Measurement of Growth-Inhibiting (Bactericidal) Antibodies in Patients with Meningococcal Meningitis and in Subjects Immunized with Meningococcal Polysaccharide Vaccine by a [\textsuperscript{3}H]Thymidine Assay

M. HASSAN-KING,†‡ B. M. GREENWOOD,‡ and H. C. WHITTLE†
Department of Medicine, Ahmadu Bello University Hospital, Zaria, Nigeria

Received 16 July 1984/Accepted 12 April 1985

We measured bactericidal antibodies against meningococci by a method which depends on the inhibition of [\textsuperscript{3}H]thymidine uptake by bacteria in the presence of antibody and complement. A significant increase in antibody activity was found in sera from patients who had recovered from group A meningococcal meningitis (mean inhibition, 38.9 ± 4.4%) compared with antibody activity present in sera from patients in the acute phase (mean inhibition, 7.0 ± 2.4%) (P < 0.001). Similarly, a significant increase in inhibitory activity against group C meningococci was observed 2 weeks after immunization with group C polysaccharide. A close correlation was observed between the results of the thymidine assay and of a standard colony-counting method and hemagglutination assay. The assay is readily applicable to testing large numbers of samples.

The ability to measure bactericidal antibody is an important aspect of studying meningococcal infection, because susceptibility to systemic meningococcal disease is associated with selective deficiency of such antibodies (4). Furthermore, because bactericidal antibodies are directed against more than one meningococcal antigen, they can be used to subtype the organism (2, 3, 7). Because of the difficulties inherent in the standard colony-counting method for measuring bactericidal antibodies, alternative methods have been introduced (5). We have developed a labeled thymidine assay to measure bactericidal antibody activity against meningococci in sera from patients and vaccinated subjects. The assay is based on the principle that only viable bacteria can incorporate labeled thymidine after exposure to the action of antibody and complement. The inhibition of thymidine uptake by the organisms can therefore be used as a measure of bactericidal action.

Three strains of meningococci were used in the study, group A Neisseria meningitidis M1027, group B N. meningitidis M993, and group C N. meningitidis M1628. A loopful of an 18-h culture of test organism was inoculated into 20 ml of Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) and incubated at 37°C in a shaking water bath for up to 3 h to achieve a log-phase culture. The bacteria were washed twice at 4°C in medium 199 (Flow Laboratories, McLean, Va.) at pH 7.3 and then adjusted in the same medium to an optical density of 0.3 at 650 nm. Blood was collected from patients with group A meningococcal meningitis during the acute and convalescent phases of their illness. Samples were also obtained from patients with group A meningococcal disease who had been immunized with 50 μg of group C meningococcal polysaccharide vaccine (Institut Mérieux) at least 6 weeks after recovery, from relatives of these patients, and from unrelated control subjects. Since we had no access to a suitable source of human complement or to baby rabbit serum, three adult white rabbits provided the complement used in the study. Naturally occurring meningococcal antibodies were removed from a serum pool obtained from these rabbits. The serum was diluted 1:5 with medium 199 before absorption with killed bacteria of groups A, B, and C at the start of each experiment. After absorption for 2 h on ice, bacteria were removed from the serum by centrifugation and filtration through a membrane filter (0.45 μm pore size; Millipore Corp., Bedford, Mass.).

Reaction mixtures were set up in duplicate in sterile plastic tubes with caps (Becton Dickinson Labware, Oxnard, Calif.). The test mixtures consisted of 100 μl of inactivated (56°C for 30 min) serum, 100 μl of complement, and 100 μl of meningococcal suspension. Two sets of controls were included in each experiment: a complement control consisting of 100 μl of complement and 100 μl of meningococcal suspension and, to reaffirm the necessity for complement in the system, a control tube containing 100 μl of test serum, 100 μl of inactivated (56°C for 30 min) complement, and 100 μl of bacterial suspension. All reaction mixtures were brought to a final volume of 1 ml with medium 199. Reaction mixtures were incubated in a shaking water bath at 37°C for 30 min to allow bactericidal action to occur. At the end of this period, 1 μCi of [\textsuperscript{3}H]thymidine (specific activity, 5 Ci/μmol; Radiochemical Centre, Amersham, England) was added to each reaction mixture with a Hamilton precision syringe. The mixtures were then incubated for a further 2 h, after which the labeling reaction was terminated by washing the mixtures twice in cold phosphate-buffered saline (1,500 × g for 10 min) at 4°C. Bacteria were then extracted in 10% trichloroacetic acid for 10 min at room temperature and spun down. The precipitate obtained was washed twice in phosphate-buffered saline at 4°C and then solubilized with 200 μl of 1 N sodium hydroxide for 30 min at room temperature. The lysate (100 μl) was then added to 10 ml of scintillation fluid made up of toluene-based NE233 (Nuclear Enterprises) containing 20% solubilizer NE520 to incorporate water in the sample. Each sample was neutralized by the addition of 10 μl of 1 N glacial acetic acid. After 30 min at room temperature, thymidine uptake was measured by counting for 1 min in a Nuclear Enterprises (model LSCI) β-counter. A blank sample consisting of 100 μl of 1 N

* Corresponding author.
† Present address: Medical Research Council Laboratories, Fajara, Banjul, The Gambia.
sodium hydroxide in 10 ml of scintillation fluid, neutralized with 10 μl of glacial acetic acid, was prepared for each assay, and the counts per minute obtained with the blank were subtracted from the results for each sample.

Bactericidal activity in the test samples was expressed as percentage inhibition, calculated as [(cpm in control - cpm in test sample)/cpm in control] × 100. The heat-inactivated complement control with added test serum was used as the control in this calculation to achieve the same protein concentration in both test and control samples.

The relationship between the standard and the radioactive bactericidal assays was studied with the same reaction mixtures. At the end of the 2-h incubation period, two 100-μl portions were withdrawn from the reaction mixture with an Eppendorf pipette. One was prepared for liquid scintillation counting, and the other was plated out in 10-fold tube dilutions onto well-dried Mueller-Hinton agar plates. The plates were incubated for 24 h in 5% carbon dioxide, and colonies were counted the next morning. Hemagglutination antibody titers in patients and vaccinated subjects were measured by using human group O erythrocytes coated with group A or group C purified meningococcal polysaccharide antigen. Correlation coefficients were used to test the relationship between the results of the thymidine-labeled assay for measuring bactericidal activity, the standard colony-counting test, and the hemagglutination antibody test. Differences between means were analyzed by Student's t test. Results were considered statistically significant at P < 0.05.

To avoid the necessity of making serum dilutions, it was decided to use a single serum dilution in the assay and to express its bactericidal activity as a percentage of the inhibition of thymidine uptake. Preliminary experiments with 10 immune serum samples obtained from patients who were convalescing from group A meningococcal disease showed that maximum inhibition was obtained at a final serum dilution of 1:100, and this dilution was used in subsequent experiments. The specificity of the thymidine uptake assay was studied by absorbing 10 immune serum samples with the homologous group A meningococcus. The mean inhibition of the 10 samples before absorption, 60.4 ± 9.2%, fell to 17.6 ± 3.3% after absorption. The reproducibility of the assay was investigated by testing a group A and a group C antiserum against the homologous organisms on 10 occasions over a 2-month period. The group A antiserum gave a mean inhibition of 61.5%, with a variation of less than 4% and a standard deviation of 1.3%. Corresponding figures for the group C antiserum were a mean inhibition of 53.7%, with a variation of less than 3% and a standard deviation of 1.1%. Good correlations were obtained between the thymidine uptake assay and the standard colony-counting method results and also with a hemagglutination antibody assay (P < 0.001 in each case).

The results of thymidine uptake assays undertaken on samples from patients with group A meningococcal disease taken during the acute and convalescent phases of illness (Fig. 1) showed a significant increase in bactericidal antibody activity during the convalescent phase, with a mean inhibition of 38.9 ± 4.4%, compared with 7.0 ± 2.4% during the acute phase (P < 0.001). Bactericidal activity against group A N. meningitidis was present in both the immunoglobulin G (IgG) and IgM fractions of pooled sera obtained from patients recovering from group A meningococcal disease. In three studies, an IgG pool gave a mean inhibition of 31.4% (range, 30.2 to 32.8%) and an IgM pool gave a mean inhibition of 26.8% (range, 26.4 to 27.2%).

A significant increase in antibody activity was observed 2 weeks after immunization with group C meningococcal vaccine in samples from patients who had recovered from group A meningococcal meningitis, from relatives of these patients, and from unvaccinated control subjects (Fig. 2). Prevaccination antibody activity did not differ significantly among the three groups, although the mean inhibition in the patients (5.2 ± 1.8%) was slightly less than in their relatives (8.9 ± 2.8%) and the controls (8.4 ± 2.9%). After vaccina-

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FIG. 1. Bactericidal activity in serum samples from patients in the acute and convalescent phases of group A meningococcal meningitis, measured as the inhibition of [3H]thymidine uptake by group A N. meningitidis M1027 in the presence of antibody and complement. Horizontal bars indicate the mean percentage of inhibition.

FIG. 2. Bactericidal activity in vaccinated subjects before (pre vac) and 2 weeks after (post vac) immunization with group C meningococcal polysaccharide vaccine, measured as the inhibition of [3H]thymidine uptake by group C N. meningitidis M1628 in the presence of antibody and complement. Horizontal bars indicate the mean percentage of inhibition.
tion, patient samples showed the highest levels of bactericidal antibody (23.3 ± 1.3%), versus 21.6 ± 1.1% for relatives and 21.9 ± 0.8% for controls. The differences between the three groups were not statistically significant. Although antibody activity in the initial serum samples was generally low, there were a few samples with initially high antibody activity. In these cases there was usually a reduction in activity after vaccination.

We have developed a [3H]thymidine uptake assay for studying antibody activity against meningococci which has some advantages over the colony-counting method. The use of one test serum dilution to express bactericidal activity offers considerable savings of time, effort, and materials compared with the usual method of serially diluting the test sera to determine a bactericidal antibody titer. The highest concentration of immune serum tested (1:10) failed to show the greatest bactericidal activity. This could be analogous to the inhibition of complement-mediated bactericidal action by excess antibody that has been observed for other gram-negative organisms (6). One hundred percent killing was not achieved with the thymidine uptake assay, presumably because some of the radiation emitted from the tritium was absorbed in the bacterial cells. The thymidine assay has a high degree of specificity, as was shown by the absorption experiment. Furthermore, good correlation was established with the standard test for measuring complement-mediated bactericidal activity. Although only meningococci were used as test organisms in our study, the assay offers a simple and sensitive practical method for measuring both natural and vaccine-induced antibodies against other gram-negative organisms susceptible to the bactericidal action of antibody and complement.

LITERATURE CITED