A Major Crossreactive Idiotype Associated with Human Antibodies to the *Haemophilus influenzae* b Polysaccharide

Expression in Relation to Age and Immunoglobulin G Subclass

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Abstract

Using idiotypic analysis, we examined the variable (V) region diversity of human antibodies specific for the capsular polysaccharide of *Haemophilus influenzae* b (Hib PS). A goat anti-idiotypic serum (anti-Id) was prepared against anti-Hib PS antibodies isolated from the serum of an adult immunized with Hib PS. The anti-Id bound donor anti-Hib PS antibodies and inhibited Hib PS binding of donor anti-Hib PS. In contrast, the anti-Id did not bind donor or pooled Ig depleted of Hib PS antibodies, nor did it inhibit antigen binding of human antibodies to pneumococcal PS's, meningococcal A PS or diphtheria toxoid. Crossreactive idiotype (CRI), as measured by anti-Id inhibition of Hib PS binding, was found in 74 of 98 subjects (76%) vaccinated with Hib PS at 1.7–57 yr of age. 60 of these 74 subjects had > 50% of their serum Hib PS–binding activity inhibited by anti-Id. No correlation was found between age and CRI expression. In subjects showing both IgG1 and IgG2 antibody responses, CRI was most frequently detected in both subclasses (71% of subjects). CRI was limited to either IgG1 or IgG2 in 19% of subjects, a finding suggestive of independent B cell lineages. 13 of 15 infants < 17 mo of age, who responded to Hib PS–outer membrane protein conjugate vaccine, had > 50% of their serum anti-Hib PS antibody activity inhibited by anti-Id. The ability of native Hib PS and Hib PS oligomer to partially inhibit (60 and 35%, respectively) the binding between anti-Id and heterologous anti-Hib PS, indicated that some CRI determinants are in or near the combining site. In summary, our findings demonstrate a highly penetrant and frequently predominant CRI, which is expressed in both infants and adults. The results underscore the limited V region diversity of anti-Hib PS antibodies and indicate that CRI predominance is manifest early in ontogeny and is induced by both TI and TD forms of the Hib PS antigen. (*J. Clin. Invest.* 1990. 85:1158–1166.) shared idiotype • Ig variable domains

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Introduction

Haemophilus influenzae type b is the most important cause of bacterial meningitis in children in North America (1). Considerable evidence indicates that the type b capsule (Hib-PS),[1] a relatively simple antigen consisting of repeating units of 3-β-D ribose-(1 → 1)-D-ribitol-5-phosphate, is an important virulence determinant (2, 3). For example, nearly all clinical *Haemophilus* isolates from patients with meningitis elaborate this capsular polysaccharide (PS) (1), and deletion mutants that are genetically identical to the wild type parent except for production of capsular PS are no longer pathogenic in infant rats (3).

Serum antibodies to Hib PS confer protection against invasive *Haemophilus* type b disease (4–6). However, the immune response to immunization with Hib PS is characterized by a maximal antibody response to one injection and little or no increase in antibody after repeated injection, i.e., no booster response. In addition, the antibody responses of infants < 18 mo of age are poor (reviewed in reference 7), and this age group has the highest risk of developing *Haemophilus* type b meningitis (1). Studies of human IgG antibodies to Hib PS have revealed limited diversity in that they are restricted to the IgG1 and IgG2 subclasses (8, 9), express predominantly kappa light chains (10, 11), and are resolvable by isoelectric focusing (IEF) into a few distinct clonotypes that may be shared by unrelated individuals (12, 13).

Recently we described the occurrence of anti-Hib PS associated crossreactive idiotypes (CRI) in sera from five unrelated adults (14). These findings suggested that human anti-Hib PS antibodies may also have limited V region diversity. In the present study, we have extended our idiotypic analysis of the human Hib PS–specific antibody repertoire. Using a larger sample size, we examined the prevalence of CRI in naturally occurring and vaccine-induced anti-Hib PS antibodies, the relation between age of vaccination and CRI expression, and the association between CRI and IgG subclass.

Methods

Subjects and vaccines. Sera from 113 healthy children and adults who were immunized with one dose of Hib PS vaccine (n = 98), or a conjugate of Hib PS coupled to a *Neisseria meningitidis* group B outer membrane protein complex (Hib PS–OMP [15]) (n = 15) were available for this study. The serum samples were obtained immediately before immunization and 1–2 mo later and were stored at −70°C. The

1. *Abbreviations used in this paper:* A^0, absorbance; CRI, crossreactive idiotype; Hib PS, the capsular polysaccharide of *H. influenzae* b; Id, idiotype; IEF, isoelectric focusing; OMP, outer membrane protein; OOB, Office of Biologics; PS, polysaccharide; RABA, radioantigen binding assay.

principal criterion for inclusion of subjects in the present study was the availability of sufficient sera. Also, since we wanted to investigate CRI expression in vaccine-induced antibody, we selected subjects showing twofold or greater increases in anti-Hib PS antibody after vaccination (the only exceptions were five children, aged 24–27 mo, with > 1 μg/ml of anti-Hib PS antibody in postvaccination sera, but in whom prevaccination sera were unavailable). The age distributions of the subjects and the respective antibody responses to vaccination are summarized in Table I. With the exception of 10 of the adults, the antibody responses of these subjects were included in previous reports (16–19).

Group 1 consisted of 15 healthy, unrelated white infants, 2–17 mo of age, who were vaccinated with Hib PS–OMP conjugate vaccine (16). A single lot of vaccine was used (lot 1003/C/680; Merck Sharp & Dohme Research Laboratories, West Point, PA), and the dose contained 7 μg of Hib PS and 43 μg of OMP. Groups 2 and 3 consisted of 29 unrelated healthy children, age 1.7–2.5 and 3.9–6.3 yr, respectively, who were vaccinated with Hib PS. 23 were white, 4 were black, and 2 were Hispanic. 23 of the children in groups 2 and 3 were vaccinated intramuscularly with 5 μg of Hib PS prepared by Porter Anderson, University of Rochester, Rochester, NY. This dose is within the maximally immunogenic range (20). The remaining six children received 25 μg Hib PS s.c. prepared by Praxis Biologics (Rochester, NY). There were no qualitative or quantitative differences in the antibody responses of the children to the two vaccines, and therefore the data were combined (18). Group 4 consisted of 55 subjects, 13–57 yr of age. 47 of the subjects in this group were unrelated whites residing in Missouri or California. The remaining eight individuals were members of an isolated Amish community in Missouri previously described (21). Of the 55 subjects in group 4, the 47 non-Amish individuals received 25 μg Hib PS vaccine s.c. (Praxis Biologics), and the 8 Amish subjects received 5 μg Hib PS vaccine i.m. (Porter Anderson). 10 of the non-Amish subjects also received separate intramuscular injections of 23 valent pneumococcal PS vaccines (0.5 ml containing 25 μg of each of the PS's; Lederle Laboratories, Wayne, NJ), and quadrivalent meningococcal vaccine (0.5 ml containing 50 μg each of A, C, Y, and W135 PS's (Connaught Laboratories, Swiftwater, PA) given at different sites during the same visit (19). All eight Amish subjects also received a separate injection of bivalent meningococcal vaccine (0.2 ml containing 10 μg of A and C PS's; Connaught Laboratories). There were no significant differences in the anti-capsular antibody responses to Hib PS of the subjects in group 4 given different doses or different combinations of vaccines; therefore, the data were combined.

Table I. Summary of Antibody Responses to Vaccination

Subject group*	Age range (mean)	No. tested	Serum anti-Hib PS antibody (geometric mean)			
			Prevaccination	Postvaccination		
			Total‡	Total	IgG1§	IgG2
	yr			*μg/ml*		
1	0.2–1.4 (0.7)	15	0.16	4.8	1.5	0.2
2	1.7–2.5 (2.1)	16	0.24‖	4.4	0.9	0.3
3	3.9–6.3 (4.8)	13	1.00	22.0	5.7	1.7
4	13–57 (28)	69	2.24	52.5	7.7	5.3

* Group 1 received Hib PS–OMP conjugate vaccine; groups 2–4 received Hib PS vaccine. Subjects were included only if they showed twofold or more increases in total serum antibody after vaccination. The only exceptions were in group 2, where five subjects were included who had > 1 μg/ml of total antibody in postvaccination sera but in whom prevaccination sera were not available.

‡ Total antibody was measured by RABA.

§ IgG1 and IgG2 were measured by ELISA.

‖ Based on 11 of the 16 subjects in this group.

Antibody assays. Total antibody to Hib PS was measured using a radioactive antigen binding assay (RABA) with ^{125}I-labeled antigen (17). The antibody concentrations, in micrograms/milliliter, were determined from a standard curve using dilutions of the U. S. Office of Biologics (OOB) serum reference pool estimated to contain 80 μg/ml of total antibody in undiluted serum.

ELISAs were used to measure IgG1 and IgG2 antibodies to Hib PS. IgG3 and IgG4 antibody responses were not measured because previous studies have shown little or no IgG3 or IgG4 responses to Hib PS (9). For measurement of IgG1 and IgG2 antibody concentrations, microtiter plates were coated with Hib PS conjugated to poly-L-lysine, test sera were titrated in the plate, and binding of subclass-specific antibody was detected with biotinylated conjugates of secondary antibodies as previously described (8). The anti-IgG1 reagent was murine monoclonal HG11. To detect IgG2 antibodies, we used a mixture of two monoclonal antibodies (HP6014 and HP6008 from ICN, Immunobiologics [Lyle, IL]) to achieve optimal sensitivity while minimizing the λ light chain preference of MAb HP6014. The assays for IgG1 and IgG2 anti-Hib PS antibodies were quantitated in micrograms/milliliter using the OOB reference serum pool and affinity-purified IgG1 and IgG2 fractions prepared from a postvaccination serum pool as standard (8). As previously described (8), the subclass assays were highly specific and sensitive. The interassay coefficient of variation ranged from 7 to 14%.

The light chain composition of Hib PS antibodies was determined using the above described ELISA, except that goat anti-kappa and -lambda specific alkaline phosphate conjugates (Tago Inc., Burlingame, CA) were used as detecting reagents. Units of kappa and lambda anti-Hib PS activity were calculated from a standard curve obtained with the OOB reference serum, which we assigned 100 and 10 U/ml of kappa and lambda anti-Hib PS, respectively.

Purification of anti-Hib PS antibodies and production of goat anti-idiotype (Id) serum. Hib PS-specific antibodies were isolated from the serum of an adult, white male (subject 20090) who had been immunized subcutaneously 1 mo previously with 50 μg of Hib PS (Hib PS vaccine, lot 19; gift of Dr. Porter Anderson). The antibody purification method has been described (14). Briefly, a gamma globulin preparation obtained by precipitation with 50% saturated ammonium sulfate was passed over a column of Hib PS–amino-hexyl Sepharose 4B. After extensive washing, bound antibody was eluted with 3.5 M NaSCN, dialyzed against PBS, and concentrated by pressure filtration. A goat was immunized subcutaneously in multiple sites with 100 μg of pepsin-digested anti-Hib PS emulsified in complete Freund's adjuvant. After two further injections, spaced 2–3 wk apart (same dose and route except antigen was in incomplete adjuvant), the goat was bled and the gamma globulin fraction isolated using 50% saturated ammonium sulfate. This gamma globulin fraction, hereafter referred to as anti-Id, was sequentially absorbed with Sepharose to which had been coupled donor Ig depleted of Hib PS antibodies or pooled human Ig depleted of Hib PS antibodies and human myeloma proteins of the IgA, M, D, G1, G2, G3, and G4 isotypes. (Myeloma proteins were generously provided by Hans Spiegelberg, Research Institute of Scripps Clinic, La Jolla, CA.) Absorption was continued until there was little or no reactivity to these Igs as detectable by ELISA. The goat anti-Id and the goat preimmunization gamma globulin fraction were also absorbed with Hib PS–Sepharose to remove naturally occurring anti-Hib PS antibodies. Immunization of the goat with anti-Hib PS did not induce an antibody response to Hib PS. Before absorption the goat preimmune serum and the immune serum had the same levels of anti-Hib PS, 3 μg/ml. The anti-Id and the preimmune globulin fraction were adjusted to equivalent protein concentration based on absorbance at 280 nm.

Assay of CRI by anti-Id inhibition of antigen binding. CRI levels in sera were quantified by measuring the ability of anti-Id to inhibit Hib PS binding activity in either RABA or ELISA assays. In the RABA for CRI, 60 μl of a dilution of human serum equivalent to an anti-Hib PS antibody concentration of ~ 200 ng/ml (the diluent was 10% fetal calf serum in PBS throughout) was mixed with 60 μl of either 1:30 anti-Id or 1:30 goat preimmune globulin and incubated at 37°C for 2 h and

overnight at 4°C. This dilution of anti-Id was found to give maximal inhibition of Hib PS binding (see Results). Each sample was then divided into two 50-μl aliquots to which 50 μl of ^{125}I-Hib PS (20,000 cpm, sp act ~ 45 μCi/μg) was added. The samples were incubated for 4 h at 37°C and 1 h at 4°C. 100 μl of saturated ammonium sulfate was added with mixing, and after 2 h at 4°C the precipitates were harvested by centrifugation (16,000 *g*), washed once in 50% saturated ammonium sulfate, and then counted in a dry well gamma counter. Background counts per minute (i.e., ^{125}I-Hib PS bound in the presence of anti-Id or goat preimmune globulin alone), which was ≤ 5% of the total cpm added, were subtracted from the respective means of duplicate determinations. Inhibition was calculated as follows:

$$\% \text{ inhibition of Hib PS binding by anti-Id} = 100 \times \left(1 - \left[\frac{\text{mean cpm } ^{125}\text{I-Hib PS bound in presence of anti-Id}}{\text{mean cpm } ^{125}\text{I-Hib PS bound in presence of preimmune globulin}}\right]\right).$$

In the ELISA for CRI, 250 μl of a dilution of human serum (diluent was PBS, 0.5% BSA) was mixed with 250 μl of either anti-Id, goat preimmune globulin, diluent with 20 μg Hib PS/ml, or diluent alone. The tubes were incubated at 37°C for 3 h and overnight at 4°C. Samples were centrifuged (16,000 *g*) for 2 min and the supernatants assayed in duplicate for IgG1 or IgG2 specific anti-Hib PS antibody in ELISA as described above. Absorbance (A^0) developed in the presence of Hib PS was subtracted from A^0 values obtained in the test samples. Percent inhibition was calculated as indicated above except A^0 units were used instead of cpm. Anti-Id and preimmune globulin were used at a final dilution of 1:100. Human sera were tested at a final dilution that generated an A^0 of ~ 1 in the absence of inhibitor. Samples having A^0 values (after subtraction of background) of < 0.5 at final serum dilutions of 1:50 were excluded because they gave high nonspecific background values when tested at lower dilutions.

To measure CRI expression by antibodies to meningococcal A PS or pneumococcal PS types 3, 14, and 23, or to diphtheria toxoid, essentially the same method as described above for the Hib PS ELISA was used, except sera were tested on microtiter wells coated with diphtheria toxoid or with poly-L-lysine conjugated PS's. In the pneumococcal assays all sera were first absorbed with a capsular deficient strain of *Streptococcus pneumoniae* (SCS-2, clone 2, from John Robbins, National Institutes of Health, Bethesda, MD) to remove antibodies to pneumococcal C PS (22).

Preparation of Hib PS oligomer. Purified Hib PS (12 mg) was hydrolyzed in 0.01 N sulfuric acid for 6 min at 100°C as previously described (23). The neutralized hydrolysate was passed through a column of G-25sf Sephadex (1.5 × 100 cm) equilibrated with 0.1 M ammonium acetate, pH 7.0. 2-ml fractions were screened for reducing activity using a modification of the phenol-sulfuric acid reaction (24). Five peaks of activity were found, V_e/V_0 = 1.00, 1.06, 1.15, 1.28, and 1.49, as described by Hetherington (23), and the peaks having a V_e/V_0 of 1–1.15 were pooled, lyophilized, and solubilized in water. Analysis of this preparation for ribose using the Dische modification of the orcinol reaction (25), and for reducing sugar using the Park and Johnson ferricyanide method (25), indicated an average oligosaccharide size of eight repeat units (i.e., total ribose/reducing ribose = 8). The concentration of Hib PS oligomer was calculated assuming a mole fraction of ribose/Hib PS monomer of 0.44.

Hib PS inhibition of anti-Id → Id. To determine whether the binding of Hib PS to anti-Hib PS would inhibit the subsequent binding of anti-Id, the following test system was used. Anti-Hib PS antibody (> 80% CRI as determined by anti-Id inhibition of RABA) was purified from the post-Hib PS vaccination serum of an adult unrelated to the Id donor, and labeled with ^{125}I using chloramine-t to a sp act of 10 μCi/μg. A fixed amount of ^{125}I-anti-Hib PS was mixed with varying amounts of Hib PS, Hib PS oligomer, or BSA-PBS. After 4 h at 37°C the mixtures were transferred to wells of flexible, polyvinyl chloride microtiter plates that had been previously coated overnight with affinity-purified anti-Id (0.45 μg/ml PBS) and blocked with BSA-PBS. Anti-Id antibodies were purified from the anti-Id serum using Sepharose coupled with anti-Hib PS isolated from serum of an adult who was unrelated to the Id donor and who was vaccinated with Hib PS. The final input of ^{125}I-anti-Hib PS/well was 1.5 × 10^5 cpm in a vol of 0.1 ml. The plates were incubated overnight at room temperature, then washed 5 times with PBS-Tween. The wells were excised and counted in a gamma counter. In the absence of inhibitor (i.e., in the presence of BSA-PBS) ~ 10,000 cpm of ^{125}I-anti-Hib PS were bound. Percent inhibition by Hib PS was calculated as follows:

$$100 \times \left(1 - \left[\frac{\text{mean cpm bound in the presence of Hib PS or oligomer}}{\text{mean cpm bound in the presence of BSA-PBS}}\right]\right).$$

IEF and Western blotting. Serum samples (~ 5 μl) were focused in 0.8-mm polyacrylamide gels at 5°C using a horizontal electrofocusing unit (Bio-Rad Laboratories, Richmond, CA) equipped with a circulating water bath. The gels (100 × 125 mm) that consisted of 5% acrylamide, 0.15% bisacrylamide, 2% pH 3.5–9.5 ampholytes (Pharmacia Fine Chemicals, Piscataway, NJ) and 5% glycerol, were focused for 2 h at 7.5 W and 1 h at 1,800 V. The electrolytes were 1 N phosphoric acid and 1 N sodium hydroxide. The focused samples were electrically blotted onto nitrocellulose in 0.7% acetic acid. Protein was detected using amido black. For visualizing Hib PS antibodies, the blot was blocked in 1% BSA-PBS, reacted with ^{125}I-Hib PS (5 × 10^5 cpm/ml), washed with PBS-0.1% Tween 20, and then exposed to x-ray film with intensifying screens. To detect material reacting with anti-Id, the blot was blocked as described above, incubated with anti-Id (1:3,000), washed in PBS-Tween, and incubated with swine anti-goat Ig coupled with alkaline phosphatase (1:2,000, Caltag Laboratories, South San Francisco, CA). After washing with PBS-Tween, the blot was incubated for ~ 30 min with substrate solution: 15 mg 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt and 30 mg *p*-nitroblue tetrazolium chloride (Bio-Rad Laboratories) in 100 ml 0.1 M $NaHCO_3$, 1 mM $MgCl_2$, pH 9.8.

Results

Anti-Id reacts specifically with anti-Hib PS antibodies. A goat anti-Id serum was prepared against affinity-purified anti-Hib PS antibodies from an immunized adult. After exhaustive absorption as described in Methods, the specificity of the reagent was evaluated. The anti-Id reacted with a predominant anti-Hib PS clonotype present in the donor's postvaccination serum as determined by Western blot analysis of an IEF gel (Fig. 1). The anti-Id showed no reactivity with the donor postvaccination serum that had been absorbed with Hib PS–Sepharose to remove anti-Hib PS antibodies (Fig. 1). Similarly the anti-Id did not react with pooled Ig depleted of anti-Hib PS antibodies (data not shown). Using ELISA, there was also no anti-Id reactivity with myeloma proteins representative of the IgA, M, D, G1, G2, G3, or G4 isotypes, or with pooled Ig absorbed with Hib PS–Sepharose (data not shown).

The ability of the anti-Id to inhibit Hib PS–binding in postvaccination sera was determined by RABA and ELISA (Fig. 2). In the RABA, the anti-Id gave dose-dependent inhibition of the Id donor's anti-Hib PS antibodies and the serum anti-Hib PS antibodies of another Hib PS–immunized adult who was unrelated to the Id donor. Thus, this second subject shares a predominant idiotype that is crossreactive with the donor's idiotype. In contrast, no inhibition was observed with the serum anti-Hib PS antibodies from a third, unrelated immunized adult (Fig. 2). Using a subclass-specific ELISA, we observed similar dose-dependent anti-Id inhibition patterns of IgG1 and IgG2 anti-Hib PS antibodies from the respective three sera (Fig. 2). In subsequent experiments, for the RABA, the anti-Id was used at a 1:60 final dilution during the incuba-

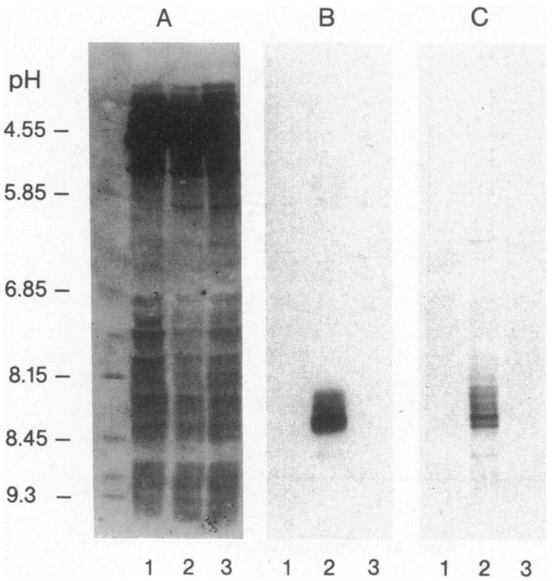

Figure 1. Western blot of an IEF gel of Id donor serum. Prevaccination serum (lane *1*), postvaccination serum (lane *2*), and postvaccination serum absorbed with Hib PS–Sepharose (lane *3*). 5 μl of serum were applied to each lane. *A*, Total protein visualized with amido black; *B*, anti-Hib PS antibody visualized with ^{125}I-Hib PS autoradiography; *C*, anti-Id–reactive material visualized with goat anti-Id and anti-goat Ig alkaline phosphatase.

tion with test sera and before addition of ^{125}I-Hib PS. For the ELISA, the anti-Id was used at a 1:100 final dilution during the incubation step with the test sera before addition to PS-coated microtiter wells.

Anti-Id inhibition of antigen binding was specific for Hib PS. Postvaccination sera from 14 adults and 11 children immunized with Hib PS conjugated to diphtheria toxoid (Prohibit vaccine; Connaught Laboratories) and from 19 adults immunized with meningococcal or pneumococcal PS vaccines, were assayed for CRI using ELISAs that measured total serum antibodies to diphtheria toxoid, IgG antibodies to pneumococcal types 3, 14, and 23 PS's, or IgG1 or IgG2 antibodies to meningococcal A PS. As shown in Fig. 3, no significant anti-Id inhibition of antigen binding was observed with any of these serum antibodies, whereas some of these same sera had high levels of anti-Id inhibitable anti-Hib PS antibody. Since the values of the percent inhibition of pneumococcal, meningococcal, and diphtheria toxoid antibodies by anti-Id centered around zero and ranged from −12 to 11% (mean percent inhibition±SD = 0.86±5), we chose a value of > 20% inhibition by anti-Id (i.e., ~ 4 SD) as the criterion for CRI positivity.

Serum CRI expression increases after vaccination with Hib PS and is predominant in most vaccinated adults. As a measure of CRI expression, we examined the ability of anti-Id to inhibit Hib PS–binding in pre- and post–Hib PS vaccination sera of adults unrelated to the donor. Using the RABA we tested pre- and postvaccination sera from 68 subjects, 13–57 yr of age (group 4, Table I). 39 of the 68 subjects (57%) showed significant CRI expression in prevaccination sera (i.e., > 20% anti-Id inhibition of Hib PS-binding). The median percent anti-Id inhibition in this group was 52.5%. In these 39 subjects there was no correlation between percent inhibition by anti-Id

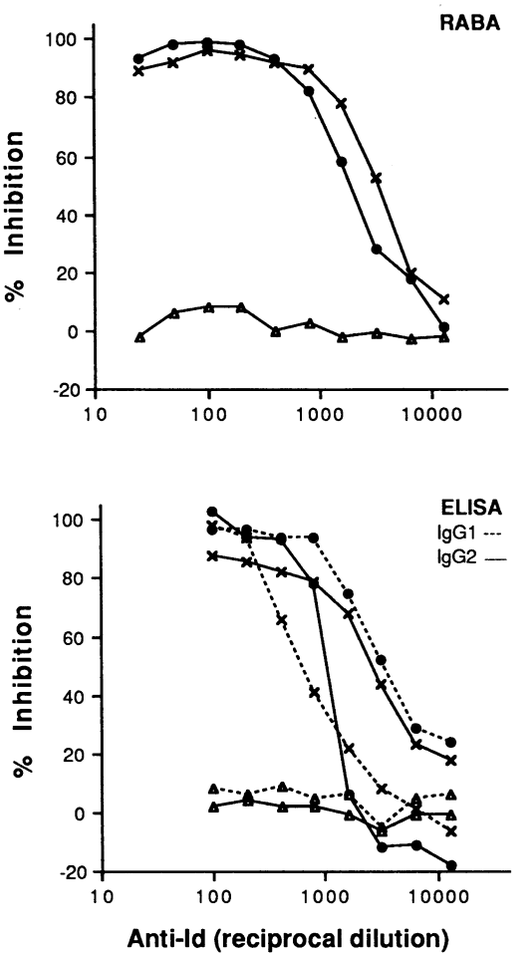

Figure 2. Anti-Id inhibition of Hib PS–binding in serum from adults immunized with Hib PS. ×, Id donor; •, subject 6053; △, subject 6213.

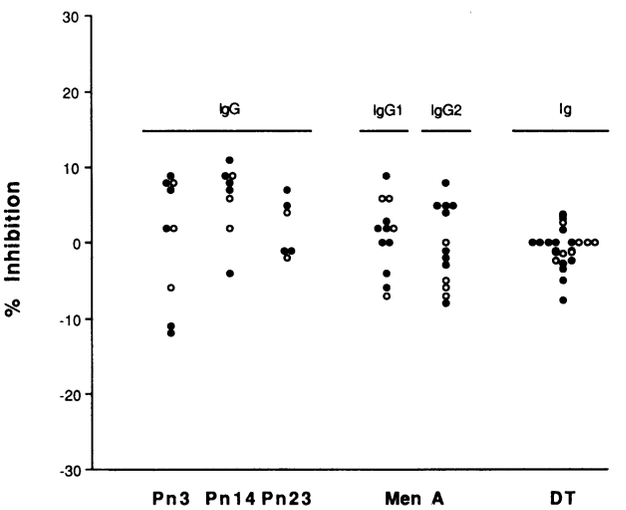

Figure 3. Failure of anti-Id to inhibit vaccine-induced antibody binding to pneumococcal types 3, 14, and 23, and meningococcal A PS's (*Men A*) and diphtheria toxoid (*DT*) in ELISA assays. Sera were tested at dilutions giving A^0 values of ~ 1 after subtraction of background. Subjects were immunized with Hib PS and meningococcal A or pneumococcal 23 valent vaccines, or with Hib PS–diphtheria toxoid conjugate. •, Subjects with > 50%; ○, subjects with < 50% of Hib PS–binding inhibitable by anti-Id.

Table II. Change in CRI Expression after Vaccination of Adults with Hib PS

Prevaccination serum CRI*	No. tested‡	Postvaccination serum: No. with CRI (%)	Postvaccination serum: Median % anti-Id inhibition (range)§
Absent	29	18 (62)	71 (22–94)
Present‖	39	34 (87)	85 (28–100)
Probability		0.02 (chi square)	NS (Mann-Whitney U test)

* Measured by RABA: CRI was considered absent when anti-Id inhibition of Hib PS–binding was ≤ 20%, and was considered present when inhibition was > 20%.

‡ CRI was measured in prevaccination sera from 68 of the 69 adults in group 4.

§ Of subjects expressing CRI.

‖ Median percent anti-Id inhibition in prevaccination sera was 52.5% (range, 21–98)

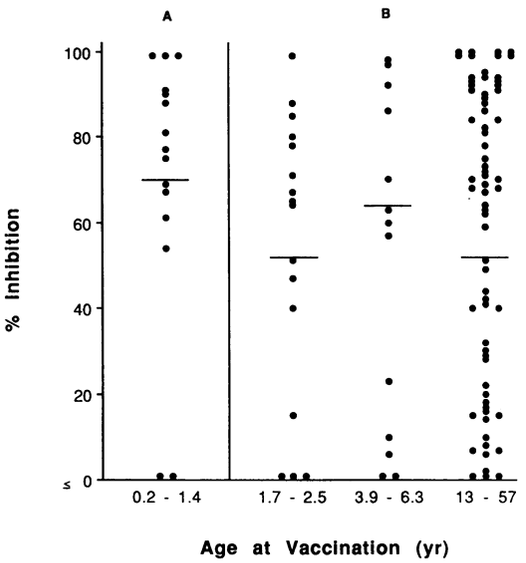

Figure 4. CRI expression after vaccination. Each circle represents the percent anti-Id inhibition of Hib PS–binding of an individual serum as measured by RABA. *A*, Infants vaccinated with Hib PS–OMP conjugate; *B*, older children and adults vaccinated with Hib PS. No significant differences between the groups given Hib PS ($P > 0.3$ by analysis of variance). Mean percent of inhibition of each group indicated by horizontal line.

and the log of the anti–Hib PS antibody concentration ($r = -0.21$, $P > 0.2$). Of the 29 subjects who did not have significant CRI in prevaccination sera, 18 became positive after vaccination with Hib PS. The median percent inhibition of the postvaccination sera in these 18 subjects was 71%. 34 of the 39 subjects with CRI in prevaccination sera also had CRI detected in their postvaccination sera. The median percent anti-Id inhibition of these 34 individuals increased from 52.5% before vaccination to 85% after vaccination ($P = 0.001$, Mann-Whitney test). The one adult whose prevaccination serum was not available for testing for CRI also had a high level of CRI detected in postvaccination serum (100% inhibition). Thus, a total of 53 of 69 adults (77%) expressed significant CRI after vaccination with Hib PS. Among these 53 subjects there was no significant correlation between the percent inhibition by anti-Id and the log of the anti–Hib PS antibody concentration in postvaccination sera ($r = -0.02$; $P = 0.90$).

CRI expression is not related to age at vaccination. Fig. 4 shows the distribution of CRI in postvaccination sera of children and adults immunized with Hib PS at different ages (Fig. 4 *B*). There were no significant differences in the distribution of CRI in the three age groups. The means of the percent inhibition by anti-Id were 52.5, 63.8, and 57.1, respectively, for groups 2, 3, and 4 ($P > 0.3$ by analysis of variance). CRI also predominated in the serum anti–Hib PS response of 2–17-mo-old infants immunized with Hib PS–OMP conjugate vaccine: 13 of 15 vaccinated infants had > 50% of their anti–Hib PS antibody inhibitable by anti-Id (Fig. 4 *A*).

CRI is expressed in both IgG1 and IgG2 anti-Hib PS antibodies. 72 of the 98 children and adults immunized with Hib PS developed both IgG1 and IgG2 antibody responses of sufficient magnitude to measure subclass-specific CRI by ELISA inhibition. Fig. 5 shows the relationship between IgG1 and IgG2 CRI expression in individual sera. 51 of the 72 subjects (71%) had detectable CRI in both IgG1 and IgG2 antibody (i.e., > 20% inhibition of Hib PS–binding by anti-Id). In 7 subjects (10%) CRI was not detected in either subclass. Of the remaining 14 subjects CRI was detected exclusively in IgG1 in 10 or exclusively in IgG2 in 4.

Correlation of CRI detected by RABA and ELISA. There were 55 subjects immunized with Hib PS who had CRI detected by RABA and who made both IgG1 and IgG2 antibody responses. In every instance, CRI as measured in ELISA was detected in this group in IgG1, IgG2, or in both subclasses. In contrast, of 20 subjects who were typed CRI-negative by RABA, 9 had > 50% of either IgG1 or IgG2 anti–Hib PS anti-

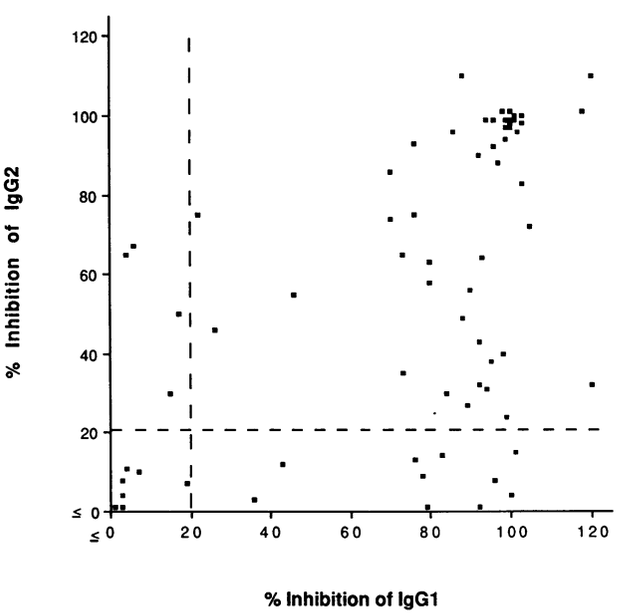

Figure 5. CRI expression by IgG1 and IgG2 anti–Hib PS antibodies in individual postvaccination sera from subjects vaccinated with Hib PS. Sera were selected based on having IgG1 (≥ 1 μg/ml) and IgG2 (≥ 0.6 μg/ml) anti–Hib PS antibody (72 of 98 of the subjects with a mean±SD age of 23.8±11.8 yr; range, 1.8–52).

body inhibitable by anti-Id in ELISA. In these nine subjects, where CRI was detected by ELISA and not by RABA, the CRI-positive subclass represented a minor isotypic component of the total anti-Hib PS antibody response. For example, the post-Hib PS vaccination serum of one individual (subject 20091) had a total anti-Hib PS level of 51 μg/ml. This subject was typed as CRI negative, since using the RABA only 7% of the serum anti-Hib PS binding activity was inhibitable by anti-Id. However, using the IgG subclass-specific ELISA, this serum, which had 3.5 and 13 μg/ml of IgG1 and IgG2 anti-Hib PS, respectively, had 78% of the IgG1 Hib PS-binding activity and 9% of the IgG2 Hib PS-binding activity inhibited by anti-Id. Thus, in contrast to the RABA result, this subject would be considered CRI positive with respect to his IgG1 anti-Hib PS population. Therefore, the estimates of CRI positivity based on RABA anti-Id inhibition should be considered minimal estimates.

Comparison between anti-Hib PS antibodies of CRI-negative and -positive subjects. In a systematic analysis we compared the pre- and post-Hib PS vaccination antibody concentrations, and the isotype concentrations of anti-Hib PS antibodies in CRI-negative adults ($n = 5$) and CRI-positive adults ($n = 16$). For purposes of this analysis, we excluded individuals with intermediate or borderline CRI expression. CRI-negative subjects were defined based on those with < 15% CRI in prevaccination sera and < 15% anti-Id inhibition of their total, IgG1, and IgG2 Hib PS-binding activity in post-Hib PS vaccination sera. Subjects were considered CRI positive if they had > 85% of their post-Hib PS vaccination serum Hib PS-binding activity inhibitable by anti-Id in RABA. The CRI-negative subjects had higher preimmunization anti-Hib PS antibody levels (geometric means: 7.24 vs. 1.12 μg/ml, $P < 0.001$ by t test), but the difference in the geometric mean postvaccination antibody concentrations was not statistically significant ($P = 0.14$). There were no significant differences in the respective geometric mean IgG1, IgG2, IgA, and kappa antibody concentrations in postimmunization sera ($P \geq 0.2$). However, CRI-negative adults had higher lambda antibody responses than CRI-positive subjects (geometric means: 66.1 vs. 6.6 U/ml, $P < 0.001$). The corresponding geometric mean kappa/lambda antibody ratios were 0.58 and 5.0 ($P = 0.03$). A similar comparison was done with the respective antibody concentrations of six CRI-positive and six CRI-negative children whose CRI status was defined as in the adults. The children had been immunized with Hib PS at 2–6 yr of age. In contrast to the adults, no statistically significant differences in the respective antibody concentrations were found ($P \geq 0.2$ for each of the comparisons).

Absorption of anti-Id with heterologous anti-Hib PS. To investigate further the shared nature of the anti-Hib PS associated Ids, we absorbed the anti-Id serum with either of two Sepharose preparations that had been coupled with Hib PS antibodies isolated from sera of two adults immunized with Hib PS (a1, subject 20966; a2, subject 21357). These two adults had ≥ 80% of their Hib PS-binding activity inhibitable by anti-Id and were unrelated to the Id donor. Absorption of the anti-Id with anti-Hib PS antibodies from either of the two subjects completely abrogated the ability of the anti-Id to inhibit Hib PS-binding of Id donor antibodies as well as the Hib PS-binding of antibodies in postvaccination sera of six adults and six children immunized with Hib PS, and six infants immunized with Hib PS-OMP (Table III). All of these individuals were unrelated to the Id donor and to the two subjects whose antibodies were used for the absorption.

Hib PS-binding to anti-Hib PS partially inhibits the reaction between anti-Id and CRI. To determine whether occupancy of the binding site with Hib PS would affect the subsequent recognition of CRI determinants by anti-Id we incubated heterologous, ^{125}I-labeled anti-Hib PS antibody (CRI) with varying concentrations of native Hib PS or with a Hib PS oligomer having an average of eight repeat units. These mixtures were then transferred to microtiter wells coated with affinity-purified anti-Id. The inhibition of ^{125}I-CRI binding to anti-Id was determined as described in Methods. Fig. 6 shows that 60 and 35% inhibition of the binding of CRI to anti-Id was obtained with native Hib PS and the Hib PS oligomer, respectively, at concentrations of ~ 100 μg/ml. An approximately 30-fold lower concentration of native Hib PS was required to achieve the same degree of inhibition as the oligomer.

Anti-Id does not inhibit Hib PS-binding of rabbit or burro antibodies. Two individual burro and four individual rabbit antisera, obtained from animals immunized with Hib organisms or Hib PS coupled to diphtheria toxoid and having high concentrations of anti-Hib PS, were tested in the RABA to determine whether the anti-Id would inhibit Hib PS binding. No inhibition of Hib PS binding was observed with either the burro antibodies (4.3 and −3.2%) or the rabbit antibodies (0.3, −4, −2, and 1.5%).

Discussion

We have confirmed and extended our earlier observations using a murine polyclonal anti-Id, which showed that human

Table III. Measurement of CRI Using Anti-Id Absorbed with Heterologous Anti-Hib PS

	Anti-Id inhibition of Hib PS-binding in serum from subject*																				
Anti-Id absorbed with:	Id	a1	a2	a3	a4	a5	a6	a7	a8	c1	c2	c3	c4	c5	c6	i1	i2	i3	i4	i5	i6
	%																				
0	95	93	78	98	93	52	73	98	58	79	68	85	86	99	59	78	58	84	97	99	87
a1 anti-Hib PS	5	5	0	4	0	0	0	0	0	0	0	0	0	5	11	2	0	6	17	0	0
a2 anti-Hib PS	0	3	0	8	0	1	0	0	0	0	0	0	0	5	8	0	0	0	6	12	1

CRI was determined by measuring anti-Id (1:60 final dilution) inhibition of Hib PS-binding in RABA as described in Methods. * Subjects: *Id*, Id donor; *a1-8*, adults immunized with Hib PS; *c1-6*, children (1.7–2.5 yr of age) immunized with Hib PS; *i1-6*, infants (0.2–1.4 yr of age) immunized with Hib PS-OMP.

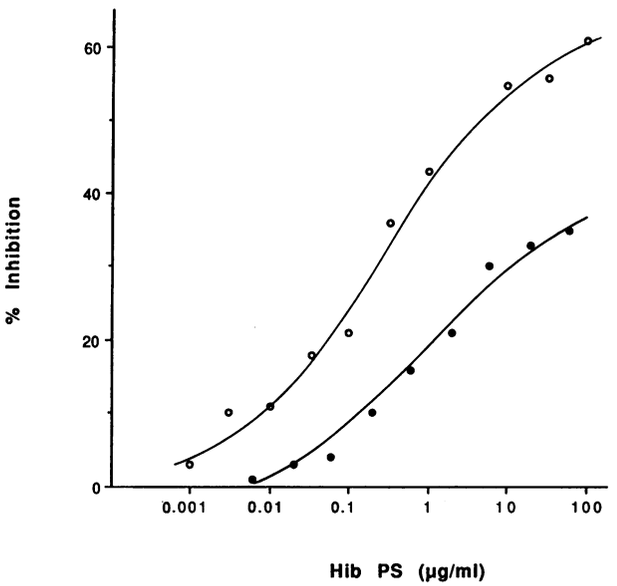

Figure 6. Inhibition of binding of anti-Id to CRI by native Hib PS (○) or Hib PS oligomer (●). ^{125}I-Anti-Hib PS (CRI) was incubated with Hib PS or Hib PS oligomer and then added to anti-Id–coated microtiter wells. Inhibition of ^{125}I-CRI binding to anti-Id was determined as described in Methods.

antibodies to Hib PS express CRI (14). In the present study a specific goat anti-Id reagent was prepared against the vaccine-induced serum anti–Hib PS antibodies of an adult subject. This individual was chosen as the Id donor since his anti–Hib PS antibody population consisted of a predominant IEF clonotype, indicative of pauciclonality, and since in our previous study using a murine polyclonal anti-Id this individual was found to express a Hib PS–specific idiotype that was shared with four other unrelated adults. The ability of this anti-Id reagent to inhibit specifically heterologous Hib PS–binding, presumably by interacting with Ids in or near the antigen combining site, provided a convenient means to measure the expression of CRI and to estimate the minimum extent to which CRI predominated in the Hib PS–specific antibody population of individual sera.

The most striking findings of this study were that the anti–Hib PS associated CRI was both highly prevalent and predominant. Approximately 75% of subjects expressed significant CRI in their vaccine-induced anti–Hib PS antibodies. Moreover, among this group CRI-positive antibodies dominated the vaccine-induced population since the median inhibition of Hib PS binding by anti-Id was ~ 80% (Table II). Drawing from the extensive studies of other CRI systems (26–28), we suggest that the CRI defined by our anti-Id reagent is serving as a serological marker for a family of structurally related V domains. The high prevalence of the CRI in an outbred population indicates a lack of genetic restriction and suggests that the genetic elements encoding the CRI are prevalent in the human germ line. The exceptionally high predominance of the CRI among vaccine-induced antibodies indicates restricted V region usage and is consistent with previous reports showing a limited number of similar, isoelectrically resolvable anti–Hib PS clonotypes in unrelated individuals (12, 13, 29). Further structural analyses will be required to define precisely anti–Hib PS V region diversity. However, it appears from our data and the aforementioned IEF studies that the human anti–Hib PS antibody repertoire that is induced by vaccination derives from only a few distinct B cell clones, which more often than not express conserved, germ line–encoded V_H and/or V_L elements.

Some of the CRI determinants are probably in or very near the combining site since occupancy of the site by either native Hib PS or Hib PS oligomer partially inhibited the recognition of CRI by the anti-Id (Fig. 6). However, the anti-Id reagent is a polyclonal serum and therefore it may react with several Ids associated with Hib PS antibodies. Despite this caveat, absorption of the anti-Id with anti–Hib PS antibodies from either of two individuals unrelated to the Id donor removed all of the anti-Id activity when tested on postvaccination sera of 18 individuals (Table III). These data indicate extensive sharing of Ids among unrelated individuals.

Further evidence that human anti–Hib PS antibodies use a limited V region repertoire is provided by a recent sequence analysis of clonotypically pure anti–Hib PS antibodies (30). These authors showed that all H chain sequences were of the V_HIII subgroup, whereas the light chains were V_KI, V_KII, V_KIII, and V_λ. Interestingly, 14 of the 21 Hib PS antibodies were V_KII, suggesting that a single V_k chain may dominate the antibody response to Hib PS. These findings taken with our observation that CRI-negative subjects had significantly higher lambda anti–Hib PS responses than did CRI-positive subjects, suggest that the anti-Id may recognize a particular V_k Id. However, we have preliminary evidence that both kappa and lambda anti–Hib PS antibodies can express CRI since the anti-Id can inhibit Hib PS binding in ELISA of both kappa and lambda anti–Hib PS. Thus, V_H-encoded Ids or combinatorial determinants may also contribute to CRI expression. An alternative interpretation of the observed high frequency of Id crossreactivity among unrelated individuals is that the anti-Id reagent is behaving as a so-called internal image of Hib PS. While this possibility cannot as yet be ruled out, we think it is unlikely since the anti-Id does not inhibit binding of rabbit or burro antibodies to Hib PS.

Notable differences were observed between the CRI expression of pre- vs. postvaccination anti–Hib PS antibody populations in that the number of CRI-positive subjects as well as the predominance of the CRI increased significantly after vaccination with Hib PS (Table II). Thus, immunization apparently selects for CRI-positive subsets inasmuch as the levels of serum antibody reflect the quantity of clonal amplification. One possible explanation that might account for this shift toward CRI predominance after immunization is that CRI-positive B cells have higher affinity for Hib PS than do CRI-negative B cells and thus are preferentially selected and expanded. Recently it has been shown that immunization of adults with Hib PS generally results in an increase in antibody avidity (31). An interesting group of subjects were the five adults who were negative for CRI in both pre- and postimmunization sera. This group had nearly 10-fold higher antibody levels before immunization than did the 16 adults with > 85% CRI in their postimmunization sera. This finding suggests that before vaccination this CRI-negative group had been exposed to either Hib or, more likely, to PS crossreactive with Hib PS. Thus, natural exposure, presumably via the mucosal route, may selectively stimulate CRI-negative B cell clones. This hypothesis is consistent with our preliminary analysis of sera from two adults

immunized with Hib PS crossreactive *Escherichia coli* K 100 PS (gift of Dr. Richard Insel, University of Rochester, Rochester NY), which showed no detectable CRI.

Analysis of the relation between CRI and IgG subclass showed that the majority of individuals who responded to Hib PS vaccination with IgG1 and IgG2 antibodies express CRI in both subclasses. This finding is consistent with the same idiotype/V region being associated with different C_H regions (32, 33). However, a small but significant number of subjects did express CRI exclusively in either the IgG1 or IgG2 subclasses, which raises the possibility that the IgG1 and IgG2 isotype responses to Hib PS in these individuals are derived from separate B cell lineages that differ in their potential for isotype expression. Evidence that this phenomenon occurs in humans has been described in the response to Hib PS (29) and phosphorylcholine where antibodies of distinctive fine specificity are frequently confined to a particular subclass (34).

Another important finding reported here is that the anti-Hib PS CRI is expressed independently of age. Vaccine-induced antibodies of children and adults did not differ with respect to either the degree of CRI penetrance or predominance. Perhaps even more significant was the observation that 15 of 17 infants immunized with Hib PS conjugated to *Neisseria* outer membrane protein responded with high levels of CRI-positive anti-Hib PS. Therefore, at least among vaccine responders, CRI dominance of the anti-Hib PS repertoire is established relatively early in ontogeny. One might have predicted that immunization with this so-called T-dependent form of Hib PS would have resulted in a more heterogeneous idiotypic response by recruiting B cells not normally activated by the T-independent form of the antigen. On the contrary, however, the anti-Hib PS populations induced in infants by immunization with the T-dependent Form of Hib PS may have been more idiotypically restricted than the responses of older children and adults immunized with Hib PS vaccine (Fig. 4). Responsiveness to Hib PS, as with other purified capsular PS's, is known to be age dependent (20, 35). The mechanisms responsible for this phenomenon are unknown, but absence of specific B cells cannot be the explanation since infants, who are generally unresponsive to free Hib PS, do indeed produce antibody after immunization with Hib PS conjugated to carrier proteins (7). Our findings show that the CRI, which presumably reflects a similar dominant V domain(s), is expressed in both infants and adults. This result indicates that unresponsiveness of infants to Hib PS cannot be accounted for by an inability to express this particular V region(s).

Several features of the anti-Hib PS CRI system such as clonal dominance, apparent germ line encoding, and presence as natural antibodies evoked by environmental antigens, are similar to murine CRI systems, which are thought to function via regulatory idiotypes (36–39). It will be interesting to determine whether idiotypic interactions play a role in the ontogeny and/or regulation of the anti-Hib PS CRI. Further analysis of the anti-Hib PS CRI should provide insight into the inheritance and diversity of the human V region repertoire.

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