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Hemagglutination Inhibition for Serogrouping of *Neisseria meningitidis*

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A hemagglutination inhibition (HI) test has been studied as an alternative to bacterial agglutination (BA) for serogrouping strains of *Neisseria meningitidis* isolated from clinical specimens. The HI test consists of polysaccharide antigens adsorbed to sheep red blood cells which were then agglutinated by group-specific antisera. Supernatant fluids from suspensions of meningococci were used to inhibit the agglutination. Results of the two tests agreed for 381 (80%) carrier strains. Of the remaining 95 strains, 82 (86%) were identified by HI although they were nongroupable by BA. Thus, the HI test has been shown to be more highly specific and sensitive and to be more economical of reagents and time than the BA test.

Serogroups of *Neisseria meningitidis* are identified on the basis of bacterial agglutination (BA) with specific antisera (2). Although other methods, such as precipitation, have been used for identifying new serogroups (12) or studying relationships among the serogroups (3), the BA test has been the standard procedure used in most laboratories for the examination of meningococci isolated from clinical specimens.

The BA test has a number of disadvantages, among the most prominent being the large volume of antisera needed and the long time required when survey studies provide hundreds of isolates for testing. Also, there is often considerable cross-agglutination among the various antisera (3, 10), probably due to common antigens unrelated to the serogroup-specific polysaccharides.

With the recent development of improved methods to isolate and characterize meningococcal polysaccharide antigens (6) and antibodies (7), it was possible to develop a hemagglutination inhibition test (HI) to identify the serogroup C meningococci isolated from clinical materials (8). The present study extends the method to include eight different serogroups and compares the HI and BA serogrouping tests.

MATERIALS AND METHODS

The test is essentially the inhibition by the unknown of a battery of standard passive hemagglutination tests.

Antigens. The following strains of meningococci from the Walter Reed Army Institute of Research collection were used to prepare antigens: group A (A-4), group B (99M), group C (9M), group Y (135M), group 29E (60M), group 135 (135-III), and Slaterus X and Z. Crude polysaccharide antigens were prepared by the methods of Edwards and Driscoll (4). Washed cells were sedimented at 600 × *g* from 250 ml of an 18-hr Mueller Hinton broth culture and suspended in 10 ml of 0.15 M NaCl. The suspension was adjusted to *p*H 11.0 with 1 N NaOH, incubated 1 hr at room temperature, and adjusted to *p*H 6.5 with 1 N HCl. The precipitate collected at 3,000 × *g* after the addition of five volumes of absolute ethanol was resuspended in 20 ml of 0.15 M NaCl, the insoluble residue was separated and discarded, and the clear supernatant fluid was used as antigen. Optimal sensitizing concentrations of each batch of antigen were determined by checkerboard titration against serogroup homologous immune serum. Specificity was determined by using homologous and heterologous antisera. Although purified polysaccharides prepared by the method of Gotschlich (6) were satisfactory, such preparations were only available for serogroups A, B, and C. Therefore, the less purified antigens were prepared for all serogroups studied.

Erythrocytes. Pyruvic aldehyde-fixed sheep red blood cells (SRBC) were prepared by the method of Ling (11).

Sensitization of RBC. SRBC were washed three times in phosphate-buffered saline, *p*H 7.2 (PBS), and suspended to 4% (v/v) in PBS. The cells were sensitized by mixing equal volumes of washed 4% cells with antigen diluted to its predetermined optimal concentration in PBS. The mixture was incubated at 37 C for 1 hr and washed five times in PBS to remove excess antigen. The sensitized cells were then diluted to 0.5% in PBS containing 0.5% (w/v) bovine serum albumin (BSA). Sensitized cells were stable for at least 1 week when stored at 4 C.

Antisera. Bacterial suspensions were prepared in 0.15 M NaCl from an overnight Mueller Hinton agar culture to match a MacFarland no. 10 barium sulfate standard. New Zealand White rabbits were inoculated by the following schedule: day 1, 0.5 ml of Formalin-treated suspension; days 3 and 5, 1 ml of Formalin-treated suspension; and days 15, 17, 19, 1 ml of living cell suspension. Sera were collected on day 29. Hemagglutination titer of each serum was determined by methods previously described (1). Four units of antisera were used for the HI test.

Cultures. Meningococcal carrier surveys were performed as previously described in a study of Army basic trainees and laboratory personnel (5). The selective growth medium consisted of Mueller Hinton agar containing 5% (v/v) defibrinated sheep blood, chocolated at 80 C to which 6 μg of Lincocin per ml and 25 units of polymixin B sulfate per ml were added.

All suspected meningococcal cultures were confirmed by demonstration of oxidase activity, gram-negative diplococci in Gram stains, and fermentation of glucose and maltose but not sucrose.

Bacterial suspensions in PBS-1% (w/v) BSA were prepared from positive cultures, either from the original plate or after one or two transfers, and inactivated by heating at 56 C for 2 hr.

The killed suspensions were centrifuged for 10 min at 2,500 $\times$ g, and the clear supernatant fluids were used. These fluids could be used immediately or after storage for at least 6 months at -20 C.

HI test. A 0.05-ml amount of the supernatant fluid was mixed with 0.05 ml of each of the eight group-specific meningococcal antisera in "U" bottom Microtiter plates (Linbro Chemical Co.). Each antiserum contained four units per 0.05 ml. Each unknown was tested against eight different antisera in separate wells and incubated at 37 C for 30 min. Then 0.05 ml of sensitized cells [0.5% (v/v)] was added; the plates were sealed with transparent tape, gently mixed, and incubated at room temperature on a vibration damping platform for 2 hr. Hemagglutination patterns which were difficult to read at 2 hr could be improved by further incubation overnight at 4 C.

A positive test was indicated by complete inhibition of agglutination as shown by a clear small button of cells in the bottom of the well surrounded by a clear supernatant fluid. Negative reactions were indicated by any pattern of agglutination.

BA tests. For the standard bacterial agglutination (BA) tests, moderately heavy suspensions of cells in 0.15 M NaCl were made from the same cultures used for preparing suspensions for the HI test. BA was performed by using plastic trays. One drop of each of eight different group-specific rabbit antisera was placed in one of each of eight different wells, and one drop of the bacterial suspension of unknown serological group was placed in each of these wells. Each drop of antiserum contained four agglutinating units of antibody. The mixtures were shaken at room temperature for 3 min, and agglutination was read. Agglutination was graded from + to 4+, but any agglutination was considered positive.

RESULTS

A number of preliminary experiments were performed to define the optimum conditions for the HI test.

Fresh, formaldehyde-fixed (9), pyruvic aldehyde-fixed (11), and formaldehyde-pyruvic aldehyde-fixed (9) erthrocytes were compared. Pyruvic aldehyde-fixed SRBC were selected for use because they were stable at 4 C for at least 6 months, displayed considerable affinity for the antigens, and yielded more homogeneous suspension and better defined agglutination patterns than the other erythrocyte treatments.

Living, Formalin treated, β-propiolactone-treated, or heat-killed bacterial cell suspensions were investigated. Living cell suspensions were considered unsatisfactory because of the obvious safety hazards involved.

In the HI test, supernatant fluids prepared from Formalin-treated suspensions resulted in patterns of complete inhibition (settling of SRBC as buttons in all wells). Inactivation of suspensions with β-propiolactone resulted in complete agglutination of SRBC (no inhibition). Heat killing of bacterial suspensions was used and gave clear patterns of specific HI.

Extraction of group-specific antigens from unknown cultures was carried out at 56 C by using various times and various suspending fluids, including 0.15 M NaCl, PBS, PBS-BSA, and 0.3% (w/v) Trypticase in 0.15 M NaCl. Best results were obtained with PBS-1% (w/v) BSA as suspending fluid and heating for 2 hr.

A summary of the results of BA and HI serogrouping on 476 carrier strains of meningococci is given in Table 1.

There was agreement between the two tests for 381 (80%) of the strains. Of these, 218 strains were identified as a specific serogroup; 163 strains were nongroupable by both methods (Table 2). Of the nongroupables, 74 were smooth nonagglutinable and 89 were multiply agglutinated or rough by the BA scheme and noninhibitory in the HI assay. Another set

TABLE 1. *Comparison of HI and BA tests on 476 meningococcal isolates*[a]

Result	No. of strains	Per cent of total
BA and HI agree	381	80.0
HI groupable, BA nongroupable	82	17.2
BA and HI disagree	13	2.8

[a] HI, hemagglutination inhibition; BA, bacterial agglutination.

TABLE 2. *Distribution of serogroups identified by BA and HI tests*[a]

Result		No. of strains of indicated serogroup								
		A	B	C	Y	29E	WR135	X	Z	Non-groupable
BA and HI agree		2	27	110	69	9	0	1	0	163
HI groupable	{MA[b]	0	2	16	5	0	2	2	2	
BA nongroupable	{RO	0	3	1	0	1	0	1	1	
	{NA	0	25	5	3	11	2	0	0	

[a] BA, bacterial agglutination; HI, hemagglutination inhibition.
[b] MA, multiple agglutination; RO, rough, agglutinates in saline; NA, smooth, nonagglutinable.

of 82 strains was identified as to serogroup by HI test but could not be identified by BA. Of these, 46 were nonagglutinable and 36 were multiply agglutinable or rough. Strains of all but group A were found in this category (Table 2).

Disagreement between the results of the two tests was observed in only 13 (2.8%) of the cultures. Of these strains, six were nongroupable by BA and gave double reactions by HI. The HI results suggest simultaneous carriage of multiple serogroups. Six other strains were groupable by BA and not groupable in HI tests. Finally, only one strain was grouped differently in both assays. Tests on this strain were repeated three times. Each time the HI test gave the same result (serogroup B). In one test, BA was recorded as serogroup Y; in the other two trials, the culture was nongroupable.

HI and BA tests were performed on 24 meningococcal cultures derived from blood or cerebrospinal fluid. Each of these strains had been serogrouped by BA prior to lyophilization but, at the time of reculture, four strains were multiply agglutinated in the BA test. All cultures were serogroupable by the HI test.

DISCUSSION

HI is a well known serological tool for demonstrating similarities between antigens and has been particularly valuable in studies of polysaccharide antigens derived from bacteria. A limited HI system was used several years ago in this laboratory to identify group C meningococci in a large field study with good results (8). With the use of eight different polysaccharide hemagglutinating systems described herein, it has been feasible to develop a precise method for serogrouping meningococci. This system has a number of practical advantages. Grouping antisera are used at high dilution (HA titers are at least 100-fold greater than BA titers), thus conserving this reagent. Sensitized red cells are stable for at least 1 week, and experiments are in progress to test lyophilized red cells. Greater specificity and sensitivity of the HI test over the BA assay is indicated by the current results. The presence of only six strains that typed in two groups by HI as compared to 125 that were multiply agglutinable or rough and agglutinable in saline by BA demonstrates the superior specificity of the HI test. In addition, the 46 strains that were typed by HI but smooth and nonagglutinable by BA demonstrate the greater sensitivity of the HI test. Finally, many cultures were grouped by HI from the initial plate, whereas BA grouping often required several transfers in the laboratory.

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