Integrin-mediated Localization of *Bordetella pertussis* within Macrophages: Role in Pulmonary Colonization

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Summary

The adherence of *Bordetella pertussis* to human respiratory cilia is critical to the pathogenesis of whooping cough but the significance of bacterial attachment to macrophages has not been determined. Adherence to cilia and macrophages is mediated by two large, nonfimbrial bacterial proteins, filamentous hemagglutinin (FHA), and pertussis toxin (PT). PT and FHA both recognize carbohydrates on cilia and macrophages; FHA also contains an Arg-Gly-Asp (RGD) sequence which promotes bacterial association with the macrophage integrin complement receptor 3 (CR3). We determined that virulent *B. pertussis* enter and survive in mammalian macrophages in vitro and that CR3 is important for this uptake process. We then determined the relative contribution of CR3 versus carbohydrate-dependent interactions to in vivo pulmonary colonization using a rabbit model. *B. pertussis* colonized the lung as two approximately equal populations, one extracellular population attached to ciliary and macrophage surface glycoconjugates and another population within pulmonary macrophages. Loss of the CR3 interaction, either by mutation of FHA or treatment with antibody to CR3, disrupted accumulation of viable intracellular bacteria but did not prevent lung pathology. In contrast, elimination of carbohydrate-bound bacteria, either by a competitive receptor analogue or an anti-receptor antibody, was sufficient to prevent pulmonary edema. We propose that CR3-dependent localization of *B. pertussis* within macrophages promotes persistence of bacteria in the lung without pulmonary injury. On the other hand, the presence of extracellular bacteria adherent to cilia and macrophages in carbohydrate-dependent interactions is associated with pulmonary pathology.

Bordetella pertussis is a Gram-negative coccobacillus that is the causative agent of whooping cough. It has long been recognized that *B. pertussis* establishes pulmonary colonization by adhering specifically to human ciliated epithelial cells, and this interaction has been studied in detail in vitro (1). Recently, however, in vitro studies have shown that *B. pertussis* also adheres specifically to human macrophages (2) and can enter and survive within tissue culture cells (3–5). The purpose of this study was to determine the importance of the interaction between *B. pertussis* and macrophages during pulmonary infection.

Bacterial adherence to cilia and macrophages is mediated by two nonfimbrial bacterial proteins, filamentous hemagglutinin (FHA)[1] and pertussis toxin (PT) which are expressed only by virulent cells (1, 2). These proteins are functional either when bound to the bacterial surface or when secreted into the surrounding medium during growth and serve as bifunctional ligands bridging the bacterial surface and glycoconjugates on eukaryotic cell membranes (1, 6). Both adhesins are unusually large molecules with multiple binding affinities, features reminiscent of eukaryotic extracellular matrix proteins. PT is a 105-kD hexameric protein composed of five different subunits with binding affinities for several carbohydrates (6–9). Subunits 2 and 3 bear eukaryotic-like carbohydrate recognition domains (9). FHA is a 220-kD protein that binds galactose-containing glycoconjugates on both cilia and macrophages (2, 6). In addition to a carbohydrate recognition domain, an Arg-Gly-Asp (RGD) triplet at position 1097-9 of FHA (10) promotes adherence of *B. pertussis* to the macrophage integrin CR3 (CD11b/CD18) (2). It is known that particles phagocytosed via CR3 do not engender an oxidative burst (11), suggesting this route of entry might promote survival of *B. pertussis* within the macrophage resulting in a sequestered reservoir of bacteria within the large airways.

In this study we determined that CR3 contributes to the localization of *B. pertussis* within the alveolar macrophage in vivo and that intracellular bacteria constitute as much as half of the total viable number of bacteria in the rabbit lung early in the course of colonization. However, intracellular bacteria do not appear to contribute to pulmonary pathology. In contrast, *B. pertussis* bound to the surfaces of cilia and macro-

[1] *Abbreviations used in this paper:* FHA, filamentous hemagglutinin; PT, pertussis toxin; RGD, Arg-Gly-Asp sequence.

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phages in a carbohydrate-dependent fashion effectively induce pulmonary pathology.

Materials and Methods

Bacterial Strains. The strains used are derivatives of the virulent, phase I *B. pertussis* strain 536 (2, 10, 12). BP-TOX6 contains a deletion of the 3.4-kb BstEll fragment encompassing the PT operon. BP101 contains an inframe 2.4-kb deletion within *fhaB* which eliminates carbohydrate and integrin recognition. BP1098 carries a site-directed mutation within *fhaB* resulting in substitution of Ala for Gly at position 1098 of the RGD site. BP101-TOX6 and BP1098-TOX6 are derivatives into which the TOX6 mutation was introduced. Strains were grown for 3 d at 37°C on Bordet Gengou agar supplemented with 15% sheep blood.

Colonization Assays. New Zealand White rabbits (2 kg; Hare Marland, Nutley, NJ) were anesthetized with ketamine and xylazine, the trachea was cannulated (19 G 7/8 butterfly infusion set; Abbott Laboratories, North Chicago, IL) through a tracheostomy, and 10^8 bacteria (in 0.2 ml saline) were introduced intratracheally as described in a model of pulmonary colonization (13, 14). To interrupt carbohydrate or CR3-dependent events, some animals were treated intratracheally with one of the following within 10 min of the bacterial challenge: lactose or sialic acid (0.1 M); mAbs recognizing CR3 (mAb IB4, 0.5 mg/kg; courtesy of Merck Sharpe & Dohme, Inc., Rahway, NJ, [reference 15]), or the blood group determinants Lewis a (mAb 23.24) or A (mAb 69.4) (0.2 mg/kg; courtesy of Ciba-Geigy, Basel, Switzerland, [reference 6]). 24 h after the bacterial challenge, the lungs were harvested, weighed, homogenized, and titered for bacterial CFUs. Pulmonary edema was measured by comparing lung wet weight to that of uninfected lungs (normal = 4.7 ± 0.4 g). To ascertain if bacteria were within macrophages, excised tracheas were lavaged (10, 11) to harvest intra-airway macrophages, and lavage fluids were centrifuged, washed and placed on two slides. One slide was fixed in buffered formalin (16) and stained with anti-*Bordetella* fluorescent antibody (1:40, Bacto-FA *Bordetella pertussis*; Difco Laboratories, Inc., Detroit, MI) to visualize extracellular bacteria. The duplicate slide was fixed in methanol (16) and stained with the same fluorescent antibody to visualize both intra- and extracellular bacteria. The difference in the number of bacteria visualized by the two preparations was taken as a measure of the number of intracellular bacteria. Values are expressed as the mean ± SD of the number of bacteria associated with 25 cells. Statistical significance was determined by student's *t* test.

In Vitro Adherence Assays. Quantitation of viable intracellular bacteria was performed using pulmonary alveolar macrophages from healthy rabbits or human peripheral blood monocytes at day 4–6 in tissue culture (2). Bacteria and eukaryotic cells were incubated in medium RPMI (Sigma Chemical Co., St. Louis, MO) at a relative density of 100:1 for 30 min on a tumbler at 37°C. Suspensions were centrifuged to sediment the cells and the supernatant fluid containing the majority of nonadherent, extracellular bacteria was discarded. The cells were then incubated at 37°C in RPMI supplemented with gentamicin (50 μg/ml) to kill extracellular bacteria. To determine the number of viable intracellular bacteria, samples were taken at 30 min intervals over 6 h and the gentamicin was removed by centrifugation of the cells at 10,000 *g*. Cells were resuspended in 0.1% Triton X-100 and viable bacteria released from the cells were titered by CFUs. In parallel, samples were also taken at hourly intervals, fixed with formalin or methanol, and stained as described above to visually confirm the number of intra- versus extra-cellular bacteria.

Results

CR3 Promotes Accumulation of Viable B. pertussis Within Macrophages In Vitro. *B. pertussis* were incubated in vitro with macrophages cultured from human peripheral blood or harvested from rabbit lungs. The presence of intracellular bacteria after a 30-min coincubation was determined by quantitation of bacteria surviving incubation of the bacteria-macrophage mixtures with gentamicin. 1, 2, and 4 h after addition of gentamicin, the number of viable wild type BP536 recovered per 10 human macrophages was 3.1 ± 0.1, 3.6 ± 0.4, and 5.6 ± 0.7, respectively. No avirulent BP537 were recovered under the same conditions. Thus, *B. pertussis* appeared to enter, survive, and slowly multiply within macrophages.

The determinants of intracellular localization were examined by comparing the relative abilities of mutant strains to enter the macrophages. Recovery of viable bacteria from macrophage lysates differed greatly between strains. All strains carrying the FHA RGD region were recovered from within macrophages of both species while those lacking this region were not, even if carbohydrate-dependent adherence of bacteria to the macrophages was intact (Table 1). This was even more apparent when values were normalized for the ability of each strain to adhere to the macrophage surface. For instance, galactose inhibits adherence of BP536 by half but recovery from within cells remained high (Table 1) and intracellular bacteria were seen by direct visualization (Fig. 1, row *B*, number of bacteria in $M > F$). In contrast, BP1098/TOX6, which also adheres half as well as the wild type, showed very low recovery from within cells (Table 1) and few visually discernable intracellular bacteria (Fig. 1, row *A*, number of bacteria in $M = F$). These results indicated that bacterial uptake and survival were promoted through the interaction of the RGD region, as opposed to the carbohydrate recognition domain, of FHA with macrophages.

CR3 Promotes Accumulation of Viable B. pertussis Within Macrophages In Vivo. To determine if virulent *B. pertussis* localized within intra-airway macrophages early in the course of colonization of the rabbit lung, intracellular versus extracellular bacteria were distinguished by differential fixation of cells from bronchoalveolar lavage fluids. In samples fixed with formalin (Fig. 2, column *F*), numerous virulent bacteria were seen adherent to the surface of the macrophages (12 ± 3 bacteria/cell). However, stained bacteria were clearly more abundant (>25 bacteria/cell) and could be seen clustered within macrophages upon fixation of duplicate specimens with methanol (Fig. 2, column *M*). Thus, virulent *B. pertussis* appeared to be present inside alveolar macrophages within the first 24 h of infection of the rabbit tracheobronchial tree. Inhibition experiments were then performed to assess the relative contribution of CR3-dependent and carbohydrate-dependent adherences of *B. pertussis* to uptake into macrophages in vivo. The CR3-dependent interaction was eliminated by aerosol treatment with antibody to CR3, leaving the carbohydrate-dependent binding interactions intact. Although extracellular

Table 1. *Determinants of Intracellular Localization and Survival of Bordetella pertussis within Mammalian Macrophages*

Strain	Active adhesin*	AI‡	Viable intracellular bacteria (percent of wild type at 2 h)	
			Rabbit alveolar macrophage	Human blood macrophage
BP536	FHA + Tox	100	100§	100
BPTOX6	FHA RGD + CRD	100	52 ± 7	43 ± 6
BP536 + galactose	FHA RGD	54	72 ± 12	64 ± 16
BP1098/TOX6	FHA CRD	58	3 ± 8	7 ± 9
BP101/TOX6	None		4 ± 8	6 ± 6

* Tox = pertussis toxin; RGD = Arg-Gly-Asp sequence of FHA; CRD = carbohydrate recognition domain of FHA.
‡ Adherence index expressed as percent of wild type adherence to human macrophages as published in reference 2.
§ 100% = 3.6 ± 0.4 bacteria/10 macrophages. Values are means ± SD from three experiments.

bacteria were somewhat reduced in number (4.3 ± 3 bacteria/cell; Fig. 2, panels *B* and *F*) as compared to the wild type, no additional intracellular bacteria were visualized upon fixation with methanol (4.7 ± 2 bacteria/cell) (Fig. 2, panels *B* and *M*), indicating reduced bacterial accumulation within the airway macrophage. In contrast, inhibition of carbohydrate-dependent binding by treatment with the competitive receptor analog lactose or the anti-receptor antibody mAb 23.24, re-

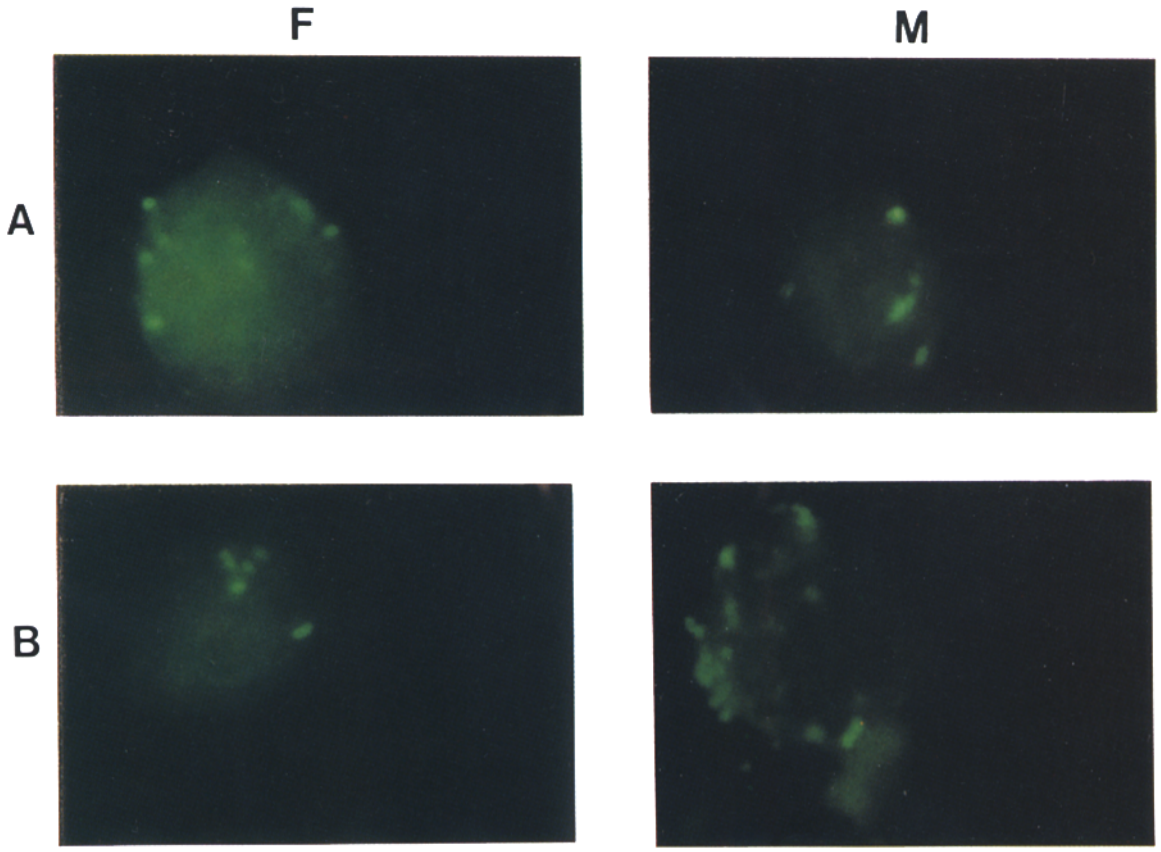

Figure 1. Effect of interruption of RGD versus carbohydrate-dependent attachment on uptake of bacteria into macrophages. The RAD-containing strain BP1098TOX6 (row *A*) or wild-type BP536 plus galactose (50 mM) (row *B*) were incubated with human macrophages. Extracellular attached bacteria (visualized by fixation of cells with formalin [*F*]) and total intracellular and extracellular bacteria (visualized by fixation of cells with methanol [*M*]) were stained with fluorescent anti-*Bordetella* antibody. Uptake into the macrophage (no. of bacteria in *M–F*) was blocked by the RAD mutation but not the carbohydrate competitive inhibitor.

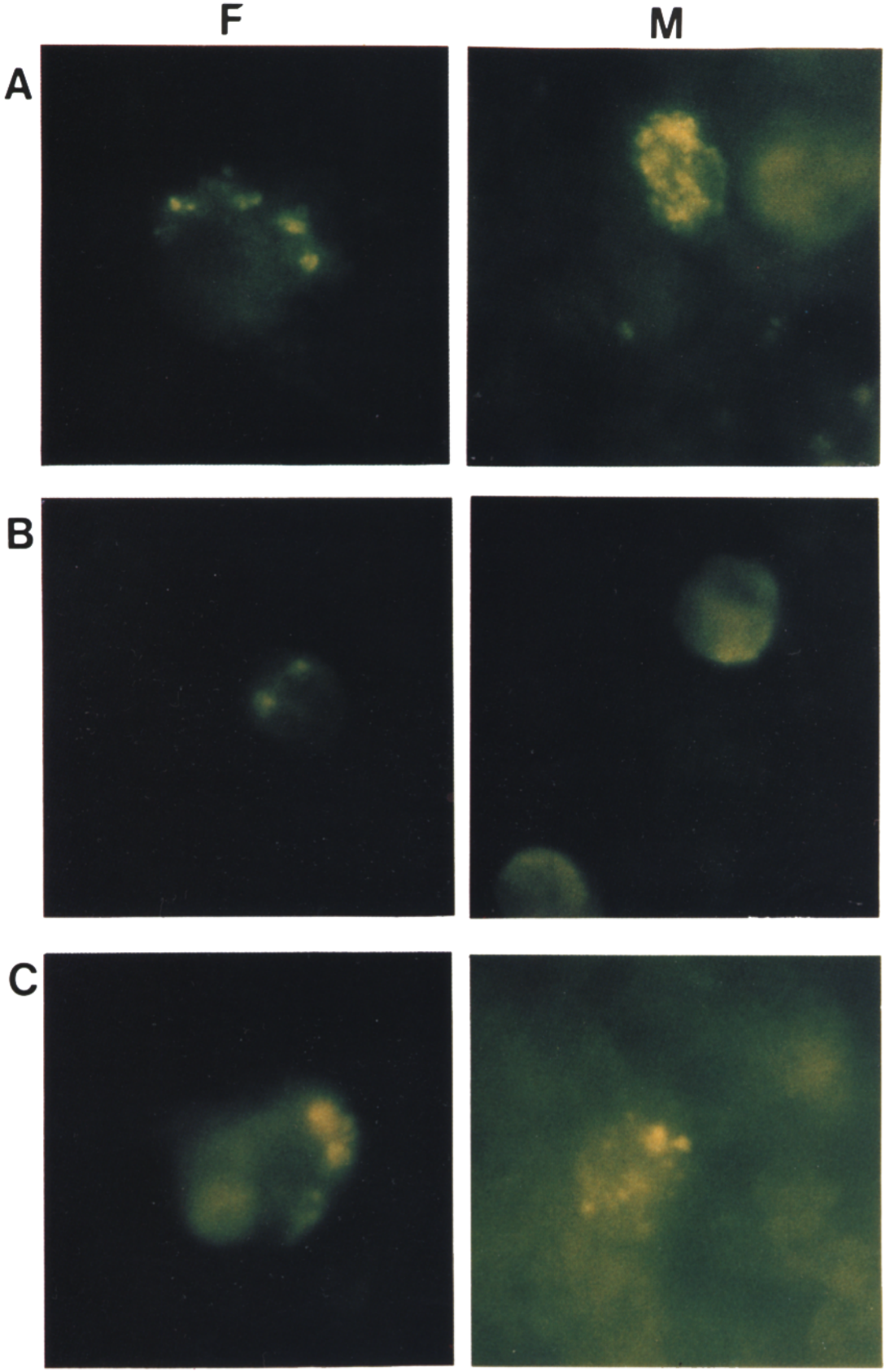

Figure 2. Fluorescence micrographs of alveolar macrophages obtained by lung lavage 24 h after infection. Left and right hand panels in each row are duplicate samples fixed with either formalin (*F*) allowing staining of only extracellular *B. pertussis*, or methanol (*M*) allowing staining of intra- and extracellular bacteria. Animals were challenged with the parental strain BP536 (row *A*), BP536 plus anti-CR3 antibody (row *B*), or BP536 plus either lactose or anti-receptor antibody anti-Lewis a (row *C*).

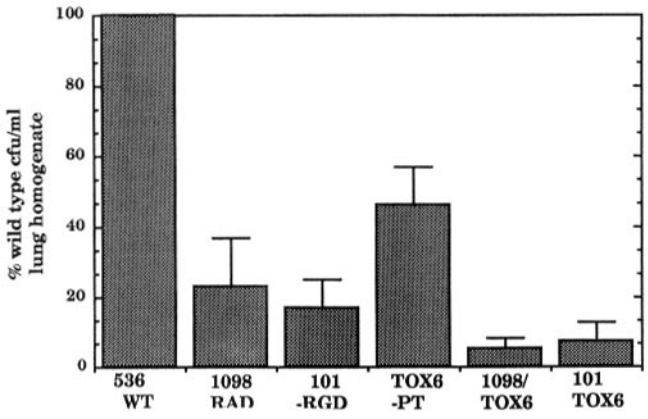

Figure 3. Effect of mutation of FHA on the number of bacteria in lungs at 24 h. Rabbits (at least four per group) were challenged with the designated strain and the total viable bacterial number was determined from lung homogenates and expressed as a per cent of the wild type (WT) strain 536.

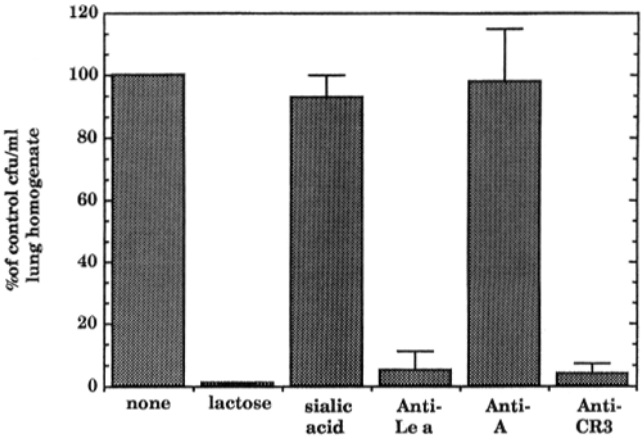

Figure 4. Effect of receptor blockade on pulmonary colonization. Rabbits (at least four per group) were challenged with BP536 plus the designated anti-adherence modalities. Lactose and anti-Le a antibody are known to block carbohydrate dependent adherence while sialic acid or anti-A antibody are ineffective. Anti-CR3 is known to block the CR3 interaction.

duced the number of extracellular bacteria (3.6 ± 3 bacteria/cell) but uptake persisted (15 ± 5 bacteria/cell) (Fig. 2, row *C*). The specificity of these treatments was indicated by the lack of effect on number or distribution of adherent wild type bacteria in animals receiving the noncompetitive carbohydrate sialic acid or antibody 69.4 which does not recognize the *B. pertussis* receptor (data not shown). Thus, CR3 appeared to be of greater importance to directing the adherent bacteria into the macrophages than the carbohydrate-dependent interaction.

Pathophysiologic Significance of Intracellular B. pertussis. To determine the viability of bacteria residing within macrophages during natural infection and the contribution of this population to pulmonary pathology, *B. pertussis* deficient in intracellular localization were compared to wild-type strains for the ability to colonize the pulmonary tract of the rabbit and cause pulmonary edema (Fig. 3). Virulent BP536 colonized the lung in high titer ($3.2 \pm 0.4 \times 10^6$ cfu/ml lung homogenate) and produced pulmonary edema, while the FHA-PT-double mutant (BP101TOX6) did not ($< 10^3$ cfu-ml lung homogenate). Elimination of all FHA-mediated adherence (BP101), decreased colonization to 18 ± 12% of parental values, reflecting the dominance of this adhesin in vivo. To determine the location of bacteria adherent by FHA, animals were challenged with BPTOX6, a mutant deficient in adherence to cilia but not macrophages (1, 2). FHA-dependent adherence to macrophages (by RGD and carbohydrate-dependent mechanisms) contributed significantly to the total lung bacterial load as evidenced by persistent lung colonization (46 ± 7%). This half of the lung population was predominantly involved in RGD- rather than carbohydrate-dependent interactions as indicated by the loss of colonization upon loss of the RGD site in mutant BP1098TOX6 (adheres only in a carbohydrate-dependent fashion to macrophages; Fig. 3).

We interpret these studies with the mutant strains to suggest that the association via FHA of *B. pertussis* with alveolar macrophages contributes substantially to the total bacterial load in the lung, with macrophage- and cilia-associated populations reaching approximately equal numbers early in the course of colonization of the lung. This is consistent with the similar and substantial decreases in total lung colony counts in rabbits receiving anti-carbohydrate (lactose or anti-Lewis a antibody) or anti-CR3 (mAb IB4) directed treatments (Fig. 4). Although numerically similar, the cilia- versus macrophage-associated populations of *B. pertussis* did not contribute equally to pulmonary disease. When pulmonary edema was assessed in animals challenged with the various mutants (Fig. 5), interruption of the RGD interaction by the RAD mutation or antibody to CR3 did not decrease the generation of pulmonary edema. Only treatment of animals with lactose or anti-Lewis a antibody blocked pulmonary edema.

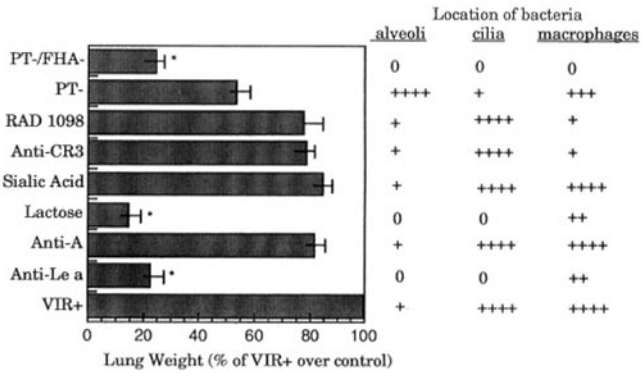

Figure 5. Effect of anti-adherence modalities on pulmonary edema and location of bacterial colonization. Rabbits were challenged as in Figs. 3 and 4 and lung weight was determined at 24 h as a measure of pulmonary edema. Control lung weight was 4.7 ± 0.4 g while the parental strain (VIR+, BP536) induced pulmonary edema with a lung weight of 6.1 ± 0.5 g (* = $p < 0.01$). The location and relative density of bacteria (0 = none, + = few, + + + + = many) were determined by differential staining of lavage fluids or sections of trachea or distal lung as in Figs. 1 and 2.

Discussion

B. pertussis has been shown to invade and survive within tissue culture cells (3, 4), and preliminary evidence suggested these bacteria could multiply in leukocytes in vitro (5) and could be found within the alveolar macrophages of some patients with HIV disease subjected to bronchoalveolar lavage for diagnosis of pulmonary infection (K. Bromberg, Downstate Medical Center, Brooklyn, NY, personal communication). Our results demonstrate that these findings are relevant to the pulmonary tract where *B. pertussis* resides throughout the natural course of whooping cough. In vitro, *B. pertussis* was shown to enter cultured human macrophages and freshly harvested rabbit alveolar macrophages by subverting a natural leukocyte-specific phagocytic apparatus involving the integrin CR3. Uptake was followed by bacterial survival and slow multiplication over 4 h. In a rabbit model of infection, differential staining of lavage fluids indicated that *B. pertussis* established an intracellular state within alveolar macrophages early in the course of pulmonary colonization. CR3 appeared to promote uptake of bacteria into macrophages in vivo as evidenced by the lack of intracellular bacteria in lavage specimens from animals challenged with the RAD mutant or from animals challenged with the wild-type strain in conjunction with anti-CR3 antibody. Since CR3 is a leukocyte-specific integrin, such intracellular localization is not likely to extend to other cells of the respiratory epithelium.

Approximately half of the total bacterial population recoverable from the lung appeared to reside within macrophages. However, pulmonary pathology could not be attributed to this population since even a 90% decrease in total lung bacterial number achievable by treatment with anti-CR3 antibody for 24 h did not diminish pulmonary edema. Reduction of pulmonary edema required elimination of carbohydrate-dependent adherence suggesting that the extracellular bacterial population associated with ciliary and macrophage glycoconjugates was the primary source of bacterial products, such as pertussis toxin and adenylate cyclase toxin, causing lung injury. We propose that agents such as receptor analogues or anti-receptor antibodies, which promote clearance of the extracellular bacterial population, may represent potential therapeutic modalities capable of decreasing lung tissue damage in natural disease (6).

We conclude that CR3 promotes internalization of *Bordetella* within macrophages in vitro and in vivo. This uptake does not lead to bacterial killing but rather results in a net enhancement of the number of bacteria persisting in the lung. The fact that intracellular bacteria did not contribute to pulmonary pathology indicates that either the intracellular reservoir may not permit release of bacterial toxins or, alternatively, that the intracellular environment may induce the bacteria to modulate to the avirulent phenotype and cease toxin production. This latter possibility is suggested by preliminary results measuring a cessation of adenylate cyclase toxin production by *B. pertussis* upon entry into alveolar macrophages in vitro (R. Masure, Rockefeller University, NY, personal communication). The intracellular population may be a reservoir from which virulent bacteria can reemerge later in the course of infection. This hypothesis would explain two features of natural infection: (*a*) the reappearance of tracheal colonization many days into the course of infection in a rat model of disease (17), and, (*b*) the protracted 6-wk course of human disease despite apparent disappearance of culturable bacteria from cilia after 2 wk of overt infection.

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