

Risk of Invasive *Haemophilus influenzae* Type b (Hib) Disease in Adults with Secondary Immunodeficiency in the Post-Hib Vaccine Era

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Prior to the introduction of *Haemophilus influenzae* type b (Hib) conjugate vaccines, invasive Hib disease affected almost exclusively children. According to some recent studies, in the postvaccine era, adults, the elderly, and immunocompromised persons can be affected more often than children. As the production of type-specific anti-capsular polysaccharide antibodies is the major defense mechanism against Hib, individuals with defects in humoral immune responses have high susceptibility to infections caused by Hib. We hypothesized that nonvaccinated adults with chronic conditions causing immunosuppression may lack protective antibody to Hib. We assessed serum anti-Hib IgG levels and bactericidal activity in 59 patients with chronic renal failure, 30 patients with type 2 diabetes mellitus, 28 patients with chronic obstructive pulmonary disease (COPD), and 20 patients with multiple myeloma compared to 32 healthy controls of similar age. Considering antibody at >0.15 $\mu\text{g/ml}$ as the protective correlate in unvaccinated individuals, we detected subprotective Hib antibody levels in 29% of chronic renal failure, 20% of diabetes, 14% of COPD, and 55% of myeloma patients compared to 3% of healthy controls. Additionally, 70% of myeloma and 58% of chronic renal failure patients did not have detectable serum bactericidal activity against Hib. Among individuals with severe diseases causing secondary immunodeficiency, patients with multiple myeloma and chronic renal failure are at an increased risk of invasive Hib disease. Considering that Hib continues to circulate in the population, this study provides a rationale for the immunization of some adult patients with secondary immunodeficiency with the pediatric Hib vaccine to achieve protective immunity.

Haemophilus influenzae is a common Gram-negative human-restricted bacterial pathogen that frequently colonizes the nasopharynx in healthy individuals and can cause local infections, such as otitis media, sinusitis, pneumonia, or exacerbations of chronic obstructive pulmonary disease (COPD). When the bacteria breach the epithelial barriers, they are able to cause invasive disease, including meningitis, sepsis, and epiglottitis (27, 28). Most invasive infections are caused by encapsulated strains, in particular *H. influenzae* type b (Hib), characterized by a polyribosylribitol phosphate (PRP) capsule, which is an important virulence factor. In immune individuals, circulating anti-PRP antibodies effectively protect against the disease by activating the classical complement pathway, as well as opsonizing bacteria for phagocytosis (41).

Prior to the introduction of vaccination against Hib, the pathogen was the major cause of bacterial meningitis in children (43). A dramatic decrease in the incidence of invasive Hib disease has rapidly followed the introduction of Hib protein-conjugated vaccines in Western countries since the beginning of the 1990s (48). In Canada, a conjugate Hib vaccine first became available in 1988 for children over 18 months of age; the routine vaccination of infants beginning at 2 months of age with the current vaccine (PRP conjugated to tetanus toxoid) started in 1992 (1). In the post-Hib vaccine era, invasive Hib disease affects adults, especially the elderly and immunocompromised individuals, more often than children (12, 35, 39, 44). As adults born before the 1990s have not been vaccinated, their natural immunity may be insufficient to prevent invasive disease if they have an immune defect. In addition, low Hib circulation rates due to the vaccine's "herd effect" may account for reduced maintenance of natural anti-Hib immunity in nonvaccinated populations (14). Severe cases of invasive Hib disease affecting adults have been reported (6, 40). As Hib

continues to circulate in countries with high pediatric Hib vaccine coverage (31), the public health guidelines recommend adult vaccination for some high-risk groups (anatomical or functional asplenia, congenital antibody, or complement deficiency) (34).

In modern Western society, the number of adults with secondary immunodeficiency states resulting from aging, severe chronic diseases, or an immunosuppressive therapy is increasing. Such individuals are not routinely immunized against Hib, and it is unclear whether they may be at risk of developing invasive Hib disease if exposed to the pathogen. To address this question, we studied a group of patients with common clinical conditions known to lead to immunosuppression. Because circulating antibodies to Hib capsular polysaccharide are the major defense mechanism against invasive Hib disease, we studied the antibody levels and functional activity as indicators of protection.

MATERIALS AND METHODS

Patient population. We recruited 59 patients with chronic renal failure, 30 patients with type 2 diabetes mellitus, 28 patients with COPD, 20 patients with multiple myeloma, and 32 age-matched healthy controls. All patients with chronic renal failure were undergoing hemodialysis at the Renal Services, Thunder Bay Regional Health Sciences Centre (TBRHSC), Thunder Bay, Ontario, Canada. The COPD patients were recruited at a

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TABLE 1 Demographic characteristics of studied groups

Group	n	Age (yr)		No. (%)	No. (%) ≥60 yr of age
		Mean (median) ^a ± SD	Range		
Chronic renal failure	59	62.6 (63) ± 13.5	29–91	23 (38)	24 (41)
Diabetes mellitus	30	60.5 (61) ± 11.2	33–80	18 (60)	16 (53)
COPD	28	69.8 (72) ± 8.8	45–81	19 (68)	25 (89)
Multiple myeloma	20	68.7 (70) ± 10.1	43–84	9 (45)	16 (80)
Controls	32	63 (61) ± 8.2	53–80	19 (59)	19 (59)

^a No statistically significant difference between any patient group and controls; $P < 0.05$ between chronic renal failure and COPD and between diabetes and COPD patients. SD, standard deviation.

time when they did not have disease exacerbation and were undergoing the outpatient respiratory rehabilitation program at St. Joseph's Care Group (Thunder Bay, Ontario, Canada). Patients with diabetes and multiple myeloma were attending the outpatient clinics in Thunder Bay and Sault Ste. Marie, Ontario, Canada (Algoma District Cancer Program), respectively. All patients undergoing hemodialysis at the Renal Services or attending the involved physicians' offices at the time of the study (May to August 2009) were invited to participate, and those who were able to give informed consent, were included. Most of the multiple myeloma patients were on intermittent chemotherapy (pulsed therapy given every 3 weeks), and COPD patients received inhaled corticosteroids, but not oral corticosteroids; the remaining patients did not receive immunosuppressive therapies at the time of the study. None of the study participants had been immunized against Hib. Clinical data for all the patients were analyzed with respect to duration of disease, comorbidities, and history of infections. The demographic characteristics of the studied groups are shown in Table 1. No statistically significant differences in age between any patient group and controls were present. Serum samples were obtained from the participants under informed consent and stored at -80°C prior to use. This study was approved by the research ethics boards of all involved institutions.

Anti-Hib ELISA. Serum anti-PRP IgG antibody concentrations were determined by using a VaccZyme Human Anti *Haemophilus influenzae* type b enzyme-linked immunosorbent assay (ELISA) kit (The Binding Site, Birmingham, United Kingdom) according to the manufacturer's protocol. Briefly, serum samples diluted 1:100 were added in duplicate to microwells precoated with PRP conjugated to human serum albumin. The reaction was developed using peroxidase-conjugated anti-human IgG secondary antibody; the bound IgG was detected using a colorimetric substrate read at 450 nm with an automated microplate reader (BioTek Powerwave XS; VT). Concentrations of anti-PRP IgG antibody were determined using standard calibrators supplied with the kit and expressed in $\mu\text{g/ml}$. The lower and upper limits of detection were 0.11 and 9 $\mu\text{g/ml}$, respectively. For statistical analysis, antibody concentrations below the lower limit of quantitation were assigned half the lower limit of detection, i.e., 0.055 $\mu\text{g/ml}$. Samples with higher than 9- $\mu\text{g/ml}$ antibody levels were further diluted and rerun to obtain accurate results. Following dilution, the upper limit of detection was 90 $\mu\text{g/ml}$.

Serum bactericidal assay. For the serum bactericidal assay (SBA), we used a Hib strain isolated from the cerebrospinal fluid of an infant with meningitis, kindly provided by R. S. W. Tsang. Bacteria were grown on brain heart infusion agar supplemented with 10 $\mu\text{g/ml}$ hemin chloride (factor X) and 1 $\mu\text{g/ml}$ NAD (factor V) in a humidified incubator at 37°C and 5% CO_2 overnight. The SBA was essentially performed as previously described (10, 37). Briefly, Hib was cultured, harvested, and diluted to a concentration of approximately 10^4 CFU/ml in SBA buffer, which consisted of Hank's buffered salt solution supplemented with 10 $\mu\text{g/ml}$ factor X and 1 $\mu\text{g/ml}$ factor V. Serum samples were incubated in a water bath at 56°C for 30 min to inactivate complement and serially diluted 2-fold 11 times beginning at a ratio of 1:8. Ten microliters of each serum dilution

was mixed with 20 μl of bacterial suspension and incubated for 10 min at 37°C and 5% CO_2 . Next, 10 μl of baby rabbit complement (Pel-Freeze, AR) and 40 μl of SBA buffer were added to the mixture and incubated for 1 h at 37°C and 5% CO_2 . The viable bacteria were determined by drop plating following overnight incubation at 37°C and 5% CO_2 . The SBA titer was defined as the reciprocal of the highest dilution able to kill $>50\%$ of bacteria compared to a negative serum control, which contained SBA buffer in place of serum. Titers below the lower detection limit of 8 were reported as 4 for statistical analysis. As a positive control, we used anti-Hib reference serum 96/536 (National Institute for Biological Standards and Control, Potters Bar, United Kingdom). For quality control purposes, a postimmunization serum from the same healthy adult control was included in each experiment and consistently yielded an SBA titer of $1,024 \pm 1$ dilution.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The sample size was calculated based on 20% difference in means and 30% coefficient of variation (two-sided alternatives) with 80% statistical power and 5% significance (49). Geometric mean antibody concentrations (GMC), SBA geometric mean titers (GMT), and 95% confidence intervals (CI) were calculated for each group. Serum antibody levels and SBA results were compared between patients and controls using the Mann-Whitney rank sum test and Student's t test as appropriate. Analysis of variables was performed using Pearson's χ^2 test. Associations were assessed by linear regression analysis or Spearman's correlation. P values of < 0.05 were considered significant.

RESULTS

To assess naturally acquired immunity against Hib, we studied IgG anti-PRP antibody levels in sera of unvaccinated adult individuals. Among healthy controls, 97% had anti-PRP IgG levels of >0.15 $\mu\text{g/ml}$, which has been considered an immunological correlate of natural protection against invasive Hib disease in unvaccinated individuals (3, 19, 33), in comparison to 71% of chronic renal failure, 80% of diabetes, 86% of COPD, and 45% of myeloma patients (Table 2). The relative risk of having anti-PRP IgG antibody levels below 0.15 $\mu\text{g/ml}$ was significantly higher in all the patient groups than in healthy controls, with the exception of COPD ($P = 0.059$) (risk ratio ranging from 6.4 in diabetes to 17.6 in myeloma patients) (Table 3).

Among all patients, the multiple myeloma group had the lowest GMC of IgG antibody, i.e., 0.22 $\mu\text{g/ml}$ (95% CI, 0.09 to 0.45), which was significantly lower than in healthy controls (1.50 $\mu\text{g/ml}$; 95% CI, 1.06 to 2.13; $P < 0.0001$), followed by chronic renal failure patients (0.56 $\mu\text{g/ml}$; 95% CI, 0.34 to 0.90; $P = 0.02$). In one myeloma and two chronic renal failure patients, high anti-PRP antibody levels were detected (>10 $\mu\text{g/ml}$), potentially reflecting a recent exposure to the pathogen (Fig. 1a). In patients with diabetes mellitus and COPD, the antibody levels did not significantly differ from those of controls, i.e., 0.93 $\mu\text{g/ml}$ (95% CI, 0.50 to 1.75, $P = 0.4$) and 1.12 $\mu\text{g/ml}$ (95% CI, 0.56 to 2.23; $P = 0.5$), respectively (Table 2 and Fig. 1a).

A decline in antibody levels against bacterial polysaccharide antigens may occur with aging. However, we observed a significant negative correlation of antibody level with age in ≥ 60 -year-old controls ($r = -0.45$; $P = 0.03$), but not in patients ($r = 0.023$; $P = 0.42$) (Fig. 2), suggesting that a lack of naturally acquired antibody in severely ill individuals is attributable to their general immunosuppression, rather than to advanced age. No differences in antibody levels between males and females in any group were detected (data not shown). Further analysis indicated that IgG anti-PRP antibody levels did not depend on the length of dialysis

TABLE 2 Serum antibody levels and bactericidal activity against Hib in patients with secondary immunodeficiency states and healthy controls

Parameter	Value for group				
	Chronic renal failure	Diabetes mellitus	COPD	Multiple myeloma	Controls
Anti-PRP IgG					
GMC ($\mu\text{g/ml}$) (95% CI)	0.56 ^b (0.34–0.90)	0.93 (0.50–1.75)	1.12 (0.56–2.23)	0.22 ^c (0.09–0.54)	1.50 (1.06–2.13)
No. (%) >0.15 $\mu\text{g/ml}$	42 (71)	24 (80)	24 (86)	9 (45)	31 (97)
No. (%) ≥ 1.0 $\mu\text{g/ml}$	26 (44)	16 (53)	13 (46)	3 (15)	26 (81)
No. (%) ≥ 5.0 $\mu\text{g/ml}$	6 (10)	7 (23)	7 (25)	2 (10)	1 (3)
SBA					
GMT (95% CI)	20.72 ^b (12.22–35.14)	55.72 (25.34–122.5)	36.22 (15.89–82.61)	12.55 ^b (5.2–30.33)	36.44 (16.31–81.44)
No. (%) $\geq \text{MDA}^a$	25 (42)	20 (67)	16 (57)	6 (30)	18 (56)

^a MDA, minimum detectable activity.^b $P < 0.05$ compared with control group (see precise P values in the text).^c $P < 0.01$ compared with control group (see precise P values in the text).

or comorbidities (in chronic renal failure) or the number and severity of infectious episodes (in all patient cohorts) (data not shown).

Antibodies detected by ELISA may have different functional capabilities due to their specific chemical and genetic characteristics. To assess the functional activity of the anti-PRP antibody, we employed a serum bactericidal assay, which measures the killing of bacteria by anti-Hib specific antibody mediated by the complement activation (37). Among healthy controls, 56% of serum samples were able to kill >50% of Hib bacteria in the presence of baby rabbit complement in contrast to only 30% of sera from myeloma patients ($P = 0.032$) (Table 2). The relative risk of the lack of bactericidal antibody against Hib in myeloma patients was 17.6 ($P < 0.0001$) (Table 3). The SBA GMT was significantly lower in myeloma patients than in the controls, i.e., 12.55 (95% CI, 5.2 to 30.33) versus 36.44 (95% CI, 16.31 to 81.44), respectively ($P = 0.048$). Also, patients with chronic renal failure had lower SBA GMT than controls, i.e., 20.72 (95% CI, 12.22 to 35.14; $P = 0.029$).

However, no statistically significant differences in SBA GMT were found among the diabetes mellitus, COPD, and control groups (Table 2 and Fig. 1b). No correlation was detected between the SBA titers and IgG anti-PRP antibody concentrations measured by ELISA for healthy controls ($r = -0.3$; $P = 0.05$) or patients ($r = 0.05$; $P = 0.56$) (Fig. 3). Because aging is associated with decreased functional antibody activity (13), we compared SBA titers in older and younger individuals. No significant differences in SBA GMT between individuals <60 and ≥ 60 years of age among healthy controls or patient groups were detected (data not shown).

Hence, the results of our study demonstrated that among individuals with severe chronic conditions causing secondary immunodeficiency, patients with multiple myeloma and chronic renal failure show both decreased IgG anti-PRP antibody levels and

a defect in antibody functional capabilities; however, no association of antibody deficiency with age was found.

DISCUSSION

In 97% of healthy unvaccinated adults, circulating IgG antibodies against Hib capsular polysaccharide were above the level ensuring long-term protection against invasive Hib disease, i.e., 0.15 $\mu\text{g/ml}$ (3); in 56% of them, functionally active serum antibodies were detectable. Moreover, 81% of healthy individuals had antibody levels of ≥ 1.0 $\mu\text{g/ml}$, a correlate of protection in the vaccinated population (19). These data suggest that the general adult popu-

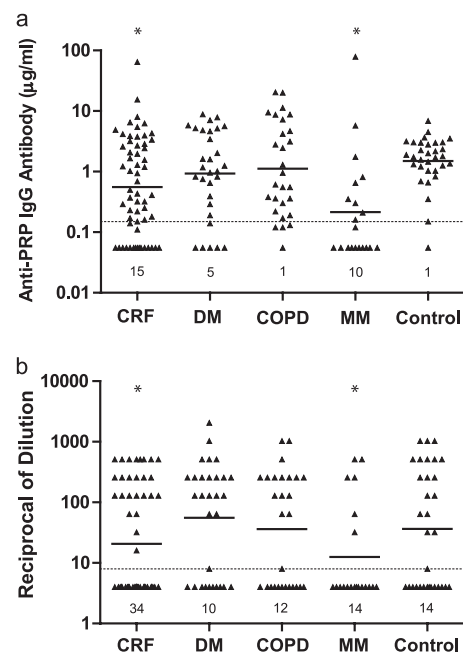


FIG 1 (a) Distribution of individual anti-PRP IgG antibody levels in patients with secondary immunodeficiency and healthy controls (geometrical mean concentrations are indicated for each group). The dashed line indicates the correlate of protection (0.15 $\mu\text{g/ml}$). (b) Antibody-mediated bactericidal activity in patients and controls. The dashed line indicates the lower limit of detection. CRF, chronic renal failure; DM, diabetes mellitus; MM, multiple myeloma. *, $P = 0.029$ for CRF and $P = 0.048$ for MM compared to controls. The numbers of individual samples with antibody levels or SBA scores below the lower limit of detection are indicated on each graph.

TABLE 3 Relative risk of having anti-PRP IgG antibody levels below 0.15 $\mu\text{g/ml}^a$ among patients with secondary immunodeficiency

Group	Relative risk (95% CI)	P value (Pearson's χ^2 test)
Chronic renal failure	9.22 (1.29–66.17)	0.002
Diabetes mellitus	6.4 (0.82–50.12)	0.018
COPD	4.57 (0.54–38.56)	0.059
Multiple myeloma	17.6 (2.46–26.2)	<0.0001

^a Minimum concentration required for protection against invasive disease.

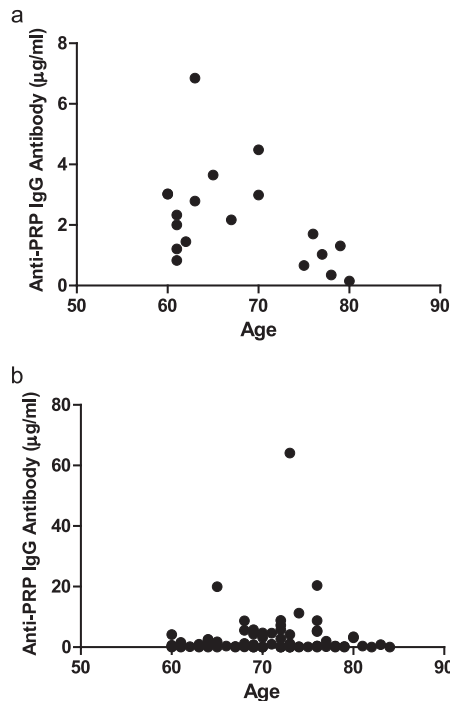


FIG 2 Correlation of anti-PRP IgG antibody levels with age among ≥ 60 -year-old individuals. (a) Healthy controls. (b) All patient cohorts.

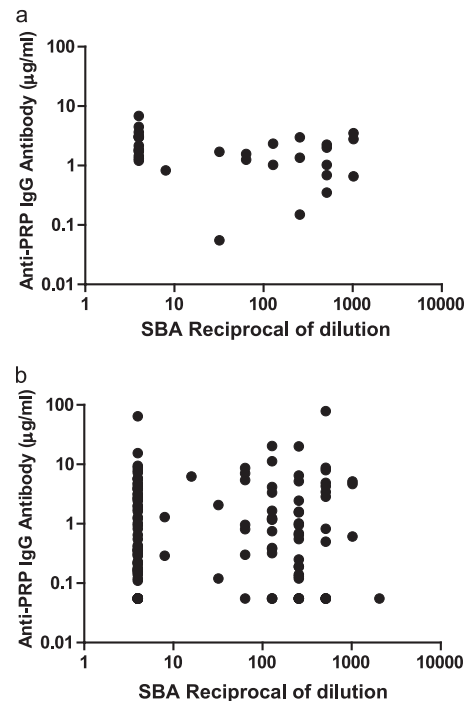


FIG 3 Correlation of anti-PRP IgG antibody levels with serum bactericidal assay geometric mean titers. (a) Healthy controls. (b) All patient cohorts.

lation is well protected against invasive Hib disease, which is indeed extremely rare in healthy adults (46). Because none of the subjects had been vaccinated against Hib, natural anti-Hib antibodies may have been induced by exposure to some common environmental bacteria that carry antigens cross-reacting with PRP, such as *Escherichia coli* K100 (15).

In compliance with earlier studies, we considered serum IgG anti-PRP levels the major indicator of protection against Hib invasive disease (19). However, multiple factors may contribute to clinical protection against this infection, i.e., antibody affinity/avidity, IgG subclass distribution, GM allotype, or idiotype. A specific idiotype (HibId-1, a marker of the V κ II-A2 chain) has been identified as the prevalent idiotype in the postvaccination adult anti-PRP antibody repertoire (22). According to our previous studies, unvaccinated adults may lack this idiotype despite high levels of natural anti-PRP, potentially induced by some cross-reactive antigens (47). In the present study, we did not see a correlation between anti-PRP IgG levels and serum bactericidal activity. A similar discordance between anti-capsular IgG levels and their functional activity had previously been documented in response to vaccination against Hib and *Neisseria meningitidis* serogroup C (2, 24). These studies identified antibody avidity as an important source of variability in their functional activity (2). As results obtained from ELISA may be relatively independent of antibody avidity (2), this may explain the lack of correlation between IgG antibody levels and SBA. In the case of natural antibody in unvaccinated individuals induced by cross-reactive antigens, the discordance between antibody levels and their functional activity may depend on the use of different V genes rendering varying avidity. Discordance between IgG anti-PRP and SBA was also observed in unvaccinated Alaskan adults and healthy elderly individuals (11, 21).

In contrast to healthy individuals, a proportion of our patients (ranging between 14% in COPD and 55% in myeloma) lacked protective anti-Hib antibody levels. Secondary immunodeficiency could be due to multiple reasons, e.g., profound metabolic disturbances and malnutrition due to uremia, as well as the immunosuppressive effect of hemodialysis in chronic renal failure (9, 18), impaired respiratory function, chronic inflammation, and therapy with corticosteroids in COPD (8, 42) or metabolic disorders and associated obesity in type 2 diabetes mellitus (16). The lowest anti-Hib immunity was detected in myeloma patients, likely caused by a decreased synthesis of normal immunoglobulins due to both malignant transformation of plasma cells and the immunosuppressive effect of chemotherapy (30). Multiple myeloma patients have a recognized high susceptibility to bacterial infections with common pathogens, such as *Streptococcus pneumoniae* and *H. influenzae* (30). However, an earlier study found that among 46 patients with multiple myeloma, prevaccination anti-PRP levels were comparable to that of the healthy United Kingdom adult population (36). A recent paper reported that 80% of multiple myeloma patients had anti-Hib IgG levels of >0.15 $\mu\text{g/ml}$ (17). In contrast, in our study, only 45% of myeloma patients had antibody levels above this cutoff. Such discrepancies may be due to different patient populations, progression/length of disease, or ELISA technique variability. Despite the fact that vaccination of multiple myeloma patients against Hib remains controversial (30), our findings suggest that the majority of such patients lack protective immunity against Hib and should be vaccinated. Chronic renal failure patients also have significantly decreased IgG anti-PRP levels, as well as functional antibody activity, and hence may have high susceptibility to Hib infection. Cases of peritonitis caused by Hib in chronic renal failure patients undergoing peritoneal dialysis have been reported (7, 29). However, to the best

of our knowledge, no specific defects in the immunological defense against Hib have been previously identified in adults with chronic renal failure.

Humoral immune defects represent an important component of immunosenescence (5). Mechanisms behind B-cell defects in the elderly include reduced antibody diversity, defects in isotype switching, and somatic mutation resulting in low-affinity antibody production, as well as deficiency in IgM memory B cells (32, 50). However, our data suggest that severe chronic diseases have a larger negative impact on natural immunity to Hib than aging, as we found an age-associated decline in anti-PRP levels in older healthy adults, but not in patients. This implies that general immunosuppression may play a greater role than senescence in the observed lack of protective anti-Hib antibody levels. However, the correlate of protection against invasive Hib disease in older adults is unknown. Several studies indicate that antibody against bacterial capsular polysaccharides present in the elderly may lack functional activity (21, 32). On the other hand, the existence of B memory cells, a result of previous exposure to Hib or cross-reactive bacteria, may contribute to protection against invasive disease in older individuals.

Although the antibody levels required to prevent nasopharyngeal carriage have not been accurately determined, it has been suggested that they may be as high as 5 to 10 µg/ml (20, 38). Considering that only 3% of healthy controls in our study had ≥ 5 µg/ml of IgG anti-PRP, most of them are potential carriers. Although only a small fraction will be susceptible to developing Hib invasive disease, such individuals may nevertheless transfer the pathogen supporting Hib circulation within the population. According to recent studies, invasive Hib disease still exists in countries with high pediatric anti-Hib vaccination coverage, and the disease affects adults more often than children (4, 23, 39, 44).

This study has several limitations. The multiple myeloma group was smaller than an estimated sample size because a limited number of patients were available at the time of our study. Also, the small sample size could potentially influence the analysis of the effects of age and sex. In general, the interpretation of immunological data with regard to clinical protection against Hib disease is complex. The analysis of humoral immunity does not account for the presence of immunological memory against Hib that can develop following natural exposure to whole bacteria expressing protein antigens along with PRP. Under these circumstances, the anti-polysaccharide immune response can acquire T-cell-dependent properties that may potentially contribute to long-lived populations of memory B cells capable of developing a secondary immune response following repeated exposure to the pathogen. However, despite these limitations, our findings point to the lack of immunological protection in a substantial proportion of adults with severe chronic conditions causing secondary immunodeficiency, particularly in multiple myeloma and chronic renal failure patients.

Conclusion. Our study has demonstrated that in the era of universal pediatric immunization against Hib, healthy adult individuals typically have protective immunity against invasive Hib disease, but over 90% of them have the potential for pathogen carriage. In contrast, we have found a lack of protective immunity against Hib in adults suffering from multiple myeloma and chronic renal failure. Such individuals may be at risk of developing invasive Hib disease if exposed to the pathogen.

Considering that Hib continues to circulate in Western coun-

tries despite the high vaccine coverage of infants and that individuals born before the beginning of the 1990s have not been immunized, adult patients with multiple myeloma and chronic renal failure can benefit from immunization with a Hib conjugate vaccine. Previous studies have established that immunization of adults, including immunodeficient individuals, with pediatric Hib vaccines is safe and highly effective (21, 25, 26). Although it is critically important to maintain herd immunity via adequate and universal pediatric immunization, our findings, along with recent data on emerging invasive Hib disease in unimmunized adults, provide a rationale for extended indications for immunization of vulnerable groups of adults against Hib (39, 45).

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