ELSEVIER

Immunology Letters 68 (1999) 213–217

Immunology Letters

Interferon-γ mediated immune effector mechanisms against *Bordetella pertussis*

Bernard P. Mahon [a,*], Kingston H.G. Mills [b]

[a] *Mucosal Immunology Laboratory, Biology Department, National University of Ireland, Maynooth, Kildare, Ireland*
[b] *Infection/Immunity Group, Biology Department, National University of Ireland, Maynooth, Kildare, Ireland*

Received 4 February 1999; accepted 15 February 1999

Abstract

The role of IFN-γ in reducing the intracellular load of *Bordetella pertussis* in murine macrophages in vitro has been examined. The results demonstrate that exposure to IFN-γ can reduce bacterial load in viable macrophages and that this is associated with production of nitric oxide (NO). These observations provide a mechanism by which IFN-γ may mediate its antimicrobial effect and support an important role for activated alveolar macrophages in the elimination of *B. pertussis* from the respiratory tract. Using intracellular iron chelation, it is shown that intracellular survival of *B. pertussis* is dependant on iron availability and suggest that iron restriction may be an important mechanism by which IFN-γ influences bacterial survival within mouse macrophages. It is also shown that IFN-γ may mediate its effect through NO independent mechanisms and that *B. pertussis* is sensitive to agents that stimulate the respiratory burst. Finally, it is shown that the concentration of L-tryptophan may be a limiting step in the intracellular survival of *B. pertussis* and that the induction of tryptophan degrading enzymes may be an additional mechanism through which IFN-γ exerts its antimicrobial effects against *B. pertussis*.

Keywords: Pertussis; Interferon-γ; Nitric oxide; Iron; Indoleamine 2,3-dioxygenase

1. Introduction

Infection of the respiratory tract by the gram-negative bacterium *Bordetella pertussis* results in whooping cough, an important cause of morbidity and mortality in human infants. It is well known that during colonization of the respiratory tract this bacterium can specifically adhere to ciliated epithelium, however, it is now clear that *B. pertussis* can exploit both extracellular and intracellular niches during infection. Persistence of *B. pertussis* within murine and rabbit alveolar macrophages has been described [1,2], and the ability of *B. pertussis* to invade and survive within human macrophages and other cell types has recently been documented by a number of groups [3–6], suggesting that intracellular localization may be an important mechanism in the disease process.

Protective immunity induced by previous infection or immunization with whole cell pertussis (Pw) and new generation acellular pertussis (Pa) vaccines can protect against whooping cough. The roles of B cells, T cells, and IFN-γ in immunity to *B. pertussis* using normal mice or gene knockout mice have previously been examined, and it was demonstrated that protective immunity involves both cell mediated and humoral mechanisms [7–11]. Adoptive transfer of $CD4^+$ Th1 cells from convalescent mice conferred protection against *B. pertussis* challenge in T cell deficient athymic mice providing evidence that T cells play a significant role in protection. Aerosol challenge of naive IFN-γ receptor knockout mice resulted in an atypical disseminated disease, demonstrating an important role for this cytokine in confining the bacterium to the respiratory tract during infection of naive animals. Furthermore, studies in wildtype mice and in $Ig^{-/-}$ mice prior to or after bacterial challenge revealed that protection early after immunization with Pa is mediated by antibody against multiple protective antigens [10]. However, the

* Corresponding author. Tel.: +353-1-7083835; fax: +353-1-7083845.
E-mail address: bpmahon@may.ie (B.P. Mahon)

0165-2478/99/$ - see front matter
PII: S0165-2478(99)00070-X

more complete protection conferred by previous infection or following immunization with Pw reflects the induction of Th1 cells [9]. In the present study the in vitro mechanisms by which IFN-γ may mediate these effects have been examined.

2. Materials and methods

2.1. Growth of B. pertussis

B. pertussis W28 phase I were grown as previously described [10–12], all cultures were in exponential-phase growth at the time of use.

2.2. B. pertussis infection of murine macrophages

The mouse macrophage-like cell line J774 in exponential growth phase was used in all studies. Cells were washed out of culture medium and resuspended by agitation in a small volume of *B. pertussis* transport medium containing freshly prepared bacteria at a ratio of 10 bacteria per macrophage, a ratio which did not induce macrophage apoptosis. After a 1-h incubation, cells were washed three times in antibiotic-free RPMI 1640 (Gibco, Paisley, UK) to remove unbound bacteria. Cells were then incubated for 40 min at 37°C in a Gentamycin (50 μg/ml)/Polymyxin B (20 μg/ml) solution in RPMI 1640, which killed extracellular bacteria (5 $\log_{10}$ CFU reduction) but was found to have limited effect on numbers of intracellular bacteria (0.5 $\log_{10}$ CFU reduction). Infected macrophages were then washed a further six times before culturing for 48 h at 37°C, 5% CO_2. In test cultures different concentrations of recombinant murine IFN-γ (a kind gift from A. Meagher, NIBSC, Herts, UK) were added to macrophage cultures. At 48 h cells were harvested and the bacterial load assessed. Macrophages remained $>$ 80% viable using this protocol except when high concentrations of IFN-γ were used ($>$ 400 ng/ml), this was important as loss of macrophage viability can lead to artefacts in bactericidal assays [10]. Macrophage viability was determined by acridine orange/ethidium bromide viable cell counting from sample wells.

2.3. NO_2^- assay

Cell free supernatants from macrophage cultures were sampled 24 h after establishment of cultures using the Greiss assay as previously described [13]

2.4. Evaluation of arginine analogues on the bactericidal activity of IFN-γ

Infected and uninfected macrophage cultures were also performed in the presence of 100 μM *N*-monomethyl-L-arginine (L-NMMA) or *N*-monomethyl-D-arginine (D-NMMA) (Calbiochem, Nottingham, UK) added to cultures at 0, 6 and 12 h after infection.

2.5. Evaluation of L-tryptophan, and iron chelation on the bactericidal activity of IFN-γ

Infected and uninfected macrophage cultures were also established in the presence of either 1 mM L-tryptophan (Sigma, Dorset, UK); or 50 μM deferoxamine mesylate (Sigma) an intracellular iron chelator [14]. Using these concentrations no decrease was seen in macrophage viability over the course of the experiment.

2.6. Induction of the respiratory burst by methylene blue

Methylene blue (Sigma), an agent that induces the respiratory burst [15], was added to selected cultures 1 h after infection to a concentration of 10^{-4} M, a concentration which did not influence macrophage viability.

2.7. Assessment of bacterial load

Bacterial load was assessed by removal of 100 μl of lysed macrophages from individual cultures. These were spotted in triplicate onto each of three Bordet–Gengou agar plates and the number of CFU was estimated after 5 days of incubation. Results are reported as the mean viable *B. pertussis* from at least three samples per experimental group, and are expressed as CFU per viable macrophage. This avoided potential artefacts due to loss of macrophage viability during the course of bactericidal assays [16].

3. Results

3.1. IFN-γ reduces macrophage bacterial load in vitro.

The mechanisms underlying the established role of IFN-γ in limiting *B. pertussis* infection were investigated in vitro. The murine macrophage-like cell line J774 was infected with virulent *B. pertussis* at a ratio of 10 bacteria per macrophage and exposed to varying concentrations of murine IFN-γ. Fig. 1 shows that at concentrations between 3.2 and 80 ng/ml IFN-γ reduced the intracellular load of *B. pertussis* over 48 h of in vitro culture. At high concentrations (400 ng/ml and above) the bacterial load appeared to increase, however, this was accompanied by a loss of macrophage viability and presumably bacterial release into the culture supernatant.

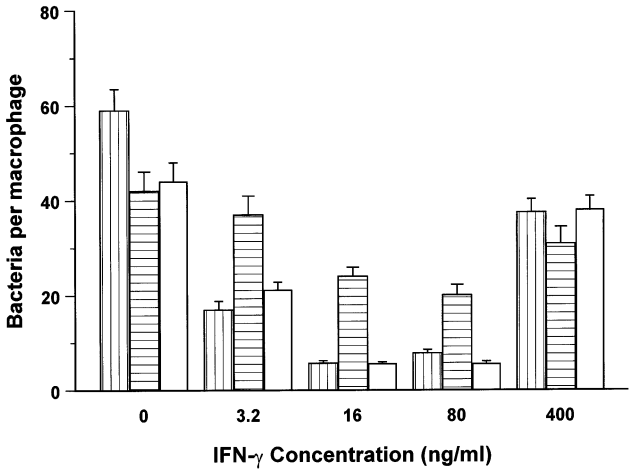

Fig. 1. IFN-γ reduces macrophage bacterial load in vitro. *Bordetella pertussis*-infected macrophages were cultured with recombinant murine IFN-γ at concentrations ranging between 0 and 400 ng/ml, in the absence (vertical shading) or presence of 100 μM *N*-monomethyl-L-arginine (L-NMMA) (horizontal shading) or 100 μM *N*-monomethyl-D-arginine (D-NMMA) (open bars).

3.2. IFN-γ induces nitric oxide (NO) release from B. pertussis infected cells in vitro

Exposure of *B. pertussis* infected macrophages to IFN-γ resulted in the production of NO as measured by NO_2^- release (Fig. 2), which was suppressed by addition of the competitive inhibitor L-NMMA, but not by D-NMMA [17]. Addition of these compounds to cultures of infected macrophages in the presence or absence of IFN-γ (Fig. 1) showed that D-NMMA had

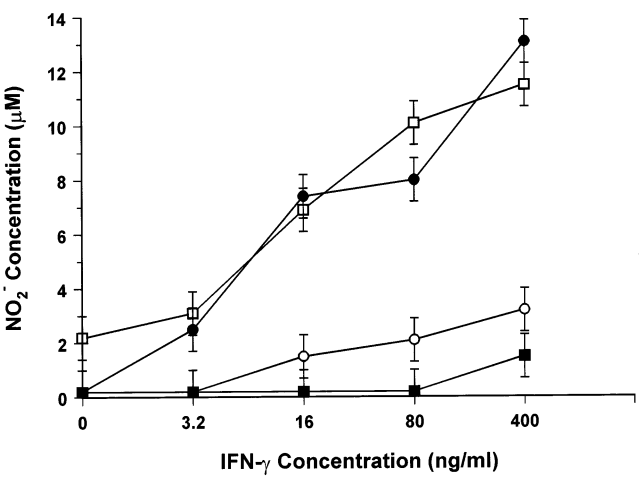

Fig. 2. IFN-γ induces nitric oxide (NO) release from *Bordetella pertussis*-infected cells in vitro. NO_2^- was measured by Greiss assay from 24 h supernatants sampled from *B. pertussis*-infected macrophages, cultured with recombinant murine IFN-γ at concentrations ranging between 0 and 400 ng/ml (open squares). Similar samples were taken from non-infected control cultures (solid squares) or infected cultures incubated in the presence of 100 μM *N*-monomethyl-L-arginine (L-NMMA) (open cicles) or 100 μM *N*-monomethyl-D-arginine (D-NMMA) (solid circles).

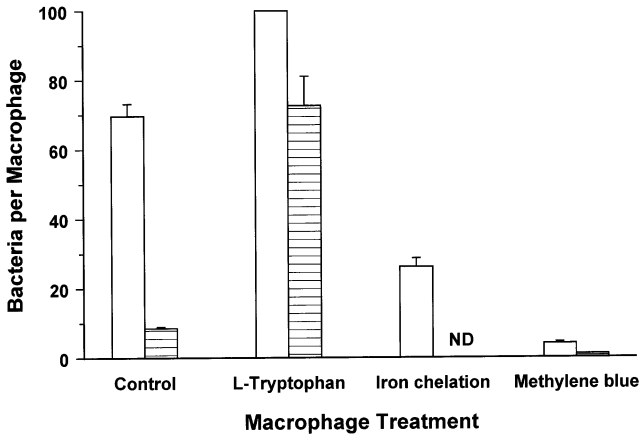

Fig. 3. IFN-γ acts through more than one mechanism. *Bordetella pertussis*-infected macrophages were cultured in the absence (open bars) or presence (horizontal shading) of 50 ng/ml recombinant murine IFN-γ. To determine the mechanisms of IFN-γ action macrophages were treated with medium (control), or with exogenous 1 mM L-tryptophan, the intracellular iron chelator deferoxamine mesylate (iron chelation) or methylene blue an inducer of the respiratory burst. ND, not determined.

little or no effect on bacterial load. Incorporation of L-NMMA resulted in an increase in bacterial load compared to controls, however, it did not fully suppress the effect of IFN-γ. These results suggest that while the production of NO may contribute to *B. pertussis* killing there are likely to be other bactericidal mechanisms in operation.

3.3. IFN-γ may also act through NO-independent mechanisms.

Treatment of macrophages with methylene blue, an electron carrier and an inhibitor of guanylate cyclase [15], enhanced the killing of intracellular *B. pertussis* (Fig. 3). However, addition of catalase, superoxide dismutase or mannitol, inhibitors of the effects of superoxide anions, hydrogen peroxide or hydroxyl radicals had no effect on bacterial recovery (data not shown). Treatment of mouse macrophages with the intracellular iron chelator, deferoxamine mesylate, inhibited the intracellular growth of *B. pertussis* (Fig. 3). Taken together these results indicate that the intracellular survival of *B. pertussis* is dependant on iron availability and suggest that iron restriction may be an important mechanism by which IFN-γ influences bacterial survival within mouse macrophages. IFN-γ is also known to induce intracellular L-tryptophan depletion by activation of indoleamine 2,3-dioxygenase [18,19]. Addition of L-tryptophan to infected macrophage cultures in the absence of IFN-γ, resulted in an increased bacterial load compared to controls, although this was reduced in the presence of IFN-γ. This suggests that the concentration of L-tryptophan may be a limiting step in

the intracellular survival of *B. pertussis* and that this may be an additional mechanism through which IFN-γ exerts its antimicrobial effect.

4. Discussion

Like most successful bacterial pathogens, *B. pertussis* appears to have adapted adversarial strategies to delay, overcome or evade most of the immune effector mechanisms deployed by the host (Table 1). Previously the authors and others have described an important role for IFN-γ in the immune response to pertussis [7–11,20], in the present study the potential mechanism by which these effects may be mediated in vitro have been examined. The demonstration that IFN-γ reduced the intracellular load of *B. pertussis* over 48 h of in vitro culture is consistent with findings by others who have described an antimicrobial effect for IFN-γ [20–23]. It has been shown that this effect was associated with the production of NO, a potent antibacterial agent, as measured by nitrite production. The results using an intracellular iron chelator suggest that the intracellular survival of *B. pertussis* is dependant on iron availability. It is known that reactive nitrogen intermediates cause intracellular iron loss by inhibiting various iron-dependent enzymes, it may be that iron restriction is an important mechanism by which IFN-γ influences bacterial survival within murine macrophages. Whilst the association between the reduction of bacterial load and NO in this study, and between NO and Pw by others [24,25], suggests an antibacterial role for reactive nitrogen intermediates, other IFN-γ induced mechanisms are also likely to be in operation. Incorporation of L-NMMA, a competitive inhibitor for NO production [17], but not the analogue D-NMMA, dramatically reduced nitrite concentrations in infected macrophage cultures but did not completely abolish the bactericidal effects of IFN-γ. Furthermore production of high concentrations of NO in response to high levels of IFN-γ, resulted in reduced viability of cultured macrophages. This suggests that NO may be inducing macrophage death. Interestingly recent reports have implicated NO in *B. pertussis*-mediated pathology to the respiratory epithelium [26].

Table 1
Probable evasion strategies employed by *Bordetella pertussis*

Immune effector mechanism	Probable evasion strategy	References
Muco-ciliary clearance	Attachment/colonization factors, destruction of ciliated cells by tracheal cytotoxin	[28,29]
Complement (classical/alternative)	BRK-A and BRK-B	[30,31]
Phagocyte function	Avoidance of respiratory burst, neutrophil intoxication	[32–34]
B cells/Antibody	Intracellular niche, PT mediated immunomodulation	[2–6,35–37]
Cell mediated immunity	Altered costimulation, migration and antigen presentation	[5,38,39]

Methylene blue, an electron carrier and an inhibitor of guanylate cyclase, known to stimulate the respiratory burst [15] enhanced the killing of intracellular *B. pertussis*. However, addition of inhibitors of the effects of superoxide anions, hydrogen peroxide or hydroxyl radicals, important mediators of the respiratory burst, had no effect on bacterial recovery. Another mechanism through which IFN-γ can mediate its antimicrobial effect is by activation of indoleamine 2,3-dioxygenase which depletes intracellular L-tryptophan [18,19]. Addition of L-tryptophan to *B. pertussis*-infected cultures resulted in enhanced bacterial load indicating that the concentration of L-tryptophan may be a limiting step in the intracellular survival of *B. pertussis*. This may be an additional mechanism through which IFN-γ exerts its antimicrobial effects against this pathogen.

Recent studies have shown that depletion of alveolar macrophages results in increased numbers of *B. pertussis* in the lungs during the early phase of infection [27] and a critical role for IFN-γ during this period has previously been described [11]. The results described herein, provide a mechanism by which IFN-γ may mediate this antimicrobial effect and support an important role for activated alveolar macrophages in the elimination of *B. pertussis* from the respiratory tract.

Acknowledgements

The authors acknowledge the technical help of Ms Fiona Griffin. This work was supported by grants from the Wellcome Trust.

References

[1] C. Cheers, D.F. Gray, Immunology 17 (1969) 875–887.
[2] E. Saukonen, C. Cabellos, M. Burroughs, S. Prasad, E. Tuomanen, J. Exp. Med. 173 (1991) 1143–1149.
[3] H.R. Masure, Microb. Pathog. 14 (1992) 253–260.
[4] K. Bromberg, G. Tannis, P. Steiner, Infect. Immun. 59 (1991) 4715–4719.
[5] J.S. Boschwitz, J.W. Batanghari, H. Kedem, D.A. Relman, J. Infect. Dis. 176 (1997) 678–686.
[6] C.A. Ewanowich, A.R. Melton, A.A. Weiss, R.K. Sherburne, M.S. Peppler, Infect. Immun. 57 (1989) 2698–2704.
[7] A. Barnard, B.P. Mahon, J. Watkins, K. Redhead, K.H.G. Mills, Immunology 87 (1996) 372–380.

[8] K.H.G. Mills, A. Barnard, J. Watkins, K. Redhead, Infect. Immun. 61 (1993) 399–410.
[9] K. Redhead, A.L. Barnard, J. Watkins, K.H.G. Mills, Infect. Immun. 61 (1993) 3190–3198.
[10] K.H.G. Mills, M. Ryan, E. Ryan, B.P. Mahon, Infect. Immun. 66 (1998) 594–602.
[11] B.P. Mahon, B.J. Sheahan, F. Griffin, G. Murphy, K.H.G. Mills, J. Exp. Med. 186 (1997) 1843–1851.
[12] Y. Sato, K. Izumiya, H. Sato, J.L. Cowell, C.R. Manclark, Infect. Immun. 29 (1980) 261–266.
[13] L.C. Green, D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok, S.R. Tannenbaum, Anal. Biochem. 126 (1982) 131–138.
[14] J. Park, Y. Rikihisa, Infect. Immun. 60 (1992) 3504–3508.
[15] X. Jiang, B. Leonard, R. Benson, C.L. Baldwin, Cell Immunol. 151 (1993) 309–319.
[16] J. Warwick-Davies, J. Dhillon, L. O'Brien, P.W. Andrew, D.B. Lowrie, Clin. Exp. Immunol. 96 (1994) 214–217.
[17] H.W. Murray, R.F. Teitelbaum, J. Infect. Dis. 165 (1992) 513–517.
[18] S.K. Halonen, F. Chiu, L.M. Weiss, Infect. Immun. 66 (1998) 4989–4993.
[19] D. Alberati-Giani, P. Malherbe, P. Ricciardi-Castagnoli, C. Kohler, S. Denis-Donini, A.M. Cesura, J. Immunol. 159 (1997) 419–426.
[20] J. Barbic, M.F. Leef, D.L. Burns, R.D. Shahin, Infect. Immun. 65 (1997) 4904–4908.
[21] D. Torre, F. Speranza, A. Pugliese, L. Perversi, P. Marone, G. Ferrario, J. Infect. Dis. 174 (1996) 786–789.
[22] D. Torre, G. Ferrario, G. Bonetta, L. Perversi, R. Tambini, F. Speranza, FEMS Immunol. Med. Microbiol. 9 (1994) 183–188.
[23] D. Torre, A. Pugliese, F. Speranza, G.P. Fiori, L. Perversi, P. Marone, R. Tambini, J. Infect. Dis. 167 (1993) 762–765.
[24] S. Sakurai, K. Kamachi, T. Konda, N. Miyajima, M. Kohase, S. Yamamoto, Infect. Immun. 64 (1996) 1309–1313.
[25] D.K. Xing, C. Canthaboo, M.J. Corbel, Vaccine 16 (1998) 16–23.
[26] L.N. Heiss, T.A. Flak, J.R. Lancaster Jr., M.L. McDaniel, W.E. Goldman, Infect. Agents Dis. 4 (1993) 173–177.
[27] N. Mielcarek, I. Nordstrom, N. vanRooijen, F.D. Menozzi, C. Locht, J. Holmgren, Immunologist 6 (Suppl. 1) (1998) 295.
[28] E. Tuomanen, A. Weiss, J. Infect. Dis. 152 (1985) 118–125.
[29] K.E. Luker, J.L. Collier, E.W. Kolodziej, G.R. Marshall, W.E. Goldman, Proc. Natl. Acad. Sci. USA 90 (1993) 2365–2369.
[30] R.C. Fernandez, A.A. Weiss, Infect. Immun. 62 (1994) 4727–4738.
[31] R.C. Fernandez, A.A. Weiss, FEMS Microbiol. Lett. 163 (1998) 57–63.
[32] D.R. Cundell, K. Kanthakumar, G.W. Taylor, W.E. Goldman, T. Flak, P.J. Cole, R. Wilson, Infect. Immun. 62 (1994) 639–643.
[33] R. Pearson, P. Symes, M. Conboy, A.A. Weiss, E.L. Hewlett, J. Immunol. 139 (1987) 2749–2754.
[34] B.D. Meade, P.D. Kind, C.R. Manclark, Dev. Biol. Stand. 61 (1985) 63–74.
[35] A.B. Lyons, Immunol. Cell. Biol. 75 (1997) 7–12.
[36] A.S. Kong, S.I. Morse, J. Immunol. 116 (1976) 989–993.
[37] N. Mielcarek, G. Riveau, F. Remoue, R. Antoine, A. Capron, C. Locht, Nat. Biotechnol. 16 (1998) 454–457.
[38] P. McGuirk, B.P. Mahon, F. Griffin, K.H.G. Mills, Eur. J. Immunol. 28 (1998) 153–163.
[39] G.J. Spangrude, B.A. Araneo, R.A. Daynes, J. Immunol. 134 (1985) 2900–2907.