Fc Receptor-Mediated Immunity Against *Bordetella pertussis*[1]

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The relevance of specific Abs for the induction of cellular effector functions against *Bordetella pertussis* was studied. IgG-opsonized *B. pertussis* was efficiently phagocytosed by human polymorphonuclear leukocytes (PMN). This process was mediated by the PMN IgG receptors, FcγRIIa (CD32) and FcγRIIIb (CD16), working synergistically. Furthermore, these FcγR triggered efficient PMN respiratory burst activity and mediated transfer of *B. pertussis* to lysosomal compartments, ultimately resulting in reduced bacterial viability. Bacteria opsonized with IgA triggered similar PMN activation via FcαR (CD89). Simultaneous engagement of FcαRI and FcγR by *B. pertussis* resulted in increased phagocytosis rates, compared with responses induced by either isotype alone. These data provide new insights into host immune mechanisms against *B. pertussis* and document a crucial role for Ig-FcR interactions in immunity to this human pathogen. *The Journal of Immunology,* **2001, 167: 6545–6551.**

Bordetella pertussis is the causative agent of whooping cough. It produces numerous toxins and adhesins such as pertussis toxin, adenylate cyclase-hemolysin toxin, tracheal cytotoxin, dermonecrotic toxin, filamentous hemagglutinin (FHA),[4] pertactin, fimbriae, and *Bordetella* resistant to killing protein, which have all been implicated to play a role in disease pathogenesis (1, 2). Both whole cell and acellular pertussis vaccines induce high levels of specific Abs directed against several virulence factors. Although the mechanisms by which Abs contribute to host protection are largely unclear, experimental data support their relevance for the induction of immunity to *B. pertussis* (3–5).

B. pertussis resides at the mucosa of the respiratory tract during infection. It is capable of attaching to epithelial as well as immune cells, such as mononuclear leukocytes (MN) (6–10). Attachment to respiratory tract epithelia proposedly precedes invasion of epithelial cells, which might ensure bacterial immune evasion during host infection (11, 12). In addition, *B. pertussis* may evade innate host defense by favoring specific, nonbactericidal interaction with MN and polymorphonuclear leukocytes (PMN). It has been shown that complement receptor 3 (CR3/(CD11b/CD18)) on monocytes can serve as a "docking site" for *B. pertussis* by binding FHA (13, 14). In addition, bacterial constituents including fimbriae, pertussis toxin, and FHA up-regulate CR3 expression, suggesting that nonopsonized *B. pertussis* may stimulate its own attachment to immune cells (9, 13, 15). Both in vitro and in vivo studies provided evidence that uptake via CR3 does not lead to efficient bacterial killing by phagocytes, thus favoring intracellular survival (7, 16, 17).

The presence of specific Abs may alter the interaction of *B. pertussis* with phagocytes. Both MN and PMN express several classes of Ig receptors (FcR) which, upon interaction with immune complexes such as opsonized bacteria, are capable of initiating potent cellular effector functions. Leukocyte IgG receptors (FcγR) are grouped into three classes (FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16)), each of which encompasses a number of subclasses (18, 19). Members of all FcγR classes can be expressed on mononuclear cells (i.e., FcγRIa, FcγRIIa, and FcγRIIIa), whereas PMN constitutively express FcγRIIa and FcγRIIIb. In addition, MN and PMN also express an IgA receptor (FcαRI (CD89)) which is capable of triggering antibacterial effector functions (20–22).

Insight into the relevance of specific Abs for phagocytosis of *B. pertussis* is incomplete and previous studies have yielded conflicting results (23–28). In this study, we examined the relevance of FcR for the induction of Ig-triggered cellular effector functions against *B. pertussis*.

Materials and Methods

Bacterial strains and growth conditions

B. pertussis strain B213, a streptomycin-resistant derivative of Tohama, was used in this study. The virulent *B. pertussis* B213 were transformed with plasmid pCW505 (Ref. 25; kindly supplied by Dr. Weiss, Cincinnati, OH) which induces cytoplasmic expression of green fluorescent protein (GFP) without affecting growth or Ag expression (25). Bacteria were stored at −70°C and recovered by growth on Bordet-Gengou (BG) agar plates at 35°C for 3 days. Virulent bacteria were subsequently plated on BG plates, cultured overnight, and used in phagocytosis experiments. Green fluorescence of GFP-modified bacteria was checked in every experiment.

Antibodies

mAb 22 (mouse (m) IgG$_1$, anti-human (h) FcγRI), mAb IV3 (mIgG2b and F(ab′), anti-hFcγRII), and mAb 3G8 (mIgG1 and F(ab′)$_2$, anti-hFcγRIII) were purchased from Medarex (Annandale, NJ). FcαRI-blocking Ab My43 (mIgM) was a generous gift from Dr. L. Shen (29). mAbs against human

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[4] Abbreviations used in this paper: FHA, filamentous hemagglutinin; CR3, complement receptor 3; MN, mononuclear leukocyte; PMN, polymorphonuclear leukocyte; GFP, green fluorescent protein; BG, Bordet Gengou; m, mouse; h, human; LAMP, lysosome-associated membrane protein; ITAM, immunoreceptor tyrosine-based activation motif.

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lysosome-associated membrane protein (LAMP)-1 (mIgG1) and human LAMP-2 (mIgG1) were from BD PharMingen (San Diego, CA).

IgG fractions from pooled sera of pertussis patients[5] with high titers against *B. pertussis* (as measured by ELISA; Ref. 30) were obtained using protein G (Pharmacia Biotech, Uppsula, Sweden) chromatography followed by acidic elution. Sera of pertussis patients[5] with high *B. pertussis*-specific IgA titers (measured by ELISA; Ref. 31) were pooled and IgA was purified using Affi-T (Biozym, Landgraaf, The Netherlands; Ref. 32) and size chromatography (Superdex 200; Pharmacia Biotech). Isotype purity of IgG and IgA fractions was verified by electrophoresis on 4–15% SDS PAGE (Phast Gels; Pharmacia Biotech), followed by Coomassie brilliant blue staining. Samples of purified IgG, monomeric IgA, and secretory IgA (all from ICN, Zoetermeer, The Netherlands) were run in parallel lanes and served as controls. Western blot analyses were performed to exclude the presence of contaminating isotypes. Polyclonal rabbit anti-*B. pertussis* antiserum was generated by immunizing rabbits with pertussis whole-cell vaccine (National Institute of Health and the Environment, Bilthoven, The Netherlands) as described elsewhere (17). Briefly, rabbits were immunized and boostered at 3 and 6 wk. Sera were collected 7 wk after primary immunization. Rabbit IgG was isolated by protein G chromatography (Pharmacia Biotech) and samples were checked by 4–15% SDS PAGE.

Cells

Peripheral blood PMN were isolated from heparinized venous blood using Ficoll-Histopaque (Sigma-Aldrich, St. Louis, MO) gradient centrifugation. Polymorphic leukocytes were harvested and the remaining erythrocytes removed by hypotonic lysis. Cell viability was >99% as determined by trypan blue exclusion. Before functional assays, PMN were washed twice with RPMI 1640 medium supplemented with 10% heat-inactivated FCS, resuspended, and used immediately. All experiments described in this study were conducted with freshly isolated PMN lacking FcγRI expression, as monitored by FACS analysis with FITC-conjugated anti-FcγRI mAb 22. (33)

Phagocytosis

Phagocytosis of *B. pertussis* was evaluated as in Ref. 34 with minor modifications. Briefly, wild-type or GFP-expressing *B. pertussis* were grown overnight on BG agar plates and resuspended in RPMI 1640 medium containing 10% FCS. Bacteria were opsonized with human IgG or human IgA or both human IgG and human IgA (IgG/IgA ratio 2:1, 1:2, or 1:1 w/w) for 30 min at 37°C. Initial pilot experiments showed *B. pertussis* phagocytosis to be dose-dependent in the range of 0–200 μg/ml. For additional experiments, 200 μg/ml IgG or IgA were used for opsonization, unless specified otherwise. Aliquots were analyzed by flow cytometry after incubation with PE-conjugated goat F(ab′)$_2$ of anti-human IgG or PE-conjugated goat F(ab′)$_2$ of anti-human IgA (from Southern Biotechnology Associates, Birmingham, AL). After washing, opsonized and nonopsonized bacteria were incubated with phagocytic cells in a 70:1 ratio for 45 min at 4°C to allow binding of bacteria to PMN. Nonopsonized bacteria served as a control in all phagocytosis experiments. In select experiments, 200 ng/ml cytochalasin D (Sigma-Aldrich) was added to inhibit phagocytosis (35). After extensive washing to remove nonattached bacteria, cells were split in two aliquots and further incubated for 30 min, either at 4 or 37°C. Next, remaining cell surface-bound opsonized bacteria were detected by incubation (30 min at 4°C) with PE-conjugated goat F(ab′)$_2$ of anti-human IgG or PE-conjugated goat F(ab′)$_2$ of anti-human IgA. In experiments performed with nonopsonized bacteria, surface-bound bacteria were detected by incubation with rabbit anti-*B. pertussis* IgG, followed by incubation with PE-conjugated goat F(ab′) of anti-rabbit IgG (Molecular Probes, Eugene, OR). After washing, samples were analyzed by flow cytometry. Five-thousand cells were analyzed per sample. Green and red fluorescence intensities of cells maintained at 4°C throughout served as control for bacterial binding (i.e., 0% phagocytosis). The decrease in red fluorescence of green positive cells after incubation at 37°C reflects bacterial phagocytosis, as confirmed microscopically (see below). Phagocytosis rates were calculated from the drop in mean red fluorescence intensity of green-positive cells, as described (34).

Controls to exclude autofluorescence of PMN or unspecific binding of F(ab′) of PE-secondary Abs to PMN were included in each experiment. Phagocytosis of unlabeled bacteria was performed as outlined above, although in this experimental set-up, bacteria were detected by a two-step labeling procedure after phagocytosis. For this purpose, bacteria were incubated with polyclonal rabbit anti-*B. pertussis* antiserum, followed by incubation with FITC-conjugated goat F(ab′)$_2$ of anti-rabbit Ab (The Jackson Laboratory, Bar Harbor, ME) to label extracellular bacteria. IgG opsonins were detected as described above by incubation with PE-conjugated goat F(ab′)$_2$ of anti-human IgG.

In some experiments, FcαRI and FcγR-mediated phagocytosis were studied with and without simultaneous engagement of the other FcR class. For this purpose, PMN were incubated with both IgG (or IgA) opsonized GFP-labeled bacteria and IgA (or IgG) opsonized bacteria without a fluorescent label. Internalization of GFP-labeled bacteria was assessed as described above by incubation with PE-labeled F(ab′)$_2$ directed against the opsonin on fluorescently labeled bacteria.

In select experiments, bacterial phagocytosis was evaluated microscopically. For this purpose, cell surface-bound IgG-opsonized bacteria were detected by incubation (30 min at 4°C) with tetramethylrhodamine isothiocyanate-conjugated goat anti-human IgG$\kappa\lambda$ antiserum (Southern Biotechnology Associates).

Respiratory burst

B. pertussis was opsonized by incubation with either IgG or IgA as described above. Tubes containing 50 μl with 7×10^6 opsonized (or nonopsonized) bacteria were transferred to a luminometer (Autolumat; Wallac, Berthhold, Germany), in which chemoluminescence responses of 10^5 PMN were measured every min for 30 min at 37°C after injection of 600 μl of 180 μM of luminol (Sigma-Aldrich) (36).

Microscopic evaluation of internalization and colocalization of B. pertussis *with LAMP-1*

Phagocytosis and intracellular trafficking of *B. pertussis* were studied microscopically. Aliquots of neutrophils incubated either at 4 or 37°C during phagocytosis experiments were fixed using 3% paraformaldehyde. After fixation, PMN were washed twice with PBS and incubated for 10 min at room temperature with PBS containing 50 mM of NH_4Cl. After two washing steps, cells were permeabilized by incubation with PBS containing 0.1% saponin (Sigma-Aldrich) and 0.2% BSA for 30 min. Next, cells were incubated for 30 min at 4°C with rabbit anti-*B. pertussis* Abs and either mouse anti-human LAMP-1 or mouse anti-human LAMP-2 mAbs in the presence of 0.1% saponin and 0.2% BSA. After washing three times, PMN were incubated (20 min) with CY-3-conjugated sheep anti-rabbit IgG (The Jackson Laboratory) and FITC-conjugated F(ab′)$_2$ of goat anti-mouse IgG1 (Southern Biotechnology Associates). Finally, cells were spun on microscope slides. Microscopic analyses were performed using a confocal laser scanning microscope (Leica, Heidelberg, Germany).

Killing assay

Opsonized (either IgG, IgA, or both) or nonopsonized *B. pertussis* were allowed to attach to human PMN at 4°C for 30 min. Nonadherent bacteria were removed by washing and samples were split in two aliquots and incubated either at 4 or 37°C. To determine the number of bacteria initially attached to PMN (N_4), serial dilutions from samples kept at 4°C throughout the experiment were prepared and plated in triplicate on BG agar. Similarly, serial dilutions of samples incubated for 30 min at 37°C were prepared and plated on BG agar to determine the number of surviving bacteria after incubation at 37°C (N_{37}). The percentage of bacteria killed by PMN was calculated as follows: the percentage of killing $= 100 \times (1 - N_{37}/N_4)$. Bacterial phagocytosis rates were determined in parallel by flow cytometry as described above.

Statistics

Student's *t* tests served to assess the significance of differences. Significance was accepted at the $p < 0.05$ level.

Results

Phagocytosis of *B. pertussis* was evaluated by a two-color flow cytometric assay that provides information about both the attachment and internalization of bacteria. Importantly, this method enables the accurate determination of phagocytosis, without loss of read-out sensitivity due to quenching of fluorescence upon phagocytosis (34).

Purified IgG fractions from the sera of *B. pertussis*-infected individuals promoted efficient attachment and phagocytosis of *B. pertussis*. In the absence of Abs, bacterial attachment was much lower and no significant internalization could be observed. Fig. 1

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[5] Written informed consent was obtained before enrollment. This study was approved by the institutional review board of the National Institute of Public Health and the Environment, Bilthoven, The Netherlands.

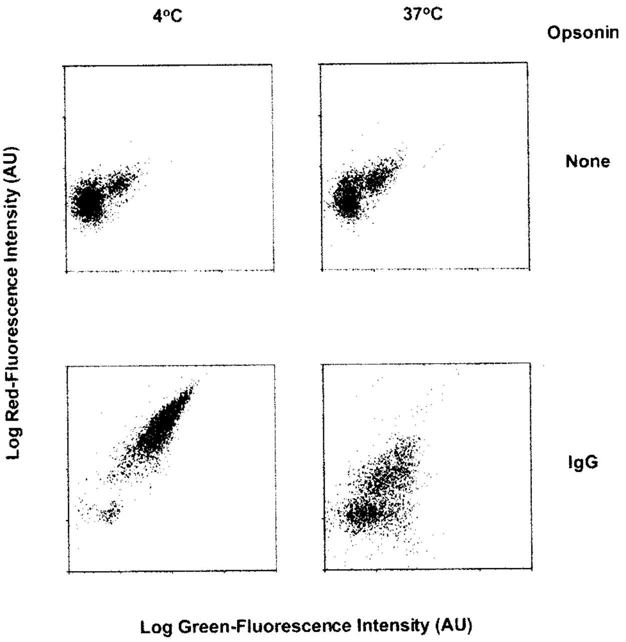

FIGURE 1. Effect of IgG opsonization on attachment and phagocytosis of *B. pertussis* by human PMN. Nonopsonized or IgG-opsonized GFP-expressing *B. pertussis* were incubated with PMN at 4°C for 30 min. Cells were split over two aliquots and subsequently incubated for 30 min at either 4 or 37°C. Remaining surface-bound IgG-opsonized *B. pertussis* were detected by addition of PE-conjugated goat $F(ab')_2$ of anti-human IgG Abs. Incubation with rabbit anti-*B. pertussis* IgG, followed by PE-conjugated goat F(ab′) of anti-rabbit IgG, was used to detect nonopsonized bacteria bound to the surface of PMN. The experiment was repeated five times with PMN isolated from different donors, yielding essentially identical results.

shows dot-plot diagrams of green and red fluorescence intensities of PMN incubated with nonopsonized, or IgG-opsonized, bacteria. Bacterial attachment is reflected by green fluorescence associated with PMN kept at 4°C, whereas phagocytosis is reflected by the decrease of PMN-associated red fluorescence after incubation at 37°C. IgG from nonimmunized individuals with undetectable Ab titers against *B. pertussis* did not induce phagocytosis. Results were comparable to those obtained with nonopsonized bacteria (data not shown)

To confirm that the drop in PE-fluorescence of PMN incubated at 37°C was attributable to bacterial phagocytosis, PMN were incubated with cytochalasin D. Similar results were obtained as with controls maintained at 4°C, confirming the decrease of red fluorescence intensity at 37°C to result from bacterial ingestion (n = 3, data not shown). We further assessed phagocytosis of *B. pertussis* microscopically. Fig. 2 shows PMN kept at 4°C to display both green and red fluorescence, whereas most of the bacteria associated with PMN incubated at 37°C display green fluorescence only (ingested bacteria). Confocal microscopy of nonopsonized *B. pertussis* showed bacteria to be attached to a low number of PMN and not to be internalized at 37°C (data not shown). Although GFP expression has been reported not to influence *B. pertussis* phagocytosis (25), wild-type bacteria (lacking the plasmid for GFP expression) were tested in control experiments. For this purpose, rabbit anti-*B. pertussis* Abs were used to label extracellular bacteria. Essentially, similar results were obtained with GFP-transformed and wild-type bacteria, confirming that GFP expression does not influence *B. pertussis* phagocytosis (data not shown, n = 3)

We next evaluated the role of FcγR in IgG-mediated phagocytosis. IgG-induced phagocytosis proved dependent on both PMN FcγRIIa and FcγRIIIb. Both attachment and phagocytosis of IgG-opsonized *B. pertussis* were significantly reduced by incubation of PMN with either F(ab′) of CD32 or $F(ab')_2$ of CD16-blocking Abs, whereas attachment of nonopsonized bacteria was unaffected. Moreover, in the presence of both F(ab′) of CD32 and $F(ab')_2$ of CD16-blocking Abs, attachment of IgG-opsonized *B. pertussis* was abolished (Fig. 3). Consistent differences in dot-blot patterns were observed when the binding of IgG-opsonized bacteria to either FcγRIIa or FcγRIIIb were selectively blocked. Blocking FcγRIIa resulted in a higher ratio of red to green fluorescence associated with PMN, compared with that detected when FcγRIIIb-mediated attachment was blocked. This result suggests highly opsonized bacteria to preferably attach to FcγRIIIb, whereas lower levels of opsonization seem to direct bacteria to FcγRIIa (Fig. 3).

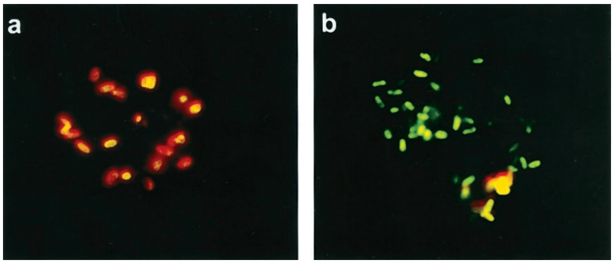

FIGURE 2. Confocal laser scanning fluorescence microscopy of *B. pertussis* phagocytosis. IgG opsonized GFP-labeled *B. pertussis* were incubated with PMN for 30 min at 4°C, washed, and further incubated for 30 min either at 4°C (*A*) or at 37°C (*B*). Cells were then stained with tetramethylrhodamine isothiocyanate-conjugated goat anti-human IgG and spun onto microscope slides for examination. Cell surface-attached bacteria exhibit both green and red fluorescence, whereas ingested bacteria exhibit green fluorescence only. Representative panels of one of four independent experiments are shown.

Both IgG and IgA isotypes have been implicated in host defense against bacterial infections (17, 22, 37). IgA represents the most prevalent Ig isotype at mucosal sites. Previous studies showed IgA to induce effective phagocytosis of *B. pertussis* and other respiratory pathogens via FcαRI (22, 38). Because IgG and IgA are both present at mucosal sites (39), we assessed the capacity of IgA plus IgG to induce cellular effector functions against *B. pertussis*. Similar to IgG, and consistent with previous observations (38), IgA was found to enhance bacterial attachment and promote efficient phagocytosis (Fig. 4). FcαRI showed to be crucial for IgA-mediated functions, because incubation with mouse IgM anti-CD89 mAb My43 (29) completely prevented bacterial attachment (data not shown, n = 3). Both attachment to PMN and internalization of bacteria opsonized with both IgA and IgG increased in an additive manner, compared with bacteria opsonized with one isotype (Fig. 5). To further study interaction of FcαRI and FcγR, phagocytosis of GFP-expressing bacteria opsonized with IgA was evaluated in the presence of unlabeled bacteria opsonized with IgG, and vice versa. In this way, the effect of FcγR engagement on FcαRI-mediated phagocytosis and FcαRI cross-linking on FcγR-mediated phagocytosis was studied. Efficiency of either FcγR- or FcαR-mediated phagocytosis was not influenced by simultaneous engagement of the other FcR class at IgG/IgA ratios (w/w) of 1:1, 1:2, or 2:1 (data not shown, n = 3).

Both IgG- and IgA-opsonized *B. pertussis* induced significant respiratory burst responses in human PMN with no activity detected with nonopsonized bacteria (Fig. 6). These experiments documented the capacity of specific Abs to induce cellular effector functions against *B. pertussis*. Because some pathogenic bacteria,

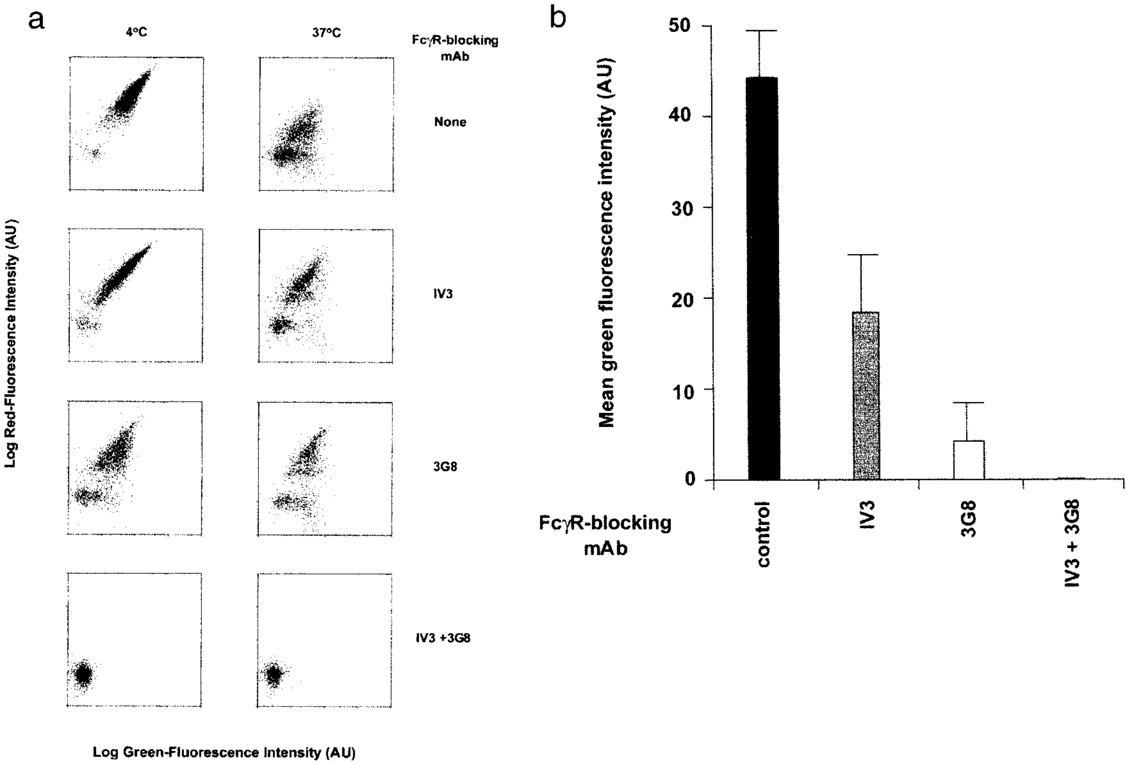

FIGURE 3. Role of FcγR in *B. pertussis* phagocytosis by PMN. *A,* IgG-opsonized GFP-expressing *B. pertussis* were incubated with PMN alone or in the presence of FcγRIIa-blocking mAb IV3, FcγRIIIb-blocking mAb 3G8, or both mAb at 4°C for 30 min. Cells were split over two aliquots and subsequently incubated for 30 min at either 4 or 37°C. Remaining surface-bound IgG-opsonized *B. pertussis* were detected by addition of PE-conjugated goat F(ab′)$_2$ of anti-human IgG Abs. *B,* Phagocytosis of IgG opsonized *B. pertussis* by PMN in the absence (control) or in the presence of F(ab′) of FcγRIIa-blocking mAb IV3, F(ab′)$_2$ of FcγRIIIb-blocking mAb 3G8, or both. Data represent the mean ± SD of four experiments with PMN from different donors. AU, arbitrary units.

including *B. pertussis*, are capable of influencing intracellular trafficking after phagocytosis (40), we next studied whether uptake of *B. pertussis* leads to transportation to phagolysosomes and cellular bactericidal activity. Aliquots of PMN with either adherent IgG-opsonized *B. pertussis* (after incubation at 4°C) or internalized *B. pertussis* (after incubation at 37°C) were stained with mAb specific for LAMP and Abs directed to *B. pertussis* to determine colocalization (thereby assessing intracellular trafficking of bacteria after uptake). At 4°C, bacteria were only detected at the PMN surface. Accordingly, no colocalization of bacteria (red fluorescence) and LAMP-1 (green fluorescence) could be observed (Fig. 7*A*). Following 30 min of incubation at 37°C, colocalization of *B. pertussis* with LAMP-1 (yellow areas) (Fig. 7*B*) as well as LAMP-2 (data not shown, $n = 2$) was observed, suggesting transport of IgG-opsonized *B. pertussis* to lysosomal compartments upon internalization. Incubation of PMN with either CD32- or CD16-blocking Abs before experiments demonstrated both FcγRIIa and FcγRIIIb to be capable of shuttling *B. pertussis* to LAMP-containing subcellular compartments. Similar results were obtained with IgA-opsonized bacteria, indicating FcαRI-IgA interaction to initiate trafficking of *B. pertussis* to lysosomal compartments as well (Fig. 7, *C* and *D*).

We next evaluated the viability of Ig-opsonized and nonopsonized *B. pertussis* after incubation with PMN. Bacterial killing was determined from the difference between the numbers of viable bacteria initially attached to PMN (cells incubated at 4°C) and after bacterial phagocytosis (cells incubated at 37°C). Both IgG- and IgA- mediated phagocytosis proved to induce efficient bacterial killing, at an average of 85% (SD = ± 11, $n = 6$), and 70% (SD = ± 7, $n = 6$), respectively. Importantly, no significant killing was observed in the absence of Abs. Microbial killing correlated well with bacterial phagocytosis rates detected flow cytometrically (data not shown).

Discussion

In this paper, we document specific Abs to efficiently induce cellular anti-*B. pertussis* effector functions via interaction with FcR

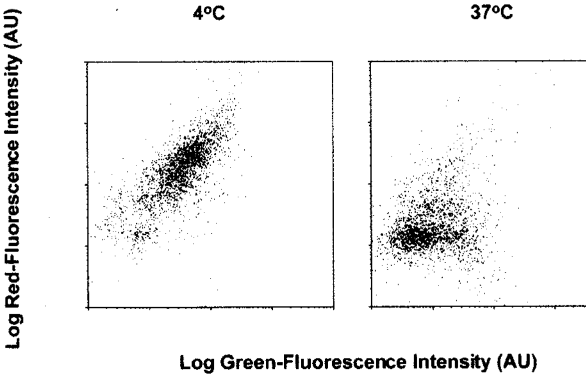

FIGURE 4. IgA-mediated PMN phagocytosis of *B. pertussis.* Isolated PMN were incubated at 4°C for 30 min with IgA-opsonized GFP-expressing *B. pertussis* and were further incubated for 30 min, either at 4 or 37°C. Remaining surface-bound *B. pertussis* were detected by the addition of PE-conjugated goat F(ab′)$_2$ of anti-human IgA Abs. Representative dot-plot diagrams are shown. The experiment was repeated five times yielding similar results.

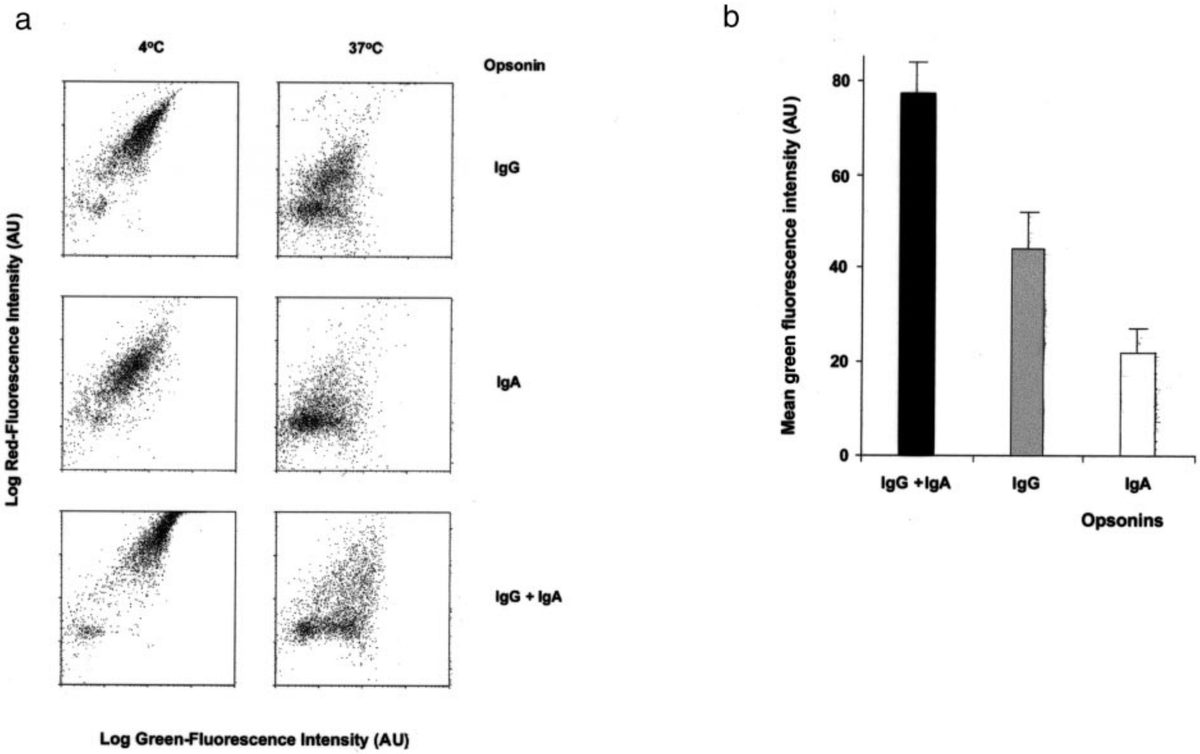

FIGURE 5. Effect of IgA and IgG opsonization on the uptake of *B. pertussis* by PMN. *A,* PMN were incubated with *B. pertussis* opsonized either with IgG, IgA, or IgG plus IgA at 4°C during 30 min. Cells were split over two aliquots and subsequently incubated for 30 min at either 4 or 37°C. The remaining surface-bound opsonized *B. pertussis* were detected by addition of PE-conjugated goat F(ab′)$_2$ of anti-human IgG Abs, PE-conjugated goat F(ab′)$_2$ of anti-human IgA Abs, or both. *B,* Phagocytosis of IgG-, IgA-, or IgG- plus IgA-opsonized *B. pertussis* by PMN. Data represent the mean ± SD of four experiments with PMN from different donors. AU, arbitrary units.

on human neutrophils. Ig-opsonization increased *B. pertussis* attachment, phagocytosis and respiratory burst activity via interaction with FcγRIIa, FcγRIIIb, or FcαRI. Furthermore, engagement of these FcR initiated transport of *B. pertussis* to lysosomal compartments. These events ultimately lead to decreased bacterial viability.

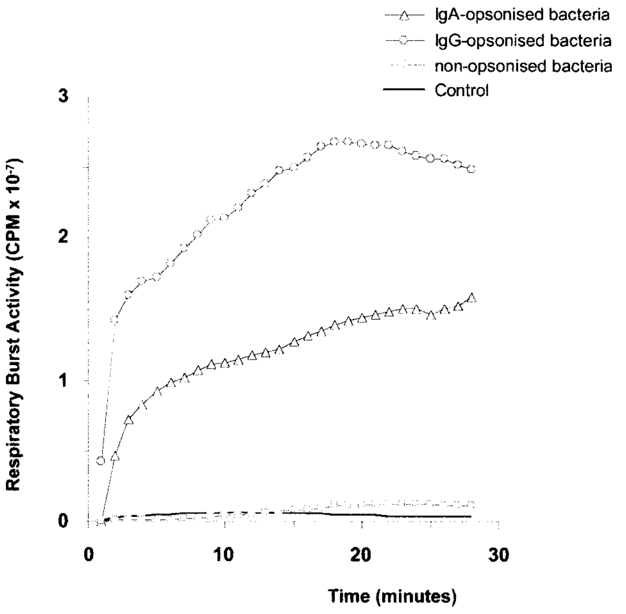

FIGURE 6. Induction of PMN respiratory burst by *B. pertussis* opsonized with either IgG or IgA. IgG-opsonized, IgA-opsonized, or nonopsonized *B. pertussis* were incubated with PMN and luminol at 37°C. PMN incubated with buffer served as a control. Chemoluminescence responses were measured every min for 30 min. Data are representative of four independent experiments.

B. pertussis represents an important respiratory pathogen causing significant morbidity, as well as mortality, in nonvaccinated infants. Although a number of vaccines have been shown to be effective in preventing disease, recent reports on pertussis outbreaks in highly vaccinated populations underline the necessity to assess the mechanisms of immunity to *B. pertussis* in better detail (41–43). Vaccination induces Abs against a number of *B. pertussis* Ags. High titers of specific Abs have been reported to induce protection (4, 5), although experimental studies exploring the relevance of Abs for immunity to *B. pertussis* yielded contradictory results. Until now, the contribution of Ig-induced phagocytosis to *B. pertussis* immunity remained unclear (23–28). To address this issue in more detail, we used a flow cytometry-based phagocytosis assay, which allows discrimination of bacterial attachment and phagocytosis, as well as quantitation of phagocytosis. Efficient phagocytosis of *B. pertussis* by PMN was induced by both IgG and IgA, the main Ig isotypes induced by vaccination. Previous studies using fluorescence microscopy techniques to assess phagocytosis mediated by human sera failed to report significant *B. pertussis* phagocytosis (23, 25). However, such techniques have the intrinsic drawback that read-out sensitivity (i.e., fluorescence of internalized bacteria) is significantly decreased by the low pH in phagolysosomes, as well as lengthy quantification procedures. Therefore, these older studies may well have underestimated Ig-mediated phagocytosis of *B. pertussis*.

Phagocytosis of *B. pertussis* was studied qualitatively with mAbs blocking specific FcR. Internalization of Ig-opsonized *B.*

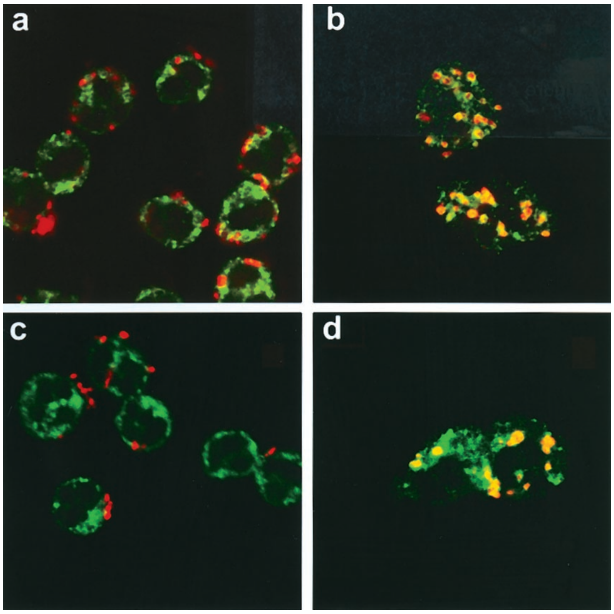

FIGURE 7. Confocal laser scanning fluorescence microscopy of uptake of IgG- or IgA-opsonized *B. pertussis* by PMN. IgG-opsonized (*A* and *B*) or IgA-opsonized (*C* and *D*) *B. pertussis* were incubated with PMN for 30 min at 4°C, washed, and further incubated for 30 min either at 4°C (*A* and *C*) or at 37°C (*B* and *D*). Cells were fixed and permeabilized before incubation with Abs against *B. pertussis* and LAMP. *A* and *C,* Red fluorescent *B. pertussis* attached to the surface of PMN. No colocalization with FITC-immunolabeled LAMP-1 is observed. *B* and *D,* Colocalization of *B. pertussis* and LAMP-1, reflected by yellow areas. Representative panels of one of four independent experiments are shown.

pertussis was mediated via both PMN IgG, as well as IgA receptors. Optimal IgG-mediated phagocytosis was shown to depend on employment of neutrophil FcγR, FcγRIIa, and FcγRIIIb. These results are in agreement with previous reports documenting heterotypic cross-linking of FcγRIIa and FcγRIIIb to induce synergistic PMN responses (44–47). Interestingly, specific FcγR subclass engagement by opsonized bacteria seems to depend on the level of bacterial opsonization. FcγRIIIb was primarily involved in the binding of highly opsonized bacteria, whereas bacteria with lower levels of opsonization attached well to FcγRIIa (Fig. 3*A*). These findings corroborate and extend previous studies documenting high valency immune complexes to activate PMN primarily via FcγRIIIb (44, 45, 48). Furthermore, IgA-opsonized *B. pertussis* was taken up efficiently via interaction with FcαRI and induced PMN respiratory burst activity. These results suggest that FcαRI constitute an important molecule for immunity not only against Gram-positive bacteria (22), but also against Gram-negative bacteria. Importantly, IgA and IgG are both relevant for host immunity to pathogens at mucosal sites (39). Therefore, phagocytosis of *B. pertussis* opsonized with both isotypes was evaluated. Simultaneous stimulation of FcγR and FcαRI induces a phagocytic response which was approximately the sum of FcγR- and FcαRI-mediated activities, suggesting simultaneous FcR cross-linking to constitute an important host-defense mechanism at mucosal sites.

A number of human pathogens are capable of evading host immunity by interfering with phagosome-lysosome fusion. *B. pertussis* has been reported to be able to survive after uptake in professional phagocytes by inhibiting the formation of phagolysosomes (24). However, results from recent studies using murine macrophages challenged this concept, because *B. pertussis* was found to be transported to lysosomal compartments after internalization and to be effectively killed upon phagolysosome formation (49). *B. pertussis* is a strictly human pathogen and mouse models may have limited value for our understanding of human immunity. Using human PMN, we found IgA- and IgG-opsonized bacteria to be efficiently transported to lysosomal compartments upon internalization. Bacteria could be observed in LAMP-containing compartments of neutrophils as soon as after 30 min. It was recently shown that the presence of an immunoreceptor tyrosine-based activation motif (ITAM) is sufficient to initiate transport of internalized particles to lysosomes (50). Both FcγRIIa and FcαRI initiate signal transduction upon cross-linking via ITAM-signaling motifs (51). However, engagement of FcγRIIIb, which lacks ITAM, induced internalization and trafficking of *B. pertussis* to lysosomal compartments as well. GPI-linked FcγRIIIb may use FcγRIIa or other associated molecules for signaling, leading to efficient transport to lysosomes, or may trigger lysosome formation via ITAM-independent pathways (45, 52, 53).

Finally, killing assays indicated that bacterial uptake, induction of respiratory burst activity, and formation of phagolysosomes induced efficient killing of IgG- and IgA- opsonized *B. pertussis.* In the absence of specific Abs, binding of *B. pertussis* to human neutrophils was significantly lower and neither efficient phagocytosis, nor respiratory burst activity/bacterial killing could be detected. These findings suggest that internalization is crucial for bacterial killing and underline the importance of specific Abs for immunity to *B. pertussis*. Furthermore, the data indicate that *B. pertussis*-specific IgG and IgA reduce the odds for intracellular bacterial survival as suggested by previous murine infection studies (17, 38).

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