INFECTION AND IMMUNITY, July 1995, p. 2467–2472 Vol. 63, No. 7
0019-9567/95/$04.00+0
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Cellular Immunity to the P6 Outer Membrane Protein of Nontypeable *Haemophilus influenzae*

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Received 26 September 1994/Returned for modification 19 December 1994/Accepted 11 April 1995

Cellular immunity to nontypeable *Haemophilus influenzae* in a population of 10 healthy, immune adults was determined by measuring lymphocyte blast transformation and antibody secretion in response to the P6 outer membrane protein. P6 (200 μl/ml) induced lymphocyte blast transformation that peaked on day 10 of incubation. The peak induction of antibody-secreting cells occurred on day 8 of incubation. In comparison with the response to tetanus toxoid stimulation, the peak lymphocyte blast transformation response to P6 was reduced (mean counts per minute ± standard error of the mean [SEM], 3,457 ± 503 versus 9,414 ± 1,464; $P = 0.0051$) and delayed (mean days ± SEM, 10.3 ± 0.4 versus 8.4 ± 0.5; $P = 0.0169$); however, P6 was a better stimulus of antibody secretion from lymphocytes, particularly antibody of the immunoglobulin M (IgM) class (mean peak numbers of antibody-secreting cells per 10^5 peripheral blood mononuclear cells ± SEM: IgG, 85 ± 29 versus 42 ± 16 [$P = 0.0469$]; IgM, 81 ± 20 versus 25 ± 7 [$P = 0.0125$]; IgA, 24 ± 8 versus 16 ± 6 [$P = 0.0526$]). Thus, lymphocytes from immune individuals recognize P6 of nontypeable *H. influenzae* as an immunogen. These data provide a basis for future studies with otitis-prone children who fail to develop a normal antibody response to P6 antigen (N. Yamanaka and H. Faden, J. Pediatr. 122:212–218, 1993).

A significant proportion of young children are subject to recurrent episodes of otitis media (5, 7, 37). At least 46% of children have three or more episodes by 3 years of age, and up to 16% have six or more episodes (37). In more than half of the infections, a bacterial pathogen can be identified, most commonly *Streptococcus pneumoniae*, nontypeable *H. influenzae*, or *Moraxella catarrhalis*. Nontypeable *H. influenzae* is responsible for 20 to 40% of identifiable cases of otitis media (4, 6, 22, 23, 36). Nontypeable *H. influenzae* is implicated also in sinusitis (9, 34, 40), neonatal sepsis (13, 41), and bronchitis (31).

Current vaccines against *H. influenzae* are effective against only strains possessing the type b capsular polysaccharide. However, these vaccines are of no value against nontypeable *H. influenzae*, because it lacks a polysaccharide capsule. In contrast, immunity to nontypeable *H. influenzae* is directed against surface antigens in the outer membrane, which is composed of proteins and lipopolysaccharide (12, 17, 26). Efforts to develop vaccines against nontypeable *H. influenzae* have focused on the protein antigens. The outer membrane of nontypeable *H. influenzae* contains five major proteins: P1 (47,000 Da), P2 (39,000 Da), P4 (30,000 Da), P5 (37,000 Da), and P6 (16,000 Da) (3, 16, 25, 30, 39). P1, P2, P4, and P5 are targets for bactericidal antibodies (2, 14, 15, 19, 25, 28). P1, P2, and P5 are heterogeneous (2, 19, 38, 39), while P4 and P6 are antigenically stable and highly conserved (15, 16, 18, 29, 30). Antibody to the P6 protein has been detected in sera, middle ear effusions, nasopharyngeal secretions, and breast milk (42, 43). The anti-P6 antibody in nasopharyngeal secretions and breast milk prevented colonization with nontypeable *H. influenzae* (20, 21).

Since immunity to P6 appears to be important in protection against disease due to nontypeable *H. influenzae*, the present study was designed to investigate the cell-mediated immune response to P6 in naturally immune adults. Responses to P6 were measured serologically as well as by measurement of lymphocyte blast transformation and antibody-secreting cells. The data suggest that adults immune to nontypeable *H. influenzae* possess lymphocytes which recognize P6 as an immunogen.

MATERIALS AND METHODS

Laboratory assays. Peripheral blood samples were collected from 10 healthy adult volunteers.

(i) Serum separation. Five to 10 ml of whole blood was drawn into syringes and centrifuged at 725 × *g* for 20 min at room temperature. Serum was removed and stored at −70°C for serologic tests.

(ii) Lymphocyte separation. Thirty to 60 ml of whole blood was drawn into syringes containing preservative-free heparin (20 U/ml of blood). The heparinized blood was layered over Histopaque (Sigma Chemical Company, St. Louis, Mo.) and centrifuged at 600 × *g* for 30 min at room temperature. Peripheral blood mononuclear cells were collected (0.5×10^6 to 2.0×10^6 cells per ml of blood), washed three times with Hanks' balanced salt solution, and counted. Cell viability was determined by trypan blue exclusion and was >90%.

Isolation of P6. A modification of the method of Munson and Granoff (27) was used to isolate P6 as described previously (20, 21, 42, 43). The relative insolubility of P6 in 1% sodium dodecyl sulfate (SDS) with 0.1 M Tris, 0.5 M NaCl, and 0.1% 2-mercaptoethanol (pH 8.0) (buffer B) was used to separate it from lipooligosaccharide and other outer membrane proteins. Nontypeable *H. influenzae* 1479 was grown on chocolate agar plates overnight at 37°C with 5% CO_2. Cells were collected and suspended in phosphate buffer solution (PBS). The cell suspension was centrifuged at 9,000 × *g* for 30 min at 4°C. The pellet was resuspended in buffer B, incubated at 37°C for 30 min, sonicated, and centrifuged at 21,000 × *g* for 30 min at room temperature. The pellet was resuspended in buffer B with RNase A (10 μg/ml) (Boehringer Mannheim, Indianapolis, Ind.), sonicated, incubated at 37°C for 30 min, and centrifuged. This procedure was repeated once. The pellet was next suspended in buffer B without RNase A, sonicated, incubated, and centrifuged two more times. The pellet was finally suspended in buffer A (0.01 M Tris and 0.15 M NaCl [pH 7.4]) and incubated at 65°C for 30 min. The insoluble material was removed by centrifugation at 100,000 × *g* for 60 min at 30°C. The supernatant was concentrated by pressure filtration using a PM 10 membrane (Amicon, Beverly, Mass.). The protein concentration in the supernatant was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). Coomassie blue-stained SDS-polyacrylamide gel electrophoresis of the purified protein demonstrated a single band with a molecular mass of 16,000 Da.

Mitogens and antigens. (i) PHA. A stock solution of 250 μg of phytohemagglutinin (PHA) (Sigma) per ml in complete RPMI 1640 (GIBCO, Grand Island,

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N.Y.) was prepared, filtered through a sterile 0.45-μm-pore-size filter (Millipore Corp., Marlborough, Mass.), and stored at −70°C.

(ii) PWM. A stock solution of 400 μg of pokeweed mitogen (PWM) (GIBCO) per ml in complete RPMI 1640 was prepared, filtered, and stored at −70°C.

(iii) TT. An undiluted stock solution of tetanus toxoid (TT USP; Connaught, Swiftwater, Pa.) was dialyzed in PBS for 12 h at 4°C and in Hanks' balanced salt solution for 12 h at 4°C by using dialysis tubing (SPECTRA/POR, 12-14,000 MW; Spectrum, Los Angeles, Calif.), filtered, and stored at −70°C.

(iv) P6. Prepared P6 solution was filtered and stored at −70°C.

Measurement of anti-P6 antibodies. P6-specific immunoglobulin G (IgG), IgM, and IgA antibody titers in serum were determined by an enzyme-linked immunosorbent assay (ELISA) (20, 21, 42, 43). Wells of flat-bottomed microplates (Immulon Type II; Dynatech Laboratories, Alexandria, Va.) were coated with purified P6 (3 μg/ml) in carbonate-bicarbonate buffer (pH 9.6; Sigma) and incubated at 4°C overnight. The material was then aspirated, and 3% gelatin in PBS was added. After standing for 60 min at 37°C, the wells were washed with PBS containing 0.05% polysorbate 20 (Tween 20) (PBS-T). Serum diluted 1:50 in PBS-T containing 0.5% gelatin (PBS-T-G) was added to these wells. After incubation for 2 h at 37°C, the wells were washed with PBS-T. The plates were then incubated sequentially with horseradish peroxidase-conjugated rabbit anti-human IgG, IgM, or IgA antibody (Dako Corp., Santa Barbara, Calif.) diluted in PBS-T-G, at 37°C for 60 min each. After each reaction, the plates were washed with PBS-T. The wells were then reacted with *o*-phenylenediamine (Sigma). After a 15-min incubation at room temperature, the reaction was stopped by adding 5 N sulfuric acid. The optical density of each well was measured with a spectrophotometer (Multiskan Plus, MK II; Labsystems, Helsinki, Finland) at 492 nm. All ELISAs were standardized by the method of Zollinger and Bolslego (44). Anti-P6 antibodies were expressed in absolute amounts of IgG, IgM, and IgA anti-P6 antibodies as micrograms per milliliter.

Measurement of anti-TT antibody. TT-specific IgG antibody titers in serum were determined by the method of Sedgwick et al. (33). TT refined concentrate (Wyeth Laboratories, Inc., Marietta, Pa.) was diluted to 1:1,000 in carbonate-bicarbonate buffer (pH 9.6), and 50 μl was dispensed into wells of a microtiter plate (Immulon 4, 96 flat-bottommed wells; Dynatech Laboratories, Inc., Chantilly, Va.). The plate was incubated for 1 h at 37°C. After being coated with toxoid, the microtiter plate was washed with PBS-T. Serial twofold dilutions (1:10 through 1:320) were prepared in PBS-T from both the human serum (HS) pool (prepared by combining aliquots of sera from healthy donors) and individual serum specimens. Fifty microliters each of the HS pool and serum specimen dilutions was dispensed into the wells. The plate was incubated for 30 min at 37°C and then washed with PBS-T. Fifty microliters of a 1:200 dilution of horseradish peroxidase-conjugated goat anti-human IgG antibody (Fc fragment; Cappel Cooper Biomedical, Inc., Malvern, Pa.) was added to all wells, and the plate was incubated for 30 min at 37°C and washed again. Substrate-chromogen working solution, consisting of 150 μl of ABTS [2,2′-azino-di-(*s*-ethylbenzthiazoline-6-sulfonate)] stock solution in citrate buffer (pH 4.0) with 3% hydrogen peroxide, was prepared, and 100 μl was dispensed into wells. After 4 to 5 min of incubation at room temperature, optical density at 414 nm was determined with an Automatic Titertek Multiskan ELISA plate reader (Flow Laboratories, Inc., McLean, Va.). Controls included wells without serum samples (background) and noncoated wells.

Dilutions of tetanus immune globulin (250 U/ml; Cutter Laboratories, Berkeley, Calif.) were prepared in PBS-T for various dilutions. Each of these globulin preparations was then treated as a single sample, and serial twofold dilutions were made from 1:10 through 1:320. The ELISA procedure was performed with these samples, and the results were expressed as a percentage of the HS pool internal control result.

Lymphocyte transformation assay. In vitro lymphocyte transformation assays were performed by the method of Ballow et al. (1). One hundred microliters of peripheral blood lymphocytes at a concentration of 5×10^5 cells per ml in RPMI 1640 with L-glutamine (GIBCO) supplemented with 2 g of $NaHCO_3$ per liter, 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 15% pooled HS was added to wells in a U-bottomed 96-well cell culture cluster (Costar, Cambridge, Mass.). Twenty microliters of PHA (50 μg/ml), PWM (50 μg/ml), TT (1:5 dilution), or P6 (50, 100, or 200 μg/ml) or RPMI 1640 alone was added. Control wells contained cells and medium alone. After incubation for 1 to 13 days at 37°C in 5% CO_2 humidified air, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; NEN Research Products, Boston, Mass.) per well for 18 to 24 h and harvested on glass fiber filter mats with a Mash II harvester (Cambridge Technology, Cambridge, Mass.). Dots of filter paper were placed in tubes (Snap Cap Bio-Vial, Beckman, Irvine, Calif.) with 3 ml of scintillation fluid (Ecoscient O; National Diagnostics, Atlanta, Ga.). Radioactivity ([^{3}H]thymidine incorporation) was determined by using a β liquid scintillation counter (model LS9000; Beckman). The results were expressed as counts per minute or as stimulation indices (SIs) calculated as (counts per minute of cells cultured with mitogens or antigens)/(counts per minute of control cells). Lymphocytes were collected from each individual and studied on one or two occasions. In each experiment, the test wells were set up in triplicate.

Measurement of total antibody-secreting cells. (i) Lymphocyte culture. Peripheral blood lymphocytes were suspended at a concentration of 10^6/ml in 4 ml of RPMI 1640 with L-glutamine supplemented with 2 g of $NaHCO_3$ per liter, 20 mM HEPES buffer, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 15% fetal calf serum (GIBCO). The cells were cultured with PWM (final concentration, 10 μg/ml), TT (final concentration, 2.35%), or P6 (final concentration, 10 μg/ml) and without mitogens or antigens as a control. After incubation for 6, 8, 10, or 14 days at 37°C in 5% CO_2 humidified air, the cell concentrations were determined and adjusted to 1×10^5 to 20×10^5 cells per ml.

(ii) ELISPOT. The ELISPOT assay was modified from the method of Czerkinsky et al. (8). Wells of nitrocellulose membrane plates (96-well MILLITITER hemagglutination plate; Millipore) were prewet with 100 μl of PBS and allow to stand for 3 min. After the PBS was discarded, the wells were coated with 100 μl of rabbit anti-human IgG (1:200), IgM (1:200), or IgA (1:200) antibody (Dako) in PBS and incubated overnight at 4°C. The wells were emptied, washed four times with PBS, immersed in PBS for 5 min, and exposed to 200 μl of PBS containing 3% nonfat cow's milk as a blocking solution for 20 min at 37°C in 5% CO_2 humidified air. After the blocking solution was discarded, lymphocyte suspensions containing 1×10^5 to 20×10^5 cells per ml in 100 μl of medium with 5% fetal calf serum were added to duplicate antibody-coated wells and cultured for 3 h at 37°C in 5% CO_2 humidified air. The cells were removed, and the wells were washed three times with PBS and three times with PBS-T and immersed in PBS-T for 5 min. The wells were treated sequentially with 100 μl of alkaline phosphatase-conjugated goat anti-human IgG (1:1,000), IgM (1:1,000), and IgA (1:1,000) antibody (Southern Biotechnology Association, Birmingham, Ala.) in PBS-T containing 0.1% bovine serum albumin (Sigma) and incubated overnight at 4°C. The plates were then rinsed six times with PBS and immersed in PBS for 5 min. The wells were emptied of wash buffer and then exposed to 100 μl of 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium in Tris buffer solution (BCIP/NBT phosphate substrate system; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.). After standing for 15 to 20 min, the plates were rinsed with PBS. The developed plates were dried, and cells in the blue spots were counted under low magnification. The results were expressed as numbers of immunoglobulin-secreting cells per 10^5 peripheral blood mononuclear cells. Lymphocytes were collected from each individual and studied on one or two occasions. In each experiment, the test wells were set up in duplicate.

TABLE 1. Concentrations of antibodies to TT and P6

Subject	Sex	Age (yr)	IgG antibody to TT (U/ml)	Antibody to P6 (μg/ml)		
				IgG	IgM	IgA
1	Male	47	1.60	1.60	1.36	<0.1
2	Male	35	0.17	10.70	1.36	<0.1
3	Male	50	0.66	1.60	1.36	<0.1
4	Male	26	0.47	1.90	1.36	<0.1
5	Female	59	0.76	11.20	1.36	<0.1
6	Female	52	0.76	1.10	0.50	0.40
7	Female	33	0.92	1.40	1.20	0.80
8	Female	50	0.20	8.90	1.60	<0.1
9	Female	45	0.38	2.00	1.40	<0.1
10	Female	37	3.00	19.30	14.00	2.90
Mean		43.40	0.89	5.97	2.55	0.41
SD		10.23	0.85	6.25	4.03	0.91
SEM		3.24	0.27	1.98	1.28	0.29

RESULTS

Population. Ten healthy adults between the ages of 26 and 59 years (mean ± standard deviation [SD], 43.4 ± 10.2 years) were studied (Table 1). Four of the individuals were male, and six were female.

Antibody. IgG antibodies to TT and P6 were detected in all individuals, as was IgM antibody to P6 (Table 1). In contrast, only three individuals had detectable levels of IgA antibody to P6. Antibody to P6 was predominantly of the IgG class. One individual (subject 10) had extremely high levels of antibody to TT and P6.

Lymphocyte blast transformation. Lymphocyte responsiveness to PHA, PWM, TT, and P6 was evaluated over 12 days in a blast transformation assay. Optimal concentrations of PHA, PWM, and TT were based on previously established results. Different concentrations of P6 were tested in the present study. The response curves were similar for the three doses tested

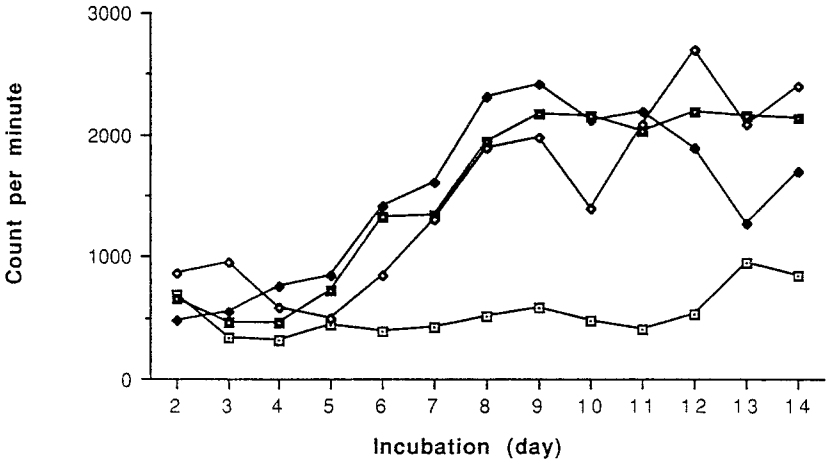

FIG. 1. Dose-response curve for lymphocyte blast transformation with P6. Peripheral blood mononuclear cells were cultured with 200 (◆), 100 (■), or 50 (◈) μg of antigen per ml or without antigen as a control (⊡).

(Fig. 1). The peak response was achieved between days 9 and 12. Higher concentrations of P6 induced peak responses earlier. Two hundred micrograms of P6 per milliliter was the concentration selected for all subsequent studies. Peak responses with each stimulus are displayed in Tables 2 and 3. Lymphocytes from each individual responded to every stimulus. Peak stimulation with TT was inversely related to age ($r = 0.65$; $P < 0.05$). The kinetics of the peak responses for each day evaluated are displayed in Fig. 2. Responses were greatest for PHA and PWM. P6 induced the weakest response. The peak SIs with PHA, PWM, TT, and P6 occurred on days 5, 7, 8, and 10, respectively. In comparison with the response to TT stimulation, the peak lymphocyte blast transformation response was reduced (mean counts per minute ± standard error of the mean [SEM], 3,457 ± 503 versus 9,414 ± 1,464; $P = 0.0051$) and delayed (mean days ± SEM, 10.3 ± 0.4 versus 8.4 ± 0.5; $P = 0.0169$, Wilcoxon signed rank test).

Antibody-secreting cells. Cells secreting IgG, IgM, and IgA were examined after 6, 8, and 10 days of incubation. Peak responses to stimulation with PWM, TT, and P6 occurred on day 8. The peak responses of each subject are displayed in Table 4. Unstimulated cells secreted predominantly IgG, followed by IgM and IgA. Stimulation with PWM, TT, and P6 resulted in increased numbers of secreting cells of each immunoglobulin class. IgG-secreting cells remained the most prominent. PWM was the best stimulus, followed by P6 and TT. In general, there was a direct relationship between the number of antibody-secreting cells in the control and the response to a stimulus. P6 was a better stimulus than TT of antibody secretion from lymphocytes, particularly antibody of the IgM class (mean numbers of antibody-secreting cells per 10^5 peripheral blood mononuclear cells ± SEM: IgG, 85 ± 29 versus 42 ± 16 [$P = 0.0469$]; IgM, 81 ± 20 versus 25 ± 7 [$P = 0.0125$]; IgA, 24 ± 8 versus 16 ± 6 [$P = 0.0526$, Wilcoxon signed rank test]).

DISCUSSION

Nontypeable *H. influenzae* is a common inhabitant of the upper respiratory tract and a frequent cause of otitis media in young children (5, 7, 37). Previous studies have demonstrated the presence of antibody to nontypeable *H. influenzae* in the sera of newborns (11). The level of IgG anti-nontypeable *H. influenzae* antibody declines to the lowest level at 6 months of age and remains relatively low until 2 years of age; this period

TABLE 2. Lymphocyte blast transformation of individual subjects determined as counts per minute

Subject	P6 (200 μg/ml)		TT (1:5)		PWM (50 μg/ml)		PHA (50 μg/ml)	
	cpm	Day	cpm	Day	cpm	Day	cpm	Day
1	4,178	12	10,899	8	13,329	10	40,295	5
2	2,386	11	13,145	8	20,441	8	48,112	6
3	3,099	11	4,251	7	11,687	7	16,883	3
4	3,348	10	13,557	9	15,153	7	42,915	6
5	1,805	10	12,949	9	3,860	6	16,152	5
6	3,971	8	13,228	8	14,891	9	28,861	5
7	5,498	10	12,040	7	10,958	8	20,357	4
8	1,448	12	3,363	9	17,143	7	21,971	5
9	2,431	11	3,098	11	11,456	9	16,873	6
10	6,406	8	7,610	8	13,033	8	29,117	6
Mean	3,457	10.3	9,414	8.4	13,195	7.9	28,154	5.1
SD	1,590	1.4	4,393	1.2	4,375	1.2	11,844	1.0
SEM	503	0.4	1,464	0.5	1,384	0.5	3,745	0.4

TABLE 3. SI of lymphocyte blast transformation of individual subjects

Subject	P6 (200 μg/ml)		TT (1:5)		PWM (50 μg/ml)		PHA (50 μg/ml)	
	SI	Day	SI	Day	SI	Day	SI	Day
1	3.3	8	21.4	8	58.9	10	81.3	4
2	4.9	11	15.2	7	94.1	6	274.1	6
3	6.4	11	8.5	7	23.4	5	46.5	4
4	14.2	10	65.7	8	64.3	7	235.2	5
5	10.3	12	16.2	10	39.5	8	35.0	5
6	13.4	11	34.4	8	38.6	8	334.3	5
7	18.7	9	33.5	7	34.9	6	82.8	3
8	7.3	11	7.0	6	25.0	7	41.2	3
9	2.4	11	3.1	11	25.2	7	70.4	4
10	33.5	8	39.5	8	68.1	8	108.9	6
Mean	11.4	10.2	24.4	8.0	47.2	7.2	131.0	4.5
SD	9.3	1.4	19.1	1.5	23.3	1.4	108.5	1.1
SEM	3.0	0.4	6.4	0.7	7.4	0.6	34.3	0.5

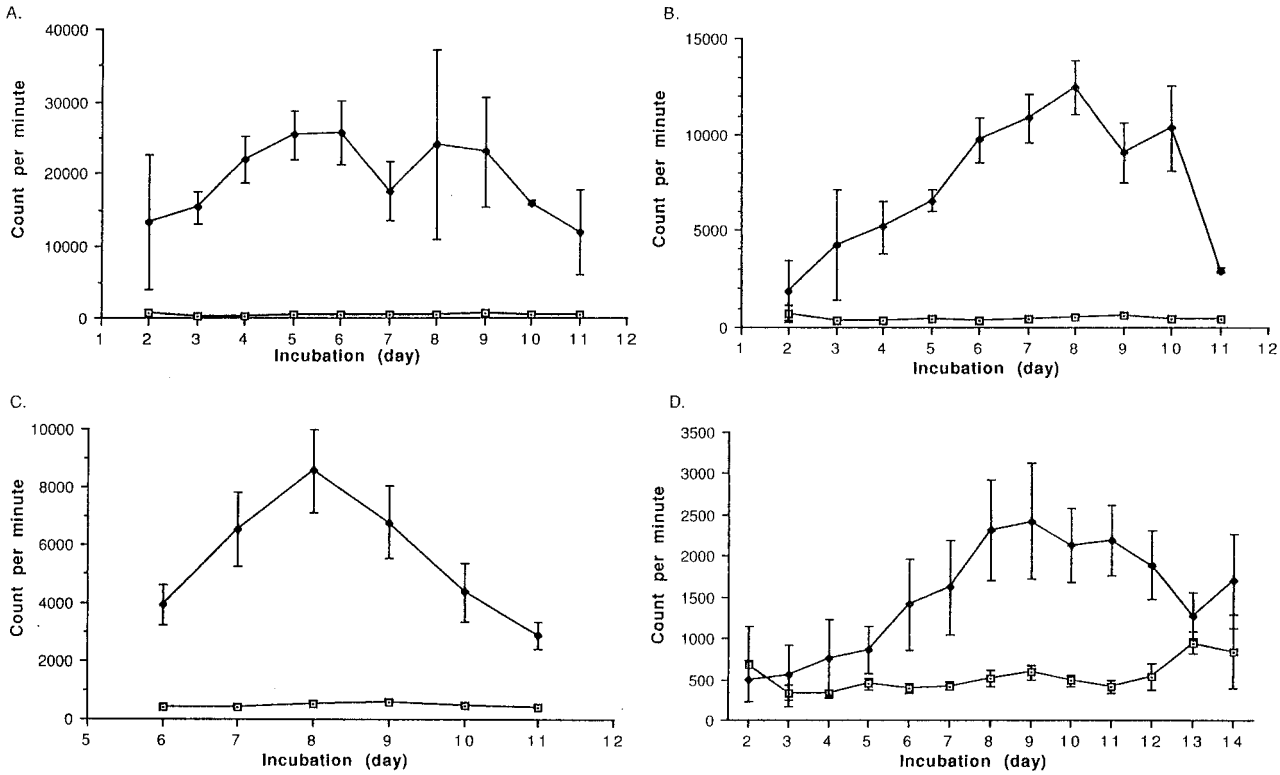

FIG. 2. Lymphocyte blast transformation. (A) PHA. (B) PWM. (C) TT. (D) P6 (200 μg/ml). Values for controls (⊡) are also shown. Each datum point indicates the mean ± SEM for 10 subjects.

corresponds to the greatest incidence of otitis media. Adult levels of IgG, IgM, and IgA antibodies to nontypeable *H. influenzae* are attained at 4 years of age (11). Shurin et al. (35) were the first group of investigators to demonstrate a relationship between the presence of specific nontypeable *H. influenzae* antibody and protection against otitis media. Subsequently, it was noted that there is a degree of strain specificity to the antibody response following infection with nontypeable *H. influenzae* (10, 23). Since the outer membrane of nontypeable *H. influenzae* is composed of a number of different proteins which serve as targets for bactericidal antibody, it is reasonable to assume that one or more of these proteins account for strain specificity in the immune response (16).

Only two of the major proteins, P4 and P6, were highly conserved (15, 16, 29, 30, 32). P6 is of particular interest, because antibody to it kills a large number of nontypeable *H. influenzae* strains and is protective in an animal model (16, 27). Longitudinal evaluation of antibody to P6 in children demonstrates a failure of otitis-prone children to develop a normal age-related rise in anti-P6 antibody despite repeated exposure to the organism (42). Furthermore, these children do not manifest an anamnestic antibody response following second episodes of infection with nontypeable *H. influenzae*. These data suggest an inability of lymphocytes from otitis-prone individuals to recognize P6 as an important immunogen.

The present study was designed to determine the normal lymphocyte response to P6 in a group of healthy adults. Each of the adults demonstrated serum IgG antibody to the P6 protein. In order to understand the immune response to P6, assays were simultaneously conducted with a well-known protein antigen, TT. Each of the adults possessed antibody to TT at the time of the lymphocyte studies.

Results from the present study demonstrated that the P6 antigen activated lymphocytes from individuals who were immune to nontypeable *H. influenzae*. The lymphocyte blast transformation response to P6 was similar to the response to TT; however, in comparison, the peak antibody response to P6 was slightly reduced and delayed. In contrast, lymphocytes secreted more antibody in response to P6 than to TT. This was true for each of the immunoglobulin classes studied; however,

TABLE 4. Peak antibody-secreting cells

Subject	Peak no. of antibody-secreting cells/10^5 PBMCs[a]											
	Control			PWM			TT			P6		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
1	2	1	4	214	250	62	22	27	14	14	12	4
2	80	32	6	535	184	105	83	33	16	278	155	12
3	23	12	4	312	229	40	30	25	3	28	76	7
4	2	1	2	288	166	87	9	6	6	52	33	14
5	15	9	11	380	277	162	15	11	13	110	116	39
6	2	4	12	38	40	24	2	2	3	5	8	4
7	5	1	4	205	14	8	21	1	1	16	3	6
8	80	43	43	996	263	149	167	54	61	216	153	75
9	62	70	13	1,760	954	299	61	67	20	90	109	32
10	2	2	5	250	812	269	5	27	21	40	141	48
Mean	27	18	10	498	319	121	42	25	16	85	81	24
SD	33	23	12	514	312	100	51	22	17	93	62	24
SEM	11	7	4	162	99	32	16	7	6	29	20	8

[a] PBMCs, peripheral blood mononuclear cells.

P6 was a particularly potent stimulus of IgM secretion. The dominant immunoglobulin class of antibody to P6 in the sera of the adults was IgG (mean anti-P6 IgG versus IgM, 5.97 versus 2.55 μg/ml). The design of the present study did not enable us to determine whether the antibody secreted in response to P6 stimulation was in fact specific for the P6 antigen. Although *H. influenzae* contains lipooligosaccharide in the outer membrane, it is unlikely that endotoxin affected the results of the lymphocyte assays, because the process of preparing P6 effectively removes endotoxin contamination.

We found no correlation between the level of specific antibody in serum and the number of antibody-secreting cells, regardless of isotypes, as observed previously by Lee et al. (24). The lack of correlation between the two parameters suggests that the rate of immunoglobulin secretion in vitro is very different from the normal state in vivo.

The results from the present study enhance our understanding of immunity to nontypeable *H. influenzae* and provide a basis for future investigations of otitis-prone children who do not recognize P6 in a normal manner. It is possible that otitis-prone individuals lack a memory cell component to their immune response to P6 and therefore fail to exhibit an anamnestic antibody response, thus enabling them to experience repeated episodes of otitis media due to nontypeable *H. influenzae* (42).

ACKNOWLEDGMENT

This study was supported by grant 19679 from the National Institute of Child Health and Human Development.

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