Fc Receptor-Mediated Immunity Against *Bordetella pertussis*

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*J Immunol* 2001; 167:6545-6551; doi: 10.4049/jimmunol.167.11.6545

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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 FcγRIIB (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 FcεRI 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.
lysosome-associated membrane protein (LAMP)-1 (mlgG1) and human LAMP-2 (mlgG1) were from BD Pharmingen (San Diego, CA).

IgG fractions from pooled sera of pertussis patients with high titers against B. pertussis (as measured by ELISA; Ref. 30) were obtained using protein G (Pharmacia Biotech, Uppsala, Sweden) chromatography followed by acidic elution. Sera of pertussis patients 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 boosted 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 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 FcεRI expression, as monitored by FACS analysis with FITC-conjugated anti-FcεRI 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 ratios 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 IgM 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')2 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')2 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 antisera, 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 then removed by acidic elution. Sera of pertussis patients with high B. pertussis-specific IgA titers were used as a control for bacterial binding, as monitored by FACS analysis with FITC-conjugated anti-FcεRI mAb 22. (33) In some experiments, FcεRI and FcγRI-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 iotamethylrhodamine isothiocyanate-conjugated goat anti-human IgG (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 × 10⁶ opsonized (or nonopsonized) bacteria were transferred to a luminometer (Autolumat; Wallac, Berthold, Germany), in which chemoluminescence responses of 10⁷ 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 NaN₃. 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 (N₀), 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 (N₃). The percentage of bacteria killed by PMN was calculated as follows: the percentage of killing = 100 × (1 − N₃/N₀). 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

9 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.
bacteria bound to the surface of PMN. The experiment was repeated three times with PMN isolated from different donors, yielding essentially identical results.

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’2) 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.

showed 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 FcγRI in IgG-opsoniated 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’2) 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’2) 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).

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 FcεRII (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). FcεRI 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 FcεRI 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 FcεRI-mediated phagocytosis and FcεRI cross-linking on FcγR-mediated phagocytosis was studied. Efficiency of either FcγR- or FcεRI-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,
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. 7A). Following 30 min of incubation at 37°C, colocalization of *B. pertussis* with LAMP-1 (yellow areas) (Fig. 7B) as well as LAMP-2 (data not shown, n/11005/11006) 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...
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.

*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. pertussis* was studied qualitatively with mAbs blocking specific FcR.

**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.

**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 was mediated via both PMN IgG, as well as IgA receptors. Optimal IgG-mediated phagocytosis was shown to depend on employment of neutrophil FcγRI, FcγRIIa, and FcγRIIIb. These results are in agreement with previous reports documenting heterotypic cross-linking of FcγRIIa and FcγRIIIa to induce synergistic MN responses (44–47). Interestingly, specific FcγR subclass engagement by opsonized bacteria seems to depend on the level of bacterial opsonization. FcγRIIIa was primarily involved in the binding of highly opsonized bacteria, whereas bacteria with lower levels of opsonization attached well to FcγRIIa (Fig. 3A). 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 FcαR 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 phagosomal-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 phagolysosomal 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).

References


