Taylor & Francis healthsciences

Mediators of Inflammation, **13**(1), 45–49 (February 2004)

Secretion of interleukin-8 by human-derived cell lines infected with *Mycobacterium bovis*

Patricia Méndez-Samperio[CA], Janet Palma-Barrios, Abraham Vázquez-Hernández and Elizabeth García-Martínez

Departamento de Inmunología, Escuela Nacional de Ciencias Biológicas, IPN, Carpio y Plan de Ayala, México, D.F. 11340 México

[CA]Corresponding author
Tel: +52 57 29 6000, ext. 62499
Fax: +52 53 96 35 03
E-mail: pmendezs@bios.encb.ipn.mx

***Background*: The variable efficacy of bacillus Calmette-Guérin (*Mycobacterium bovis* BCG) in protecting humans against tuberculosis has prompted a search for the mechanisms through which BCG induces chemokines. In this study, our experiments were designed to determine the role of the transcription factor nuclear factor-κB (NF-κB) and intracellular calcium in the production of interleukin (IL)-8, a main chemotactic factor, by human-derived monocytic cell line U937 and by a human epithelial HEp-2 cell line infected with *M. bovis* BCG.**

***Methods*: The concentrations of IL-8 in culture supernatants of U937 cells or HEp-2 cells infected with *M. bovis* BCG were determined by enzyme-linked immunosorbent assay. We used sulfasalazine and curcumin, which are well-described inhibitors of NF-κB activity, and we used ethylenediamine tetraacetic acid to deplete extracellular Ca^{2+} or used the cell-permeable agent 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester to chelate releasable intracellular stores of Ca^{2+} in order to investigate the mechanisms through which *M. bovis* BCG induces IL-8 secretion in our system.**

***Results*: The enzyme-linked immunosorbent assay showed that IL-8 protein secretion was elevated in *M. bovis*-infected cell lines. This effect was statistically significant ($p < 0.01$). When calcium influx was suppressed in *M. bovis*-infected cell lines, IL-8 secretion was inhibited. Notably, specific inhibitors of NF-κB (sulfasalazine and curcumin) inhibited *M. bovis*-induced IL-8 secretion from U937 cells or HEp-2 cells.**

***Conclusions*: Collectively, these results indicate that activation of NF-κB is an important signal transduction pathway in *M. bovis*-induced IL-8 secretion in monocytic or epithelial cells. Furthermore, the results showed that calcium influx had a direct effect on IL-8 secretion in U937 cells or HEp-2 cells infected with *M. bovis*.**

Key words: Bacillus Calmette-Guérin, Interleukin-8, *Mycobacterium bovis*, Nuclear factor-κB

Introduction

Human tuberculosis is a major health problem world-wide and is responsible for an estimated 1.9 million deaths annually.[1] It is predominantly caused by *Mycobacterium tuberculosis.* Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, has been widely used for vaccination against human tuberculosis despite controversy over its protective efficacy.[2] The emergence of multidrug-resistant strains of *M. tuberculosis*, the variable efficacy of the current vaccine, and the human immunodeficiency virus pandemic have all contributed to a growing global tuberculosis problem.[3] In most healthy persons, acquired immunity, mediated by T cells, controls but does not eradicate *M. tuberculosis* infection.[4,5] The early migration of activated monocytes/macrophages to the site of mycobacterial inflammations is an important step in the control of such infection. Chemokine functions in leukocyte migration and organization of inflammatory reactions are recognized.[6–8] Mycobacterial infection frequently induces increased expression of chemokines, including the CC chemokine subfamily members and the CXC chemokine subfamily members, such as interleukin (IL)-8 (CXCL-8).[9–11] However, the molecular mechanism (s) responsible for secretion of IL-8 by *M. bovis* BCG in cell lines are not understood. Although recent studies have shown that phagocytosis of mycobacteria by cells is usually accompanied by activation of the transcription factor nuclear factor-κB (NF-κB),[12–14] little is known about

ISSN 0962-9351 print/ISSN 1466-1861 online/04/10045-05 © 2004 Taylor & Francis Ltd
DOI: 10.1080/09629350410001664743

the effect of the transcription factor NF-κB on *M. bovis*-induced IL-8 secretion by monocytic cells or epithelial cells. In the present study, we used specific inhibitors to investigate the effect of the transcription factor NF-κB in IL-8 production by human monocytic cell line U937 or by human epithelial cell line HEp-2 infected with *M. bovis*. In addition, we investigated whether depletion of extracellular calcium reduced the secretion of IL-8 in these cells.

Materials and methods

Reagents

M. bovis BCG, Danish Strain 1331, was provided by Dr J. Ruiz-Puente (Birmex, México). Sulfasalazine, curcumin, ethylenediamine tetraacetic acid (EDTA), and 1,2-bis (2-aminophenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra (acetoxymethyl) ester (BAPTA/AM) were purchased from Sigma-Aldrich (St Louis, MO, USA).

In vitro infection of cell lines

The human epithelial HEp-2 cell line and the human monocytic cell line U937 were originally acquired from the American Type Culture Collection (Rockville, MD, USA). The HEp-2 cells were maintained in minimum essential medium Eagle with 2 mM of L-glutamine, 1 mM of sodium pyruvate, 0.1 mM of non-essential amino acids, and Earle's BSS adjusted to contain 1.5 g/l of sodium bicarbonate and 10% heat-inactivated foetal bovine serum (Gibco-BRL, Rockville, MD, USA). U937 cells were maintained in complete medium (RPMI 1640 [Gibco-BRL] with 10% heat-inactivated foetal bovine serum, sodium pyruvate, non-essential amino acids, 2 mM of L-glutamine, penicillin G [100 IU/ml], and streptomycin [100 μg/ml]). The U937 cells were treated with 4 nM of phorbol myristate acetate (Sigma) for 72 h to induce differentiation into macrophage-like cells and were washed with phosphate-buffered saline three times. Differentiated U937 cells or HEp-2 cells were incubated overnight at 37°C in a humidified atmosphere containing 5% CO_2. After the overnight incubation, cells (10^5/ml) were infected with mycobacteria using an opsonized bacteria-to-cell ratio of 3:1. Control cultures with no mycobacteria were always included.

Inhibitor treatment

To observe the effect of NF-κB inhibitors and agents to chelate releasable intracellular stores of Ca^{2+} on the secretion of IL-8 by human-derived monocytic cell line U937 or by a human epithelial cell line, 2 mM of sulfasalazine, 20 μM of curcumin, 3 mM of EDTA, or 30 μM of BAPTA/AM was added into different cultures 1 h prior infection with *M. bovis*. Following further culturing for 48 h, the supernatants from U937 cells or HEp-2 cells were collected and analysed.

Cytokine assay

IL-8 levels were measured in supernatants from U937 cells or HEp-2 cells infected with *M. bovis* BCG. Supernatants were harvested from cultures of U937 cells infected with mycobacteria and frozen at − 20°C before they were assayed. The frozen supernatants were thawed at room temperature, and chemokine levels were measured with commercial IL-8 assay kits (Amersham Life Science, Aylesbury, UK), according to the manufacturer's instructions.

Statistical method

For determination of statistical significance, analysis of the results between control and experimental groups was performed using the Statgraphics program (Statistical Graphics Co., Rockville, MD, USA). Statistical significance of enzyme-linked immunosorbent assay (ELISA) data was analysed using Student's *t*-test.

Results

Involvement of NF-κB pathway signalling in *M. bovis*-induced IL-8 secretion from U937 cells and HEp-2 cells.

In our experiments, we used the ELISA to measure the IL-8 concentrations in culture supernatants from U937 cells after infection with *M. bovis*. As shown in Fig. 1A, IL-8 secretion in *M. bovis*-infected U937 cells was markedly increased after infection. IL-8 secretion from infected U937 cells varied with multiplicity of infection (MOI), and higher concentrations of *M. bovis* yielded similar results (data not shown). Since the IL-8 gene promoter contains a number of binding sites, including that of NF-κB,[15] we determined whether NF-κB nuclear transcription factor participates in IL-8 production by *M. bovis*-infected U937 cells. We treated cells with sulfasalazine. Results showed that, on exposure to 2 mM of sulfasalazine, IL-8 secretion was markedly downregulated in these cells (Fig. 1A).

To further examine the involvement of NF-κB in the *M. bovis*-induced IL-8 secretion by U937 cells, we treated cells with 20 mM of curcumin, another specific inhibitor of NF-κB. Results showed an interesting and consistent tendency that this concentration of curcumin could significantly inhibit the

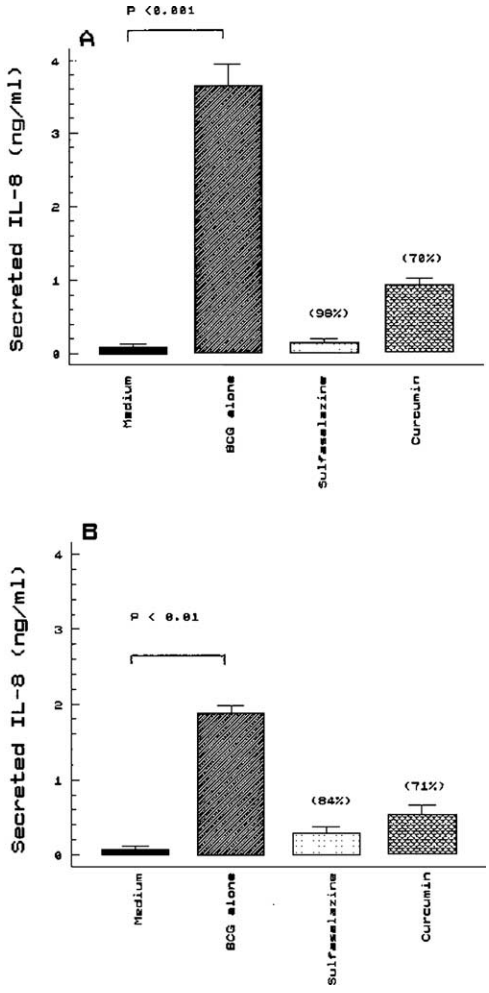

FIG. 1. Effect of NF-κB on *M. bovis*-induced IL-8 secretion from U937 cells and HEp-2 cells. U937 cells (A) or HEp-2 cells (B) were incubated with medium alone, or treated with sulfasalazine (2 mM) or curcumin (20 mM) prior to infection with *M. bovis*. Following further culturing for 48 h, the cell culture supernatants were harvested and the IL-8 protein was measured by ELISA. The mean and standard deviation of triplicate experiments are shown in each graph. Results in parentheses indicate the percentage of inhibition with respect to BCG cultures.

secretion of IL-8 by human-derived monocytic cell line U937 infected with *M. bovis* (Fig. 1A).

Since in the earliest stages of mycobacterial infection, production of IL-8 from epithelial cells might induce early recruitment of T lymphocytes, in this study parallel experiments were performed to measure IL-8 production by the human epithelial HEp-2 cell line infected with *M. bovis* (Fig. 1B). It is important to note that significant differences were observed between IL-8 secreted by epithelial cells (Fig. 1B) ($p < 0.01$) and human monocytic cells (Fig. 1A) ($p < 0.001$) infected with *M. bovis*.

Additionally, the effects of *M. bovis* on HEp-2 cells were inhibited by sulfasalazine and curcumin, two well-described inhibitors of NF-κB activity (Fig. 1B). Thus, these experiments support our aforementioned results that activation of nuclear transcription factor NF-κB may contribute to the induction of IL-8 in infection with *M. bovis*.

Calcium influx regulates *M. bovis*-induced IL-8 secretion by U937 cells or HEp-2 cells

Calcium signaling has been implicated in the activation of the NF-κB in many systems.[16] Therefore, it was considered possible that calcium influx signalling was transducing the regulation of *M. bovis*-induced IL-8 secretion. To address this, we investigated whether depletion of extracellular calcium reduced the IL-8 secretion from U937 cells infected with *M. bovis*. U937 cells were treated with 3 mM of EDTA or cultured in complete medium for 1 h prior to infection with *M. bovis* for 48 h. Indeed, U937 cells infected with *M. bovis* in the presence of EDTA (Fig. 2A) had reduced levels of IL-8 secretion. This is a finding consistent with BAPTA/AM acting as a negative regulator, since pretreatment with 30 μM of BAPTA/AM also reduces the production of IL-8 levels (Fig. 2A).

HEp-2 cells incubated in EDTA showed a decreased in the level of *M. bovis*-induced IL-8 secretion by approximately 30% compared with control cells

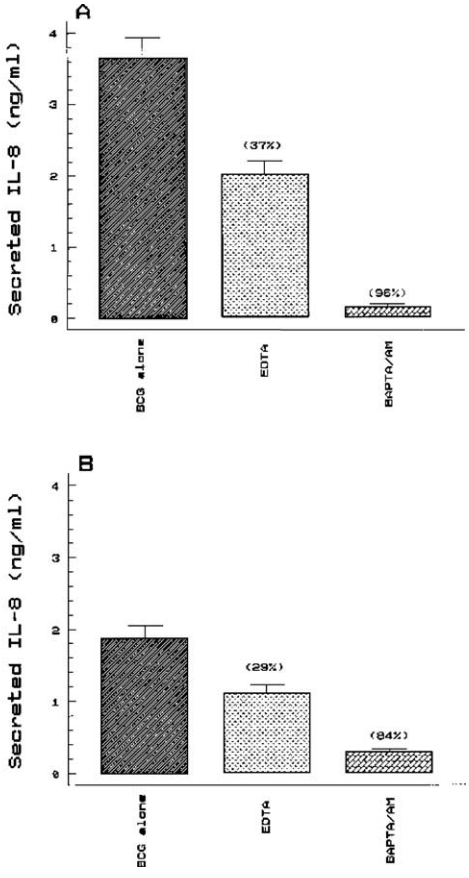

FIG. 2. Regulation of IL-8 production by calcium influx. U937 cells (A) or HEp-2 cells (B) were pretreated with 3 mM of EDTA or 30 μM of BAPTA/AM for 1 h prior to infection with *M. bovis* for 48 h. IL-8 protein levels in culture supernatants were measured by ELISA. The mean and standard deviation of triplicate experiments are shown. Results in parentheses indicate the percentage of inhibition with respect to BCG cultures.

(Fig. 2B). When epithelial cells were incubated with BAPTA/AM, the ability of *M. bovis* to induce IL-8 secretion was significantly inhibited (84%) (Fig. 2B).

Taken together, these results indicate that the levels of IL-8 production by human-derived monocytic cell line U937 and by the human epithelial HEp-2 cell line are predominantly controlled by calcium influx signals.

Discussion

IL-8 is a chemokine that has a central role in leukocyte recruitment to areas of granuloma formation in tuberculosis.[17] In addition, IL-8 is a molecule that is also chemotactic for T lymphocytes, and it has an important role in controlling cellular influx into sites of mycobacterial infection.[18] Recent studies have shown that phagocytosis of *M. tuberculosis* by monocytic cells is an important stimulus for IL-8 production.[19,20] Furthermore, elevated IL-8 concentrations in sera of patients with tuberculosis have been demonstrated.[21] In our experiments, we used the human monocytic cell line U937 as an *in vitro* host model and found that infection with BCG, currently administered as the only available vaccine for the prevention of tuberculosis in humans, led to significant IL-8 production in U937 cells.

In this paper we examined whether there was evidence for a role of NF-κB in *M. bovis*-induced IL-8 production by U937 cells. To this purpose, we describe the use of sulfasalazine and curcumin to block IL-8 production by inhibiting NF-κB activity in the human monocytic cell line U937. These inhibitors may act at several steps of the pathway between *M. bovis* infection and IL-8 production. Sulfasalazine is a well-described inhibitor that inhibits phosphorylation of IκBα,[22] whereas curcumin interacts directly with the p50 subunit of NF-κB, thus blocking degradation of IκBα.[23] We found that these specific inhibitors of NF-κB inhibited *M. bovis*-induced IL-8 secretion from U937 cells. Our data are consistent with a previous study that demonstrated that activation of NF-κB in monocytes is found in mycobacterial infection.[24]

In this study, blocking of calcium influx using EDTA induced suppression of IL-8 production following phagocytosis of *M. bovis* by U937 cells. These results were confirmed using BAPTA/AM to chelate releasable intracellular stores of Ca^{2+}. These data suggest that calcium influx selectively negatively regulates IL-8 production in our system.

Epithelial cells have been shown to secrete IL-8 following infection by pathogenic respiratory viruses.[25] A previous study has demonstrated that epithelial cells are considered the major cellular source of chemokines in the lung.[26] This study is the first to demonstrate secretion of IL-8 from human epithelial cells following infection with *M. bovis*. Thus, it is probable that although phagocytosis of *M. bovis* by human monocytic cells may initiate host immune responses by cytokine secretion, epithelial cells subsequently have a pivotal role by chemokine production. We found that IL-8 secretion in response to *M. bovis* from epithelial cells was regulated by NF-κB.

Our results with *M. bovis* are in keeping with a recent study reporting that secretion of IL-8 by *Helicobacter pylori* is regulated by NF-κB in epithelial cells.[27] In addition, our results demonstrated regulation of *M. bovis*-induced IL-8 secretion from HEp-2 cells by calcium influx.

Although little is known about the mechanisms controlling IL-8 secretion by mycobacterial infection, a recent study indicated that IL-8 secretion by *M. tuberculosis* is regulated by protein tyrosine kinases.[28] Our results do not exclude the possibility that different members of the *src* PTK family are involved in IL-8 secretion induced by *M. bovis*. Thus, experiments are currently being carried out to investigate the effect of *M. bovis* on the activation of the *src* PTK members in IL-8 secretion by epithelial cells.

In summary, this study demonstrates expression of IL-8 in monocytic and epithelial cells infected with *M. bovis*. In addition, this chemokine secretion was regulated by NF-κB and calcium influx. Further studies are needed to clarify the underlying mechanisms involved in IL-8 secretion by *M. bovis in vivo*, but understanding the molecular mechanism of the production of IL-8 by human monocytic and epithelial cells infected with *M. bovis* BCG may lead to new approaches to regulate inflammatory reaction during human tuberculosis.

ACKNOWLEDGEMENTS. This work was supported by a grant from The Coordinación General de Posgrado e Investigación (CGPI). P.M.-S. is an EDI, COFAA, and SNI fellow. The authors thank Dr J. Ruiz-Puente for providing *M. bovis*.

References

1. World Health Organization. *Tuberculosis Fact Sheet Number 104*. Available from: http://www.who.int/mediacentre/factsheets/who104/en/index.html [Online].
2. Fine PEM. Variation in protection by BCG: implications of and for heterologous immunity. 1995. *Lancet* 1995; **346**: 1339–1345.
3. Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. W.H.O. Global Surveillance and Monitoring project. *JAMA* 1999; **282**: 677–686.
4. Kaufmann SH. Immunity to intracellular bacteria. *Annu Rev Immunol* 1993; **11**: 129–163.
5. Kaufmann SH. How can immunology contribute to the control of tuberculosis? *Nat Rev Immunol* 2001; **1**: 20–30.
6. Zlotnik A, Yoshie O. Chemokines: a new classification system and their role in immunity. *Immunity* 2000; **12**: 121–127.
7. Sadek MI, Sada E, Toosi Z, Schwander SK, Rich EA. Chemokines induced by interferon of monocuclear phagocytes with mycobacteria and present in lung alveoli during active pulmonary tuberculosis. *Am J Respir Cell Mol Biol* 1998; **19**: 513–521.

8. Moser B, Loetscher P. Lymphocyte traffik control by chemokines. *Nat Immunol* 2001; **2**: 123–128.
9. Mohammed KA, Nasreen N, Ward MJ, Mubarak KK, Rodríguez-Panadero F, Anthony VB. Mycobacterium-mediated chemokine expression in pleural mesothelial cells: role of C-C-chemokines in tuberculosis pleurisy. *J Infect Dis* 1998; **178**: 1450–1456.
10. Lin YG, Gong J, Zhang M, Xue W, Barnes PF. Production of monocyte chemoattractant protein 1 in tuberculosis patients. *Infect Immun* 1998; **66**: 2319–2322.
11. Riedel DD, Kaufmann SH. Chemokine secretion by human polymorphonuclear granulocytes after stimulation with *Mycobacterium tuberculosis* and lipoarabinomannan. *Infect Immun* 1997; **65**: 4620–4623.
12. Wickremasinghe MI, Thomas LH, Friedland JS. Pulmonary epithelial cells are a source of IL-8 in the response to *Mycobacterium tuberculosis*: essential role of IL-1 from infected monocytes in a NF-κB-dependent network. *J Immunol* 1999; **163**: 3936–3947.
13. Ott M, Lovett JL, Mueller L, Verdin E. Superinduction of IL-8 in T cells by HIV-1 tat protein is mediated through NF-κB factors. *J Immunol* 1998; **160**: 2872–2880.
14. Toossi Z, Xia L, Wu M, Salvekar A. Transcriptional activation of HIV by *Mycobacterium tuberculosis* in human monocytes. *Clin Exp Immunol* 1999; **117**: 324–330.
15. Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol* 1989; **143**: 1366–1377.
16. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. *Nature* 1998; **392**: 933–936.
17. Larsen CG, Thomsen MK, Gesser B, Thomsen PD, Deleuran BW, Nowak J, *et al*. The delayed-type hypersensitivity reaction is dependent on IL-8. Inhibition of a tuberculin skin reaction by an anti-IL-8 monoclonal antibody. *J Immunol* 1995; **155**: 2151–2157.
18. Amexia C, Friedland JS. Down-regulation of interleukin-8 secretion from *Mycobacterium tuberculosis*-infected monocytes by interleukin-4 and -10 but not by interleukin-13. *Infect Immun* 2001; **69**: 2470–2476.
19. Lin Y, Zhang M, Barnes PF. Chemokine production by a human alveolar epithelial cell line in response to *Mycobacterium tuberculosis*. *Infect Immun* 1998; **66**: 1121–1126.
20. Van Diujn C, Jr. Tuberculosis in fish. *J Small Anim Pract* 1981; **22**: 391–411.
21. Juffermans NP, Verbon A, van Deventer SJ, van Deutekom H, Belisle JT, Ellis ME, *et al*. Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: a possible role for mycobacterial lipoarabinomannan. *Infect Immun* 1999; **67**: 4295–4297.
22. Wahl C, Liptay S, Adler G, Schmid RM. Sulfasalazine: a potent and specific inhibitor of nuclear factor κB. *J Clin Invest* 1998; **101**: 1163–1174.
23. Brennan P, O'Neill AJ. Inhibition of nuclear factor κB by direct modification in whole cells—mechanism of action of nordihydroguaiaritic acid, curcumin and thiol modifiers. *Biochem Pharmacol* 1998; **55**: 965–973.
24. Toossi Z, Hamilton BD, Phillips MH, Averill LE, Ellner JJ, Salvekar A. Regulation of nuclear factor-kappa B and its inhibitor I kappa B-alpha/MAD-3 in monocytes by *Mycobacterium tuberculosis* and during human tuberculosis. *J Immunol* 1997; **159**: 4109–4116.
25. Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus: induction of cytokine release and modulation of susceptibility to infection. *J Clin Invest* 1995; **96**: 549–554.
26. Zhang Y, Broser M, Cohen H, Bodkin M, Law K, Reibman J, Rom WN. Enhanced interleukin-8 release and gene expression in macrophages after exposure to *Mycobacteriun tuberculosis* and its components. *J Clin Invest* 1995; **95**: 586–592.
27. Sharma SA, Tummuru MK, Blaser MJ, Kerr LD. Activation of IL-8 gene expression by *Helicobacter pylori* is regulated by transcription factor nuclear factor-kappa B in gastric epithelial cells. *J Immunol* 1998; **160**: 2401–2407.
28. Amexia C, Friedland JS. Interleukin-8 secretion from *Mycobacterium tuberculosis*-infected monocytes is regulated by protein tyrosine kinases but not by ERK 1/2 or p38 mitogen-activated protein kinases. *Infect Immun* 2002; **70**: 4743–4746.

Received 28 November 2003
Accepted 10 December 2003