Role of nuclear factor-κB in the regulation of intercellular adhesion molecule 1 after infection of human bronchial epithelial cells by *Bordetella pertussis*

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Received 28 March 2003; received in revised form 9 June 2003; accepted 10 June 2003

Abstract

Previous work has demonstrated that infection of human bronchial epithelial cells by *Bordetella pertussis* up-regulates intercellular adhesion molecule-1 (ICAM-1) gene and protein expression. It has also been shown that interaction of the Arg-Gly-Asp (RGD) site of filamentous hemagglutinin (FHA) with host cell very late antigen (VLA)-5 (α5β1 integrin) is required for the up-regulation of epithelial ICAM-1 expression, and that pertussis toxin (PT) impairs this response. We therefore examined the molecular mechanisms leading to *B. pertussis*-induced ICAM-1 up-regulation in BEAS-2B human bronchial epithelial cells. A colorimetric nuclear factor κB (NF-κB) activation assay demonstrated that NF-κB was activated in response to infection of these cells with *B. pertussis*. This activation occurred in an FHA(RGD)-dependent manner, and was blocked by an antibody against VLA-5, implying that binding of the RGD to VLA-5 integrin is involved in NF-κB activation. Western blot analysis revealed that the activation of NF-κB by *B. pertussis* was preceded by degradation of IκBα, a major cytoplasmic inhibitor of NF-κB. Pretreatment of the BEAS-2B cells with the NF-κB inhibitors pyrrolidine dithiocarbamate (PDTC), MG-132, and SN50 resulted in a marked decrease in *B. pertussis*-induced ICAM-1 expression, implying the involvement of NF-κB in ICAM-1 expression. Purified PT abrogated both NF-κB activation and IκBα degradation. These results suggest that ligation of VLA-5 integrin by FHA induces RGD-dependent NF-κB activation, thus leading to the up-regulation of epithelial ICAM-1 expression, and that a PT-sensitive G protein may be involved in this signaling pathway.

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Keywords: *Bordetella pertussis*; Integrin; Human epithelial cells; ICAM-1; NF-κB; IκBα; Filamentous hemagglutinin; Pertussis toxin

1. Introduction

Respiratory epithelial cells interact directly with bacteria in the environment, and play a critical role in host inflammatory responses through the secretion of chemokines and the expression of adhesion molecules [1,2], including intercellular adhesion molecule 1 (ICAM-1; CD54) [3]. The expression of ICAM-1 on respiratory epithelial cells can be up-regulated in response to microbial infection, and this process is involved in leukocyte accumulation and activation at the site of infection [4–6].

*Bordetella pertussis* is the causative agent of whooping cough, a highly contagious infection of the respiratory tract in humans [7]. This Gram-negative coccobacillus initiates colonization of the respiratory tract by adhering to ciliated epithelial cells, thereby causing local tissue damage, increased mucus secretion and inflammatory cell infiltration [8]. *B. pertussis* produces a number of potent virulence factors [9,10], among which filamentous hemagglutinin (FHA) is the dominant adhesin for bronchial epithelial cells. FHA contains several distinct binding sites, including glycosaminoglycan and carbohydrate binding sites, together with an arginine-glycine-aspartate (RGD) sequence which is involved in binding to the very late antigen-5 (VLA-5; α5β1 integrin) of bronchial epithelial cells [9–11]. *B. pertussis* also produces several toxins, including pertussis toxin (PT) [12], which belongs to the A-B toxin family. PT has ADP-ribosyltransferase activity and inactivates the Gia subunit of GTP-binding heterotrimers, thereby disrupting Gi-associated signaling cascades [13]. We have recently shown that infection of human bronchial epithelial cells by *B. pertussis* up-regulates ICAM-1 mRNA and surface protein expression [14]. Furthermore, we showed that
interaction of the RGD site of FHA with host cell VLA-5 integrin is required for the up-regulation of ICAM-1 expression, whereas PT impairs epithelial cell response [14]. However, little is known about the molecular mechanisms involved in VLA-5-triggered up-regulation of ICAM-1 expression. VLA-5 integrin participates in the ‘outside-in’ signaling pathway that leads to gene activation, cellular differentiation and proliferation [15,16]. Several lines of evidence have suggested that engagement of VLA-5 integrin promotes a nuclear factor-\( \kappa \)B (NF-\( \kappa \)B)-dependent program of gene expression that is important in inflammation [17–19]. NF-\( \kappa \)B is a transcriptional activator that plays a central role in the activation of several proinflammatory genes, including ICAM-1, in human respiratory epithelial cells [20,21]. The NF-\( \kappa \)B family of proteins consists of homo- or heterodimeric subunits of the Rel family, including p50 and p65 [22]. In resting cells, NF-\( \kappa \)B is mainly localized in the cytoplasm as a complex with an inhibitory protein, I\( \kappa \)B [23]. Upon stimulation, I\( \kappa \)B is phosphorylated, ubiquinated, and subsequently degraded by the proteasome machinery [24,25]. This allows NF-\( \kappa \)B to translocate to the nucleus, bind to DNA, and transactivate the transcription of specific genes.

In this study, we found that infection of human bronchial epithelial cells by \textit{B. pertussis} induces the degradation of I\( \kappa \)B and the subsequent activation of NF-\( \kappa \)B, which in turn up-regulates ICAM-1 gene and protein expression. Ligation of VLA-5 integrin by the RGD sequence of FHA may trigger the signaling sequence for such NF-\( \kappa \)B activation, thus leading to transcriptional activation of the ICAM-1 gene. In contrast, PT impairs this epithelial response, implying a role for a PT-sensitive G protein in this signaling pathway.

2. Results

2.1. Infection of BEAS-2B cells with \textit{B. pertussis} induces NF-\( \kappa \)B activation

Our previous work has shown that infection of human bronchial epithelial cells with \textit{B. pertussis} up-regulates ICAM-1 mRNA and surface protein expression [14]. The transcription factor NF-\( \kappa \)B regulates ICAM-1 gene expression [26,27]. To determine whether infection of human bronchial epithelial cells with \textit{B. pertussis} activates NF-\( \kappa \)B, BEAS-2B cells were infected with the virulent \textit{B. pertussis} strain BP536 at a multiplicity of infection (MOI) of 100, and NF-\( \kappa \)B DNA-binding activity in nuclear extracts prepared from the infected cells was measured in a colorimetric NF-\( \kappa \)B activation assay. As shown in Fig. 1A, infection of BEAS-2B cells with \textit{B. pertussis} resulted in increased NF-\( \kappa \)B DNA-binding activity. This binding activity reached a maximum at 2 h, then gradually declined.

NF-\( \kappa \)B activation involves the phosphorylation of I\( \kappa \)B\( \alpha \), which is followed by I\( \kappa \)B\( \alpha \) degradation and the subsequent translocation of NF-\( \kappa \)B from the cytoplasm to the nucleus [24,25]. To determine whether the degradation of I\( \kappa \)B\( \alpha \) precedes the activation of NF-\( \kappa \)B, we assayed the kinetics of I\( \kappa \)B\( \alpha \) degradation by western blot analysis in BEAS-2B cells after infection with \textit{B. pertussis} BP536. As expected, \textit{B. pertussis}-induced degradation of I\( \kappa \)B\( \alpha \) was apparent 1 h after infection, and had returned to the base-line level by 3 h (Fig. 1B upper panel). Time-dependent re-synthesis of I\( \kappa \)B\( \alpha \) is a common finding, which appears to reflect activation of the I\( \kappa \)B\( \alpha \) gene by NF-\( \kappa \)B as part of a feedback loop [28]. Treatment of cells with cycloheximide, a protein synthesis inhibitor, blocked the restoration of I\( \kappa \)B\( \alpha \) to the basal level, confirming the re-synthesis of I\( \kappa \)B\( \alpha \) (Fig. 1B lower panel).

2.2. Subunit composition of the NF-\( \kappa \)B DNA-binding complex induced by \textit{B. pertussis} infection

The transcription factor family Rel includes several NF-\( \kappa \)B protein subunits [22]. To identify the subunit composition of the NF-\( \kappa \)B complex induced by \textit{B. pertussis} infection, we performed a colorimetric NF-\( \kappa \)B DNA-binding assay using antibodies against different members of the NF-\( \kappa \)B family (p50, p65, c-Rel, p52 or RelB subunits) as the primary antibodies. BEAS-2B cells were infected with strain BP536 at a MOI of 100. Two hours after infection, nuclear extracts were prepared and NF-\( \kappa \)B DNA-binding activities were assessed. As shown in Fig. 2, infection of BEAS-2B cells with \textit{B. pertussis} resulted in marked increases in p65 and p50 DNA binding activities and a slight increase in c-Rel DNA binding activity. In contrast, there were no substantial increases in p52 or RelB DNA binding. These results suggest that, in BEAS-2B cells, \textit{B. pertussis} infection activates NF-\( \kappa \)B complexes composed mainly of p65 and p50, and partly of c-Rel.

2.3. The RGD sequence of FHA is essential for NF-\( \kappa \)B activation, whereas PT production impairs this response

We have previously demonstrated that \textit{B. pertussis} infection up-regulates epithelial ICAM-1 expression in an RGD-dependent manner, and that PT impairs this response [14]. To ascertain the roles of the RGD sequence of FHA and of PT in NF-\( \kappa \)B activation, BEAS-2B cells were infected with various isogenic mutant strains of \textit{B. pertussis} and NF-\( \kappa \)B activation was assessed 2 h after infection. As shown in Fig. 3A, infection of BEAS-2B cells with the virulent strain BP536 resulted in a significant increase in NF-\( \kappa \)B DNA-binding activity. The specificity of the NF-\( \kappa \)B DNA-binding activity was confirmed by diminished binding in the presence of a wild-type probe containing the NF-\( \kappa \)B-consensus binding sequence, while a mutated probe did not affect binding. The mutant strain BP1098, which carried the site-directed chromosomal mutation G1098A in the RGD site of FHA, showed markedly reduced NF-\( \kappa \)B DNA-binding activity (p < 0.05) compared to the virulent parental strain BP536, implying involvement of the RGD.
sequence of FHA in NF-κB activation (Fig. 3B). Strain BP-TOX6, which was deficient in PT, induced significantly higher (p < 0.05) levels of NF-κB DNA-binding activity than the parental strain BP536. In contrast, the double mutant strain BP1098-TOX6, in which the RGD sequence was mutated to RAD (i.e. Ala was substituted for Gly) and the PT gene was deleted, showed little NF-κB DNA-binding activity. B. pertussis BPA2-6, which was deficient in adenylate cyclase (AC) activity, activated NF-κB to a similar extent as the parental strain BP536. These results show that the RGD sequence of FHA plays a role in NF-κB activation, but that the production of PT may suppress this response.

2.4. Host cell VLA-5 integrin is involved in NF-κB activation

Our previous study also showed that interaction of the RGD sequence of FHA with host cell VLA-5 integrin leads to up-regulation of epithelial ICAM-1 expression [14]. To ascertain that signals emanating from VLA-5 integrin are responsible for the induction of NF-κB DNA-binding activity, we investigated the inhibitory effects of integrin antagonists, including an RGD-containing synthetic peptide and a monoclonal antibody (mAb) against VLA-5, on NF-κB activation. BEAS-2B cells were preincubated with one of the synthetic peptides (5 mM) for 30 min at 37°C and then infected with TP536. As shown in Fig. 4, the ability of BP536 to induce NF-κB DNA-binding activity was significantly reduced (p < 0.01) in the presence of GRGDSP peptide. In contrast, no inhibition was observed when the non-active peptide GRGESP was present. When BEAS-2B cells were preincubated with antibodies against VLA-5 mAb (50 μg/ml) in a similar way, significant inhibition of BP536-induced NF-κB activation was observed (p < 0.01). In contrast, a mAb against αV integrin had no inhibitory effect.

These results reveal that engagement of VLA-5 integrin with an RGD sequence is a prerequisite for NF-κB activation in response to B. pertussis infection.

2.5. Adherence of BEAS-2B cells to FHA(RGD)-coated wells induces NF-κB activation as well as up-regulation of ICAM-1 expression

Ligation of VLA-5 integrin by the RGD sequence of FHA is sufficient to produce epithelial ICAM-1 expression [14]. We investigated whether the same applies to NF-κB activation, and if so, whether this event is related to the up-regulation of ICAM-1 expression. BEAS-2B cells were infected with strain BP536. BEAS-2B cells were left uninfected or infected with B. pertussis BP536 at a MOI of 100. Two hours after infection, NF-κB DNA-binding assay were performed using antibodies against the p65, p50, c-Rel, p52 or RelB NF-κB subunits. The values represent the means ± S.E. of four experiments.
exposed to bovine serum albumin (BSA)-, FHA(RGD)- or FHA(RAD)-coated wells; IκBα degradation, NF-κB activation, ICAM-1 mRNA expression and ICAM-1 surface expression were then assessed after 1, 2, 4, and 24 h of incubation. As shown in Fig. 5, BEAS-2B cells incubated in FHA(RGD)-coated wells exhibited increased levels of NF-κB activation (Fig. 5C) and IκBα degradation (Fig. 5D), whereas no alteration was seen in cells incubated in BSA- or FHA(RAD)-coated wells. Increments in ICAM-1 gene and protein expression also occurred in an RGD-dependent manner (Fig. 5A and B), demonstrating that RGD-dependent NF-κB activation is closely related to the up-regulation of ICAM-1 expression. These results suggest that adhesion to the RGD sequence of FHA induces activation of NF-κB, which is responsible for the up-regulation of ICAM-1 expression in BEAS-2B cells.

2.6. NF-κB inhibitors abrogate up-regulation of ICAM-1 expression in response to B. pertussis infection

To further confirm the participation of NF-κB activation in B. pertussis-induced up-regulation of ICAM-1 expression, we examined the effects of NF-κB inhibitors on B. pertussis-induced ICAM-1 expression. BEAS-2B cells were pretreated with 200 µM pyrrolidine dithiocarbamate (PDTC) for 2 h, 10 µM carbenoxyl-L-leucyl-L-leucinal (MG-132) for 30 min, or 100 µM SN50 for 1 h, before infection. As shown in Fig. 6, treatment with either PDTC, MG-132, or SN50 abrogated the up-regulation of epithelial ICAM-1 expression in response to BP536. These inhibitors had no effect on the viability of BEAS-2B cells (data not shown). These findings demonstrate a critical link between NF-κB activation and up-regulation of epithelial ICAM-1 expression in response to B. pertussis infection.

2.7. PT inhibits B. pertussis-induced NF-κB activation and IκBα degradation in BEAS-2B cells

PT production has been shown to impair FHA-mediated up-regulation of ICAM-1 expression [14]. To investigate the inhibitory role of PT in NF-κB activation, BEAS-2B
cells were preincubated with 10 ng/ml of either PT or its inactive B oligomer for 1 h at 37°C, then infected with strain BP-TOX6 at an MOI of 100. IκBα degradation and NF-κB activation were assessed 1 and 2 h after infection, respectively. As shown in Fig. 7, PT prevented both NF-κB activation (<p>0.05) and IκBα degradation in response to strain BPTOX-6, whereas the inactive B oligomer did not. Thus, B. pertussis-induced IκBα degradation and NF-κB activation require PT-sensitive G protein-associated signaling.

3. Discussion

ICAM-1 is an adhesion protein that plays a critical role in inflammatory cell recruitment and activation after bacterial infection [4–6]. Previous work has shown that infection of BEAS-2B human bronchial epithelial cells with B. pertussis up-regulates ICAM-1 expression [14]. The B. pertussis-induced increase in epithelial ICAM-1 expression involves interaction of the RGD sequence of the bacterial FHA with human VLA-5 integrin. Furthermore, this up-regulation of surface ICAM-1 protein expression is accompanied by an increase in ICAM-1 mRNA [14], suggesting regulation of gene expression at the transcriptional level. The transcription factor NF-κB has been implicated in the regulation of proinflammatory responses, including ICAM-1 gene expression [20,21]. In the present study, we explored the role of NF-κB in B. pertussis-induced up-regulation of ICAM-1 expression. Our results show that B. pertussis infection induces NF-κB activation in BEAS-2B cells,

![Fig. 5. Exposure of BEAS-2B cells to FHA(RGD)-coated wells induces up-regulation of epithelial ICAM-1 surface protein and mRNA expression, NF-κB activation and IκBα degradation. BEAS-2B cells were allowed to spread on wells coated with bovine serum albumin (BSA), or with FHA containing a normal or mutated RGD sequence [FHA(RGD) or FHA(RAD)]. ICAM-1 surface protein expression was assessed by flow cytometry after 24 h of incubation (A). The results are expressed as the fold increase over uninfected cells. ICAM-1 mRNA expression was determined after 4 h of incubation by a real-time reverse transcriptase-polymerase chain reaction technique (B). NF-κB activation was determined after 2 h of incubation by a colorimetric NF-κB assay (C). IκBα degradation was analyzed after 1 h of incubation by western blotting (D). The values represent the means ± S.E. of four experiments. *p < 0.05 vs control BSA.

Fig. 6. Effects of NF-κB inhibitors on ICAM-1 expression in BEAS-2B cells in response to strain BP536. BEAS-2B monolayers were incubated with either 200 μM pyrrolidine dithiocarbamate (PDTC) for 2 h, 10 μM carbenoxolone-ε-leucyl-ε-leucinal (MG-132) for 30 min, or 100 μM SN50 for 1 h, prior to the addition of bacteria. Twenty-four hours after infection, ICAM-1 surface expression was assayed by flow cytometry. The values represent the means ± S.E. of four experiments. **p < 0.01 vs control.
confirming previous finding that *B. pertussis* infection induces transcription of genes involved in NF-κB pathway as assessed by DNA microarrays [29]. Transient degradation of IkBα was also observed in response to *B. pertussis* infection; this is consistent with the finding that degradation of IkBα precedes the activation of NF-κB [25]. The interaction of the RGD sequence of FHA with host cell VLA-5 integrin appeared to be a prerequisite for *B. pertussis*-induced NF-κB activation because this response was inhibited by treatment with either an RGD-containing peptide or an anti-VLA-5 mAb. *B. pertussis* adheres to and invades respiratory epithelial cells through interaction of the RGD sequence of its FHA with host cell VLA-5 integrin [11]. In our study, treatment of cells with cytochalasin D, an inhibitor of microfilament-dependent bacterial invasion, failed to inhibit the activation of NF-κB (data not shown), which is consistent with our previous finding that the invasion step is not essential for the up-regulation of ICAM-1 expression. We found that adhesion of BEAS-2B cells to FHA(RGD)-coated wells resulted in IkBα degradation and subsequent NF-κB activation. Sequential increases in mRNA and surface protein levels of ICAM-1 were also noted. In agreement with these results, previous studies have demonstrated that engagement of VLA-5 (α5β1) integrin with an RGD motif activates the NF-κB pathway in fibroblasts [30], endothelial cells [18] and T cells [17]. Taken together, these findings indicate that ligation of VLA-5 integrin by the RGD sequence of FHA is sufficient to initiate NF-κB activation, and that actual bacterial uptake might not be required for this process. FHA contains multi-binding sites, including glycosaminoglycan and carbohydrate binding sites, together with the RGD sequence [9–11]. We do not rule out the possibility that another sequence of FHA could act in synergy with the RGD sequence. Since the extent of NF-κB activation in *B. pertussis*-infected BEAS-2B cells was slightly higher than that in cells adhered to FHA(RGD)-coated wells, it is likely that other bacterial factor(s) may also contribute to the RGD-dependent NF-κB activation.

PDTC potently inhibits NF-κB activation and/or interaction of NF-κB with its upstream regulatory binding site, thereby preventing NF-κB-mediated transcriptional activation [31,32]. The proteasome inhibitory peptide MG-132 is also a potent inhibitor of NF-κB [33], although MG-132 may affect the degradation of proteins other than IkBα; e.g. c-Jun and IL-2 receptor complex [34,35]. SN50 peptide, which contains the nuclear localization sequence (NLS) for the p50 NF-κB subunit, inhibits selectively the translocation of cytosolic NF-κB into the nucleus [36]. These NF-κB inhibitors abrogated *B. pertussis*-induced ICAM-1 expression, indicating that NF-κB plays a central role in increasing epithelial ICAM-1 expression in response to *B. pertussis* infection.

NF-κB exists as a variety of homo- and heterodimers. The ICAM-1 promoter contains an NF-κB binding site that binds strongly to p65 and c-Rel homodimers and to heterodimers of these subunits with p50, but only weakly to p50 homodimers [37]. Both p65 and c-Rel are capable of transactivating the κB enhancer in the ICAM-1 promoter [37]. In agreement with this observation, we showed that infection of BEAS-2B cells with *B. pertussis* induced the activation of NF-κB consisting mainly of p65 and p50, and partly of c-Rel. Similarly, it has been demonstrated that the most important members of the NF-κB family that mediate rhinovirus-induced NF-κB element binding for ICAM-1 transcription in human respiratory epithelial cells are p65, c-Rel and p50, with p65 being the major component of the homo- or heterodimers formed [38].

The signaling pathways involved in VLA-5-triggered NF-κB activation remain uncertain; however, our previous [14] and present studies have demonstrated that PT abrogates VLA-5 integrin-triggered NF-κB activation and up-regulation of epithelial ICAM-1 expression. Since PT has ADP-ribosyltransferase activity and inactivates the Giα subunit of heterotrimeric G proteins [13], it is conceivable...
that a PT-sensitive G protein is involved in this signaling pathway. Several integrins have been shown to form complexes with neighboring non-integrin cell surface proteins, and these integrin/integrin associated protein complexes may in turn be linked to Gi-associated signaling [39,40]. In particular, VLA-5 integrin associates with the urokinase receptor and caveolin, which regulate the functions of integrins [40]. Caveolin is a membrane protein that binds to cholesterol as well as a number of signaling molecules, such as the Src family of kinases, heterotrimeric G proteins and H-ras [41]. Further studies will be necessary to elucidate any possible roles of caveolin and caveolin-coupled components in VLA-5 integrin-triggered NF-κB activation.

In conclusion, the results of this study demonstrate that infection of human bronchial epithelial cells with B. pertussis results in NF-κB activation leading to up-regulation of ICAM-1 expression. Outside-in signaling mediated by the ligation of VLA-5 integrin by the RGD sequence of FHA requires IkBx degradation and subsequent activation of NF-κB to induce ICAM-1 expression. Furthermore, a PT-sensitive G protein appears to be involved in this signaling pathway. Further elucidation of the molecular mechanisms involved in the regulation of NF-κB activation that lead to ICAM-1 expression will provide clues to the nature of the inflammatory response during B. pertussis infection and shed more light on the pathogenesis of this organism. In addition, NF-κB and ICAM-1 may represent new targets for potential therapeutic intervention in B. pertussis-induced respiratory inflammation.

4. Materials and methods

4.1. Bacterial strains

B. pertussis BP536 is a streptomycin-resistant derivative of BP338, a virulent-phase (Vir + ) member of the Tohama I lineage [42]. All of the B. pertussis strains used in this study were derivatives of BP536. BP-TOX6 contained a complete deletion of the PT operon [42]. BP1098 carried a site-directed mutation in fhaB that effected substitution of Ala for Gly within the Arg-Gly-Asp site at amino acid positions 1097-9 [43]. The double mutant strain BP1098-TOX6 contained both of these mutations [44]. BPA2-6 contained an amino acid substitution in the CyaA AC catalytic site (K58M), resulting in loss of enzymatic activity [45]. All bacteria were cultured for 2 days on Bordet–Gengou (BG) agar plates (Difco Laboratories, Detroit, MI) supplemented with 15% sheep blood. Before use, the bacteria were harvested from the plates and suspended in Dulbecco’s phosphate-buffered saline (PBS; pH 7.4) to a concentration of 1 × 10^8 colony forming units (CFU) per ml as estimated by optical density at 600 nm. Actual concentrations of viable bacteria were determined by colony counts after plating on BG agar.

4.2. Antibodies, peptides and reagents

Mouse mAbs against human integrin αV (NKI-M9, IgG1) and VLA-5 (JBS5, IgG) were purchased from Chemicon International Inc. (Temecula, CA). Purified PT and its inactive B oligomer were obtained from Biomol Research Lab, Inc. (Plymouth Meeting, PA). The six-amino-acid peptides GRGDSP and GRGESP were purchased from Takara Biochemicals (Otsu, Japan). The lyophilized peptides were dissolved in EMEM medium and filter sterilized; aliquots were stored at −80 °C. PDTC was purchased from Sigma Chemicals Co. (St Louis, MO). MG-132 and NF-κB SN50 were obtained from Calbiochem (La Jolla, CA).

4.3. Preparation of purified FHA protein

FHA(RGD) and mutant FHA(RAD) proteins were isolated and purified from B. pertussis strains BP-TOX6 and BP1098-TOX6, respectively, using previously published techniques [44]. These preparations were then concentrated with Centricon-3 concentrators (Amicon, Beverly, MA). FHA-coated wells were prepared as described previously [44] with a slight modification. Briefly, 24-well tissue culture plates were coated overnight at 4 °C with 1 ml of PBS containing 5 μg/ml of BSA, FHA(RGD) or FHA(RAD) protein. The wells were blocked with 1 mg/ml BSA and washed with PBS prior to use.

4.4. Cell culture

The human bronchial epithelial cell line BEAS-2B was obtained from the American Type Culture Collection (CRL-9609). The cells were cultured in EMEM medium (BioWhittaker, Inc.) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/ml sodium penicillin G and 100 μg/ml streptomycin.

4.5. Infection

BEAS-2B cells were seeded into 24-well tissue culture plates at a density of 1 × 10^5 cells/well 18 h before infection. The epithelial cell monolayers grown in the tissue culture plates were washed three times and incubated with antibiotic-free medium for 2 h prior to bacterial challenge. The monolayers were infected with 1 × 10^7 bacterial CFU and incubated at 37 °C in 5% CO2 for 2 h to allow bacterial entry to occur [14]. For longer incubations, the monolayers were washed three times to remove extracellular bacteria, then incubated for an additional period in the presence of 100 μg/ml gentamicin to kill any remaining extracellular, but not intracellular, bacteria.
4.6. Preparation of nuclear cell extracts and colorimetric NF-κB assay

Nuclear cell extracts were prepared from BEAS-2B cells plated at a density of 1 × 10^6 cells in a six-well plate using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). The extracts were kept frozen at −70 °C. NF-κB DNA-binding activity was detected using a TransAM NF-κB family transcription factor assay kit (Active Motif) according to the manufacturer’s protocol. Briefly, microwells precoated with a double-stranded oligonucleotide containing the NF-κB consensus sequence were incubated with the nuclear cell extracts for 1 h at room temperature with mild agitation. The microwells were washed three times with washing buffer to remove any unbound proteins. The captured active transcription factor bound to the consensus sequence was incubated for 1 h with a specific primary antibody, then for an additional hour with a secondary horseradish peroxidase-conjugated antibody. Unless otherwise noted, anti-p65 was used as the primary antibody. After washing, the wells were exposed to developing solution for 10 min before adding stopping solution. The optical density of each well was measured and normalized relative to uninfected cells.

4.7. Western blotting for IκBα

Whole cell lysates (20 μg of protein) prepared from infected cells were electrophoresed on sodium dodecylsulfate-10% polyacylamide gels, then transferred to Immobilon-P membranes (Millipore, Marlborough, MA). The membranes were blocked with a solution of 5% (w/v) dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. They were then probed with a murine mAb against IκBα (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using an ECL Plus detection kit (Amersham, Arlington Heights, IL) before exposure to Hyperfilm ECL (Amersham, Arlington Heights, IL).

4.8. Flow cytometric analysis of surface ICAM-1 expression

ICAM-1 surface expression was determined by flow cytometry, as described previously [14]. The infected cells were detached by incubation with trypsin/EDTA solution (BioWhittaker, Inc.). They were then washed and resuspended in PBS containing 1% BSA and incubated with saturating amounts of fluorescein isothiocyanate-conjugated anti-human ICAM-1 (CD54) antibody or an isotype-specific control antibody (Serotec, Oxford, UK) for 30 min at 4 °C in the dark. After washing, 10^4 cells were analyzed for fluorescence by single color flow cytometry on a FACScan analyzer (Becton Dickinson, San Jose, CA) equipped with Lysis II software. The mean fluorescence intensity was measured and normalized relative to uninfected cells.

4.9. Real time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from the epithelial cell monolayers after bacterial infection using the SV total RNA isolation system (Promega Corp., WI), and reverse transcribed using a first-strand cDNA synthesis kit (Roche Molecular Biochemicals). Real time RT-PCR was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany) with LightCycler-FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals) as described previously [14]. The expression level of the ICAM-1 gene was estimated from the ratio of ICAM-1 mRNA to β-actin mRNA.

References


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