***In Vitro* Induction of IgG1 and IgG2 Secretion by B Cells of Children Who Developed Invasive *Haemophilus* Disease despite Vaccination**

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ABSTRACT. Lymphocytes from seven patients who developed *Haemophilus influenzae* type b (Hib) disease after being vaccinated with Hib polysaccharide vaccine and from one patient who developed disease after conjugate vaccination were investigated for the ability to secrete IgG1 and IgG2, *in vitro*, in response to a combination of pokeweed and *Staphylococcus aureus* Cowan I mitogens. The eight patients were selected because they had low anticapsular antibody responses to Hib disease. Only one of the eight children had a history of previous severe, recurrent infections. This child (in whom a polysaccharide vaccine failed) had a deficiency of the second component of complement and also had a subnormal serum concentration of IgG4. Only one of the eight children, an otherwise healthy 54-mo-old with normal serum Ig concentrations, had subnormal mitogen-induced B-cell secretion of IgG1 and/or IgG2. When this child's lymphocytes were separated into T- and B-cell fractions and cocultivated with the respective fractions of the father's lymphocytes, the child appeared to have an intrinsic B-cell defect and normal T cells. There were no significant differences ($p > 0.3$) in the respective geometric means of the *in vitro* secretion of IgG1 or IgG2 of the B cells from the children with polysaccharide vaccine failure and those of 14 healthy controls of similar ages as the patients (IgG1, 1524 versus 3497 ng/mL per 10^5 lymphocytes; IgG2, 79 versus 89 ng/mL per 10^5 lymphocytes). Thus, despite the presence of impaired serum anticapsular antibody responses to Hib disease, most children who develop Hib disease after Hib polysaccharide vaccination have normal *in vitro* B-cell secretion of IgG1 and IgG2 in response to mitogens. (*Pediatr Res* 30: 124-129, 1991)

Abbreviations

Hib, *Haemophilus influenzae* type b
PS, polysaccharide
PBMC, peripheral blood mononuclear cell
SRBC, sheep red blood cell
PWM, pokeweed mitogen
C2, second component of complement
PRP, polyribysl ribitol phosphate

Hib PS vaccine was first licensed in the United States in 1985. It soon became apparent that this vaccine was incompletely protective in children ≥ 24 mo of age, the principal age group targeted for immunization (1-3). The reasons why vaccination failed to protect some children are unclear (1-4). Most of the children with vaccine failure were found not to have underlying conditions, such as sickle cell disease, previous splenectomy, or hypogammaglobulinemia, which predispose to an increased risk of Hib disease (1, 4). Although children with vaccine failure had normal antibody responses to protein antigens, such as tetanus toxoid (1), many were found to have impaired serum antibody responses to the Hib PS capsule, both after recovery from invasive Hib disease (1) and after reimmunization with Hib PS vaccine (5). These observations suggested a selective antibody deficiency to the Hib PS antigen in some of the children. However, the antibody unresponsiveness of the children could be overcome by vaccination with Hib PS conjugated to protein carriers (5, 6), and the antibody elicited expressed the same, recently described, dominant cross-reactive idiotype on antibody elicited by Hib vaccination of most healthy children and adults (7, unpublished data).

The underlying cellular immune defects involved in the impaired serum anticapsular antibody responses of patients who did not respond to Hib PS are unclear. PS-protein conjugate vaccines are thought to confer thymic-dependent properties to the PS component of the vaccine (8). Thus, the ability of the children with vaccine failure to respond to Hib PS-protein conjugate vaccines, but not to unconjugated Hib PS, favors the hypothesis that these children possess B cells that are able to expand and differentiate into Hib PS antibody secreting cells with the help of T cells. But, for reasons that remain unexplained, their B cells do not respond normally to the thymic-independent form of the Hib PS antigen.

Measurement of *in vitro* Ig production by B cells stimulated with mitogens has been used to evaluate patients suspected of having a variety of immunodeficiencies, including common variable immunodeficiency (9-11) and IgG subclass deficiency (12-14). In addition, children with recurrent bacterial infections associated with normal serum Ig concentrations but abnormal antibody responses to specific antigens have been reported to show *in vitro* abnormalities of T- and B-cell function, including abnormal secretion of IgG subclasses in response to mitogen stimulation (15, 16). Little is known about the function of T and B cells in patients with Hib vaccine failure. The purpose of our present study was to determine whether B cells from such patients demonstrate abnormal *in vitro* secretion of IgG1 and IgG2 in response to mitogen stimulation.

MATERIALS AND METHODS

Subjects. The protocol was approved by the Human Studies Committee of Washington University School of Medicine. The

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principal patient group consisted of six children with a history of culture-proved invasive Hib disease, with the onset of the disease occurring 3 to 11 mo after Hib PS vaccination. As described in the results, except for minor illnesses, these six children were otherwise healthy, and none of the six had a history of previous episodes of bacteremia. Data from two additional patients who developed Hib disease after vaccination are included in this report, but because one developed disease after conjugate vaccination and the other had recurrent invasive bacterial disease and a deficiency of C2, the data from the two were analyzed separately.

At the time of our study, the eight children ranged in age from 48 to 84 mo (median = 60 mo). All eight children resided in Missouri or Illinois and were selected based on their availability and the willingness of their parents to consent to these studies. In addition, all eight children had $<1 \mu\text{g/mL}$ of anticapsular antibody in serum obtained 2 to 11 mo after the onset of Hib disease (median = 6 mo). The serum antibody responses of seven of the eight children to Hib conjugate vaccines have been described in previous reports (5, 6).

The control subjects for the B-cell studies consisted of 14 healthy children, ranging in age from 43 to 92 mo (median = 62 mo). All of the control children resided in the St. Louis area and were the same ages as the patients with vaccine failure. As an additional control group, lymphocytes from a healthy parent of each patient and each control child were fractionated and cultured *in vitro* on the same day as those of the respective child. This group of adults was included to control for variability in cell separation and culture techniques that might result in poor Ig secretion by the child's lymphocytes. In addition, as described below, the parents' lymphocytes were used for the cocultivation experiments with the B and T cells from their children.

General Immunologic Evaluation. Serum concentrations of IgG, IgA, and IgM were measured by nephelometry (Beckman Instrument, Inc., Fullerton, CA). Serum concentrations of IgG1, IgG2, IgG3, and IgG4 were measured by particle concentration fluorescence immunoassay, as previously described (17). In five patients, enumeration of lymphocyte populations was performed by incubating peripheral blood mononuclear cells, separated by Ficoll-Hypaque density sedimentation, with FITC-conjugated MAb and examining the cells by fluorescent microscopy (Labophot; Nikon, Tokyo, Japan). The MAb used were anti-Leu 16, anti-Leu 5B, anti-Leu 3A, and anti-Leu 2A (Becton-Dickinson, Immunocytometry Systems, Mountain View, CA) to quantitate B cells, T cells, CD4 cells, and CD8 cells, respectively. In the remaining three patients, enumeration of lymphocyte populations was performed on heparinized blood, using the commercially available Coulter Immuno-Prep method (Coulter Diagnostics, Hialeah, FL). After lysis of the red cells, membrane stabilization, and fixation of the lymphocytes, the FITC-conjugated MAb described above were used to determine the respective lymphocyte surface markers by flow cytometry (Coulter-Epics Profile, Hialeah, FL). Cells from two patients were enumerated by both flow cytometry and the manual method described above, and there was good agreement between the respective results.

Measurement of In Vitro Secretion of IgG1 and IgG2 by Mitogen-Stimulated B Cells. Lymphocyte separation. PBMC were obtained from heparinized blood by Ficoll-Hypaque density sedimentation as described (18). Except where noted, each patient and control were studied on one occasion. Partial depletion of monocytes was achieved by adherence of the PBMC to plastic surfaces. Briefly, PBMC at 2×10^6 cells/mL were incubated in 15 mL of RPMI 1640 (J.R. Scientific, Ballwin, MO) containing 10% FCS (Hyclone Laboratories, Logan, UT) in 75-cm² polystyrene flasks at 37°C for 30 min. Nonadherent cells were incubated overnight with neuraminidase-treated SRBC. The next morning, the spontaneous rosettes formed by T lymphocytes with the SRBC were separated from the B-cell fractions by density sedimentation on Ficoll-Hypaque. The B-cell fraction was harvested from the interface between the Ficoll-Hypaque and the RPMI

1640 medium and washed once with Hanks' balanced salt solution (J.R. Scientific) and resuspended in RPMI 1640. The rosetted T cells were harvested from the pellets, washed with 1% NH₄Cl buffer, pH 7.4, to lyse the SRBC, and then centrifuged for 10 min at $350 \times g$. The T-cell fraction in the pellet was washed once with Hanks' balanced salt solution and then resuspended in RPMI 1640.

Enumeration of lymphocyte subpopulations. Total T cells, B cells, and monocytes were quantitated in the T- and B-cell fractions, using FITC-conjugated MAb anti-OK T₃ (Ortho Diagnostic Systems, Raritan, NJ), anti-Leu 16, and anti-Leu M₃ (Becton-Dickinson), respectively. A total of 2×10^6 cells were incubated with 20 μL of each MAb for 30 min at 4°C, followed by fixation with 1% paraformaldehyde and examination by fluorescent microscopy. The T-cell fractions contained 80 to 90% T cells ($<2\%$ monocytes, $<2\%$ B cells). The B-cell fractions contained 40 to 55% B cells, 10 to 25% monocytes, and $<2\%$ T cells.

Culture conditions. Equal numbers of lymphocytes in the T- and B-cell fractions from patients were cocultivated together or with counterpart lymphocytes from a healthy parent in the presence or absence of mitogen in 96-well microtiter plates (Falcon Microtest III, Maryland Heights, MO). A total of 1×10^5 cells per well were cultured in triplicate in a final volume of 200 μL of RPMI 1640 medium supplemented with L-glutamine (2 mM), gentamicin (50 $\mu\text{g/mL}$), and 10% heat-inactivated FCS (Hyclone). Incubation was for 7 d in a humidified atmosphere at 37°C with 5% CO₂. Cultures were stimulated with a mixture of two mitogens: PWM (Sigma Chemicals, St. Louis, MO) at dilutions of 1:1000 and 1:3000 in RPMI 1640 and *Staphylococcus aureus* Cowan I (Enzyme Center, Inc., Malden, MA) at 0.02 and 0.005% vol/vol, respectively. The specific dilutions of the combination of the two mitogens were selected based on the maximal Ig secretion response to a series of mitogen dilutions performed in earlier experiments. Supernatants from triplicate wells were pooled and stored at -70°C until they were assayed.

IgG Subclass ELISA. Concentrations of IgG1 and IgG2 were measured in the supernatants, using a "capture" ELISA as previously described (19). MAb HG11 (20) was used for the detection of IgG1. HP6016, a MAb specific for the human isoallotype G4m(b), present on all IgG2 molecules and on a subset of IgG4 molecules, was used to detect IgG2 (21, 22). The latter antibody was selected instead of two other commonly used MAbs, HP6014 or GOM1 (which are specific for IgG2), to avoid the λ -light-chain bias of HP6014 (17) and the low affinity of GOM1 (22). For our purposes, detection of the G4m(b) isoallotype was found to be suitable for measurement of IgG2 secretion because the concentrations of IgG4 in the supernatants were so low that essentially all of the G4m(b) detected was IgG2.

Briefly, Dynatech Immunolon 2 plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated with 10 $\mu\text{g/mL}$ of the respective MAb in 10 mM Tris saline buffer, pH 8.5, and incubated for 4 h. Plates were washed with 20 mM Tris saline buffer, pH 7.4, containing 0.05% Tween 20. Uncoated sites were blocked with 1% BSA diluted in the Tris saline wash buffer. Serial 4-fold dilutions of the culture supernatants were added, starting at 1:40 for the IgG1 assay and 1:4 for the IgG2 assay, and incubated overnight at 4°C. After washing and blocking, bound IgG1 and IgG2 were detected, using an alkaline phosphatase-conjugated goat antihuman IgG, which had been adsorbed to remove reactivity with mouse Ig (Sigma Chemical Co., St. Louis, MO). In addition, we added normal mouse serum and normal goat serum (2% vol/vol) to the alkaline phosphatase conjugate of goat antihuman IgG to decrease nonspecific reactivity in the assay. *p*-Nitrophenyl phosphate disodium at 1 mg/mL in diethanolamine buffer, pH 9.8, was used as the substrate. A human purified IgG1 myeloma protein was used as the standard in the IgG1 assay. Because of insufficient quantity of a purified IgG2 myeloma protein, dilutions of a secondary standard, consisting of a human serum pool designated HSP-3 (17), were used

as the reference serum in the IgG2 assay. The lower limit of detection of IgG1 and IgG2 was 10 ng/mL. Culture supernatants from each patient were assayed in parallel with those of controls of similar age. For quality control, two control pools of culture supernatants were tested in every assay. In eight assays, the mean IgG1 and IgG2 concentrations of pool 1 were 3350 and 171 μ g/mL, and those for pool 2 were 20 and 15 μ g/mL. The mean variation of values for replicate samples tested on different days was 16% for the IgG1 assay and 10% for the IgG2 assay.

Statistical Analyses. The *in vitro* IgG1 and IgG2 secretion values were transformed logarithmically, and the mean logs of the values of the patients and controls were compared by the *t* test. In addition, the median values of the logs were compared by the Robust analysis.

RESULTS

Clinical Characteristics of Patient Group. Table 1 summarizes the clinical characteristics of the patients with vaccine failure. All of the patients were Caucasian, and 50% were male. Two of the patients with PS vaccine failure had a history of mild asthma, and a third patient had a history of recurrent episodes of otitis media. Three children were healthy before and after recovering from invasive Hib disease. As noted in Materials and Methods, two additional patients with vaccine failure differed clinically from the six patients described above, and, therefore, the data from the two were analyzed separately. Patient 7 had a history of severe recurrent bacterial infections and was known to have a deficiency of C2. The recurrent infections in this child included sinusitis, otitis media, pneumonia, and two previous episodes of *Streptococcus pneumoniae* bacteremia. Patient 8 was an otherwise healthy child who developed invasive Hib disease 5 mo after being immunized with Hib PS-diphtheria toxoid (PRP-D) conjugate vaccine.

Immunologic Evaluation. Anticapsular antibody concentrations. The mean age at vaccination of the seven children with Hib PS vaccine failures was 28 mo, and the mean age at onset of invasive Hib disease was 35 mo. The one child with conjugate vaccine failure was 18 mo of age at vaccination and 23 mo of age at onset of disease. After recovery from Hib disease, all of the eight patients had <1 μ g/mL of anticapsular antibody in convalescent serum (Table 1). Of note, before onset of Hib disease, patient 7 had been vaccinated twice with Hib PS vaccine at 24 and 30 mo of age. His serum antibody concentrations before and after vaccination and revaccination were 0.07, 0.08, and 0.08 μ g/mL, respectively. At 38 mo of age, he developed periorbital cellulitis and Hib bacteremia. Two mo after onset of the disease, his serum antibody level was 0.14 μ g/mL.

Response to reimmunization. The eight patients were reimmunized with Hib PS vaccine and/or Hib conjugate vaccine at a median interval of 6 mo after onset of Hib disease. Four

children were given Hib PS vaccine, and only one responded with antibody >1.0 μ g/mL (patient 2, 3.3 μ g/mL of antibody). The three nonresponders (patients 4–6) had 0.40, 0.07, and 0.58 μ g/mL of antibody, respectively, after revaccination. These three patients and the four remaining patients who were not reimmunized with Hib PS vaccine were given one or two doses of Hib conjugate vaccine (PRP-meningococcal outer membrane complex conjugate vaccine, four patients; PRP-D conjugate vaccine, three patients). As previously reported (5, 6), all seven children showed 5-fold or greater increases in serum antibody concentration, including the three patients who did not respond to reimmunization with Hib PS vaccine.

Serum Ig concentrations and circulating T and B cells. The seven patients with Hib PS failure had normal serum concentrations of IgM, IgA, and IgG. In contrast, patient 8, the child with the PRP-D conjugate vaccine failure, had a low serum IgM concentration (31 mg/dL) and a borderline IgA concentration (19 mg/dL). Table 2 summarizes the serum IgG subclass concentrations. The IgG subclass concentrations were normal except for a borderline subnormal concentration of IgG4 in patient 1 and a subnormal concentration of IgG4 in patient 7, the child with C2 deficiency. The percentage of circulating T3, T4, and T8 cells and the percentage of B cells were normal in all eight patients (Table 2).

***In vitro* secretion of IgG1 and IgG2.** Table 2 shows the concentrations of IgG1 and IgG2 secreted by B cells stimulated, *in vitro*, with the combination of *S. aureus* Cowan I and PWM. Only one of the eight patients (patient 5) had subnormal *in vitro* secretion of IgG1 and IgG2. Of note, patient 1, who had a borderline subnormal serum IgG4 concentration, and patient 8, who had a subnormal serum concentration of IgM and a borderline concentration of IgA, had normal *in vitro* B-cell secretion of IgG1 and IgG2. Because of the small quantities of culture supernatant and because *in vitro* secretion of IgG4 is below the detection limit of our assay, *in vitro* secretion of IgM, IgA, or IgG4 was not measured.

Figure 1 shows the individual B-cell secretion of IgG1 and IgG2 for the six patients with "primary" Hib PS vaccine failure and the corresponding values for the 14 healthy control children and 20 healthy adults. The median B-cell secretion of IgG1 was similar in the control children and healthy parents (8085 versus 6150 ng/mL per 10^5 lymphocytes, $p < 0.9$). In this analysis, the data from the parents of the patients and the parents of the control children were combined because there was no significant difference in the values obtained for these two groups of adults. Examination of the individual IgG1 secretion values of the patients with vaccine failure and control children also indicates that there is considerable overlap between the two groups. There was a trend for lower IgG1 secretion in the patients, but the difference was not statistically significant (geometric means of 1524 versus 3497 ng/mL per 10^5 cells, $p = 0.32$ by *t* test; medians

Table 1. Clinical characteristics of patients with Hib vaccine failure

Patient no.*	Sex	Age at Hib vaccination (mo)	Age at disease (mo)	Hib disease	Convalescent anti-PRP antibody† (μ g/mL)	Clinical history before Hib disease
1	M	24	34	Meningitis	0.15	Healthy
2	F	27	31	Epiglottitis	0.15	Healthy
3	M	45	48	Meningitis	0.16	Asthma
4	F	25	36	Epiglottitis	0.52	Asthma
5	M	24	28	Meningitis	0.07	Recurrent otitis media
6	F	24	29	Meningitis	0.92	Healthy
7	M	24	38	Cellulitis	0.14	C2 deficiency; bacteremia; recurrent sinusitis; pneumonia
8	F	18	23	Meningitis	0.19	Healthy

* Patients 1–7 had Hib PS vaccine failure. Patient 7 also had a C2 deficiency. Patient 8 had Hib conjugate vaccine failure. All patients were Caucasian.

† Serum sample obtained 2 to 11 mo after onset of Hib disease (median = 6 mo).

Table 2. Results of immunologic evaluation of patients with Hib vaccine failure

Subject no.*	Age at study (mo)	Serum IgG subclasses ($\mu\text{g/mL}$)				Peripheral blood lymphocyte population					In vitro secretion (ng/mL/ 10^5 cells)	
		IgG1	IgG2	IgG3	IgG4	Total lymphocyte count/ mm^3	T3 cells (%)	T4 cells (%)	T8 cells (%)	B cells (%)	IgG1	IgG2
1	48	400	109	54	1	2688	75	41	21	16	9530	569
2	65	572	208	32	27	3800	80	33	24	9	2695	204
3	84	630	120	67	5	3200	78	42	29	5	2000	100
4	71	601	151	60	26	2806	70	46	27	16	2136	44
5	54	376	64	48	17	1862	80	36	16	5	150†	<10†
6	50	785	64	52	6	7920	81	42	32	6	760	77
7	53	501	97	81	<0.4†	3523	58	35	16	37	360	35
8	30	404	67	28	3	3864	73	30	17	24	5156	148

* Patients 1–7 had Hib PS vaccine failure. Patient 7 also had a C2 deficiency. Patient 8 had Hib conjugate vaccine failure. All patients were Caucasian.

† ≥ 2 SD below mean value for age.

Fig. 1. Secretion of IgG1 and IgG2, *in vitro*, by peripheral blood lymphocytes cultured with a combination of PWM and *S. aureus* Cowan 1. Each point represents the results of studies of cells from an individual subject. The bars represent the respective medians. The data from patient 7 with recurrent infection and C2 deficiency and patient 8 with conjugate failure are excluded (see text). The data for the parents of the control children and the parents of the patients were not significantly different, and, therefore, for calculation of the medians, the data from the two groups were combined. The children with vaccine failure and the control children had lower IgG2 values than those of the adults ($p < 0.05$). The difference in the IgG1 secretion between the children with vaccine failure and the control children was not significant ($p > 0.2$). Note that the control child and the control parent with very low IgG1 values are not related and were studied on different days.

of 2067 versus 8085 ng/mL per 10^5 lymphocytes, $p = 0.20$ by Robust analysis). In this analysis, we compared the respective medians in addition to the geometric means because of the outlier value in a control child with a very low IgG1 secretion (Fig. 1). The values for the B-cell secretion of IgG2 for the children with vaccine failure and the control children were very similar (geometric means of 79 and 89 ng/mL per 10^5 lymphocytes), but both groups had lower geometric mean IgG2 secretions than that of the B cells of the healthy adults (geometric mean = 302, $p < 0.05$ and 0.01 , respectively). For IgG2, there were no outliers among the controls, and the median values of the three groups (89, 104, and 277) were very similar to the respective geometric means.

Because of the trend for lower IgG1 secretion by the lymphocytes from the patients, we cocultivated T- or B-cell fractions from the patients with lymphocytes from their respective parents and measured mitogen-induced secretion of IgG1. The lymphocytes from the healthy parent served as the source of "normal"

lymphocytes in this experiment to minimize the allogenic effect that occurs during mixing of lymphocytes from unrelated donors (23). In these experiments, we examined the combination of the patient's B cells and T cells, the patient's B cells and the respective parent's T cells and the patient's T cells and the respective parent's B cells. The results are summarized in Figure 2 and are expressed as the concentrations of IgG1 per 10^5 lymphocytes secreted by the T- and B-cell combination tested. For comparison, we show the geometric mean $\pm$ SD of the IgG1 concentrations in the respective combinations from the healthy control children and their parents.

Lymphocytes from only one of the patients with vaccine failure (patient 5) had subnormal B-cell secretion of IgG1 (>2 SD below the mean for age). T and B cells from two other children with Hib PS failure (patient 6 and patient 7 with C2 deficiency and recurrent bacteremia) showed values >1 SD below the mean for age and $<30\%$ of the IgG1 secretion of the B and T cells of the respective parent measured on the same day (Fig. 2). The corresponding number of healthy control children with IgG1 values $<30\%$ of that of their respective parent was two of 14 (14%). The results of the different mixing experiments suggested that the low IgG1 secretion of the lymphocytes from patient 5 may have been a result of an intrinsic B-cell defect (*i.e.* the parent's B cells and the patient's T cells showed normal IgG1 secretion, whereas the patient's B cells with the parent's T cells still had low IgG1 secretion). The results from patient 7 showed a similar pattern. In contrast, the results with the lymphocytes of patient 6 were consistent with possible excessive T suppressor activity. In patient 6, to distinguish between excessive suppressor activity or a lack of T-cell help, we also examined the effect of the addition of T cells from this patient on the secretion of IgG1 by the respective parent's B and T cells. The value obtained with the parent's B and T cells was 5900 ng/mL per 10^5 lymphocytes. Upon the addition of the patient's T cells, secretion of IgG1 decreased to 975 ng/mL per 10^5 lymphocytes (84%).

DISCUSSION

We investigated secretion of IgG1 and IgG2 by mitogen-stimulated lymphocytes of children who developed invasive Hib disease despite previous vaccination. The patients were selected based on the presence of low serum anticapsular antibody responses to Hib disease. This latter finding was particularly noteworthy because the children developed disease between 2 to 4 y of age and, therefore, would have been expected to have high anticapsular antibody responses (1). As previously reported (1, 4), their poor anticapsular antibody responses could not be explained by the presence of hypogammaglobulinemia or serum IgG subclass deficiency.

Fig. 2. T- and B-cell fractions from the patients were cocultivated together or with counterpart lymphocytes from the respective healthy parent in the presence or absence of mitogens (see Materials and Methods). After 7 d of incubation, IgG1 was assayed in the supernatants. The results are shown as the concentration of IgG1 per 10^5 lymphocytes secreted by the different T- and B-cell combinations. Respective data from 12 control child-parent pairs are shown as geometric means $\pm$ SD. In this analysis, data from the remaining two control pairs with outlier IgG1 values in a child or a parent were omitted (Fig. 1).

Among the patients, the median B-cell secretion of IgG2 in response to PWM and *S. aureus* Cowan I was nearly identical to that of the B cells of the control children (Fig. 1). However, for IgG1, there was a trend for the children with vaccine failure to have a lower median or geometric mean B-cell secretion than that of the healthy control children (Fig. 1). The difference was not statistically significant ($p > 0.2$), but it is possible that a true difference exists in the average IgG1 secretion, and it went undetected because of the small sample size of the patients with this rare condition.

Of the eight patients studied, only patient 5 had subnormal (>2 SD) secretion of IgG1 and IgG2 and, in cocultivation experiments, the lymphocytes from this patient showed evidence of an intrinsic B-cell defect (Fig. 2). As noted above, the serum concentrations of IgG1 and IgG2 in patient 5, which reflect B-cell responses to both antigen and mitogen stimulation, were within the normal range (Table 2). In contrast, in the *in vitro* assay, which measures B-cell responses to mitogens alone, secretion of IgG1 and IgG2 was abnormal. Shackelford *et al.* (13) reported that lymphocytes from children with subnormal serum concentrations of IgG2 and impaired antibody responses to Hib PS had subnormal *in vitro* production of not only IgG2 but also IgG1 and IgG3. These findings were noteworthy because the *in vitro* data suggested that the immunoregulatory problem in these patients was not limited to IgG2 but involved other IgG subclasses, despite the presence of normal serum concentrations of these subclasses. Thus, despite the presence of normal serum Ig concentrations in patient 5, the subnormal *in vitro* secretion of IgG1 and IgG2 by his mitogen-stimulated B cells suggests the possibility of an underlying immune defect associated with the vaccination failure. Note, however, that lymphocytes from healthy individuals also occasionally can show low B-cell secretion of IgG1 in response to mitogens (Fig. 1). Indeed, lymphocytes from the one adult control with low IgG1 secretion were cultured *in vitro* on two other occasions over a 4-mo period and were again found to show low IgG1 secretion (unpublished data). Thus, the possible relationship between subnormal B-cell Ig secretion *in vitro* and the vaccination failure in patient 5 requires additional study.

In humans, PWM is a T cell-dependent polyclonal B-cell mitogen, whereas *S. aureus* Cowan I is a direct B-cell mitogen. These two mitogens are thought to stimulate different subsets of

B cells (24, 25). In the experiments reported here, we used the combination of the two mitogens at two different dose ranges to maximize the Ig secretion compared with when the mitogens are used separately (24). Because different cell populations are thought to be stimulated by the different mitogens, it is possible that a significant difference in IgG1 or IgG2 secretion between our patients and controls might have been detected if the mitogens had been used separately. However, in other experiments, when lymphocytes were stimulated with PWM alone, we found no significant difference between patients with vaccine failure and control children in the respective IgG1 or IgG2 secretion (unpublished data). To date, the responses to *S. aureus* Cowan I alone have not been examined.

In our *in vitro* studies, we did not attempt to measure antigen-specific responses. Previous efforts to measure plaque-forming cells or specific antibody secreted by B cells stimulated *in vitro* with PS antigens demonstrated minimal (26) or no triggering of specific antibody formation or proliferative responses (27). When tested *in vitro*, Hib PS is reported to be a poor mitogen (28). Although it is possible to measure *in vitro* antibody secretion of B cells from individuals vaccinated *in vivo* with PS (26, 28–30), such B cells remain in the peripheral blood only transiently after vaccination, and, when the cells are cultured *in vitro*, the responses appear to be minimally affected by exposure to antigens (26, 27).

The biologic basis for the impaired serum anticapsular antibody responses of patients with Hib PS vaccine failure remains poorly understood. Most patients have normal serum concentrations of Ig (1, 4). The present results also suggest that most patients have normal *in vitro* B-cell secretion of IgG1 and IgG2 in response to mitogens, although, as noted above, we cannot exclude the possibility that the average IgG1 secretion in the patients is lower than that of the controls (Fig. 1). In previous studies, the most notable findings in patients with Hib PS vaccination failure were impaired serum antibody responses to Hib PS (1, 4), despite normal serum concentrations of Ig (1, 4) and normal antibody responses to tetanus toxoid (1) and pneumococcal type 3 PS (Granoff DM, unpublished data). Furthermore, as noted earlier, the antibody unresponsiveness to Hib PS can be overcome by vaccination with Hib PS-protein conjugate vaccines (5, 6). Taken together, these data suggest that the impaired serum antibody responses to Hib PS are a result of either an exaggerated

physiologic delay in the normal age-related development of antibody response to this antigen or, possibly, to transient tolerance induced by vaccination with Hib PS. In both instances, the antibody unresponsiveness would be expected to be antigen specific, transient, and potentially capable of being overcome by vaccination with an Hib PS-protein conjugate. The conjugate vaccination is thought to provide additional signals to the B cells that are unresponsive because of either a maturational delay, or because they have been rendered unresponsive by exposure to the unconjugated PS. However, it remains possible that some of the children may have an evolving immunodeficiency. Therefore, it will be important to continue to follow their clinical courses and serum Ig concentrations to determine whether additional immunologic dysfunction develops.

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