

Invasion and Intracellular Survival of *Bordetella bronchiseptica* in Mouse Dendritic Cells

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We have studied the interaction between the respiratory pathogen *Bordetella bronchiseptica* and murine spleen dendritic cells, important antigen-presenting cells that are found in the airway epithelium. Wild-type *B. bronchiseptica* 5376 attached very efficiently to dendritic cells, whereas the *bvg* mutant ATCC 10580, wild-type strain BB7865, and its spontaneous $\Delta bvgS$ mutant BB7866 bound less efficiently. However, all tested *B. bronchiseptica* strains were able to invade dendritic cells and survive intracellularly for at least 72 h. These results suggest that *bvg*-independent or *bvg*-downregulated products are involved in the uptake and intracellular survival. Transmission electron microscopic analysis revealed that bacteria grew and replicated intracellularly and were present in typical phagosomes, which fused with lysosomes during the initial infection period. However, in later infection stages some bacteria seemed to escape into an unfused endocytic compartment, where individual bacteria were tightly surrounded by a membrane. The in vitro interaction of *B. bronchiseptica* with dendritic cells reported here may be relevant to natural infections caused by this organism that lead to chronicity or an altered immune response.

Bordetella bronchiseptica is a respiratory pathogen that has been isolated from many warm-blooded animals (12). Unlike other *Bordetella* species, *B. bronchiseptica* exhibits a wide host spectrum and produces a variety of pathologic syndromes (12). It is responsible for considerable economic losses amounting to millions of dollars every year by causing epizootic respiratory diseases in confinement-reared animals (12). On the other hand, humans are not natural hosts, although a number of cases of human bordetellosis have been reported, mostly in animal caretakers (12).

B. bronchiseptica synthesizes several products that have been correlated with its potential to cause disease, namely, filamentous hemagglutinin (FHA), adenylate cyclase (14), dermonecrotic toxin which seems to be involved in vivo in the suppression of antibody responses (17) and the production of nasal and lung lesions (32), pili (1), and flagella (12). As in other *Bordetella* species, the production of most of the virulence-associated determinants that have been described is coordinately regulated by the *bvg* locus during antigen modulation and phase variation (26, 48).

Bordetella spp. have been described as noninvasive pathogens, and the major pathogenic role has been ascribed to the release of toxins following attachment and colonization of the respiratory tract epithelial cells (29). However, in recent years evidence that strongly supports the invasive potential of *B. bronchiseptica* (34, 35) and other *Bordetella* spp. has accumulated (3, 4, 7, 8, 11, 22, 31, 33). It has been also proposed that interactions of *Bordetella pertussis* with macrophages could lead to persistence in this reservoir and/or altered clearance functions (11, 33).

Dendritic cells (DC) are a highly specialized subset of antigen-presenting cells (19, 20, 42, 43). They are part of a system that is widely distributed in nonlymphoid tissues and

the T-cell-dependent areas of lymphoid organs (42) and which is connected by movement and homing (42, 43). They are the most efficient antigen-presenting cells and play a key role in the immune system. DC are the major source of epitopes for specific T-cell clones and the main activators of resting T cells and can initiate primary immune responses in vitro and in vivo (42).

Peripheral DC are juxtaposed to macrophages in the mouse and human airway epithelium, lung parenchyma, and pleura (27, 36). Moreover, it has been demonstrated that lung DC pulsed in vivo with antigens can activate T cells in vitro (27). It is therefore interesting to analyze the interaction between a pathogen with a respiratory portal of entry like *B. bronchiseptica* and DC, which are main determinants of the systemic and local immune responses.

DC can be purified in small quantities from animal tissues and are viable for a limited period (42). The quantities thereby obtainable, the limited viability, and lack of homogeneity render primary DC rather unsuitable for many types of experiments. However, a protocol for immortalization of fully functional DC lines has been described recently (28). A clonal population of such cells should be particularly suitable for analyzing interactions between DC and respiratory pathogens and their role in the pathophysiology of infective processes. In the present work, a murine spleen DC line was used to analyze the interactions between *B. bronchiseptica* and DC. It was shown that attachment led to invasion, cellular spread, and persistent infection.

MATERIALS AND METHODS

Bacterial strains and media. *B. bronchiseptica* 5376 (46), ATCC 10580 (*bvg*), BB7865 and its spontaneous $\Delta bvgS$ avirulent-phase derivative BB7866 (13, 26), *B. pertussis* BP338 (48), *Escherichia coli* HB101 (2), and *Staphylococcus aureus* SA1 (isolated from a burn wound; from the strain collection of the Institute of Microbiology, University of Genova) were used in this work.

Bordetella strains were grown on Bordet-Gengou agar base

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(Difco Laboratories, Detroit, Mich.) supplemented with 1% glycerol and 15% (vol/vol) defibrinated horse blood or in SS broth (39), and *E. coli* and *S. aureus* strains were grown in brain heart infusion broth or agar (Difco Laboratories).

Tissue culture methods and invasion assays. The spleen DC line CB1, obtained from DBA/2 mice (28), was maintained in Iscove's modified Dulbecco's medium (Sigma Chemie GmbH, Deisenhofen, Germany) supplemented with 5% fetal calf serum and 5 mM glutamine (GIBCO Laboratories, Eggenstein, Germany) in an atmosphere containing 5% CO₂ at 37°C. Cells were seeded at a concentration of approximately 5×10^4 per well in 24-well Nunclon Delta tissue culture plates (Inter Med NUNC, Roskilde, Denmark). The plates were incubated for 18 h; then, they were washed twice with complete medium. For infection assays, bacteria were grown for 24 h on agar plates, recovered with sterile swabs, and suspended in complete Iscove's medium, and the suspensions were adjusted spectrophotometrically to an optical density at 540 nm corresponding approximately to 2×10^7 CFU ml⁻¹; 0.5 ml of the suspension was then added to each well, and the plates were incubated under static conditions for 1 h. The supernatant fluids were discarded, and cells were washed twice with phosphate-buffered saline (PBS) to remove nonadherent bacteria. The medium was replaced with 0.5 ml of complete Iscove's medium supplemented with 100 µg of gentamicin ml⁻¹ (Sigma Chemie GmbH) and incubated at 37°C for different periods to kill the remaining extracellular bacteria. Cells were washed twice with PBS and then lysed by addition of 0.5 ml of water to each well, and the number of CFU recovered from each well was determined by plating 10-fold dilutions on agar with a Spiral Plater model C (Spiral Biotech, Inc., Bethesda, Md.). The results reported are mean values of three independent assays \pm standard deviations.

Fluorescence staining of cellular matrix proteins and *B. bronchiseptica*. Cells were grown on round 13-mm Thermanox coverslips in 24-well Nunclon Delta tissue culture plates (Inter Med NUNC). After infection with *B. bronchiseptica* bacteria and incubation with Iscove's medium containing gentamicin, the coverslips were rinsed with PBS, and the cells were fixed in 3.7% formaldehyde in PBS and permeabilized to allow entry of antibodies by a 5-min treatment with 0.2% (vol/vol) Triton X-100 in PBS. Coverslips were processed for single fluorescence microscopy with rabbit polyclonal antibodies against α -actinin diluted 1:50 or antimyosin diluted 1:10 (Sigma Chemie GmbH) as first antibodies. For double fluorescence, rabbit polyclonal antibodies raised against whole *B. bronchiseptica* cells (gift of L. Ständner, GBF) were used at a 1:200 dilution, together with either tetramethyl-rhodamine-isothiocyanate-labelled phalloidin (Sigma Chemie GmbH) used at a 1:50 dilution (F-actin labelling), monoclonal antibodies (MAbs) to vinculin (clone VIN-11-5; Sigma Chemie GmbH) at a 1:50 dilution, MAb against tropomyosin (clone TM 311, Sigma Chemie GmbH) at a 1:400 dilution, or MAb against α -tubulin (clone DM1A; Sigma Chemie GmbH) at a 1:400 dilution. Coverslips were then incubated for 30 min at 37°C, carefully rinsed with PBS, treated for 30 min with the second antibodies diluted 1:50 (either rhodamine or fluorescein isothiocyanate-conjugated rabbit anti-mouse or goat anti-rabbit immunoglobulins [Dianova, Hamburg, Germany]), washed three times, and then mounted for microscopy with a Zeiss Axiophot microscope equipped with epifluorescence.

Determination of *B. bronchiseptica* attachment and internalization by immunofluorescence. The experiments were performed with a bacterium/cell ratio of 100 and double fluorescence as previously described (15). Cells were grown on round 13-mm Thermanox (Inter Med NUNC) coverslips as described

above for the fluorescence staining of cellular matrix proteins. Adherence and internalization were expressed as the mean number of bacteria adhering or internalized per cell \pm standard deviation. The mean was calculated by averaging the number of bacteria present in 25 cells.

Transmission electron microscopy (TEM). Infected cells were fixed with a solution containing 3% glutaraldehyde and 5% formaldehyde in cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M MgCl₂, 0.01 M CaCl₂, pH 6.9) for 45 min on ice, washed with cacodylate buffer, fixed for a further hour at room temperature with 1% (wt/vol) aqueous osmium tetroxide solution, washed with cacodylate buffer, and scraped off the culture flask. After 2 min of centrifugation at $2,000 \times g$, infected cells were embedded in 1.5% agar, after which the agar was cut into small cubes and embedded in Spurr resin (38). Ultrathin sections were cut with a glass knife and counterstained with 4% aqueous uranyl acetate (pH 4.5) and lead citrate. Sections were examined with Zeiss transmission electron microscope EM 910 at an acceleration voltage of 80 kV at calibrated magnifications.

Immunoelectron microscopy. MAb produced by rat hybridoma MAC-3 clone ATCC TIB168 was used to selectively label the lysosomal membrane of DC (18). Samples were fixed and embedded as previously described (47). Immunolabelling was made with a 1:5 dilution of the protein G-Sepharose-purified MAC-3 MAb (80 µg of immunoglobulin G protein ml⁻¹). Ultrathin sections attached to Formvar-coated nickel grids were incubated on drops of the diluted antibodies for 12 h at 4°C. After grids were washed with PBS, the bound antibodies were labelled for 1 h with goat anti-rat antibody-gold complexes (10-nm gold particle size; Plano GmbH, Marburg, Germany). Grids were subsequently rinsed with PBS containing 0.01% Triton X-100 and then distilled water. After air drying, the sections were counterstained with 4% aqueous uranyl acetate (pH 4.5) for 5 min.

Scanning electron microscopy. Infected cells on round 13-mm Thermanox coverslips (Inter Med NUNC) were fixed in a solution containing 3% glutaraldehyde and 5% formaldehyde in PBS for 45 min on ice and then washed with PBS, dehydrated in a graded series of acetone, and subjected to critical point drying with CO₂. Samples were then covered with a 10-nm gold film and examined with a Zeiss DSM 940 scanning electron microscope.

RESULTS

Attachment to and invasion of mouse DC by *B. bronchiseptica*. As can be seen in the scanning electron microscopy analysis presented in Fig. 1, *B. bronchiseptica* 5376 attaches very efficiently to CB1 cells during a 2-h incubation (Fig. 1A, B, and C). In similar experiments with *B. bronchiseptica* BB7865, BB7866 (*bvgS*), and ATCC 10580, fewer attached bacteria were observed (Fig. 1D and E). *B. bronchiseptica* bacteria exhibited a preferential avidity for the cell periphery (Fig. 1C) and lamellipodia (dendritic cell processes, Fig. 1A and B); this disposition pattern was particularly evident in side views (Fig. 1C). Almost 100% of DC observed exhibited attached bacteria, and all had a similar attachment pattern. Similar results were obtained by immunofluorescence of attached bacteria when DC were infected with the strains 5376, ATCC 10580, BB7865, and BB7866 for 45 min. The mean number of bacteria attached per cell \pm standard deviation was 24 ± 5 , 6 ± 2 , 5 ± 2 , and 7 ± 2 , respectively.

In order to investigate invasion of DC by *B. bronchiseptica*, samples were analyzed by epifluorescence microscopy after double immunolabelling. Approximately 24 ± 7 , 20 ± 7 , $23 \pm$

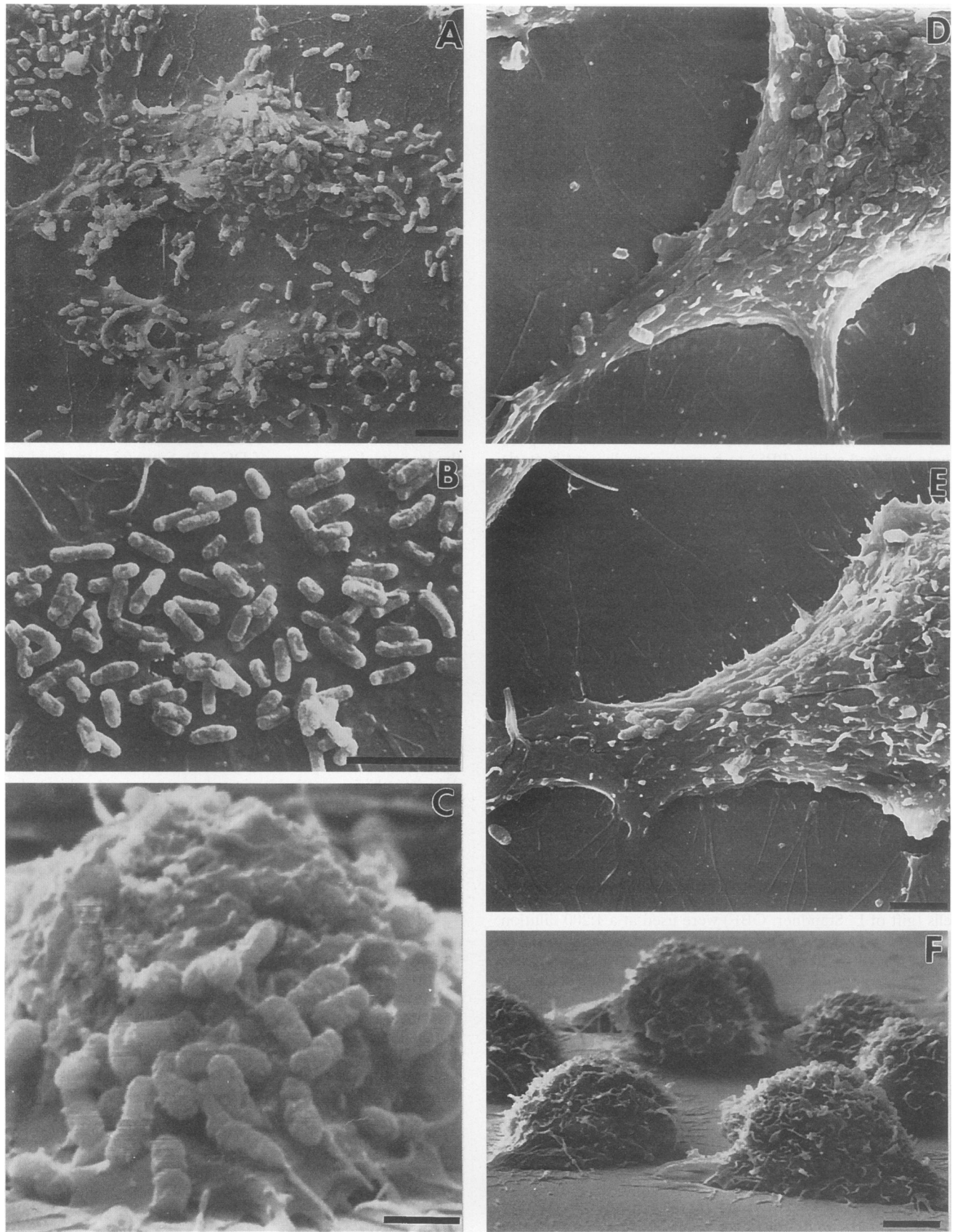


FIG. 1. Scanning electron microscopic analysis of DC infection by *B. bronchiseptica* strains. CB1 cells were infected with the strains *B. bronchiseptica* 5376 (A to C), BB7865 (D), and BB7866 (E). In panel F, uninfected cells are shown. Panels A, B, D, and E are top views, whereas panels C and F are tilted views of the sample (75°). Bars, 3 μm (A, B, D, E, and F) or 1 μm (C).

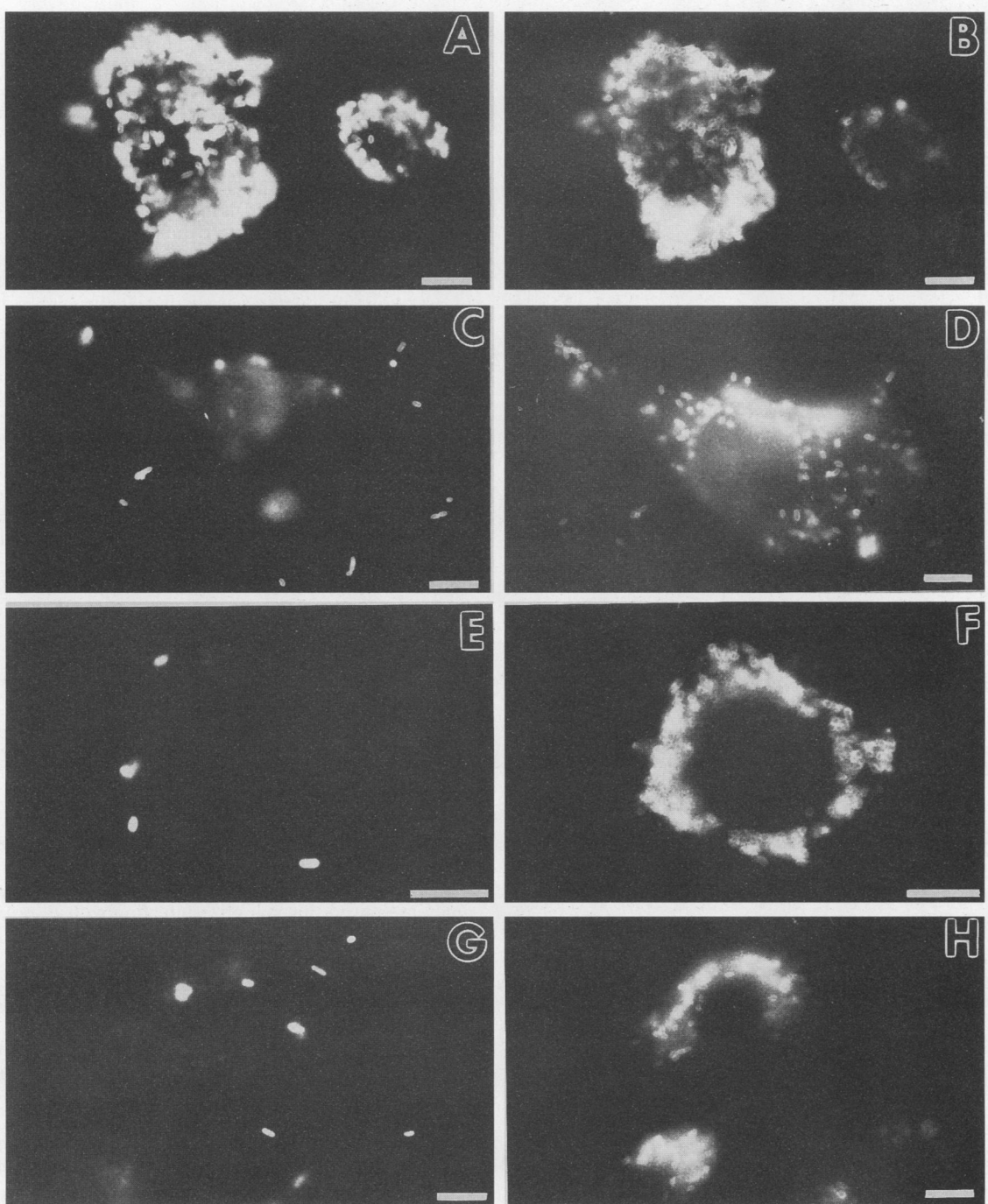


FIG. 2. Localization of *B. bronchiseptica* on infected cells. DB1 cells were processed for double immunofluorescence 2 h after infection with *B. bronchiseptica* 5376 (A and B), BB7865 (C and D), BB7866 (E and F), and ATCC 10580 (G and H). In panels A, C, E, and G, extracellular bacteria are labelled, whereas in panels B, D, F, and H both intracellular and extracellular bacteria are labelled in the corresponding microscopic fields. Bars, 5 μ m.

7, and 30 ± 10 internalized bacteria, respectively, were present per DC after 45 min of infection with strains 5376, ATCC 10580, BB7865, and BB7866. Therefore, *B. bronchiseptica* 5376, which attaches very efficiently, and wild-type BB7865 or the *bvg* mutants ATCC 10580 and BB7866, which exhibit weaker binding, were efficiently internalized by CB1 cells (Fig. 2). Invasion of DC seems thus to depend primarily either on

host cell processes or on bacterial products synthesized independently of the *bvg* regulatory system.

Time course of DC infection by *B. bronchiseptica*. Immunofluorescence is not able to discriminate between live and dead bacteria; therefore, to analyze the kinetics of *B. bronchiseptica* uptake by CB1 cells and to establish whether the internalized bacteria were viable, time course internalization experiments

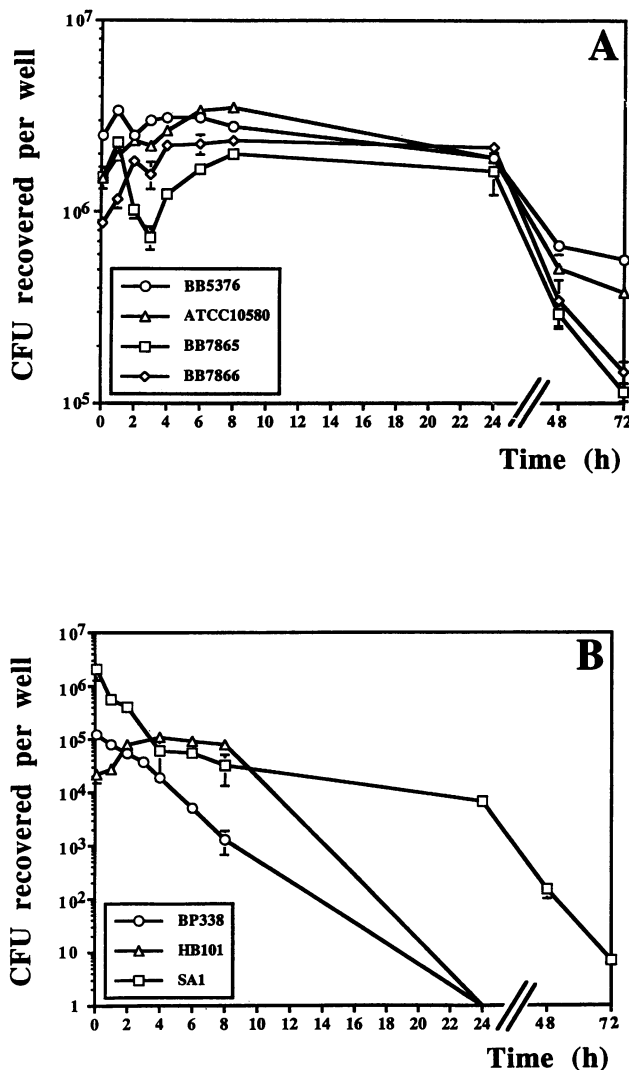


FIG. 3. Intracellular survival of *B. bronchiseptica*. CB1 cells were infected with *B. bronchiseptica* 5376, ATCC 10580, BB7865, and BB7866 (A) and *B. pertussis* BP338, *E. coli* HB101, and *S. aureus* SA1 bacteria (B). After 1 h with gentamicin-containing medium, cells were lysed at different intervals and the number of CFU recovered per well was determined. Results are presented as the means \pm standard deviations of three independent experiments.

were performed (Fig. 3A). *B. bronchiseptica* wild-type strains and the *bvg* mutants were all efficiently internalized by CB1 cells and retained viability. After 1 h of incubation with gentamicin (time zero), between 1 and 5.6% of the inocula was recovered from cells. The differences in attachment efficiencies detected by scanning electron microscopy and epifluorescence (see above) probably account for the small differences in the number of intracellular bacteria of the different strains at time zero. Invasion curves exhibited a typical pattern for all strains with an initial increment of approximately 59% in the number

of the viable bacteria during the first 1 to 2 h followed by a reduction over the next few hours, which was more evident for virulent *B. bronchiseptica* strains. The second phase is followed by a further increment in the number of viable bacteria over the next few hours. Samples taken 24 to 72 h after infection clearly showed that all *B. bronchiseptica* strains persist in DC without killing infected cells (remained trypan blue exclusion positive). This persistence was due not only to cell-to-cell spread during infection, as almost 100% of the cells were infected at time zero (immune fluorescence studies). The small reduction in the number of microorganisms recovered after 48 h might be due to reduced cell viability caused by the presence of metabolic products accumulated at the high cell densities reached.

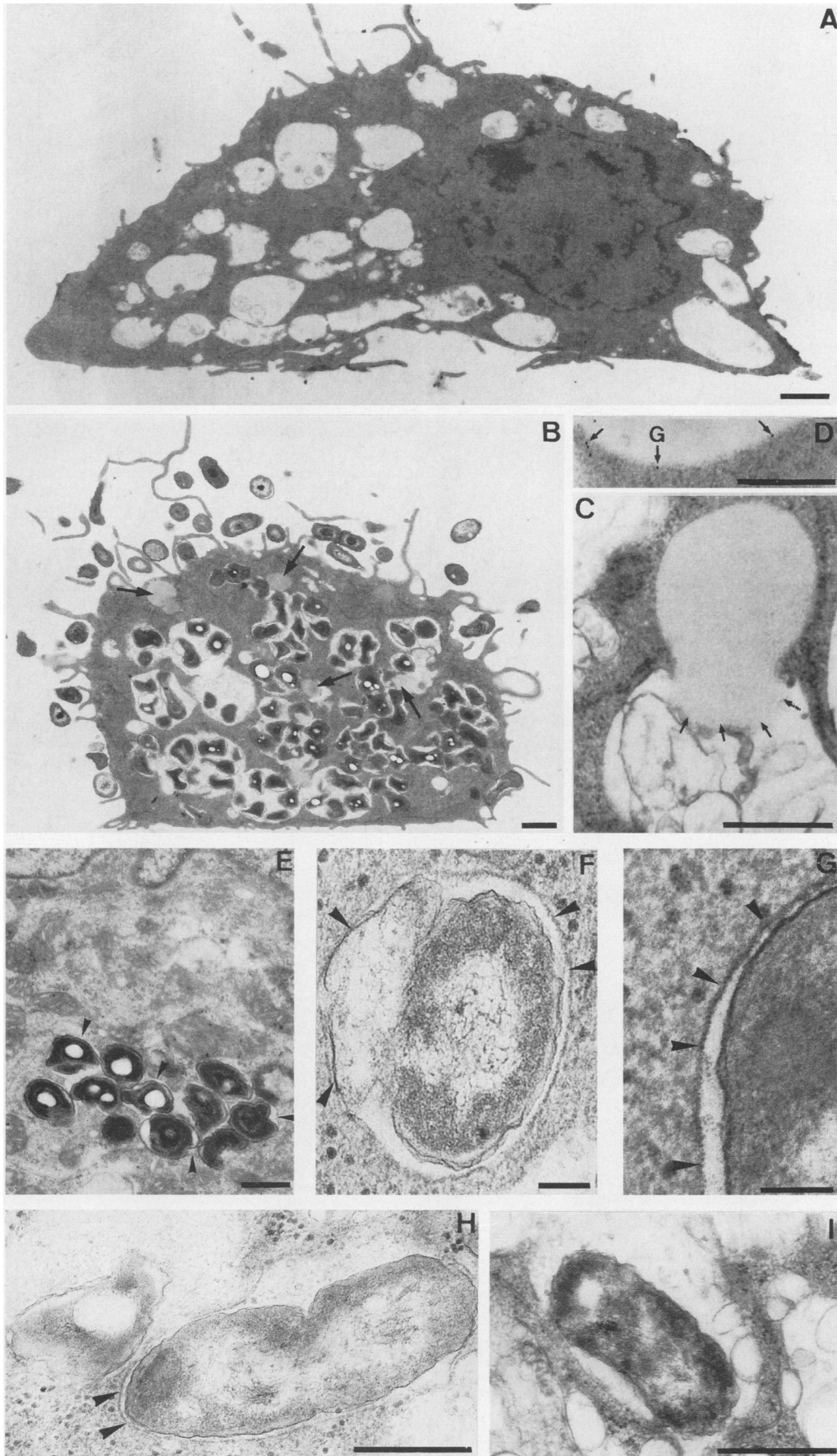
In order to analyze whether long-term intracellular survival is a specific property of *B. bronchiseptica* or a property of bacteria in general in the interaction with DC, similar experiments were performed with strains *B. pertussis* BP338, *E. coli* HB101, and *S. aureus* SA1. The results reported in Fig. 3B demonstrate that intracellular survival is a specific property of *B. bronchiseptica*: BP338 and HB101 strains were internalized at least 1 order of magnitude less efficiently than *B. bronchiseptica* strains, and after 24 h of infection, no viable bacteria were recovered. On the other hand, *S. aureus* SA1 was taken up as efficiently as *B. bronchiseptica*, but its intracellular survival was very short: the number of viable bacteria was greatly reduced after 4 h of infection and fell by 2 to 4 orders of magnitude after 24 to 72 h.

A number of invasive pathogens cause major alterations in the distribution of host cell cytoskeletal proteins following invasion (5, 9). However, in the CB1-*B. bronchiseptica* system, fluorescent labelling of cytoskeletal proteins and bacteria did not reveal any specific alterations in the distribution patterns of vinculin, actin, α -actinin, tropomyosin, myosin, or tubulin after 4 or 24 h of infection (data not shown). This could in part explain the ability of *B. bronchiseptica* to infect DC persistently without causing lethal damage.

TEM analysis of DC infection by *B. bronchiseptica*. TEM analysis showed that 4 h after infection with *B. bronchiseptica* bacteria were either attached to the cell surface or enclosed within cytoplasmic vesicles with the characteristics of phagosomes (Fig. 4B and Fig. 5A and D). Phagosomes were generally surrounded by round vesicles, some of which were in the process of fusing (Fig. 4B and C and Fig. 5A and D). Immunoelectron microscopy performed with the MAC-3 MAb specific for a glycoprotein of the lysosomal membrane (18) demonstrated that these vesicular structures were lysosomes (Fig. 4D).

Lysosomes were not detected or reduced in number in samples obtained after 24 h postinfection (Fig. 4E to I and Fig. 5B, C, E, and F). At this point, many vacuoles with resident *B. bronchiseptica* cells exhibited specific morphological changes: membranes of phagosomes containing bacteria were in tight contact with the bacterial surface (Fig. 4E to H and Fig. 5G), and many *B. bronchiseptica* cells were segregated with one cell per vacuole (Fig. 4F to H); this pattern was more conspicuous for strain 5376. The presence of single bacteria within the vacuoles of cells infected with *B. bronchiseptica* would be consistent with the notion that intravacuolar multiplication

FIG. 4. TEM analysis of dendritic cell infection by *B. bronchiseptica* 5376. (A) Control uninfected cells; (B to I) cells infected with *B. bronchiseptica* 5376 at 4 h (B to D), 24 h (E to H), and 72 h (I) after infection. Lysosomes are indicated by arrows, whereas phagosome membranes are identified by arrowheads. In panel C, a phagolysosome fusion and, in panel D, gold particles (G) bound to lysosomal membrane are indicated by small arrows. Bars, approximately 1 μ m (A and B), 0.5 μ m (C, D, E, H, and I), and 0.1 μ m (F and G).



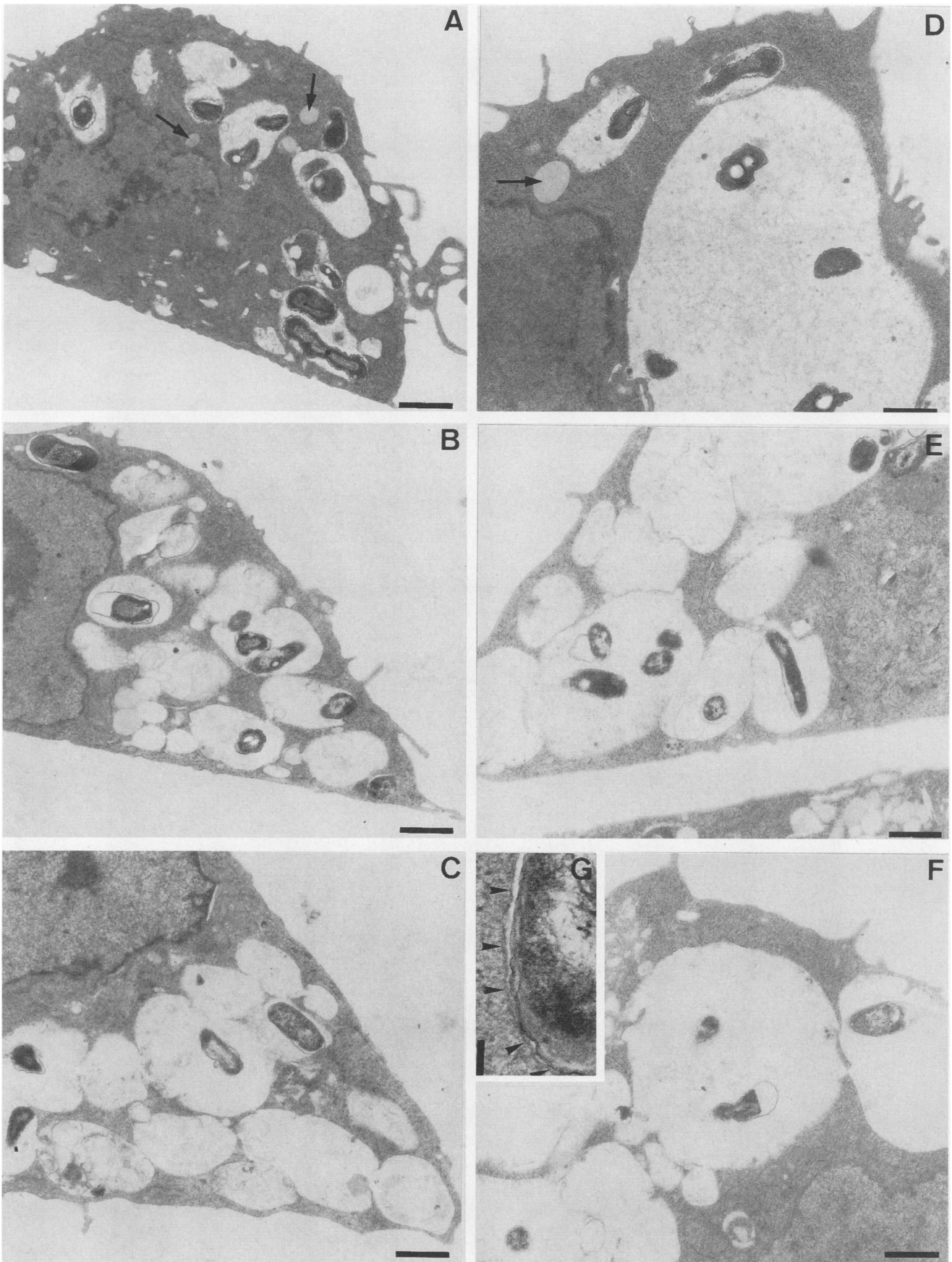


FIG. 5. TEM analysis of cells infected with *B. bronchiseptica* BB7865 and BB7866. Cells were infected with strain BB7865 (A to C) or BB7866 (D to G), and samples were processed after 4 h (A and D), 24 h (B and E), or 72 h (C, F, and G). The phagosome membrane is indicated by arrowheads (panel G), whereas lysosomes are indicated by arrows. Bars, 1 μm (A to F) and 0.1 μm (G).

does not occur but that bacteria are apparently able to grow and replicate within the host cell (Fig. 4H and Fig. 5A). At low magnifications (Fig. 4E) and by time-lapse microscopy (data not shown), bacteria in phagosomes with the tightly apposed membranes appeared as free intracytoplasmic bacteria. However, higher magnifications revealed that they are enclosed by a double membrane (Fig. 4F to H and Fig. 5G). Some phagosomes containing membranous debris, probably derived from killed bacteria, were observed in cells infected with all strains (Fig. 4B, C, and I and Fig. 5C).

DISCUSSION

Bordetella spp. have been classified as noninvasive pathogens. However, this conclusion is based on experiments with a limited number of cells that do not represent all those present in the respiratory tract during natural infections (29). The data presented here demonstrate that DC are permissive host cells for *B. bronchiseptica* invasion and replication and raise the possibility that interactions between DC and *Bordetella* spp. may be relevant in natural infections. In the light of these data and recent reports from other laboratories using other cells (34, 35), the invasive potential of *B. bronchiseptica* should be reevaluated as this microorganism seems to behave like true invasive bacteria. It would seem prudent to assess the role of invasiveness *in vivo* in the pathogenesis of bordetellosis.

Our results suggest that *B. bronchiseptica* may attach extensively to one or more surface structures and, to a lesser extent, via another *bvg*-independent adhesin(s) to different receptors, which in turn trigger the internalization process. In fact, DC are practically devoid of complement receptors which mediate uptake of *Bordetella* spp. by macrophages via the *bvg*-dependent FHA (16, 31).

The initial reduction in the number of viable bacteria between 1 and 4 h after infection might result from damage caused by the virulent strain provoking either a cell response leading to bacterial killing or cell lysis with release of the invading microorganisms into the gentamicin-containing medium. This reduction corresponds with the presence of damaged bacteria as shown by TEM and suggests that after a latency period following internalization one or more genes specifying products involved in *in vivo* survival are expressed. Alternatively, two bacterial subpopulations, one resistant and one sensitive to intracellular killing, may be present during the first intracellular stages. *B. bronchiseptica bvg* bacteria as well as the wild type had survived intracellularly after 72 h postinfection. Therefore, a *bvg*-independent function, or basal levels of a *bvg*-regulated protein, permits survival of *B. bronchiseptica* inside DC. After entry into the host cells, different environmental signals may result in up- or downregulation of several bacterial genes (25), such that intracellular survival is favored by modulation to a quasiavirulent phenotype characterized by production of virulence factors at minimum levels and/or by the synthesis of new products which are *bvg* independent or downregulated.

Invasive bacteria that do not escape from the phagosome are able to survive intracellularly by their (a) resistance to lysosomal enzymes, (b) inhibition of phagolysosome fusion and/or respiratory burst, or (c) uptake by a pathway that does not lead to phagolysosome fusion (9). It has been proposed that *B. pertussis* can survive in polymorphonuclear cells in part by inhibiting phagolysosome fusion, since respiratory burst occurs at normal levels (40, 41). The morphological evidence presented here suggests that, following a normal phagolysosome fusion in the initial period, *B. bronchiseptica* can escape from the fused vesicle to another unfused compartment where its

envelopes are in close contact with the vesicle membrane. In their morphological study of *B. pertussis* invasion of HeLa cells, Ewanovich et al. (7) also showed a close proximity between bacterial and vacuole membranes. This aspect of the endocytic vacuole containing a single bacterium also closely resembles that described for *Yersinia pestis* and *Mycobacterium tuberculosis* (24, 44) and may be related to resistance to killing and intracellular survival. It has been reported recently that *B. bronchiseptica* is able to escape from phagosomes of epithelial cells after 3 days (35), although we did not observe any free cytoplasmic bacteria in DC. The disappearance of lysosomes that are typical of the initial infection period suggests that *B. bronchiseptica* synthesizes products that affect not only phagolysosome fusion but also lysosome number and/or distribution. Other mechanisms may also be involved in intracellular survival, such as the synthesis of products that render bacteria resistant to lysosomal enzymes.

The role of *Bordetella* spp. virulence factors in uptake and intracellular survival is controversial. It has been reported that *bvg* mutants of *B. pertussis* and mutants defective in pertussis toxin (PT) or FHA are less invasive for HeLa cells than wild-type strains (7). However, Lee et al. (21) showed that PT- and FHA-negative mutants invaded more than the parent strain. On the other hand, while PT and adenylate cyclase inhibit phagocyte respiratory burst and chemotaxis *in vitro* (10, 37), adenylate cyclase mutants survived intracellularly better than wild type, probably because of the lower intracellular levels of cyclic AMP, which increases the susceptibility of bacteria to lysosomal enzymes (16). *B. bronchiseptica* does not produce PT; therefore, this protein seems not to be involved either in bacterial entry into or in survival of *Bordetella* spp. within DC. The superoxide dismutase and catalase produced by *Bordetella* spp. seem unlikely to play an important role as *B. pertussis* was unable to survive intracellularly for extended periods.

The cells used throughout this work are fully functional and produce all molecules necessary to prime resting T cells either *in vivo* or *in vitro* (28). The results reported here may thus be pertinent to *in vivo* interactions of respiratory pathogens with DC of the airways and the consequences of these for immune responses. In the case of *B. bronchiseptica*, a pathogen that generally seems to exhibit, like a commensal, an extended interaction with the host, thereby favoring chronic processes (32, 45), these interactions may delay onset of the immune response. The presence of *B. bronchiseptica* in the DC reservoir inhibiting specific and nonspecific defense mechanisms that normally lead to bacterial clearance may also contribute to chronicity (6). Infections by *B. bronchiseptica* also predispose the host to secondary infections, which are in part responsible for the disease symptoms (23). These secondary infections might be favored by epithelial damage or by impairment of local DC functions. The clearance of infections caused by *Bordetella* spp. is a very complex phenomenon that is not yet well understood. The location of *B. bronchiseptica* in an unfused compartment of intact cells might enhance the export of antigens to the cytoplasm, thereby improving major histocompatibility complex class I-restricted presentation. This could explain the importance of cell-mediated immunity in protection against *Bordetella* infections and the predominant Th1 immune response elicited following natural infection (30).

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REFERENCES

- Bemis, D. A., and S. A. Wilson. 1985. Influence of potential determinants on *Bordetella bronchiseptica*-induced ciliostasis. *Infect. Immun.* **50**:35–42.
- Boyer, H. W., and D. Roulland-Dussoix. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* **41**:459–472.
- Bromberg, K., G. Tannis, and P. Steiner. 1991. Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. *Infect. Immun.* **59**:4715–4719.
- Cheers, C., and D. F. Gray. 1969. Macrophage behavior during the complaisant phase of murine pertussis. *Immunology* **17**:875–887.
- Clerc, P., and P. J. Sansonetti. 1987. Entry of *Shigella flexneri* into HeLa cells: evidence for directed phagocytosis involving actin polymerization and myosin accumulation. *Infect. Immun.* **55**:2681–2688.
- Conlan, W., and R. North. 1993. Neutrophil-mediated lysis of infected hepatocytes. *ASM News* **59**:563–567.
- Ewanowich, C. A., A. R. Melton, A. A. Weiss, R. K. Sherburne, and M. S. Peppler. 1989. Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. *Infect. Immun.* **57**:2698–2704.
- Ewanowich, C. A., R. K. Sherburne, S. F. P. Man, and M. S. Peppler. 1989. *Bordetella parapertussis* invasion of HeLa 229 cells and human respiratory epithelial cells in primary culture. *Infect. Immun.* **57**:1240–1247.
- Finlay, B. B., and S. Falkow. 1988. A comparison of microbial invasion strategies of *Salmonella*, *Shigella*, and *Yersinia* species. *UCLA Symp. Mol. Cell. Biol.* **64**:227–243.
- Friedman, R. L., R. L. Fiederlein, L. Glasser, and J. N. Galgiani. 1987. *Bordetella pertussis* adenylate cyclase: effects of affinity-purified adenylate cyclase on human polymorphonuclear leukocyte functions. *Infect. Immun.* **55**:135–140.
- Friedman, R. L., K. Nordensson, L. Wilson, E. T. Akporiaye, and D. E. Yocum. 1992. Uptake and intracellular survival of *Bordetella pertussis* in human macrophages. *Infect. Immun.* **60**:4578–4585.
- Goodnow, R. A. 1980. Biology of *Bordetella bronchiseptica*. *Microbiol. Rev.* **44**:722–738.
- Gross, R., and R. Rappuoli. 1989. Pertussis toxin promoter sequences involved in modulation. *J. Bacteriol.* **171**:4026–4030.
- Gueirad, P., and N. Guiso. 1993. Virulence of *Bordetella bronchiseptica*: role of adenylate cyclase-hemolysin. *Infect. Immun.* **61**:4072–4078.
- Heesemann, J., and R. Laufs. 1985. Double immunofluorescence microscopic technique for accurate differentiation of extracellularly and intracellularly located bacteria in cell culture. *J. Clin. Microbiol.* **22**:168–175.
- Hoepfman, A. I. M., and E. I. Tuomanen. 1992. Consequences of microbial attachment directing host cell functions with adhesins. *Infect. Immun.* **60**:1729–1733.
- Horiguchi, Y., H. Matsuda, H. Koyama, T. Nakai, and K. Kume. 1992. *Bordetella bronchiseptica* dermonecrotizing toxin suppresses *in vivo* antibody responses in mice. *FEMS Microbiol. Lett.* **90**:229–234.
- Inaba, K., M. Inaba, M. Deguchi, K. Hagi, R. Yasumizu, S. Ikehara, S. Muramatsu, and R. M. Steinman. 1993. Granulocytes, macrophages, and dendritic cells arise from a common major histocompatibility complex class II-negative progenitor in mouse bone marrow. *Proc. Natl. Acad. Sci. USA* **90**:3038–3042.
- Inaba, K., M. Inaba, M. Naito, and R. M. Steinman. 1993. Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guérin organisms, and sensitize mice to mycobacterial antigens *in vivo*. *J. Exp. Med.* **178**:479–488.
- King, P., and D. R. Katz. 1990. Mechanisms of dendritic cell function. *Immunol. Today* **11**:206–211.
- Lee, C. K., A. L. Roberts, T. M. Finn, S. Knapp, and J. J. Mekalanos. 1990. A new assay for invasion of HeLa 229 cells by *Bordetella pertussis*: effects of inhibitors, phenotypic modulation, and genetic alterations. *Infect. Immun.* **58**:2516–2522.
- Leininger, E., C. A. Ewanowich, A. Bhargava, M. S. Peppler, J. G. Kenimer, and M. J. Brennan. 1992. Comparative roles of the Arg-Gly-Asp sequence present in the *Bordetella pertussis* adhesins pertactin and filamentous hemagglutinin. *Infect. Immun.* **60**:2380–2385.
- Little, T. W. A. 1975. Respiratory disease in pigs: a study. *Vet. Rec.* **96**:540–544.
- McDonough, K. A., Y. Kress, and B. R. Bloom. 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect. Immun.* **61**:2763–2773.
- Mekalanos, J. J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. *J. Bacteriol.* **174**:1–7.
- Monack, D. M., B. Arico, R. Rappuoli, and S. Falkow. 1989. Phase variants of *Bordetella bronchiseptica* arise by spontaneous deletions in the *vir* locus. *Mol. Microbiol.* **3**:1719–1728.
- Nicod, L. P., M. F. Lipscomb, J. C. Weissler, and G. B. Toews. 1989. Mononuclear cells from human lungs parenchyma support antigen induced T lymphocyte proliferation. *J. Leukocyte Biol.* **45**:336–344.
- Paglia, A., G. Girolomoni, F. Robbiati, F. Granucci, and P. Ricciardi-Castagnoli. 1993. Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses *in vivo*. *J. Exp. Med.* **178**:1893–1901.
- Preston, N. 1988. Pertussis today, p. 1–19. *In* A. C. Wardlaw and R. Parton (ed.), *Pathogenesis and immunity in pertussis*. John Wiley & Sons Ltd., Chichester, United Kingdom.
- Redhead, K., J. Watkins, A. Barnard, and K. H. G. Mills. 1993. Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. *Infect. Immun.* **61**:3190–3198.
- Relman, D. A., E. Tuomanen, S. Falkow, D. T. Golenbock, K. Saukkonen, and S. Wright. 1990. Recognition of a bacterial adhesin by an integrin: macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell* **61**:1375–1382.
- Roop, M. R., II, H. P. Veit, R. J. Sinsky, S. P. Veit, E. L. Hewlett, and E. T. Kornegoy. 1987. Virulence factors of *Bordetella bronchiseptica* associated with the production of infectious atrophic rhinitis and pneumonia in experimentally infected neonatal swine. *Infect. Immun.* **55**:217–222.
- Saukkonen, K., C. Cabellos, M. Burroughs, S. Prosd, and E. Tuomanen. 1991. Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. *J. Exp. Med.* **173**:1143–1149.
- Savelkoul, P. H. M., B. Kremer, J. G. Kusters, B. A. M. van der Zeijst, and W. Gaastra. 1993. Invasion of HeLa cells by *Bordetella bronchiseptica*. *Microb. Pathog.* **14**:161–168.
- Schipper, H., G. F. Krohne, and R. Gross. 1994. Epithelial cell invasion and survival of *Bordetella bronchiseptica*. *Infect. Immun.* **62**:3008–3011.
- Serfl, K., T. Takemura, E. Tschachler, V. J. Ferrans, M. A. Kaliner, and E. M. Shevach. 1986. Dendritic cells with antigen-presenting capability reside in air-way epithelium, lung parenchyme, and visceral pleura. *J. Exp. Med.* **163**:436–451.
- Spangrude, G. J., F. Sacchi, H. R. Hill, D. E. Van Epps, and R. A. Daynes. 1985. Inhibition of lymphocyte and neutrophils chemotaxis by pertussis toxin. *J. Immunol.* **135**:4135–4143.
- Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26**:31–43.
- Stainer, D. W., and M. J. Scholte. 1970. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J. Gen. Microbiol.* **63**:211–220.
- Steed, L. L., E. T. Akporiaye, and R. L. Friedman. 1992. *Bordetella pertussis* induces respiratory burst activity in human polymorphonuclear leukocytes. *Infect. Immun.* **60**:2101–2105.
- Steed, L. L., M. Setareh, and R. L. Friedman. 1991. Host-parasite interactions between *Bordetella pertussis* and human polymorphonuclear leukocytes. *J. Leukocyte Biol.* **50**:321–330.
- Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* **9**:271–296.
- Steinman, R. M., and Z. A. Cohn. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology,

- quantitation, tissue distribution. *J. Exp. Med.* **137**:1142–1162.
44. **Straley, S. C., and P. A. Harmon.** 1984. *Yersinia pestis* grows within phagolysosomes in mouse peritoneal macrophages. *Infect. Immun.* **45**:655–659.
45. **Switzer, W. P., and D. O. Farrington.** 1981. Bordetellosis, p. 497–507. *In* D. Leman, R. D. Glock, W. L. Mengeling, R. H. C. Penny, E. Scholl, and B. Straw (ed.), *Diseases of swine*, 5th ed. Iowa State University Press, Ames.
46. **Walker, M. J., C. A. Guzmán, M. Rohde, and K. N. Timmis.** 1991. Production of *Bordetella pertussis* serotype 2 fimbriae in *Bordetella parapertussis* and *Bordetella bronchiseptica*: utility of *Escherichia coli* gene expression signals. *Infect. Immun.* **59**:1739–1746.
47. **Walker, M. J., M. Rohde, K. N. Timmis, and C. A. Guzmán.** 1992. Specific lung mucosal and systemic immune responses after oral immunization of mice with *Salmonella typhimurium aroA*, *Salmonella typhi* Ty21a, and invasive *Escherichia coli* expressing recombinant pertussis toxin S1 subunit. *Infect. Immun.* **60**:4260–4268.
48. **Weiss, A. A., and S. Falkow.** 1984. Genetic analysis of phase change in *Bordetella pertussis*. *Infect. Immun.* **43**:263–269.