In Vitro Lymphocyte Proliferation in Response to Type III Group B Streptococci

MADELINE AMBRUS LILLIE, CAROLYN S. CODY, STEVEN D. DOUGLAS, AND RICHARD A. POLIN*

Divisions of Allergy-Immunology and Neonatology, Department of Pediatrics, The Children's Hospital of Philadelphia, Joseph Stokes Jr. Research Institute, and School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Received 1 February 1982/Accepted 12 April 1982

The in vitro cell-mediated responses to group B streptococci (GBS) and the relationship of cell-mediated immunity to specific humoral immunity to type III GBS were investigated. Blood was obtained from 20 adult volunteers, and lymphocytes were isolated and cultured in microtiter plates. Each well contained 2×10^5 lymphocytes, 15% autologous serum, and either GBS (cell-to-organism ratio of 1:10, 1:1, or 1:0.1), phytohemagglutinin, streptokinase-streptodornase, or RPMI 1640. Cells were harvested at 5, 6, or 7 days, and DNA synthesis was quantitated. Serum antibody titers were determined with an enzyme-linked immunosorbent assay. Maximal lymphocyte responses occurred at 6 days of culture and at a cell-to-organism ratio of 1:1. Individuals with significant antibody titers to type III GBS, as well as those with undetectable antibody, responded to GBS (stimulation index > 10). There was a significant difference ($P < 0.001$) between mean antibody concentrations in responders (stimulation index > 10) and nonresponders (stimulation index < 10). Thus, the in vitro responses to GBS may be both to a specific antigen and to a nonspecific mitogen and may be important in host immunity to GBS.

The group B streptococci (GBS) have become a leading cause of neonatal morbidity and mortality in the last decade (1), as well as significant pathogens in the elderly and immunocompromised host. Of the five serotypes defined by Lancefield, type III GBS is the most virulent and is responsible for at least 60% of all clinical syndromes attributable to GBS (1). Since GBS has a predilection to cause clinical disease in individuals at the extremes of age, when the immune systems may not be fully intact, the study of host responses to this organism are of particular importance.

Baker and Kapser (2) have demonstrated that antibody to type III GBS is usually present in infants who are colonized but not infected with GBS, whereas it is deficient in infants with systemic infection. A protective role for antibody has not, however, been confirmed in all studies (17). The importance of functional neutrophils as well as intact classical and alternative complement pathways has been stressed by Hill and others (10).

Although cellular immune responses to group A streptococci have been well described, the role of cell-mediated immunity to the group B organisms has not been investigated. Therefore, we determined the optimal conditions to study in vitro cell-mediated responses to GBS and the

relationship between cell-mediated immunity and specific humoral immunity to type III GBS.

MATERIALS AND METHODS

Subjects. Blood was obtained from 20 healthy adult volunteers between 25 and 45 years of age.

Streptococci. Type III group B streptococci, strain D136C, were kindly provided by the late R. Lancefield. The organisms were grown overnight in Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.), quantitated by counting the number of colonies on blood agar plates, and formalinized according to previously described methods (13). The organisms were refrigerated and the sterility was checked before use. Before each experiment, organisms were freshly washed and diluted in RPMI 1640 containing 4 mM L-glutamine, 100 U of penicillin per ml, and 100 μ g of streptomycin per ml (GIBCO Laboratories, N.Y.).

Preparation of lymphocytes. RPMI 1640 was prepared from a powder (GIBCO) and supplemented with L-glutamine, penicillin, and streptomycin. Heparinized whole blood was diluted with an equal volume of RPMI 1640 and layered over an equal volume of Ficoll-Hypaque solution. The blood was centrifuged for 40 min at 1,350 rpm at room temperature, and the mononuclear cell layer interface (approximately 80% lymphocytes and 20% monocytes) was separated and washed in RPMI 1640. Cells were counted and viability was assessed by the trypan blue dye exclusion method; the cells were then diluted to appropriate

TABLE 1. Kinetics of lymphocyte proliferation to Group B streptococci^a

Day	SI ^b	cpm ^c
5	6.8 ± 1.4	1,219 ± 218
6	28 ± 7.5	4,458 ± 617
7	13.2 ± 3.7	2,392 ± 323

^a Peripheral blood lymphocytes at a cell-to-organism ratio of 1:1 were incubated for 5, 6, or 7 days, pulsed with [³H]thymidine, and harvested with an automated cell harvester. Data are given as mean ± SEM.

^b Day 6 versus day 5, $P < 10^{-6}$; day 6 versus day 7, $P < 0.005$.

^c Day 6 versus day 5, $P < 0.005$; day 6 versus day 7, $P < 0.001$.

concentrations with RPMI 1640. Viability in all cases was greater than 99%.

Lymphocyte proliferation. Lymphocyte proliferation was studied in round-bottomed microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.) in quadruplicate. Each well contained 2×10^5 lymphocytes in 15% autologous serum and RPMI 1640. Three cell-to-organism ratios of GBS were studied: 1:10, 1:1, and 1:0.1. Group B streptococci, phytohemagglutinin (PHA) (GIBCO), streptokinase-streptodornase (SKSD) (Lederle Laboratories, Pearl River, N.Y.), or RPMI 1640 was added to bring the final volume to 0.2 ml per well. The optimal doses of PHA (undiluted) and SKSD (5,000 U/ml) were predetermined by dose-response studies. Background responses of the cells in media were determined in every experiment. The plates were incubated at 37°C in 5% CO₂ for 6 days before the cells were harvested. To evaluate the kinetics of lymphocyte proliferation, lymphocytes at a cell/organism ratio of 1:1 were incubated for 5, 6, or 7 days. Before cells were harvested, [³H]thymidine (Amersham Corp., Arlington Heights, Ill.; specific activity, 2.0 Ci/mmol), diluted 1:20 in RPMI 1640, was added. The cells were further incubated for 4 to 6 h before they were harvested with an automated cell harvester (Dynatech CH-103) onto glass microfiber filters (Whatman 934AH). The filters were air dried overnight and placed in 7 ml of scintillation fluid (ACS II; Amersham) for counting in a Beckman model LS 7500 liquid scintillation counter at room temperature after at least 1 h of dark adaptation.

Values for quadruplicate samples were averaged for each study. The stimulation index (SI) was determined by dividing the average of the experimental results of each quadruplicate by the average of the background on the same plate. Responders, in whom proliferation to GBS was considered significant, were defined as those for whom the SI was greater than 10. Nonresponders had SIs that were less than 10.

Serum antibody titer. Serum antibody concentrations were determined with an enzyme-linked immunosorbent assay developed in our laboratory (14). This assay has been correlated with a radioactive antigen binding assay developed by Baker and Kasper (11). Control serum specimens whose antibody concentrations were known (determined by radioactive antigen binding assay) were tested in the enzyme-linked immunosorbent assay along with serum specimens from

TABLE 2. Effect of cell/organism ratio on lymphocyte proliferation^a

Cell/organism ratio	SI ^b	cpm ^c
1:0.1	16.2 ± 3.9	6,925 ± 1114
1:1	27.2 ± 7.1	17,925 ± 6132
1:10	11.4 ± 3.9	6,688 ± 3054

^a Peripheral blood lymphocytes were cultivated at three cell-to-organism ratios for 6 days, pulsed with [³H]thymidine, and harvested with an automated cell harvester. All data are given as mean ± SEM.

^b 1:1 versus 1:10, $P = 0.026$; 1:1 versus 1:0.1, $P = 0.09$ (NS); 1:10 versus 1:0.1, $P = 0.19$ (NS).

^c 1:1 versus 1:10, $P = 0.003$; 1:1 versus 1:0.1, $P = 0.05$ (NS); 1:10 versus 1:0.1, $P = 0.07$ (NS).

study subjects whose antibody concentrations were unknown. The concentration of "native" antibody in the control specimens ranged from 0.32 to 40.3 µg/ml. Native antibody concentrations in study specimens were calculated from the control curve. A serum native antibody concentration of 2 µg/ml or greater was considered to represent immunity to type III GBS (3).

Statistics. SI and counts per minute (cpm) on consecutive days and at different cell-to-organism ratios were compared by a Student dependent *t* test. Coefficients of correlation (*r*) were determined by linear regression analysis. Antibody concentrations in responders and nonresponders were compared by a Student independent *t* test.

RESULTS

Type III GBS. (i) Kinetics of lymphocyte proliferation. The mean ± standard error of the mean (SEM) for cpm and SI on days 5, 6, and 7 are shown in Table 1. The mean SI and cpm on day 6 were significantly greater than those on days 5 and 7. (Cpm: day 5 versus day 6, $P < 0.005$; day 6 versus day 7, $P < 0.001$. SI: day 5 versus day 6, $P < 10^{-6}$; day 6 versus day 7, $P < 0.005$).

(ii) Response of lymphocytes to various cell-to-organism ratios. The mean ± SEM for cpm and SI at the three cell-to-organism ratios (1:1, 1:10, and 1:0.1) are shown in Table 2. There was a significantly greater proliferative response at a cell-to-organism ratio of 1:1 than at 1:10. (SI, $P = 0.026$; cpm, $P = 0.003$). Neither 1:1 nor 1:10 induced a significantly greater response than did 1:0.1 ($P =$ not significant [NS]).

Comparison of lymphocyte responses to type III GBS. (i) Responses to PHA and SKSD. Every subject responded to both PHA and SKSD (mean ± SEM for SI, 19.2 ± 3.6 and 10.3 ± 3.9 , respectively). There was no correlation between the magnitude of lymphocyte responses to GBS and to either PHA or SKSD ($r = 0.20$ and 0.11 , respectively; $P =$ NS).

(ii) Correlation of lymphocyte responses with antibody concentration. The concentration of

FIG. 1. Correlation of lymphocyte responses with antibody concentration. Peripheral blood lymphocytes were cultivated at a cell-to-organism ratio of 1:1 for 6 days, pulsed with [3 H]thymidine, and harvested with an automated cell harvester. Antibody concentrations were determined with an enzyme immunoassay. Values shown represent mean $\pm$ SEM.

antibody to type III GBS ranged from undetectable ($< 0.32 \mu\text{g/ml}$) to $113 \mu\text{g/ml}$ (10 samples had concentrations $\leq 2 \mu\text{g/ml}$). There was not a significant correlation between either SI or cpm and serum antibody concentrations ($P = \text{NS}$).

The mean $\pm$ SEM serum antibody concentration in responders ($\text{SI} > 10$) was significantly greater than in nonresponders ($25.9 \pm 4.9 \mu\text{g/ml}$ versus $12.3 \pm 1.5 \mu\text{g/ml}$, $P < 0.001$) (Fig. 1). The mean SI of subjects with antibody concentrations greater than and less than $2 \mu\text{g/ml}$ were not significantly different ($P = \text{NS}$).

DISCUSSION

These studies demonstrate that type III GBS induces a proliferative response in peripheral blood lymphocytes. Maximal lymphocyte responses occurred at 6 days of culture and at a cell-to-organism ratio of 1:1. Both individuals with significant antibody titers to type III GBS and those with undetectable antibody responded to GBS, suggesting that GBS is mitogenic. There was, however, a significant difference between the mean antibody concentrations in responders ($\text{SI} > 10$) and in nonresponders. The in vitro response to type III GBS may be both to a specific antigen and to a nonspecific mitogen.

Substances known to be lymphocyte mitogens include plant lectins (9), bacterial antigens and products (5, 7, 8, 12, 15, 16), polymeric and polyionic substances, enzymes, reducing agents, heavy metal ions, ionophores, antibod-

ies, and antigen-antibody complexes. Several investigators have recently evaluated in vitro lymphocyte responses to intact organisms (4, 15, 16). Banck (4) used Formalin-killed organisms and found that lymphocytes incubated with some strains result in an SI greater than 20 (*Staphylococcus aureus* strain Cowan I, *Haemophilus influenzae* type B, *Neisseria catarrhalis*, and *Bordetella pertussis*), whereas other strains result in moderate responses with SI of 5 to 20 (*Mycobacterium tuberculosis*, *Neisseria gonorrhoeae* and *Neisseria pharyngis*, *Bacillus* spp., *Streptococcus pneumoniae* type 6, and *Streptococcus* groups A and G). These responses are considered to be mitogenic since they are most prominent at 3 to 4 days and induce polyclonal B-cell activation. *M. tuberculosis*, *B. pertussis*, and certain pneumococcal strains did stimulate a late response at 6 and 7 days in both the T cell- and B cell-enriched populations (3).

The in vitro responsiveness of our patients with negative titers to type III GBS might be explained by past exposure to antigenically related organisms such as *S. pneumoniae* type 14 (11). In addition, Boyer et al. (6) have found that immunization with polyvalent pneumococcal vaccine significantly increases the antibody titer to type III GBS in 16 of 40 vaccinees. The possible relationship between long-lasting immunity to GBS manifested by lymphocyte response and that manifested by type-specific antibody requires further investigation.

In summary, there was a significant in vitro lymphocyte response to type III GBS that peaked at 6 days, demonstrated a maximal response at a cell-to-organism ratio of 1:1, and correlated with specific antibody concentration. These data suggest that type III GBS may act as both a mitogen and an antigen and provide impetus for studying the influence of purified group B streptococcal components on lymphocyte blastogenesis.

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LITERATURE CITED

1. Baker, C. J. 1977. Summary of the workshop on perinatal infections due to group B streptococcus. *J. Infect. Dis.* 136:137-152.
2. Baker, C. J., and D. L. Kasper. 1976. Correlation of maternal antibody deficiency with susceptibility to neonatal group streptococcal infection. *New Engl. J. Med.* 294:753-756.
3. Baker, C. J., D. L. Kasper, M. S. Edwards, and G. Schiffman. 1980. Influence of preimmunization antibody levels on the specificity of the immune response to related

- polysaccharide antigens. *New Engl. J. Med.* **303**:174-178.
4. Banck, G., and A. Forgren. 1978. Many bacterial species are mitogenic for human blood B lymphocytes. *Scand. J. Immunol.* **8**:347-354.
 5. Barnetson, R., G. Bjune, and M. E. Duncan. 1976. Evidence for a soluble lymphocyte factor in the transplacental transmission of T lymphocyte responses to *Mycobacterium leprae*. *Nature (London)* **260**:150-151.
 6. Boyer, K. M., J. Theeravuthichai, L. C. Vogel, A. Orlina, and S. P. Gotoff. 1981. Antibody response to group B streptococcus type III and AB blood group antigens induced by pneumococcal vaccine. *J. Pediatr.* **98**:374-378.
 7. Dale, J. B., W. A. Simpson, I. Ofek, and E. H. Beachey. 1981. Blastogenic responses of human lymphocytes to structurally defined polypeptide fragments of streptococcal M Protein. *J. Immunol.* **126**:1499-1505.
 8. Dzairski, R. 1980. Polyclonal activation of immunoglobulin secretion in B lymphocytes induced by staphylococcal peptidoglycan. *J. Immunol.* **125**:2478-2483.
 9. Handwerker, B. S., and S. D. Douglas. 1980. The cell biology of blastogenesis, p. 609-706. *In* G. Weissmann (ed.), *Handbook of inflammation*, vol. 2. Elsevier/North Holland Publishing Co., Amsterdam.
 10. Hill, H. R., A. O. Shigeoka, R. T. Hall, and V. G. Henning. 1979. Neonatal cellular and humoral immunity to group B streptococcus. *Pediatrics* **64**(S):787-794.
 11. Kasper, D. L., C. J. Baker, R. S. Baltimore, J. H. Crabb, G. Schiffman, and H. L. Jennings. 1979. Immunodeterminant specificity of human immunity to type III group B streptococcus. *J. Exp. Med.* **149**:327-339.
 12. Pachman, L. M., and E. N. Fox. 1970. Cellular and antibody reaction to streptococcal M protein types 1, 3, 6 and 12. *J. Immunol.* **105**:898-907.
 13. Polin, R. A. 1980. Monoclonal antibodies against streptococcal antigens, p. 353-358. *In* R. K. Kennett, T. J. McKearn, and K. B. Bechtol (ed.), *Monoclonal antibodies*. Plenum Publishing Corp., New York.
 14. Polin, R. A., S. D. Douglas, D. L. Kasper, and C. J. Baker. 1982. Enzyme-linked immunosorbent assay for measurement of antibody to type III group B streptococci. *J. Clin. Microbiol.* **15**:991-993.
 15. Ringden, O., B. Rynnel-Dagoo, E. M. Waterfield, E. Moller, and G. Moller. 1977. Polyclonal antibody secretion in human lymphocytes induced by killed staphylococcal bacteria and by lipopolysaccharide. *Scand. J. Immunol.* **6**:1159-1169.
 16. Sorenson, R. U., R. C. Stern, P. Chase, and S. H. Polmar. 1979. Defective cellular immunity to gram-negative bacteria in cystic fibrosis patients. *Infect. Immun.* **23**:398-402.
 17. Wilkinson, H. W. 1978. Detection of group B streptococcal antibodies in human sera by radioimmunoassay: concentration of type-specific antibodies in sera of adults and infants infected with group B streptococci. *J. Clin. Microbiol.* **7**:194-201.