Killing of *Streptococcus pneumoniae* by capsular polysaccharide–specific polymeric IgA, complement, and phagocytes

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The role of IgA in the control of invasive mucosal pathogens such as *Streptococcus pneumoniae* is poorly understood. We demonstrate that human pneumococcal capsular polysaccharide–specific IgA initiated dose-dependent killing of *S. pneumoniae* with complement and phagocytes. The majority of specific IgA in serum was of the polymeric form (pIgA), and the efficiency of pIgA-initiated killing exceeded that of monomeric IgA–initiated killing. In the absence of complement, specific IgA induced minimal bacterial adherence, uptake, and killing. Killing of *S. pneumoniae* by resting phagocytes with immune IgA required complement, predominantly via the C2-independent alternative pathway, which requires factor B, but not calcium. Both *S. pneumoniae*–bound IgA and complement were involved, as demonstrated by a 50% decrease in killing with blocking of Fcα receptor (CD89) and CR1/CR3 (CD35/CD11b). However, IgA-mediated killing by phagocytes could be reproduced in the absence of opsonic complement by pre-activating phagocytes with the inflammatory products C5a and TNF-α. Thus, *S. pneumoniae* capsule–specific IgA may show distinct roles in effecting clearance of *S. pneumoniae* in the presence or absence of inflammation. These data suggest mechanisms whereby pIgA may serve to control pneumococcal infections locally and upon the pathogen's entry into the bloodstream.

J. Clin. Invest. **104**:1139–1147 (1999).

Introduction

At mucosal sites, the role of secretory IgA (sIgA) has been characterized as inhibition of microbial adherence and inflammation (1, 2), and neutralization of viruses, toxins, and enzymes (3–11). These local actions may serve to retard ingress of pathogens or their products into the host's internal compartment and to prevent injury of tissues and internal organs. At systemic sites, serum IgA has been reported to limit inflammation by inhibiting complement activation by IgG (12), and also to induce complement-mediated killing of Gram-negative organisms (e.g., *Neisseria meningitidis* and *Haemophilus influenzae* type B) (13). However, whether pathogen-specific IgA also plays a role in limiting or enhancing killing of invasive Gram-positive bacteria by phagocytes has not been determined.

We recently reported that up to a third of serum antibodies that are reactive with the polysaccharide capsule of an invasive mucosal Gram-positive organism, *Streptococcus pneumoniae*, are of the IgA class (14, 15). The majority of this pathogen-specific IgA in serum is in the polymeric

form (≥ 2 IgA molecules bound by a J chain) and remains polymeric long after immunization or infection (15), even though about 90% of the total IgA in serum is in the monomeric form (5). *S. pneumoniae* may colonize the nasopharyngeal mucosa without sequelae but also commonly causes serious invasive clinical syndromes, including pneumonia, bacteremia, and meningitis in children and adults (16–19). Natural infection and immunization elicit capsule-specific polymeric IgA (pIgA) responses in blood and at mucosal sites (14, 15, 20–22). Thus, pIgA may play a functional role at both mucosal and systemic sites.

Therefore, we determined levels, specificity, structure, and the functional activity of serum IgA that reacts with pneumococcal capsular polysaccharide in response to antigenic challenge. We found that immune IgA bound specifically to the capsule. However, IgA-associated binding, uptake, and killing of *S. pneumoniae* by phagocytes required either opsonization by complement in human serum, predominantly by the alternative pathway, or prior activation of phagocytes by TNF-α and C5a. That the polymeric form

of antigen-specific IgA (also found at mucosal sites as sIgA) may bind to the capsule of *S. pneumoniae*, but mediates uptake and killing of this invasive Gram-positive organism only in the presence of inflammatory products, suggests a dichotomous role of pathogen-specific IgA in quiescent and inflamed environments.

Methods

Subjects. Informed consent was obtained from all study participants according to protocols approved by the Human Subjects Subcommittees at the Minneapolis Veterans Affairs Medical Center and the University of Minnesota.

Sera were obtained from 5 healthy human volunteers (3 male and 2 female, aged 24–36 years) before and 4 weeks after intramuscular immunization with 23-valent capsular pneumococcal polysaccharide (PPS) vaccine (PNU-IMMUNE (23); a generous gift from Lederle Labs Division of American Cyanamid Company, Pearl River, New York, USA). Sera were frozen at –20°C until tested.

Purification of serum IgG and IgA. IgG was purified from whole sera using a Protein G Hi-Trap column (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) as described previously (15), followed by IgM depletion; subsequently, IgA was purified from the non-IgG, non-IgM fraction. IgM and IgA affinity columns were prepared with goat anti-human IgM or IgA (Southern Biotechnology Associates, Birmingham, Alabama, USA) coupled to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) as described (15). The IgA fractions ($n = 7$) contained 97.5–99.9% IgA, 0.1–2% IgM, less than 0.1–0.2% IgG, and no detectable type 14 capsule–specific IgG or IgM.

Total and PPS-specific Ig. Levels of total IgA, IgM, and IgG were measured by ELISA with unlabeled and labeled affinity-purified goat anti-human IgA, IgM, or IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) as the capture and detector antibodies, respectively (15). Capsule-specific antibodies against type 14 PPS were measured by ELISA as described (14, 15, 23) after samples and standards were preadsorbed overnight at 4°C with purified pneumococcal cell wall polysaccharide (Statens Serum Institut, Copenhagen, Denmark) at a final concentration of 50 μg/mL. The specificity of the ELISA for IgA against type 14 capsule was established with a competitive inhibition assay (24, 25). Increasing concentrations (0.1–100 μg/mL) of both homologous (type 14 pneumococcal capsule) and heterologous soluble antigens (type 4, 8, and 19A pneumococcal capsule; *H. influenzae* type B capsule [poly-3-D-ribose(1-1)ribitol-5 phosphate]; and tetanus toxoid) were incubated overnight at 4°C with a fixed concentration of specific IgA (optical density of 1.0 unit at 410 nm). The ability of these soluble antigens to inhibit binding of IgA to solid-phase type 14 capsule by ELISA after a 2-hour incubation at 25°C was compared.

IgA fractionation by molecular form. Purified IgA was fractionated as described previously (8, 15) by molecular sieve chromatography using a Sephacryl S-300 HR column (Pharmacia Biotech Inc., Piscataway, New Jersey, USA) that was calibrated with pIgA and monomeric IgA (mIgA) standards (generous gifts of J. Mestecky, University of Alabama, Birmingham, Alabama, USA). The polymeric structure of the capsule-specific IgA did not change with acid treatment (15), which dissociates immune complexes but not pIgA (26, 27), but did dissociate to IgA monomers under partial reducing conditions (8, 15, 27, 28). The purity of pIgA and mIgA fractions was confirmed by resolution on a 5% continuous nondenaturing polyacrylamide gel stained with Coomassie blue. The absence of secretory component in association with purified IgA fractions was determined by ELISA using anti-human IgA or purified pneumococcal type 14 capsular polysaccharide, as described above, and horseradish peroxidase–labeled antisecretory component (DAKO Corp., Carpinteria, California, USA).

Antibody-dependent killing of S. pneumoniae by complement and phagocytes. Purified IgA or control IgG were incubat-
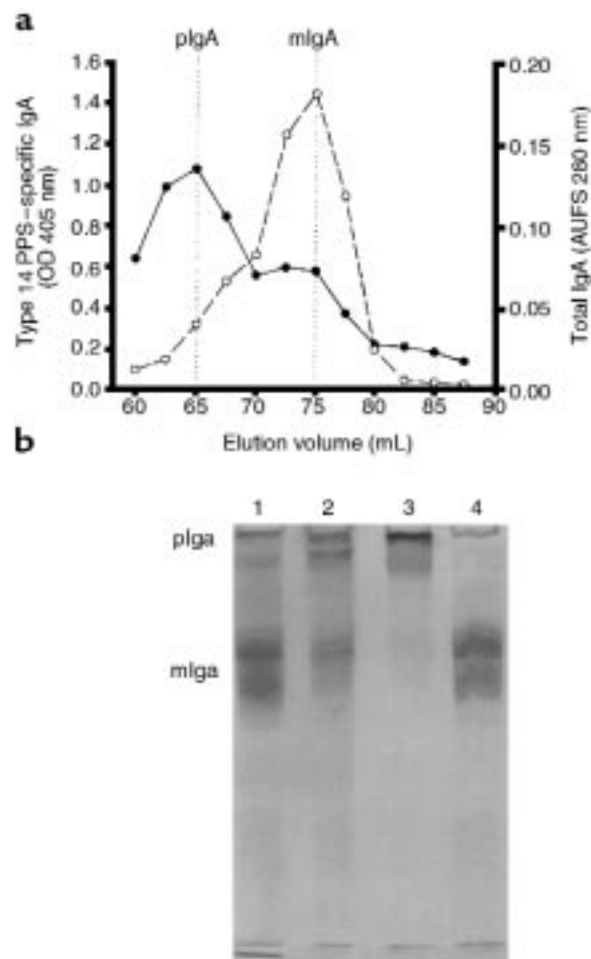

Figure 1
(**a**) Distribution of molecular forms of IgA (mIgA and pIgA) in immune serum for type 14 PPS–specific IgA (black circles; solid lines) and total serum IgA (open circles; dotted line) by gel filtration (mean of 2 experiments). Optical density (OD) represents absorbance at 405 nm by ELISA for type 14–specific IgA and absorbance at 280 nm for total serum IgA (0.2 AUFS absorbance units, full scale); the latter represents approximately 1,000-fold more protein than the former (2 μg/mL type 14–specific IgA and 2 mg/mL total IgA). Type 14–specific IgA comprised 1.2% of pIgA and 0.15% of mIgA. Vertical lines represent peaks of pIgA and mIgA standards. (**b**) Nondenaturing PAGE of purified IgA before and after size fractionation by gel filtration. Lane 1: purified IgA from serum sample before separation; lane 2: IgA standard containing both pIgA and mIgA; lane 3: purified pIga fraction pool; lane 4: purified mIgA pool.

ed with shaking for 30 minutes at 25°C with 1,000 CFU of log-phase type 14 *S. pneumoniae* (American Type Culture Collection 6314, Rockville, Maryland, USA) (29–31). Phagocytes (4×10^5) and a specified human or rabbit serum complement source (10%) were added, incubated at 37°C for 1 hour with shaking, and lysed in distilled water (1:10); serial dilutions were then plated. Phagocytes were either freshly isolated peripheral blood neutrophils (PMN) or cultured HL-60 cells (Certified Cell Line 240; American Type Culture Collection) treated with 120 mM dimethyl formamide to induce differentiation (31, 32). We verified the absence of IgG and IgA bound to purified human neutrophils by FACS® with affinity-purified FITC-labeled goat F(ab′)$_2$ anti-human Fcγ and Fcα (Southern Biotechnology Associates), respectively. Cells were also washed 3 times to remove soluble Igs from the donor. To determine the antibody specificity, killing assays were performed with and without preincubation of purified IgG and IgA with 20 μg/mL cell wall polysaccharide and type 14 PPS; control wells contained 10% heat-inactivated FCS (GIBCO BRL, Grand Island, New York, USA). Killing activity of antibodies with complement and phagocytes (or with activated phagocytes and no complement) was calculated using the following formula: percent kill = [(bacteria present with no Ig – bacteria present with IgG or IgA)/bacteria present with no Ig] × 100. To determine the receptors involved in killing, selected experiments were performed with preincubation of phagocytes with isotype controls (Southern Biotechnology Associates and PharMingen, San Diego, California, USA) and unlabeled antibodies with blocking activity against complement receptor CR1 (CD35; DAKO Corp.), CR3 (CD11b; BioSource International, Camaril-

lo, California, USA), Fcα receptor (CD89; Medarex Inc., Annandale, New Jersey, USA), Fcγ receptor I (CD64; PharMingen), Fcγ receptor II, and Fcγ receptor III (CD32 and CD16; Medarex Inc.).

Sources and treatment of complement. Complement sources were baby rabbit serum (Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) that was shown to be free of antipneumococcal activity, or human serum from a hypogammaglobulinemia patient (serum Ig in mg/mL: IgG, <2; IgM, <0.4; and IgA, <0.07), or human complement depleted of C2 or factor B (QUIDEL Corp., San Diego, California, USA). Human complement was adsorbed twice with 10^9 CFU *S. pneumoniae* per mL serum with 25 mM EDTA for 30 minutes on ice, and then dialyzed and filter-sterilized before use. Adsorbed complement sources retained complement activity and lacked detectable (by ELISA) antibodies to capsular type 14. Selected assays were performed in the presence of 10 mM EDTA (to block both classical and alternative pathways), 10 mM MgEGTA (to selectively inhibit the calcium-dependent classical pathway) (33), or factor B–depleted serum reconstituted with factor B (QUIDEL Corp.).

Receptor expression. The expression of receptors for C3bi (CR3/CD11b) and for IgA (Fcα receptor CD89) on neutrophils and HL-60 cells was examined by flow cytometry with mAbs specific for CR3 (Becton Dickinson Immunocytometry Systems, San Jose, California, USA), Fcα receptor (PharMingen), and isotype and fluorochrome controls.

Analysis of uptake of S. pneumoniae by phagocytes. Specified concentrations of antibody (purified IgA or IgG) and *S. pneumoniae* (2×10^5 CFU) were combined in a total volume of 50 μL and shaken in Immulon I wells (Dynex Technologies, Chantilly, Virginia, USA) for 30 minutes at 25°C. Baby rabbit complement and purified neutrophils (2×10^5
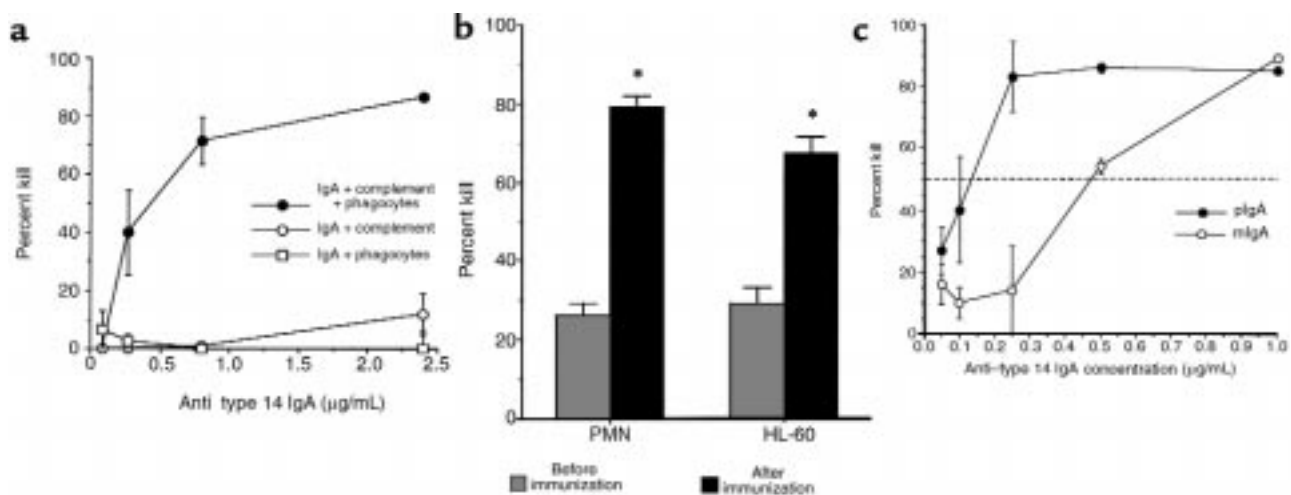

Figure 2
(**a**) IgA-mediated killing of type 14 *S. pneumoniae* by differentiated HL-60 cells and baby rabbit complement. Phagocyte/bacteria ratio was 400:1. Tests were run in duplicate; results are shown as the mean ± SEM of 3 experiments. (**b**) Killing of type 14 *S. pneumoniae* by human polymorphonuclear leukocytes (PMN) and differentiated HL-60 cells in the presence of 10% baby rabbit complement and 1.2 mg/mL of total IgA (98% IgA, <1% IgG, <2% IgM). Given in Results are means ± SEM of experiments (PMN, *n* = 4; HL-60 cells, *n* = 6) with IgA purified from serum of volunteers before and 1 month after immunization with 23-valent pneumococcal vaccine. Levels of type 14–specific IgA were 678 ng/mL before immunization and 2,445 ng/mL after immunization in these pools of purified IgA. *P < 0.0001 for percent kill before immunization vs. 1 month after immunization. (**c**) The efficacy of molecular forms of IgA on phagocytic killing of type 14 *S. pneumoniae*. Serial dilutions of purified mIgA and pIgA were tested in the presence of 10% baby rabbit complement for their ability to mediate complement-dependent killing of the organism. Percent kill is given as a function of the concentration of anti–type 14 IgA in purified mIgA and pIgA. Tests were run in duplicate; results are shown as the mean ± SEM of 2 experiments.
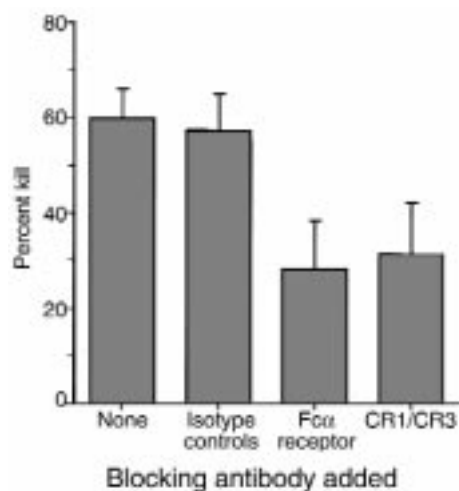
Figure 3
Effect of receptor-blocking mAbs and isotype control antibodies on killing of type 14 *S. pneumoniae* with 2 μg/mL specific IgA and 10% baby rabbit complement. Differentiated HL-60 cells were preincubated for 30 minutes at 25°C with blocking antibodies (40 μg/mL) before addition of IgA-opsonized organisms and complement. Results are shown as the mean ± SEM of 2 experiments. *P* = 0.08 for percent kill with anti-Fcα receptor antibody compared with killing without blocking antibody.

per well) were added, and the final mixture (100 μL) was incubated at 37°C for 1 hour with shaking. Well contents were either removed for cytospin (Cytospin 2, Shandon Inc., Pittsburgh, Pennsylvania, USA) or slow spun at 150 *g* to pellet neutrophils. Cytospins were stained with Wright-Giemsa for light microscopy. Cell pellets were fixed in 2.5% glutaraldehyde and processed for transmission electron microscopy as described (34). In 3 experiments, whole complement was excluded and neutrophils were preactivated with 10^{-8} M recombinant human TNF-α (R&D Systems Inc., Minneapolis, Minnesota, USA), purified 10^{-9} M C5a (CalBiochem-Novabiochem Corp., San Diego, California, USA), or both agents together.

Statistics. Mean levels of capsule-specific IgA and percent kill after immunization were compared with preimmunization levels by paired 2-tailed *t* tests. Data were analyzed using the StatView 4.0 statistical program (Abacus Concepts Inc., Berkeley, California, USA).

Results
Measurement of type 14 capsule–specific IgA in serum. Levels of IgA that were reactive with PPS 14 measured by ELISA increased 13-fold in sera from 5 subjects 1 month after immunization (0.15 ± 0.02 μg/mL before immunization vs. 2.1 ± 0.3 μg/mL after immunization). The IgA detected was known to be specific for the polysaccharide capsule because its binding was inhibited by preadsorption with soluble homologous antigen (PPS 14) but not by other heterologous antigens (not shown). The majority of PPS 14–specific IgA was in the polymeric rather than the monomeric form, even though 80–90% of total IgA in serum is mIgA (Figure 1, a and b). Capsule-specific IgA comprised approximately 1.2% of total pIgA but only 0.15% of total mIgA (an 8-fold difference). Both total and capsule-specific pIgA had little to no secretory component

detectable by ELISA (not shown). As reported previously, 80–90% of capsule-specific IgA is of the IgA1 subclass 1 month after immunization (14).

IgA-associated killing of S. pneumoniae. In the presence of complement and phagocytes, immune IgA mediated killing of the organism (Figure 2a). Killing increased with the concentration of immune IgA, required the presence of complement, and increased significantly after immunization (Figure 2b). Activity was highly correlated with PPS 14–specific IgA levels, as measured by ELISA (results not shown). IgA-associated killing of *S. pneumoniae* was caused by reactivity with the capsule — killing was inhibited 51% by preadsorption with 20 μg/mL capsular polysaccharides (PPS 14) but not with 20 μg/mL cell wall polysaccharides. Purified pIgA showed greater killing activity in the presence of complement and PMN than did mIgA (Figure 2c), although activity was similar between the 2 forms at high concentrations. The efficiency of capsule-specific pIgA was greater than that of specific mIgA, with 50% kill achieved with 0.13 μg/mL pIgA, and 0.46 μg/mL mIgA.

Killing of *S. pneumoniae* was similar with both PMN and differentiated HL-60 cells (Figure 2a), each of which expressed receptors for IgA (Fcα receptor CD89; 91% and 93%, respectively) and CR3 (CD11b/18; 78% and 100%, respectively). The density of receptor expression measured by mean fluorescence intensity was similar for the 2 cell types for CD89, whereas CR3 expression was higher on PMN cells than on differentiated HL-60 cells (mean fluorescence intensity = 202 and 99, respectively). Both Fcα receptor and CR1/CR3 contributed to IgA-mediated killing by phagocytes, as demonstrated by the fact that blocking of these receptors by specific mAbs reduced killing by about half (Figure 3). Addition of both sets of blocking antibodies simultaneously did not further inhibit killing (not shown); addition of antibodies to Fcγ receptors I, II, and III had no effect on IgA-initiated killing (not shown). Moreover, undifferentiated HL-60 cells (which expressed neither Fcα receptor nor CR1/CR3) effected no killing of the organism in the presence of complement and immune IgA or IgG.

Role of complement. Despite high levels of capsule-specific IgA detected by ELISA, immune IgA in the absence of complement mediated little or no adherence of organisms to PMNs as detected by light microscopy (Table 1). In con-

Table 1
Bacterial adherence to PMNs with and without complement by light microscopy of Wright-Giemsa–stained cytospin preparations

Complement	Antibody	Percent of PMNs with associated bacteria
–	–	4.3[A] ± 2.0
+	–	9.7[A] ± 0.7
–	IgA	9.0[A] ± 7.1
+	IgA	58.3[B] ± 14.8
–	IgG	63.5[B] ± 12.5
+	IgG	97.5[B] ± 1.5

Data are presented as mean ± SEM (*n* = 3). [A]Low numbers of visible bacteria were predominantly adjacent to external cell membrane, not internalized or in vacuoles. [B]The majority of visible bacteria were internalized, as confirmed by transmission electron microscopy. Type 14–specific antibody concentration was 1.9 μg/mL in purified IgA and 0.7 μg/mL in purified IgG.

Figure 4

Transmission electron microscopy of preparations treated with baby rabbit complement and IgA (**a**–**c**) or IgG (**d**). (**a**) The bacteria in the 3 phagosomes of a neutrophil are surrounded by electron-dense flocculent material. An empty shell is apparently all that remains of 1 bacterium (arrow). ×12,000. (**b**) Higher magnification of bacteria with coronas, in vacuoles surrounded by primary lysosomes of varying densities (arrowheads). ×25,000. (**c**) Still higher magnification shows changes in the integrity of a bacterium and membranous material that probably represents bacterial debris (arrow). ×50,000. (**d**) Three vacuoles are surrounded by electron-dense lysosomes and contain lysosomal constituents, some encasing bacteria, and bacterial debris (arrows). ×16,000.
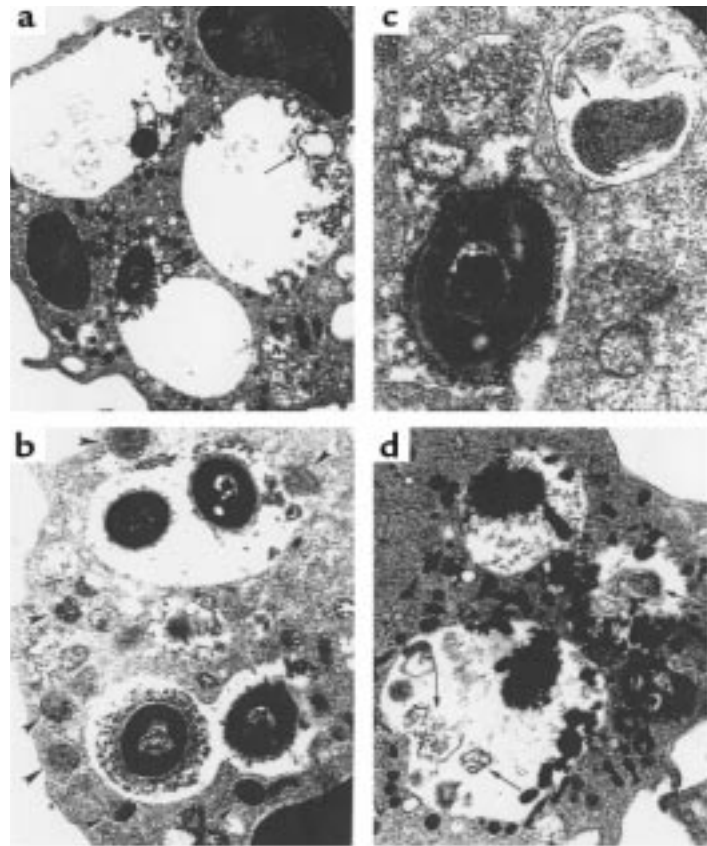
trast to results with IgA, control experiments showed that purified immune IgG was sufficient to induce both cell association and uptake of the organism into phagosomes. In the presence of complement, IgA mediated adherence, uptake, and dose-dependent killing of the organisms by PMNs (Table 1; Figure 2a). The presence of complement with both IgA and IgG increased binding and uptake of *S. pneumoniae* into phagosomes with lysosomal activity, bacterial destruction, and killing (Table 1; Figure 4).

As shown by transmission electron microscopy, in the absence of antibody and complement, only rare cells had single bacteria associated with their surfaces. In the presence of complement or IgA alone, only a few cells showed 1 or 2 large vacuoles containing individual normal-appearing bacteria, and primary lysosomes were not concentrated in the adjacent cytoplasm (not shown). Without complement, heat-inactivated immune serum or IgG led to substantially more bacterial uptake than with IgA, but virtually without evidence of fusion of primary lysosomes with phagosomes or alteration in the appearance of the bacteria. In fact, bacteria still appeared to be capable of replication while within cytoplasmic vacuoles. In the presence of complement, about twice as many PMNs contained organisms when bacteria were exposed to immune serum or IgG as when bacteria were treated with purified IgA (Table 1), but their morphological changes were qualitatively identical (Figure 4). With complement and either IgA or IgG, bacteria were found both within loose- and tight-fitting vacuoles containing electron-dense flocculent material that appeared to derive from fusion of the phagosome with primary lysosomes. The variability of electron density of the internalized bacteria suggested progressive digestion; the laminated membranous material seen in some vacuoles probably represented bacterial debris.

Complement pathway in IgA-associated killing of S. pneumoniae. Killing of IgA-opsonized pneumococci by PMNs was predominantly related to the presence of an intact alternative complement pathway. In the presence of divalent cation chelators with either baby rabbit complement or human serum from a hypogammaglobulinemic adult (both preadsorbed with whole bacteria to remove any specific antibody), IgA-associated killing of *S. pneumoniae* showed significant inhibition in the presence of EDTA, but not with MgEGTA (Figure 5). Whereas EDTA chelates Ca^{2+} and Mg^{2+} and blocks activation of both the classical and alternative pathways, MgEGTA selectively chelates Ca^{2+} and blocks only the classical pathway. In contrast to the findings with IgA-mediated killing, IgG-associated killing was inhibited by both EDTA and MgEGTA (data not shown). Consistent with these data, depletion of a specific component of the alternative pathway, factor B, from a human serum complement source resulted in significantly reduced killing of the organism with IgA and phagocytes compared with killing using whole complement (Figure 6). In contrast, selective impairment of the classical pathway in C2-depleted complement showed no significant decrease in killing with IgA. Reconstitution of factor B–deficient complement with purified factor B resulted in restoration of the ability to kill the organism. The possibility that residual IgG or IgM in the purified IgA preparation may partially account for the obtained results was excluded. First, the IgA preparation contained no type 14 capsule–specific IgG or IgM that was detectable by ELISA (not shown). Second, IgM purified from immune serum had no significant killing activity when tested at levels similar to those contaminating the purified IgA (not shown). Third, the killing efficiency of IgA preparations containing less than 0.1% IgM was similar to those containing up to 2.5% IgM (Figure 5). Fourth, no IgG was detected in the IgA fractions (limit of detection, <0.1%). Finally, complement activation by IgM typically involves the classical complement pathway, whereas as noted above, the results with IgA involved the alternative pathway.

Killing of S. pneumoniae with IgA and activated PMNs. To determine whether IgA was capable of initiating killing of *S. pneumoniae* in the absence of opsonic complement

Figure 5

Effect of cation chelators on phagocytic killing of type 14 *S. pneumoniae* by IgA in the presence of baby rabbit complement (**a**) and human complement (**b**). EDTA, which blocks both alternative and classical pathways, and MgEGTA, which blocks only the classical pathway, were added to killing reactions to determine the complement activation pathway involved in IgA-mediated phagocytic killing of pneumococci by differentiated HL-60 cells. Chelators (EDTA and MgEGTA) were added at 10 mM. All tests were run in triplicate; results are shown as the mean ± SEM of 6 experiments. *$P < 0.05$ vs. killing without EDTA. Results were the same whether individual experiments used IgA that was 98% pure (<2% IgM) or 99.9% pure (<0.1% IgM).
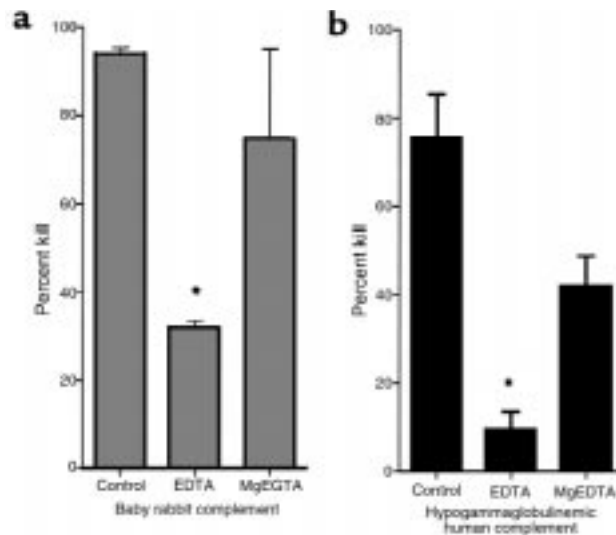
(C3bi), PMNs were activated by preincubation with products of inflammation C5a and TNF-α. PMNs activated with C5a and TNF-α killed *S. pneumoniae* that was opsonized with IgA at levels comparable to those with opsonic complement and IgA (Figure 7). Again, IgA alone mediated little killing by nonactivated phagocytes. Exposure of PMNs to C5a, TNF-α, or both did not result in spontaneous killing of the organisms in the absence of IgA (data not shown). The additive effects of C5a and TNF-α on IgA-associated killing may be related to the concentrations used. By light microscopy of cytospin preparations, the frequency of association of *S. pneumoniae* with PMNs paralleled the percent kill with each condition (data not shown).

Discussion

We demonstrate that the ability of capsule-specific IgA to mediate killing of *S. pneumoniae* by phagocytes is associated primarily with the polymeric molecular form of the antibody and participation of the complement system. Such activity promotes binding and uptake of the organism, as shown by light and electron microscopy, as well as killing of the organism. IgA-mediated killing of pneumococci by phagocytes (differentiated HL-60 cells and resting PMNs) was almost totally complement-dependent and was decreased by blocking receptors for either IgA (Fcα receptor) or for C3 fragments (CR1 and CR3). However, we also report that immune IgA can mediate uptake and killing of the organism by activated PMNs in the absence of opsonic complement. Thus, IgA may serve an active functional role in the control of pneumococcal disease in the setting of an inflammatory immune response to infection.

Previous findings have shown a significant IgA response to pneumococcal capsular polysaccharides after immunization (14, 20, 21, 35) and natural infection (15, 22). Indeed, IgA comprises an appreciable proportion of capsule-specific IgA in immune serum (14, 15), but the functional activities of these antibodies had not been characterized. The antigen specificity of the IgA-associated killing of *S. pneumoniae* is a novel feature of our results. Purified human IgA can facilitate killing of another Gram-positive organism, Group B streptococcus, by phagocytes (36). However, IgA binding to that organism was shown to be mediated by bacterial surface protein B, which attaches to the IgA Fc fragment, rather than the antigen-specific Fab fragment of IgA (37, 38). In contrast, Fc binding to the organism is an unlikely mechanism in the case of *S. pneumoniae* because IgA obtained before immunization showed little reactivity with the capsule and supported little phagocytic killing, whereas after immunization, both binding by ELISA and killing activity increased and were capsule-specific in competitive inhibition experiments.

Similarly, a surface protein of *S. pneumoniae*, SpsA, can bind to the secretory component of sIgA, but not to pIgA that lacks the secretory component (39, 40). The capsule-specific pIgA we describe in this report lacks secretory component, and therefore should not bind SpsA. In related systems, human sIgA was shown to inhibit murine mucosal infection with Group A streptococcus, but there was no bactericidal activity in vitro, despite the presence of phagocytic cells and complement (41). In that particular study, the titers of specific sIgA were low and may have been insufficient to support killing of the organism. Thus, our studies are among the first to confirm killing of a Gram-positive organism initiated by antigen-specific IgA.

The functions of the 3 distinct molecular configurations of IgA (monomeric, polymeric, and secretory) may vary. mIga comprises the majority of total IgA in serum. pIga, a union of 2 mIgA molecules joined by a J chain, comprises a minority of total IgA but the majority of pneumococcal capsule–specific IgA in serum. Indeed, capsule-specific IgA comprised a greater proportion of pIgA than of mIgA. A human IgA mAb, produced in both monomeric and polymeric forms, has been described that reacts with type 8 *S. pneumoniae* and mediates a low level of phagocyte binding in the absence of complement (42). The same antibody induced significant protection of mice when administered systemically, although the mechanism of protection was not documented. In our studies, polymeric rather than monomeric *S. pneumoniae*–specific IgA
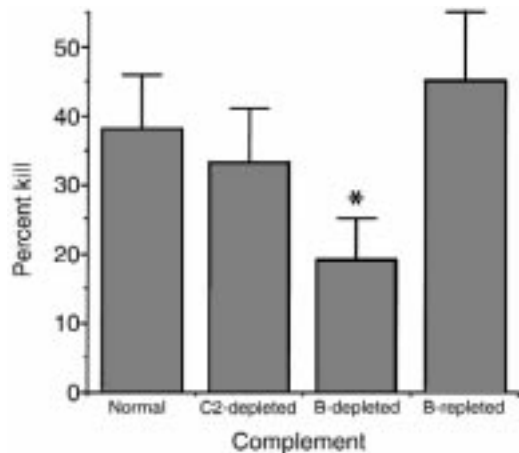
Figure 6
Effect of depletion and repletion of selected human complement components in human serum on IgA-mediated killing of type 14 *S. pneumoniae* by IgA. Organisms were incubated with 1 mg/mL of immune IgA, differentiated HL-60 cells, and a complement source consisting of 10% serum from a hypogammaglobulinemic patient, serum depleted of the classical pathway component C2, serum depleted of the alternative pathway component factor B, or serum depleted for factor B and then repleted with a physiologic concentration of factor B (200 μg/mL). All sera were preadsorbed with type 14 pneumococci before the killing assay to remove specific antibody in the complement source. Results are shown as mean ± SEM of 7 experiments. *$P < 0.05$ vs. normal complement and factor B–repleted complement.

appears to most efficiently initiate complement-mediated killing of the organism.

This complement activation is in agreement with previous observations on complement-mediated effects of IgA in other model systems (43–45). However, we also found that mIgA can significantly mediate complement-dependent killing of *S. pneumoniae*, albeit at a much higher IgA concentration than is required by the polymeric form. This observation suggests that the surface distribution of the capsular polysaccharide on *S. pneumoniae* may favor binding of contiguous IgA molecules that create conditions for IgA-mediated complement activation. The biochemical mechanism of IgA-initiated complement activation is not yet well defined. The third form of IgA, sIgA, accounts for almost all IgA at mucosal sites and is formed by the union of pIgA with the epithelial cell–derived secretory component (the pIgA receptor). Both pIgA and sIgA may have similar functions that extend beyond those of serum mIgA (2, 46, 47). Our results are consistent with other functional studies of IgA that showed that, compared with mIgA, enhanced antiviral activity was demonstrated by both pIgA and sIgA (46).

We also found that IgA-mediated killing of *S. pneumoniae* by previously resting phagocytes depends primarily on the alternative complement pathway. Killing is inhibited by EDTA, which blocks activation of both the classical and alternative pathways; however, killing proceeds in the presence of MgEGTA, which suppresses the classical pathway but does not interfere with alternative

pathway activation (33). Similarly, depletion of factor B of the alternative pathway inhibits killing by phagocytes with IgA, and repletion of factor B restores killing; depletion of the classical pathway component C2 has a limited effect. The variability in percent kill that we observed in experiments performed in the absence of functional complement may reflect low levels of PMN activation induced by the cell isolation procedures. Our finding that IgA activates complement via the alternative pathway is in agreement with the observations that IgA lacks the core C1q binding motif of IgG and IgM (which is associated with initiation of the classical pathway [48]), and that pIgA lacks the ability to activate the classical complement pathway (49). Although high concentrations of complement alone, such as those found in serum, may promote killing of *S. pneumoniae* in the absence of antibody (50, 51), levels of complement at mucosal sites are much more limited (52). At mucosal sites, complement-dependent killing may require the participation of specific antibodies such as pIgA (in its secretory form). Human sIgA with specificity for type B red cells has been shown to induce complement-mediated opsonization and lysis of these cells (53). In that study and others, both pIgA and sIgA have been proposed to activate the complement cascade by the alternative pathway under certain conditions (2, 43, 54–57).

Complement components may be produced locally by activated monocytes, mucosal macrophages (58–62), mucosal epithelial cells (63, 64), or recruited granulocytes (65) to support antibody-mediated killing of *S. pneumoniae*. Activation of these cells requires local inflammation, which may also contribute to exudation of serum proteins such as complement. Alternatively, local inflammation and associated phagocyte activation may abrogate the need for high levels of complement to
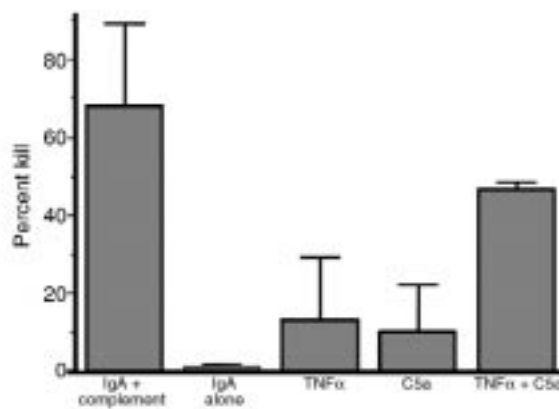

Figure 7
Effect of PMN pretreatment on IgA-mediated killing of type 14 *S. pneumoniae*. Human PMNs were preincubated with media alone (first 2 columns) or TNF-α (10^{-8}M), C5a (10^{-9}M), or both for 30 minutes before addition of log-phase organisms and purified immune IgA (1 mg/mL total IgA). Baby rabbit complement was added to untreated PMNs with IgA as a positive control (first column); IgA with untreated PMNs and no complement served as a negative control (second column). PMNs preincubated with TNF-α and C5a showed no uptake or killing of the organism in the absence of immune IgA (not shown). Results are shown as mean ± SD for 3 experiments.

effect IgA-mediated killing of mucosal pathogens.

That binding and uptake of *S. pneumoniae* is low in the absence of complement may reflect a selective 2-tiered role of IgA in immune defense. In the absence of inflammation, IgA may limit binding of pathogenic organisms, including *S. pneumoniae*, to environmentally exposed host mucosal tissues, including epithelial cells (reviewed in ref. 2; J.B. Rubins and E.N. Janoff, unpublished data). In this unperturbed setting, IgA-bound organisms also adhere poorly to inflammatory and phagocytic cells. In contrast, once an inflammatory response is initiated, IgA — particularly pIgA — may participate more actively to facilitate killing of *S. pneumoniae* by 2 mechanisms. First, IgA can promote complement binding to organisms. However, whether sufficient levels of complement are available at mucosal sites, either produced by activated phagocytes and epithelial cells or exuded from serum when sufficient tissue injury has occurred, is under investigation. Nevertheless, we have shown a second IgA-related mechanism whereby inflammatory products such as TNF-α and C5a can promote phagocyte activation with IgA-mediated uptake and killing of the pathogen. These data are consistent with the increased expression and affinity of IgA receptors on granulocytes, as well as enhanced phagocytosis (66–71), upon cytokine activation. Complement may participate directly through C3bi bound to the organism to facilitate binding, uptake, and possibly killing of IgA-opsonized *S. pneumoniae* by phagocytes. Moreover, complement products such as C5a may serve indirectly to activate phagocytes and promote killing of organisms opsonized with immune IgA alone.

In summary, we have shown that capsule-specific IgA, particularly pIgA, mediates uptake and killing of *S. pneumoniae* in the presence of an intact alternative complement pathway, or in the absence of this pathway, with activated phagocytes. In contrast to IgG, the distinct inability of IgA to mediate appreciable phagocyte interactions in the absence of complement or inflammation highlights the more passive protective role of IgA in immune exclusion. However, IgA, particularly pIgA, may participate actively in protective phagocytosis and killing of *S. pneumoniae* once the inflammatory process is initiated.

Acknowledgments

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