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Manuscript Draft

Manuscript Number:

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Article Type: Regular Research Articles

Keywords: nontypable *Haemophilus influenzae*; HI0164; vaccine; lung

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**Investigation of the potential of the novel 48 kDa HI0164 protein as a vaccine
candidate for infection against nontypable *Haemophilus influenzae***

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Running title: Characterization of the NTHi protein HI0164

Abstract

This study determined the conservation and protective efficacy of the novel nontypable *Haemophilus influenzae* (NTHi) protein, HI0164. This protein was highly conserved across the strains of NTHi examined and mucosal immunization with rHI0164 significantly reduced the numbers of viable NTHi recovered from the lung following challenge. rHI0164 induced predominantly an IgG2a antibody response that correlated with the reduction in the number of viable NTHi in the lung. These antibodies were not bactericidal against NTHi. The results suggest that HI0164 warrants further investigation as a vaccine component for NTHi disease.

Keywords

nontypable *Haemophilus influenzae*, HI0164, vaccine, lung

1. Introduction

The opportunistic pathogen, nontypable *Haemophilus influenzae* (NTHi) is commonly associated with the etiology of otitis media in children, as well as other mucosal infections including the exacerbation of chronic bronchitis (reviewed in Murphy 1987) [1]. Otitis media is one of the most common disorders of children presented to paediatricians. By 3 years of age, 80% of children have experienced at least one episode and more than 40% have suffered recurrent infections [2]. As well as a substantial burden on community health programs, the defect in hearing induced by chronic otitis media has been linked to lower scores in cognitive ability, linguistic skills and school performance [3]. While antibiotics have been the mainstays in the treatment of otitis media, such regimens are often unsuccessful due to a variety of mechanisms, including the production of bacterial-derived β -lactamases [4] and the depletion of the protective resident viridans streptococci [5]. In addition, the ability of NTHi to persist in subepithelial crypts and the formation of scar tissue in the tonsils during chronic infection decreases the efficacy of antibiotics [6, 7] and allows a reservoir of bacteria to persist. Thus, therapeutics that stimulate the immune system to specifically target NTHi would be of major benefit in combating what is a costly and debilitating disease.

Some studies have defined outer membrane protein (OMP)-specific bactericidal antibodies as fundamental mediators in the elimination of NTHi [8-10]. Therefore, considerable efforts have been invested in the use of purified OMPs as immunogens to stimulate such antibodies. However, these efforts have been somewhat thwarted by the inherent interstrain variability in NTHi OMPs, especially in the major proteins P2 and P5 [11-13]. Heterogeneity in these immunodominant molecules is regarded as a significant factor in the failure of NTHi infection to induce immune protection against reinfection and a major hurdle in vaccine development. Thus, efforts are being directed

towards investigation of the interstrain variability and vaccine potential of the less abundant proteins found in the outer membrane of NTHi.

The present study reports characterization of a novel 48 kDa NTHi protein that was identified in the *H. influenzae* Rd genome project as HI0164 [14]. Notably, HI0164 shows 76% homology with AopA from *Actinobacillus pleuropneumoniae*, the etiologic agent of pleuropneumonia in swine [15]. Immunization with an *A. pleuropneumoniae* OMP vaccine induced protection against challenge with several serotypes and stimulated an antibody response that detected the 48 kDa AopA on Western blots. In addition, sera from infected pigs, as well as a bactericidal monoclonal antibody reacted with AopA [15]. Neither the heterogeneity nor the vaccine potential of HI0164 have been reported previously.

2. Materials and Methods

2.1 *NTHi strains*

HI0164 was characterized from several isolates of NTHi [16] that were derived either from the sputum of chronic bronchitics or the middle ear fluid from children with otitis media: UC19 (Biotype I, sputum), UC103 (Biotype II, sputum), UC28 (Biotype II, otitis media) and UC63 (Biotype II, otitis media).

2.2 *Sequence analysis of HI0164*

Chromosomal DNA was prepared from the above strains, as described previously [16], and used as a template for high-fidelity PCR amplification of the HI0164 genes. The sequences of the oligonucleotides used for PCR were based on the gene encoding *H. influenzae* Rd HI0164 [14] except for the inclusion of the restriction endonucleases sites (underlined) that were used to facilitate subcloning. The forward primer, 96-14 (5'-GTAGTGCAAATGGATCCATTACAATT AAGAAAGG-3') contained a *Bam*HI site and a *Pst*I site was introduced into the reverse primer, 96-30 (5'-GATTTTTTCAACTGCAGTTTAAACCC-3'). The TCC (bold) in 96-14 replaces the methionine start site in HI0164 with a serine and the TTA triplet (bold) in 96-30 is the complementary codon for the stop site. The gene encoding HI0164 was amplified by PCR, cloned into M13mp19 for the production of a template and sequenced using a M13 primer, as well as, internal primers designed from the Rd sequence [14]. Sequencing was performed on at least two independent clones per isolate and these sequences were assembled with the MacVector program (Apple Macintosh). The predicted protein sequences were aligned with the Genetics Computer

Group PileUp program [17] from the University of Wisconsin accessed through the Australian National Genomic Information Service.

2.3 Cloning and purification of recombinant HI0164

The gene encoding HI0164 was amplified by PCR with the forward primer, 96-14 (as above) and the reverse primer, 96-15 (5'-CAAACCCATTTT**TTAG**AATTCCTTCTCAA TCTTATC-3') with chromosomal DNA prepared from the NTHi isolate UC19. The primer, 96-15 contains an *Eco*RI restriction site (underlined) and the TTA triplet (bold) is the complimentary codon to the stop site in HI0164. As the *Eco*RI site occurs just inside the reading frame, the 3' triplet encodes a phenylalanine instead of the usual glycine residue found in the native protein. The restriction sites were used to clone the PCR product into pGEX2T, which is designed to express HI0164 as a glutathione-S-transferase (GST) fusion protein with a thrombin cleavage site between GST and HI0164. Expression of the fusion protein was induced with IPTG and purified from the soluble fraction of disrupted *Escherichia coli* with Glutathione-Sepharose essentially as described by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). Recombinant HI0164 was released from the bound GST portion of the fusion protein by thrombin digestion and then further purified with a MA7Q HPLC anion exchange column (Bio-Rad, Hercules, CA, USA). Endotoxin contamination was estimated to be below 0.06 EU/ml, which was the detection limit of the ETOXATE, assay (Sigma Chem Co, St Louis, MO).

2.4 Immunization with recombinant HI0164 and bacterial challenge

Purified rHI0164 was resuspended in PBS and emulsified in an equal volume of incomplete Freund's adjuvant (Sigma Chem Co, St Louis, MO) to give a final protein concentration of either 200 or 400 µg/ml. Male 8-10 week old Wistar rats were

sensitized by Peyer's Patch immunization as described previously [18] with each rat receiving a total of either 10 or 20 µg of antigen. Rats were boosted intratracheally 14 days later with the same antigen dose used in the primary immunization in a total volume of 50 µl of PBS or with PBS only instead of the antigen for the control group. Seven days after boosting, NTHi strain, UC19 (5×10^8 CFU) was used to intratracheally challenge the rHI0164-immunized animals and after 4 h broncho-alveolar lavage fluid (BALF), serum and lung samples were taken for analysis. Lungs were homogenized and together with the BALF samples were serially diluted onto chocolate agar plates to determine the number of viable NTHi in these samples.

2.5 ELISA

rHI0164-specific antibodies were detected by ELISA as described previously [19] using plates that had been coated overnight at 4°C with 0.3 µg per well of purified rHI0164 in 100 µl of coating buffer.

2.6 Bactericidal assays

The bactericidal activity of a pool of rHI0164-specific rat serum for UC19 was determined as described previously [19]. Heat-inactivated serum from animals immunized with formalin killed UC19, complement source, and bacteria were used as a positive control. The bactericidal titer was calculated as the reciprocal of the dilution that resulted in more than a 50% reduction in bacterial viability compared to the same dilution of nonimmune serum. The assays were performed three times independently.

2.7 Western Blots of NTHi cellular extracts

HI0164 in whole cells, total membrane proteins and Zwittergent-soluble [20] and Sarkosyl-insoluble [21] OMP preparations was detected by Western immunoblot analysis. Samples from these cell fractions were separated on 4-20% SDS-PAGE and then electrophoretically transferred to a PVDF membrane. After blocking, the membrane was incubated with a pool of 1/500 diluted rHI0164-specific immune or nonimmune rat serum (from 5 rats) and bound rHI0164-specific IgG was detected with HRP-conjugated goat anti-rat IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands).

2.8 *Statistical analysis*

Data are expressed as the mean \pm standard error of the mean (SEM). Analysis for statistical significance and linear correlation between two variables in individual animals was performed with the Macintosh InStat program for Pearson analyses.

3. Results

3.1 *Conservation of HI0164 across several isolates of NTHi and the H. influenzae Rd strain*

The NTHi strains chosen for analysis (UC19, UC103, UC28 and UC63) were isolated from the sputum of patients with chronic bronchitis or the middle ear fluid of children with otitis media with effusion. Both Biotypes I and II were represented. NTHi isolate UC103 was arbitrarily selected for the standard of comparison. As the DNA encoding the first seven and last two amino acids was overlapped with the primers used for PCR amplification of HI0164, the variation in these residues is not known. Despite this uncertainty, as these amino acids are conserved in both the Rd HI0164 [14] and AopA [15], it seems likely that they would also be conserved amongst variants of HI0164. Isolates, UC19, UC28 and UC63, together with HI0164 from the Rd strain, each contained either one or two nonsynonymous (amino acid changing) mutations each. Interestingly, three of these five mutations occurred within a region of 17 amino acids (Ala146-Pro163). The gene from UC103 contained five and the Rd strain three synonymous (silent) mutations that were distributed throughout the genes. Thus, analysis of the alignment of the amino acids comprising these variants (Fig. 1) demonstrates that HI0164 is well conserved across the four NTHi isolates tested and contains very few mutations compared to a consensus sequence.

3.2 *Cloning and purification of rHI0164*

To obtain sufficient protein to assess the vaccine potential of HI0164, a recombinant form of the protein was purified from a GST-HI0164 fusion protein. The purity of rHI0164 was evaluated by SDS-PAGE (Fig. 2). High levels of expression of the fusion

protein, with an electrophoretic mobility of approximately 70 kDa (GST is approximately 26 kDa), was observed after IPTG-induction of cultures (Fig. 2 lanes 1 and 2). The fusion protein from the soluble fraction of disrupted cultures was immobilized on Glutathione-Sepharose (Fig. 2 lane 3) and after washing to remove contaminating proteins rHI0164 was released from the bound GST by cleavage with human thrombin. Some endogenous proteolytic cleavage of the fusion protein was observed during purification as demonstrated by the presence of a protein slightly smaller than GST-rP48 in lane 3. Full-length rHI0164 was further purified by anion exchange HPLC and was visualised as a single band at approximately 48 kDa (Fig. 2 lane 4), confirming a high degree of purity.

3.3 Clearance of NTHi after immunization with rHI0164

The clearance of a 5×10^8 NTHi challenge to the lung following immunization with either 10 or 20 μg of rHI0164 per rat is shown in Fig. 3. Both doses of rHI0164 resulted in significantly enhanced clearance of UC19 at 4 h from both BALF (10 μg ; 52%, $P < 0.005$, 20 μg ; 55%, $P < 0.005$) and lung homogenates (10 μg ; 69%, $P < 0.005$, 20 μg ; 69%, $P < 0.05$) compared to rats similarly treated with PBS instead of antigen. No enhanced clearance was observed in the PBS control group

3.4 rHI0164-specific antibodies in BALF and serum

Immunization with rHI0164 induced significant antigen-specific IgG and IgA antibody response in the BALF and IgG in the serum (Fig. 4). The predominant antigen-specific antibody isotype in the serum was IgG2a (Table 1). Pearson analysis for the correlation between the persistence of viable NTHi at 4 h post challenge and the rHI0164-specific antibody levels in the serum and BALF in individual animals showed a significant inverse correlation. (Fig. 5).

3.5 Bactericidal activity

The bactericidal activity of rHI0164-specific antibodies were determined against a strain which has been well characterized in our laboratories; NTHi strain UC19. Despite high levels of rHI0164-specific antibody, no serum bactericidal activity against UC19 could be detected in three independent assays. In contrast, concurrent assays with serum from animals immunized with formalin-killed UC19 demonstrated a bactericidal titer within the range of 32-64 over the three assays (Table 2).

3.6 Cellular localization of HI0164

As an indicator to the surface accessibility of HI0164, Western immunoblot analysis was performed using OMP fractions prepared using two established methods for purifying such fractions [20, 21]. Whole cells, the total membrane fraction, as well as Sarkosyl-insoluble extracts or Zwittergent-soluble extracts of OMPs from UC19 were probed with either rHI0164-specific immune or control serum. A protein corresponding to the molecular weight of HI0164 (48kDa) was detected in whole cells and the total membrane preparation (Fig. 6A, lanes 1 and 2). However, HI0164 could not be detected in either the Sarkosyl-insoluble or Zwittergent-soluble fractions in which OMPs are commonly detected (Fig. 6A, lanes 3 and 4). Some non-specific protein bands were detected by both immune and nonimmune sera.

4. Discussion

This study has characterized the NTHi HI0164 protein with emphasis on the conservation of the protein, its immunogenicity and protective efficacy in an animal model of acute lung infection.

Mucosal-directed immunization with a recombinant form of HI0164 afforded significant protection against NTHi challenge and reduced the recovery of viable NTHi from the lungs of immunized rats. rHI0164 was strongly immunogenic and elicited high titers of antibodies in both serum and BALF. Importantly, the numbers of viable NTHi recovered from both BALF and lung homogenates demonstrated a significant inverse correlation with the magnitude of antigen-specific IgG and IgA antibody in the BALF and IgG (predominantly IgG2a) in the serum. This strongly suggests that immune responses generated by immunization with rHI0164 were significantly associated with the reduced viability of NTHi in the lungs of challenged rats.

However, in contrast to serum from rats similarly immunized with formalin killed UC19 NTHi, no NTHi-bactericidal activity could be detected in the serum of rHI0164 immunized rats, suggesting that bactericidal antibody per se may not be the only factor in reducing the numbers of viable NTHi remaining after pulmonary challenge. The failure of rHI0164 to elicit bactericidal activity may be due to rHI0164-specific antibody failing to fix complement or that the cognate epitopes at the bacterial surface are not recognized by rHI0164-specific antibodies. The isotype of the antibodies (IgG2a) elicited by rHI0164 is not a likely explanation for the absence of detectable bactericidal antibodies as IgG2a is as equally efficient as IgG1 in fixing complement by the classical pathway in the rat [22]. However, a major consideration is the surface

accessibility of the HI0164 antigen to potential bactericidal antibodies. To determine if HI0164 partitions to the outer membrane and thus potentially accessible to bactericidal antibodies, both Sarkosyl-insoluble and Zwittergent-soluble membrane protein extracts were prepared from NTHi strain UC19 and localization of HI0164 in these fractions was determined by Western blot. Both methods of membrane fractionation are well characterized and widely used for the isolation of OMPs. While a protein corresponding to the same molecular weight as HI0164 was detected in the NTHi lysate and a total membrane preparation, HI0164 could not be detected in the Sarkosyl-insoluble and Zwittergent-soluble extracts. These data demonstrate that although HI0164 is membrane-associated protein, it does not partition with the outer membrane component during differential detergent fractionation.

HI0164 is part of a gene cluster that is almost identical to the *nqr* operon encoding the sodium translocating NADH-ubiquinone oxidoreduction (NQR) of the marine bacterium, *Vibrio alginolyticus* NqrA protein [15]. A Na⁺-dependent NQR has been detected in the membrane fraction of the *H. influenzae* Rd strain and inverted membrane vesicles demonstrated the presence of a respiration-driven Na⁺ pump in the cytoplasmic membrane [23]. As a protein complex encompassing HI0164 has been functionally linked to respiratory machinery embedded in the cytoplasmic membrane, this may explain why it was not possible to detect HI0164 in the OMP fractions and that antibodies potentially directing HI0164-specific bactericidal activity could not target intact NTHi.

Heterogeneity in antigens is a major barrier faced in evoking cross-protective immune responses and this is especially relevant with respect to NTHi. Analysis of the sequence of HI0164 from four isolates of NTHi, which varied with respect to the anatomical sites

from which these strains were isolated and their biotype, and the *H. influenzae* Rd strain, showed that HI0164 was conserved amongst the isolates studied with only one or two amino acid substitutions throughout each protein. Although this sequence analysis of HI0164 is far from comprehensive, the highly conserved nature of these variants suggests that this protein, either through the absence of immune pressure for mutational evasion or through a strict functional requirement for conservation, varies little in amino acid sequence.

In conclusion, mucosal-directed immunization of rats with a purified recombinant form of HI0164 affords protection against pulmonary infection by NTHi. HI0164 stimulated an IgG2a antibody response that correlated inversely with the number of viable NTHi recovered from the lung. Notably, the absence of serum bactericidal activity *in vitro*, suggests that other immune mechanisms that are independent of bactericidal antibodies contribute to the enhanced clearance of viable NTHi from immunized rat lungs. Further investigation to determine the nature of the mechanisms underlying this protective immune response is required. HI0164 was found to be membrane associated and highly conserved at the amino acid level across the NTHi isolates examined in this study. Further work will focus on investigating the genetic diversity of HI0164 across more diverse NTHi strains from distinct geographical and clinical sites. These data lend support to continuing study of this NTHi protein and its potential as a component of a vaccine for NTHi disease.

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Table 1. Isotypes of rHI0164-specific serum IgG in rats immunized with 10 µg of rHI0164.

Group (number of animals)	rHI0164-specific IgG1*	rHI0164-specific IgG2a*
PBS-treated (5)	ND	11 ± 2.5
rHI0164 immune (4)	118 ± 94	2300 ± 995

* Expressed as ELISA units (reciprocal of the serum dilution that gave an optical density between 0.4 and 0.9 in the assay)

Values are expressed as the mean ± SEM

ND-No IgG1 detected

Table 2. Bactericidal activity of whole-killed UC19-specific and UC19 rHI0164-specific immune sera against NTHi, UC19.

Antigen-specific serum	Bactericidal titre ^a
Whole killed UC19	32-64
UC19 rHI0164	ND

^a Bactericidal titre is the reciprocal of the dilution of serum that resulted in greater than 50% killing compared to the same dilution of nonimmune serum.

ND – No bactericidal activity detected.

Figure Legends

Fig. 1. Amino acid alignment of the HI0164 proteins. The amino acid sequences of HI0164 from NTHi strains, UC103, UC28, UC19 and UC63, together with HI0164 from the *H. influenzae* Rd strain were aligned with the PileUp program. Identical residues are indicated with a dot and unconserved residues are indicated with the appropriate letter. The first seven and last two amino acids in UC 103, UC28, UC19 and UC63 (indicated by a colon) are unknown as they were overlapped with the primers used for PCR, but all are conserved between AopA and the Rd sequence.

Fig. 2. SDS-PAGE of samples from the purification of rHI0164. The GST-rHI0164 fusion protein (arrow) is present in the cell lysate of the IPTG-induced culture in lane 2 compared to the uninduced culture in lane 1. The sample in lane 3 is the Glutathione bound fraction where some degradation product is evident. Recombinant HI0164 (starred arrow), which was released from the Glutathione-Sepharose after thrombin digestion, was further purified with anion exchange HPLC. Molecular weight markers are indicated on the left in kDa.

Fig. 3. Clearance of NTHi from BALF (A) and lung (B) following immunization with either 10 or 20 µg of rHI0164 per rat compared to PBS-treated rats. Both immunization doses resulted in significant clearance of UC19 at 4 h. The percentage clearance in the immunized groups (4-5 rats per group) was calculated as 100 minus the percentage ratio of the mean colony forming units (CFU) recovered from the immunized group divided by the mean of the PBS-treated group. The mean CFU of the PBS-treated group was given the value of zero percent clearance. The error bars represent the SEM.

Fig. 4. rHI0164-specific antibodies in BALF and serum after immunization with either 10 μ g or 20 μ g of rHI0164 per rat. Significant increases in IgG were present in both serum (A) and BALF (B), and increases in IgA in BALF (C) from immunized rats compared to PBS-treated rats. The error bars represent the SEM

Fig. 5. Correlation between rHI0164-specific IgG in serum (top: μ g/ml) and BALF (middle: ng/ml) and IgA (bottom: ng/ml) in BALF with colony forming units (CFU: \log_{10}) of NTHi recovered in BALF (panel A) or lung (panel B) for immune and PBS treated rats. All comparisons showed a significant inverse correlation of antibody levels with CFU. The value, P, is the probability that the gradient of the slope is significantly different from zero.

Fig. 6. Western immunoblot analysis for the presence of HI0164 in the membrane compartments of UC19 NTHi. Whole-lysed NTHi (lane 1), total membrane proteins (lane 2), Sarkosyl-insoluble OMPs (lane 3) and Zwittergent-soluble OMPs (lane 4) were probed with both HI0164-immune (A) and control serum (B). The position of rHI0164 is indicated with an arrow and the positions of the molecular weight markers are indicated on the right in kDa.

Figure 1

VACCINE Webb Figure 1

	1				50
UC103	MITIKKGLDL	PIAGKPAQVI	HSGNAVNQVA	ILGEEYVGMR	PSMKVREGDV
Rd
UC28
UC19
UC63
	51				100
UC103	VKKGQVLFED	KKNPGVIFTA	PASGTITAIN	RGEKRVLSQSV	VINVEGDEKI
Rd
UC28
UC19
UC63
	101				150
UC103	TFAKYSTEQL	NTLSSEQVKQ	NLIESGLWTA	LRTRPFSKVP	SIESEASSIF
Rd
UC28
UC19	P.....
UC63L.
	151				200
UC103	VNAMDTNPLA	ADPSVVLKEY	SQDFTNGLTV	LSRLFPSKPL	HLCKAGDSNI
Rd
UC28
UC19
UC63S.....
	201				250
UC103	PTADLENLQI	HDFTGVHPAG	LVGTHIHFID	PVGIQKTVWH	INYQDVIAVG
Rd
UC28	...N.....
UC19
UC63
	251				300
UC103	KLFTTGELYS	ERVISLAGPQ	VKEPRLVRTT	IGANLSQLTQ	NELSAGKNRV
Rd	T.....
UC28
UC19
UC63
	301				350
UC103	ISGSVLCGQI	AKDSHDYLGR	YALQVSVIAE	GNEKEFFGWI	MPQANKYSVT
Rd
UC28
UC19
UC63
	351				400
UC103	RTVLGHFSKK	LFNFTTSENG	GERAMVPIGS	YERVMLDIL	PTLLLRDLIV
Rd
UC28
UC19
UC63
	401				448
UC103	GDTDGAQELG	CLELDEEDLA	LCSFVCPGKY	EYGSILRQVL	DKIEKEG*
Rd*
UC28*
UC19*
UC63*

Figure 1

Figure 2

VACCINE Webb Figure 2

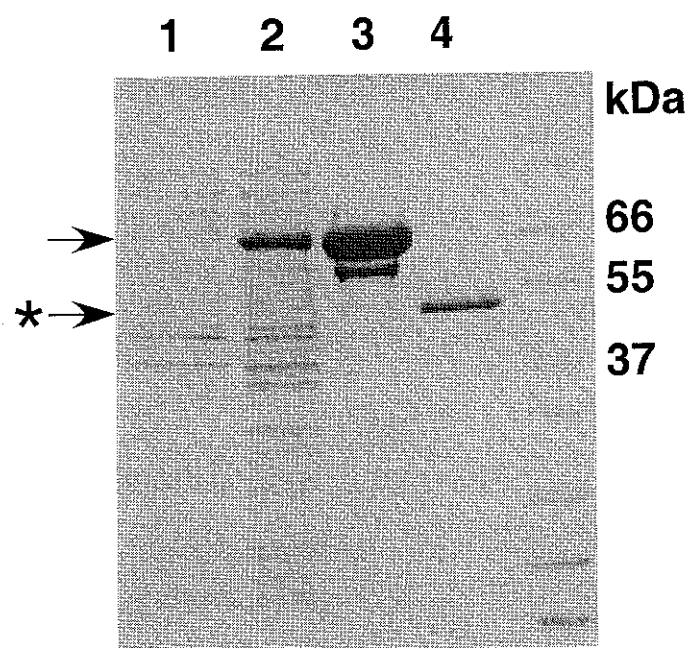


Figure 3

VACCINE Webb Figure 3

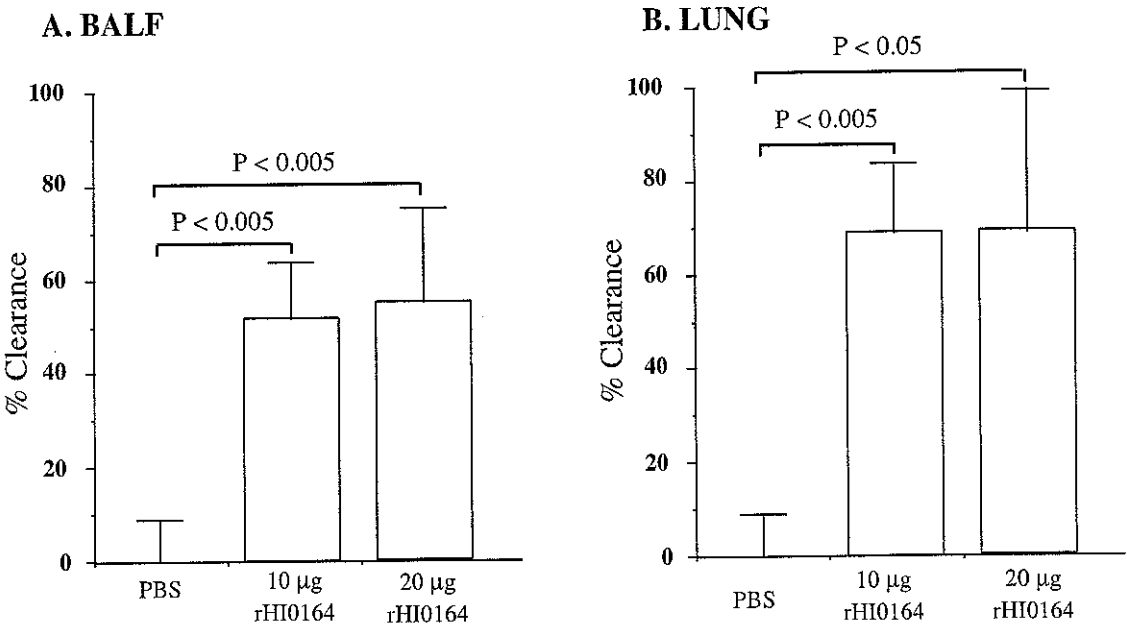


Figure 3

Figure 4

VACCINE Webb Figure 4

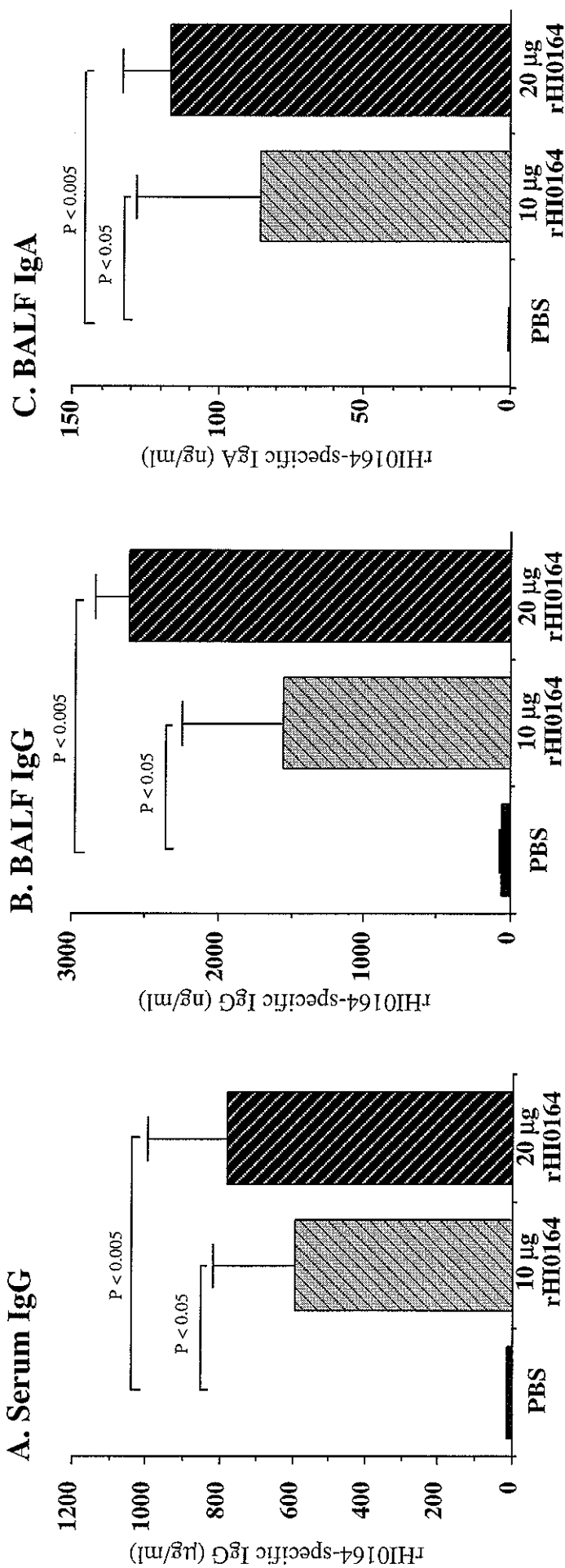


Figure 4

Figure 5

VACCINE Webb Figure 5

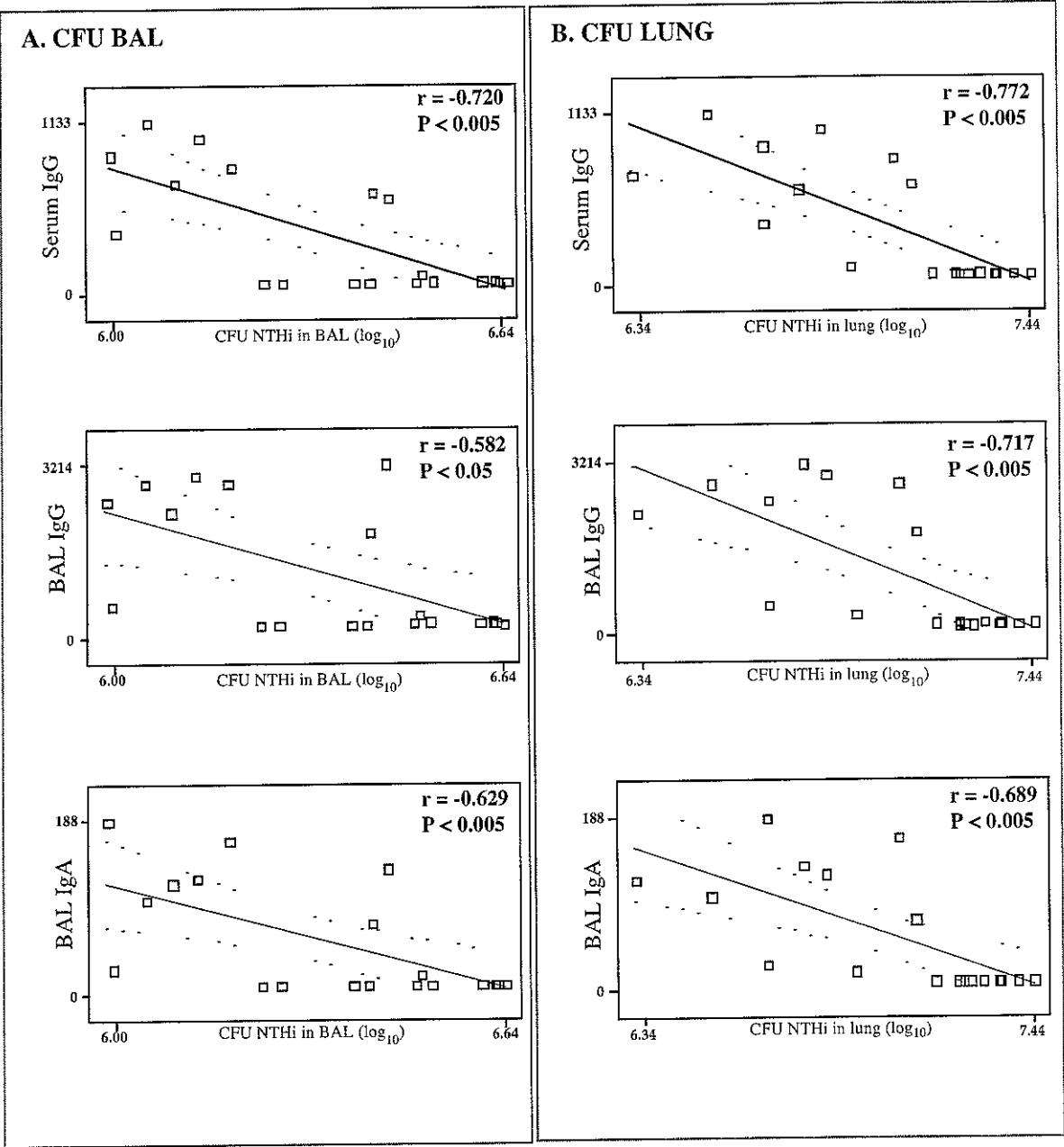


Figure 5

Figure 6

VACCINE Webb Figure 6

