of humans and animals. Within *Chlamydia trachomatis*, the biovars of trachoma and lymphogranuloma venereum (LGV) constitute major pathogens of humans with differing disease patterns that are paralleled by distinctive behavior in cell culture models (32). In particular, biovar LGV strains are highly infectious and readily propagated, while biovar trachoma strains do not easily establish productive infection in cell lines (47).

Evidence from previous studies suggests that infection of cell cultures by chlamydiae may not always lead to productive infection and that events during entry are pivotal in determining the potential for multiplication (32, 34). In this context, it is notable that inhibitors of host protein synthesis such as cycloheximide (CH) have been widely used to enhance chlamydial growth (36), with biovar differences again apparent in the marked increase of inclusion numbers observed for trachoma strains. The mechanisms of enhancement by CH are undefined but have generally been attributed to release of host competition for nutrients, thus providing a more favorable environment for chlamydial growth. However, an alternative possibility is that inhibition of host protein synthesis additionally or alternatively leads to abolition of a defense response that would otherwise have been mounted by the host cell. Indeed, the finding that productive infection of cultures can be increased either by CH treatment postinfection or by increase in inoculum concentration in the absence of CH suggests that intracellular nutrient is not the sole limiting factor (29). We have therefore begun attempts to identify and characterize potential defense responses of host cells occurring as a result of chlamydial infection.

Host cell responses to chlamydial challenge have been little examined in nonprofessional phagocyte culture models. Early work reported production of interferon (IFN) by L cells and HeLa cells in response to infection by trachoma agents (16, 23). The IFN, reported to be IFN-α/β (type I IFN), was shown to inhibit chlamydial growth by as much as 80% (11, 12, 17). More recently, Hess et al. (13) conclusively identified the production of both IFN-α and IFN-β upon infection of primary mouse fibroblast cultures with a number of facultative and obligate intracellular pathogens, including an LGV strain.

Two other cytokines thought to be important in antichlamydial immune responses (interleukin 1 and tumor necrosis factor alpha [TNF-α]) have been shown to be produced in vitro in response to chlamydial infection (20, 37). Use of recombinant cytokines identified potent antichlamydial effects of interleukin 1 and TNF-α in human cells (40, 41). Paguirigan et al. (28) reported that infection of human macrophage cultures alone induced the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase, an enzyme known to exert potent antichlamydial effects when induced in human cells by the addition of exogenous cytokines including IFN-γ and TNF-α (5, 41, 42). Antichlamydial effects induced by IFN-γ have also been reported in murine cells (7), although the effector was shown not to be indoleamine 2,3-dioxygenase. Inhibition of chlamydial growth has recently been suggested to be due to the production of the free radical nitric oxide (NO), with clear evidence of its induction in IFN-γ-treated cells during chlamydial infection (21). NO is produced from L-arginine by the enzyme nitric oxide synthase (NOS) (18, 39) and has been identified as an important molecule in cell signaling and as an antimicrobial and tumoricidal agent (24–26). Here we report the identification and partial characterization of NO production by McCoy

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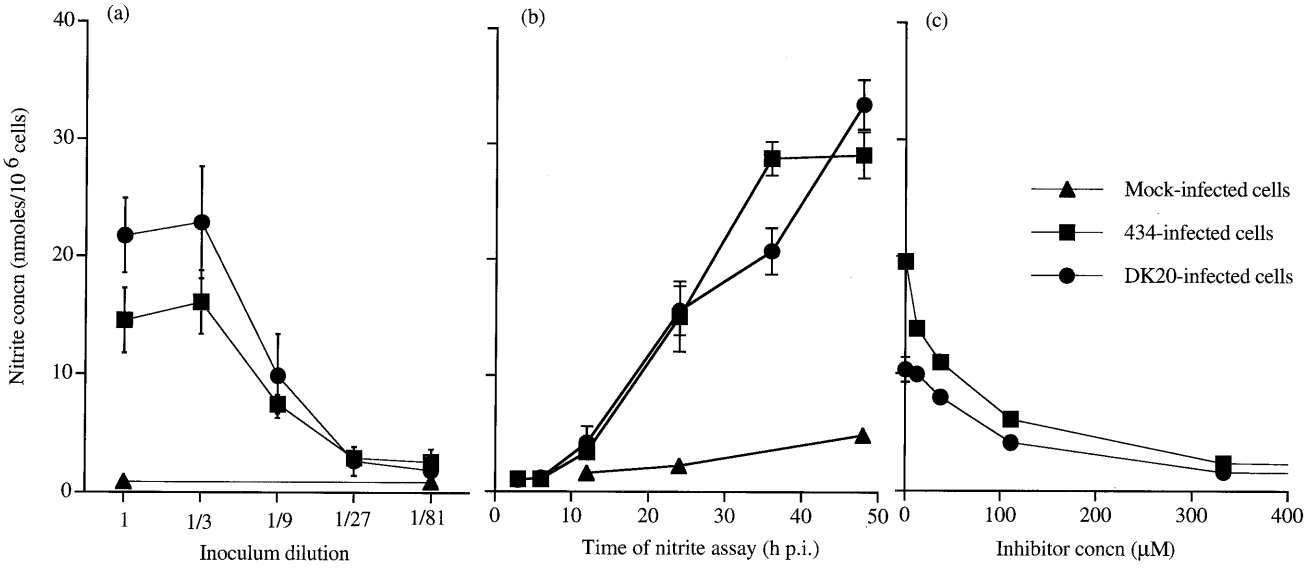

FIG. 1. Effect of chlamydial dose, time p.i. of nitrite assay, and presence of MLA upon the generation of nitrite. (a) Nitrite concentrations at 48 h p.i. McCoy cells infected with various dilutions of strain 434 or DK20 or mock infected are shown. An inoculum of 1 resulted in >95% of cells being infected. (b) Nitrite concentrations at various times p.i. from McCoy cells following mock infection or infection with strain 434 or DK20. (c) Nitrite concentrations at 48 h p.i. from McCoy cells infected with strain 434 or DK20 in the presence of various concentrations of MLA. Error bars represent standard deviations of triplicate assays.

cells (murine fibroblasts) in response to infection with *C. trachomatis* in the absence of exogenous cytokine.

MATERIALS AND METHODS

Growth and purification of chlamydiae. *C. trachomatis* L2/434/Bu (strain 434; ATCC VR-902B) and E/DK20/ON (strain DK20) (46) were grown in McCoy cells (ATCC CRL-1696) in the presence of CH (1 μg/ml) as described previously (31). Organisms were harvested postinfection at 40 h (strain 434) and 48 h (strain DK20) and purified by the batch procedure of Howard et al. (14) with Urografin 325 (Schering, Burgess Hill, United Kingdom). Stock suspensions of purified organisms were stored at −70°C in sucrose-phosphate-glutamate buffer (19).

Growth of cell monolayers and infection with chlamydiae. McCoy cells were grown in complete minimal essential medium (CMEM) containing Eagles' minimal essential medium (Gibco) supplemented with 5% fetal bovine serum, L-glutamine (2 mM), and streptomycin (100 μg/ml). HeLa 229 (ATCC CCL-2.1), HEp-2 (ATCC CCL-23), Caco-2 (ECACC 86010202), MDCK (ATCC CCL-34), L929 (ATCC CRL-6319), and WISH (ATCC CCL-25) cell lines were maintained in a similar way.

Monolayers for infection (all cell lines detailed above with the exception of L929 and WISH cells) were produced in 6- or 24-well trays (Nunc) with 1×10^6 or 2×10^5 cells, respectively. For infection, stock chlamydial suspensions were diluted in CMEM to 2×10^6 inclusion-forming units per ml (strain 434) or 2×10^7 inclusion-forming units per ml (DK20) and inoculated (200 μl for 24-well trays and 1 ml for 6-well trays) onto monolayers with centrifugation ($1{,}580 \times g$) at 37°C for 30 min; inoculum concentrations were decreased by 2-fold (strain 434) or 10-fold (DK20) when CH was present. Under these conditions, >95% of cells produced inclusions. In certain experiments, inocula were further diluted as indicated in the text. Following centrifugation, the inoculum was replaced by CMEM lacking phenol red (1.5 ml). All of the cell lines used were infectible to similar extents. For heat inactivation of chlamydiae, the diluted chlamydial suspension was held at 60°C for 15 min prior to inoculating onto cell monolayers as described above (32).

Assay of nitrite levels in the medium. As a measure of NO produced, levels of nitrite in culture medium (phenol red-free) were assayed. The medium for assay was centrifuged to pellet insoluble material ($13{,}000 \times g$) for 10 min. One milliliter of medium was added to 20 μl of 1% (wt/vol) sulfanilamide in hydrochloric acid (7.8%, wt/vol) and incubated for 10 min at room temperature prior to the addition of 20 μl of a 0.1% (wt/vol) aqueous solution of *N*-(1-naphthyl)-ethylenediamine dihydrochloride. After a further 5 min of incubation, the A_{543} was determined. Nitrite concentrations were estimated from a standard curve generated with sodium nitrite. This assay procedure reproducibly gave a low signal-to-noise ratio.

Assay of IFN activity. Mouse IFN was assayed in L929 cells challenged with Semliki Forest virus (essentially as described by Meager [22]) by measuring the inhibition of incorporation of [^{3}H]uridine into viral nucleic acids. Titers are given in international units. Assays for human IFN were carried out in WISH (human amnion) cells.

Use of inhibitors and antibodies. CH was used at a concentration of 1 μg/ml to inhibit host cell protein synthesis. Chlamydial protein synthesis was inhibited with 100 μg of chloramphenicol (CPN) per ml, a concentration reported to completely abolish chlamydial macromolecular synthesis (45).

N^G-Monomethyl-L-arginine (MLA; Sigma) was employed as a specific inhibitor of NO synthase at a concentration of 1 mM unless otherwise stated. Dilution of the inhibitor was made in CMEM as required. The inhibitor was added to cultures immediately postinoculation (p.i.).

Anti-IFN-α/β antibodies (Lee Biomolecular, San Diego, Calif.) were used to assess the role of endogenously produced IFN on the NO response. Dilutions of the antibody were made as required in CMEM and added to cultures immediately p.i.

Two sources of anti-lipopolysaccharide (anti-LPS) antibody were used in attempts to modulate chlamydial LPS action. One was a monoclonal antibody reacting with *Chlamydia*-specific LPS epitopes; the other was present in an antigonococcal serum with strong reactivity to gonococcal lipo-oligosaccharide and cross-reactive with chlamydial LPS as assessed by immunoblotting. These antibodies were kind gifts from S. J. Richmond and N. J. Parsons, respectively. Antibody and chlamydiae were incubated at 37°C for 10 min (with mixing) prior to inoculation onto monolayers as described above.

Assessment of chlamydial infectivity and yield. Inoculated monolayers (as described above) were allowed to develop to 30 to 35 h p.i. in the presence or absence of inhibitors. Inclusion numbers were assessed by fluorescence microscopy after fixation of monolayers with acid-alcohol and staining with acridine orange and examination with Zeiss Neofluar optics at ×500 magnification (43).

To assess yield of infectious chlamydiae per inclusion, a monolayer identical to that counted for infectivity in the presence or absence of inhibitors as appropriate was disrupted by sonication at 40 h p.i. and used to inoculate fresh monolayers as described above. Resultant inclusion numbers were assessed at 30 to 35 h p.i., and yield is expressed as inclusion-forming units per inclusion.

Levels of significance were calculated with the Student *t* test and a level of significance of 5% ($P < 0.05$).

RESULTS

***Chlamydia*-induced nitrite production.** Infection of McCoy cells with either strain 434 or DK20 resulted in a significant, dose-dependent production of nitrite compared with that of mock-infected cultures (Fig. 1a). Measured nitrite levels present in medium from infected monolayers were in the range of 15 to 45 nmol/10^6 cells at 48 h p.i. Levels of nitrite in mock-infected cultures were below 6 nmol/10^6 cells. Infection of cultures of the human HeLa cells, HEp-2 cells, Caco-2 cells (nonpolarized), or canine MDCK cells (nonpolarized) produced inclusion numbers comparable to those in McCoy cells,

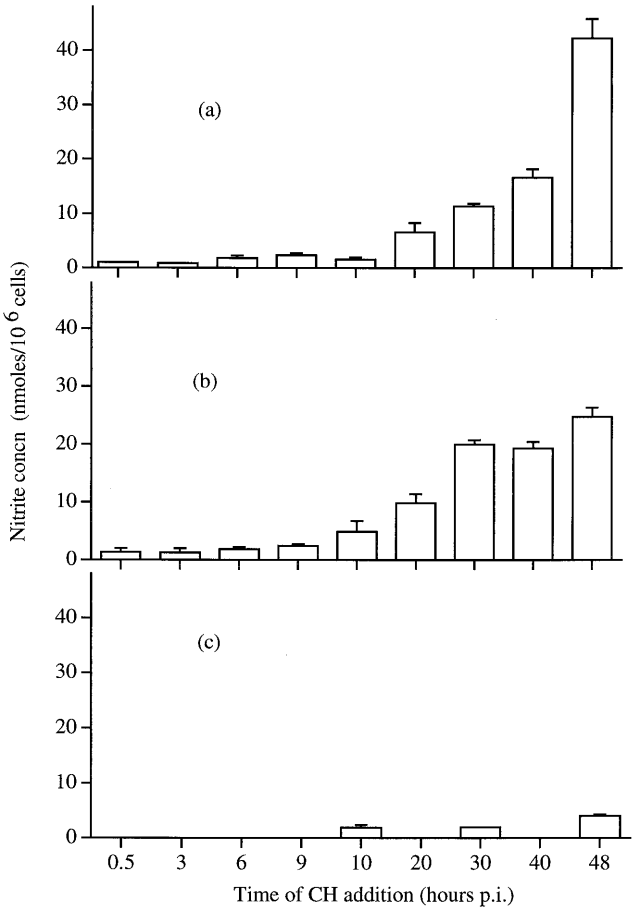

FIG. 2. Effect of CH inhibition of McCoy cell protein synthesis on evolved nitrite concentration at 48 h p.i. CH was added at 1 μg/ml to DK20-infected (a), 434-infected (b), or mock-infected (c) McCoy cell cultures at intervals p.i. Error bars represent standard deviations of triplicate assays.

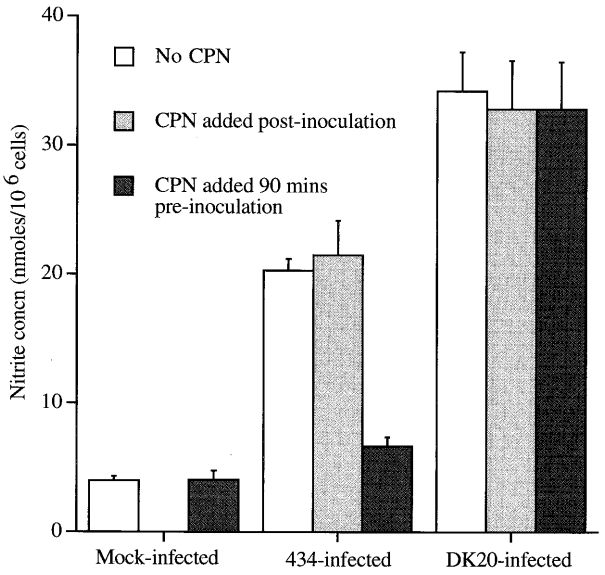

FIG. 3. Effect of CPN inhibition of chlamydial protein synthesis on nitrite production. CPN (100 μg/ml) was added to infected or mock-infected monolayers either immediately p.i. or 90 min prior to inoculation. Levels of nitrite produced as a result of chlamydial challenge were assayed at 48 h p.i. Error bars represent standard deviations of triplicate assays.

but nitrite levels were not above those in mock-infected cultures (data not shown).

Increased nitrite levels were first detectable around 10 h p.i. with both strain 434- and DK20-infected cells. Nitrite levels continued to rise throughout the infectious cycle of the organism (Fig. 1b). This continuous production (as with every other documented example of NO production) was dependent upon L-arginine. Removal of all 13 CMEM amino acids or arginine alone from culture medium abolished nitrite production in all infected cells (data not shown). In addition, MLA, a specific competitive inhibitor of NOS activity (27) reduced the nitrite response in a dose-dependent manner (Fig. 1c). Inhibition of nitrite production was complete at MLA concentrations of 400 μM and above. These results demonstrate the production of NO (detected as nitrite) by the enzyme NOS in mouse but not human cells in response to infection by *C. trachomatis* in the absence of added cytokines.

Role of cell metabolism in NO generation. Addition of CH at intervals throughout the infectious cycle resulted in decreased production of NO to the point of complete abolition of the response when added immediately p.i. (Fig. 2). Thus, production of NO was dependent upon host cell protein synthesis. The effects of CH and MLA combined are consistent with continuous synthesis of the inducible form of NOS in the generation of this response following its early induction by chlamydial infection.

The addition of CPN to infected monolayers immediately p.i. had no effect on NO production from McCoy cells infected with either strain 434 (biovar LGV) or DK20 (biovar trachoma) (Fig. 3). However, incubation of strain 434-infected monolayers with CPN from 90 min prior to inoculation to 48 h p.i. (the time of assay) resulted in a significant decrease in NO generation. No similar effect was seen with DK20-infected monolayers (Fig. 3). These results suggest that a very early event in strain 434 metabolism is essential for eliciting a NO response but that DK20 metabolism is not required. Inactivation of both strains by heat treatment (60°C for 15 min) reduced nitrite production to that of mock-infected cultures (data not shown).

Inhibition of NO production with antibodies to putative inducing factors. Previous studies in other systems have identified a number of cellular factors important in the induction of NOS by pathogen infection. The two most common of these factors are IFN-γ and LPS. Studies were therefore undertaken to assess the potential involvement of IFN and LPS in the induction of NOS in the McCoy cell system.

To test for the production of IFN by *Chlamydia*-infected McCoy cells, bioassays were carried out as described on culture medium at various times p.i. Significant amounts of IFN appeared continuously throughout the life cycle of the pathogen beginning at around 6 to 8 h p.i. (Fig. 4). Complete inhibition of the antiviral activity of the produced IFN was achieved with antibodies to IFN-α/β (1/100 dilution), thus identifying the produced IFN as IFN-α/β. No such IFN production was identified in HeLa cells as a result of chlamydial infection.

The role of this endogenously produced cytokine in the generation of the reported NO response was evaluated with anti-IFN antibodies. Inhibition of IFN activity with neutralizing antibody added to cultures decreased the levels of nitrite produced in a concentration-dependent manner (Fig. 5). However, the nitrite response was never completely inhibited, even at the antibody dilutions known to completely inhibit IFN activity (1/100). Antibody treatment of infected cells had no

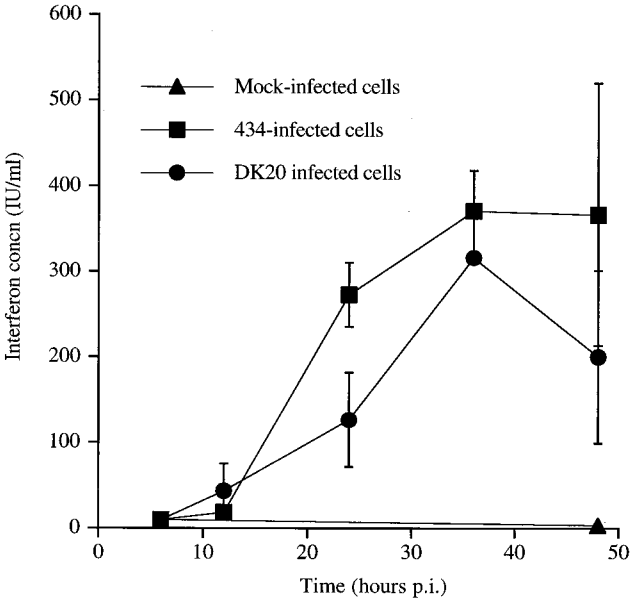

FIG. 4. IFN production by mock-infected, 434-infected, or DK20-infected McCoy cells. Error bars represent standard deviations of triplicate assays.

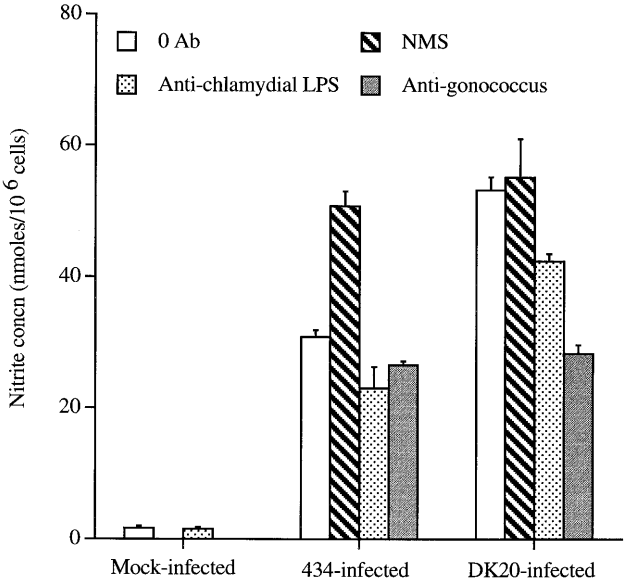

FIG. 6. Effect of antibody to LPS upon nitrite production by infected McCoy cell cultures. Chlamydiae were treated with antibody for 10 min with mixing at 37°C prior to inoculation onto McCoy cells. Nitrite levels were assessed at 48 h p.i. Normal (nonimmune) mouse serum (NMS) was used as an irrelevant antibody control. Error bars represent standard deviations of triplicate assays.

significant effect on the infectivity of the chlamydiae ($P < 0.05$).

The role of LPS in induction of NOS was evaluated by pretreatment of chlamydiae with two sources of anti-LPS antibody. Both resulted in a significant reduction in the levels of evolved nitrite from infected McCoy cells (Fig. 6) ($P < 0.05$). No effect on infection as a result of the antibody treatments was seen.

Role of NO in the control of chlamydial infection. The ability of MLA to inhibit NO production (Fig. 1c) was exploited to assess the role of NO as an anti-*Chlamydia* effector in McCoy cells. However, no consistent modulation of infection was observed when MLA (1 mM) was present p.i. in three experimental comparisons (briefly, no alteration was found for strain 434 or DK20 in one and two experiments, respectively, and DK20 was reduced by 47% in one experiment while strain 434 infectivity was enhanced in two experiments by 93 and 29%, respectively). In parallel experiments, the presence of CH consistently enhanced infectivity (94.5% for strain 434 and 320% for strain DK20). Similarly variable effects of MLA were observed on the growth of chlamydiae in HeLa cells, which functioned as control cultures since they produced neither IFN-α/β nor nitrite during infection (see above). In one experiment, MLA treatment was without effect on infectivity of either strain; in a second experiment, infectivity of both strains was decreased by about 30%. CH treatment consistently enhanced strain infectivity (109% for 434; 180% for DK20).

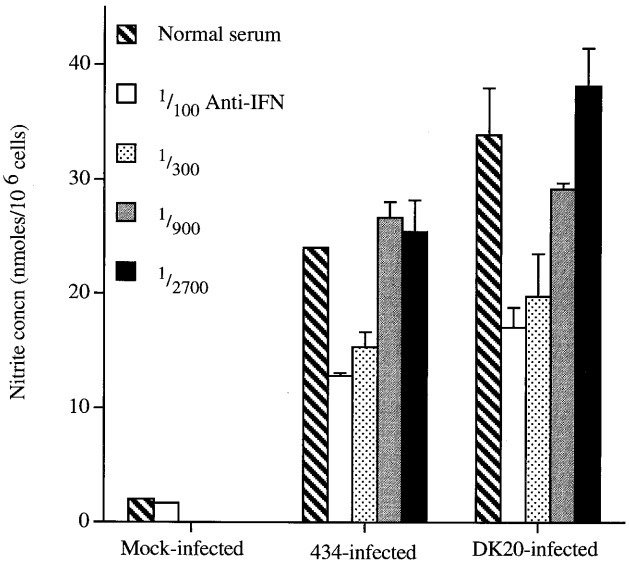

FIG. 5. Effect of antibody to IFN-α/β upon nitrite production by infected and mock-infected cultures. Antibody dilutions were added to cultures immediately p.i., and nitrite levels were assayed at 48 h p.i. Normal (nonimmune) mouse serum was used as an irrelevant antibody control. Error bars represent standard deviations of triplicate assays.

DISCUSSION

Our data demonstrate significant production of IFN-α/β from murine fibroblasts in response to chlamydial infection alone, in agreement with previous work with both mouse L cells and primary mouse fibroblasts (13, 23). However, we failed to detect IFN production from infected HeLa cells, in conflict with the findings of Jenkins and Lu (16). The reason for the discrepancy is unclear, although in these early studies the *Chlamydia*-induced IFN was characterized only partially and not identified categorically.

NO production by *Chlamydia*-infected L cells appears to function as an antiparasite defense mechanism (21). Here we demonstrate a significant NO response from murine fibroblasts (McCoy cells) following challenge with either a biovar trachoma or LGV strain of *C. trachomatis* in the absence of exogenous cytokine. The extent of the response depended on the dose of chlamydiae applied and on continuous host protein synthesis.

NO production was not detected during chlamydial infection of one canine or three different human cell lines, consistent with recent research where evidence for high-level human NO production is only beginning to emerge (3, 30, 33, 48). The reasons for species response variation are unclear but may depend on different levels of essential cofactors in human and rodent cells (38) or the requirement of human cells for very different and as-yet-unidentified cytokine patterns for stimulation (10).

Interference with the NO response by the eukaryotic protein synthesis inhibitor CH or the NOS inhibitor MLA indicates a role for the inducible isoform of the enzyme NOS (24). However, given the absence of exogenous cytokine, it may be questioned whether chlamydial metabolism could contribute to NO production. This would seem to be ruled out by the absence of nitrite production in infected cultures of human cells and unlikely given the complete suppression of nitrite by early CH addition (Fig. 2a and b) and lack of suppression of inhibition of chlamydial metabolism by CPN treatment p.i. (Fig. 3).

Antibodies to IFN or LPS significantly reduced (but did not abolish) levels of nitrite secreted from infected cells, suggesting that each is involved in induction of the inducible isoform of NOS but neither is the sole inducing factor. A similar involvement of endogenous cytokines in the induction of the antichlamydial enzyme indoleamine 2,3-dioxygenase has been observed during infection of human macrophages (28).

LPS is considered to be bioactive once released from bacteria during division when the lipid A portion becomes free (35). Although LPS may be released from the chlamydial surface in the presence of certain anti-LPS antibodies (2), it is unclear whether the antibody-neutralizing step occurred at an extracellular stage or following LPS release from the chlamydial envelope once organisms were intracellular.

When cultures were treated p.i. with CPN to inhibit chlamydial metabolism, there was no effect on nitrite production, although CPN treatment prior to inoculation significantly decreased nitrite secretion with strain 434 (but not DK20), thus implicating early metabolism of strain 434 in NOS induction. The findings may reflect intrinsic differences between members of the LGV (434) and trachoma (DK20) biovars. A possible explanation of the necessity for strain 434 metabolism depends on LPS by way of a mechanism relating to differences in intracellular fate. Strain 434 shows relatively low levels of association with the lysosome compartment during infection (32) relative to that of DK20 (fivefold greater with approximately 50% of internalized organism associated) (unpublished observations), and lysosomal degradation of organisms may provide a source of DK20 LPS for NOS induction which would be CPN insensitive. In addition, up to a 10-times-higher dose of strain DK20 than 434 was required in these experiments to provide comparable numbers of inclusion-containing cells; hence, the intracellular burden of LPS released upon DK20 internalization would be very much greater. For strain 434, with its lower inoculum and lysosomal association, a need for metabolism during the early phase of transformation may be required to provide a sufficient LPS stimulus for NOS induction. The recent findings of Ingalls et al. (15) underline the central role of chlamydial LPS in induction of inflammatory cytokines in vivo.

Whether the IFN or NO restricts infection in this culture model is open to question. Early reports have suggested that IFN-α/β inhibits chlamydial growth although host cells were treated with IFN prior to or concurrently with inoculation (11, 12, 17). Here, inhibition of endogenously produced IFN-α/β activity with antibody had no effect on the level of primary infection (inclusion number); the effect on progeny yield was not assessed. Previous work suggests that IFN treatment should prevent secondary infections, but these are a common feature of LGV-infected cultures; released IFN may restrict trachoma strains such as DK20.

When nitrite production from infected McCoy cells was abolished with the arginine analog MLA, we observed no consistent effect on the growth of either chlamydial strain. Equally inconsistent responses were found with MLA treatment of infected HeLa cells, where no evidence of NO synthesis was obtained. Variable responses to MLA may be due more to effects on other cellular processes than to NO induction. Treatment of host cells with MLA could reduce uptake of amino acids by the Y+ transporter (arginine, ornithine, and lysine), limiting supplies to the chlamydial inclusion. NOS inhibitors potently inhibit lysine influx into erythrocytes while exerting lesser effects on arginine and ornithine transport (9). The effects are difficult to assess, although chlamydiae are sensitive to changes in the nutritional environment (6). MLA itself may be weakly toxic to the growth of chlamydiae, as shown with mycobacteria (8). A number of difficulties are therefore inherent in the use of MLA to assess the effect of NO on chlamydial growth. Nevertheless, Mayer et al. (21) identified antichlamydial activity for IFN-γ-induced NO which was significantly reduced by MLA. The levels of NO produced were higher with IFN-γ treatment (four times greater than reported here), and there may be a threshold concentration of NO above which the molecule is antichlamydial. Infection alone would seem incapable of achieving a sufficient output of NO. IFN-γ treatment may result in the production of other cellular factors which, with NO, are antichlamydial via either synergistic effects or production of novel antimicrobial molecules. Interactions of NO and superoxide produce peroxynitrite, suggested to account for the toxic effects of NO (1).

Alternatively, NO may act indirectly in antichlamydial defense mechanisms. Szalay et al. (44) demonstrated inhibition of listerial growth in murine macrophages and hepatocytes by IFN-γ treatment. However, demonstrable inhibition of NO production with MLA promoted listerial growth only in macrophages, whereas with hepatocytes, induction of TNF-α was detected. Control of *Legionella* multiplication in mouse lung was by endogenously produced TNF-α and mediated, at least in part, by NO (4). Such a role for NO may be important in our system, although the effects of induced NO production may be apparent only in vivo, where NO from infected mucosal cells could stimulate production of antichlamydial cytokines from neighboring phagocytes.

Despite the lack of evidence for NO as an antichlamydial factor in HeLa and McCoy cells, the effects of CH (increasing infected cell numbers in both McCoy and HeLa cultures) leave open the possibility that as-yet-unidentified host defense factors play a role in the cell response to infection with this intracellular parasite.

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