Bactericidal Antibodies to *Hemophilus influenzae*[1] (35835)

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Fothergill and Wright (1) first showed that all sera obtained from subjects of 10 or more years of age are bactericidal for encapsulated *Hemophilus influenzae,* while sera obtained from several months to 3 to 4 years of age infrequently showed such bactericidal activity. It is now well established that the bactericidal activity of serum is due to antibody and complement (2). The development of antibodies to encapsulated *H. influenzae* at around 4 years of age has been cited as the explanation for the relative rarity of encapsulated *H. influenzae* infection in school-age children and adults (1, 3, 4). However, Norden *et al.* (3), recently reported that 8 of 29 sera collected from normal adults lacked such bactericidal antibodies for *H. influenzae,* Type B, and suggested that larger populations of adults should be studied to see if their group of adults was representative of the general population. In the bactericidal assay procedures used by Norden *et al.*, all sera were first heated for 1 hr at 56° prior to carrying out the test with added complement. It has been shown that heating serum at 56° for 30 min will inactivate complement, and will also inactivate variable amounts of antibody and result in a decrease in the bactericidal titer. This effect is not due exclusively to the lack of adequate amounts of complement since even when complement is added in excess, the bactericidal titer of the heated serum is not restored (2).

In an editorial, Weinstein (4) commented on the publication of Norden *et al.* and stated that perhaps the prevalence of *H. influenzae,* Type B, bactericidal antibodies was now much lower in the general population than in 1933, at the time of the Fothergill and Wright studies, because of generally rising hygienic conditions. He went on to urge that more extensive studies be carried out to see if the incidence of these antibodies was truly lower now, since a change in herd immunity might portend an increase in serious *H. influenzae,* Type B, infections in adults.

This study was carried out to evaluate the present incidence of bactericidal antibodies to *H. influenzae* in a group of 130 adolescents and adults. A group of 19 patients with chronic bronchitis was also included because of the prevalence of unencapsulated *H. influenzae* bronchial infections in chronic bronchitis (5). All sera evaluated for bactericidal antibodies against the *H. influenzae,* Type B, were also tested for bactericidal activity against the unencapsulated *H. influenzae* isolate.

Materials and Methods. Subjects. Sera were obtained from 130 healthy subjects ranging in age from 13 to 78 years. None of these individuals were taking antibiotics. The 19 chronic bronchitis patients ranged in age from 31 to 74 and were also not taking antibiotics at the time they were bled for these studies. All these sera were stored at —30° until used. Five sera were obtained from agammaglobulinemia patients immediately prior to their monthly replacement dose of intravenous plasmin-treated gamma globulin. These were kindly supplied by Dr. Chester A. Alper, Harvard Medical School, Boston.

The *Hemophilus influenzae,* Type B, was obtained from the American Type Culture Collection, No. 9795. This organism was confirmed to be a Type B, *H. influenzae* by using rabbit Type B antisera in the standard Quellung reaction. The unencapsulated *H.*

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TABLE I. Incidence of Hemophilus Bactericidal Antibodies in Normal Persons and Chronic Bronchitis Patients.

Population	*H. influenzae* bacteria	Bactericidal activity	
		Present	Absent
Normal	Type B	128	2 (1.5%)
	Unencapsulated	109	21 (16.2%)
Chronic Bronchitis	Type B	19	0
	Unencapsulated	19	0
Agammaglobulinemia	Type B	5	0

influenzae was isolated from a patient with chronic bronchitis, and was confirmed to be an unencapsulated organism because of the lack of a Quellung reaction when exposed to a polyvalent antiserum for *H. influenzae,* Types A through F (Difco Lab., Detroit, Mich.), and because of the absence of an iridescent sheen when cultured on Levinthal's agar (5).

Bactericidal test. The hemophilus isolates were grown in a brain heart infusion broth to which were added paper strips containing X and V factors (Baltimore Biological Lab., Baltimore, Md.). This medium supported luxuriant growth of both organisms. An overnight broth culture, containing approximately 8×10^9 organisms/ml was diluted 1:2000 in sterile brain heart infusion broth and 0.05 ml of the diluted culture was added to 0.15 ml of unheated serum. Tubes containing the bacterial suspension and serum were shaken for several minutes, incubated in a 5% CO_2 atmosphere for 18 to 24 hr at 37°, and then were subcultured on chocolate agar plates using a calibrated loop containing 0.01 ml. These plates were, in turn, incubated in 5% CO_2 for 18–24 hr and the number of colonies was determined. If one colony was present on subculture, the serum was considered to lack bactericidal antibody.

Fluorescent antibodies. These were measured to the unencapsulated *H. influenzae* using the indirect fluorescent antibody (IFA) technique (6). Fluorescein-labeled goat antihuman gamma globulin (Baltimore Biological Lab., Baltimore, Md.) and goat antihuman immunoglobulin G (Meloy Lab., Falls Church, Va.) were used in the IFA technique. The slides were viewed with a Zeiss fluorescence microscope equipped with an HB 200 mercury vapor bulb, a BG 12 exciter filter and a $\frac{50}{0}$ barrier filter.

Results. Table I shows that only 2 of the 130 sera obtained from randomly selected persons ranging in age from 13 to 78 years lacked bactericidal activity to *H. influenzae* Type B. One of these sera was from a 49-year-old man and the other was from a 41-year-old woman. Ten colonies of hemophilus were grown on subculture of the man's bactericidal test and >100 colonies from the woman's bactericidal test. Serum from the man was bactericidal for the unencapsulated *H. influenzae,* whereas the woman's serum also lacked activity against the unencapsulated organism.

Far fewer of the persons demonstrated bactericidal antibodies to the unencapsulated *H. influenzae.* The sera from 21 individuals (16%) were found to lack such bactericidal activity. From 10 to >100 colonies of hemophilus were obtained on subculture of the bactericidal test from 13 of the 21 sera lacking bactericidal activity, and there were <10 colonies present in the case of the remaining 8 sera.

All 19 of the chronic bronchitics showed bactericidal activity against both of the hemophilus isolates used.

All 5 of the agammaglobulinemic sera were bactericidal for the *H. influenzae,* Type B. Using the radial immunodiffusion method, immunoglobulin levels were determined in two of these sera and immunoglobulins A (IgA) and M (IgM) were undetectable while immunoglobulin G (IgG) levels were 440 and 390 mg/100 ml.

Using the unencapsulated *H. influenzae*

organism as the antigen in the IFA technique, we determined the hemophilus antibody titers on the following sera; 4 normal sera with no bactericidal activity against the unencapsulated isolate, 1 normal serum with no bactericidal activity against either the unencapsulated or the Type B organism, 3 normal sera with activity against both organisms and the 19 sera from the chronic bronchitis patients. The titers of the normal sera ranged from 1:10 to 1:80 and the sera which lacked bactericidal activity had just as high antibody titers as those sera which demonstrated such activity. All of the 19 sera from the chronic bronchitis patients had hemophilus antibody ranging in titer from 1:10 to 1:640.

To determine the effect of heating on hemophilus antibodies, 3 sera from a patient with chronic bronchitis were heated at 56° for 1 hr and the IFA titers to the unencapsulated hemophilus isolate were measured before and after heating the serum. All 3 sera had preheat titers of 1:640; whereas, after heating the 3 sera had reduced titers to 1:40, 1:60, and 1:80.

Discussion. These results indicate that sera obtained from a randomly selected group of persons ranging in age from 13 to 78 years contain a high incidence of bactericidal antibodies to *H. influenzae*, Type B (98.5%) and agree with the earlier findings of Fothergill and Wright (1) and do not support the hypothesis of Weinstein (4) that herd immunity to *H. influenzae*, Type B may have declined. The failure of Norden *et al.* (3) to find such antibodies in 8 of 29 healthy adults is probably due to the fact that they heated their sera at 56° for 1 hr prior to testing. As has been shown by Muschel and Treffers (2), heating at 56° for even 4 min can decrease antibody titer. We have demonstrated a striking fall in the immunofluorescent antibody titer to an unencapsulated *H. influenzae*, when sera are heated at 56° for 1 hr demonstrating the heat lability of these antibodies. There is some controversy (see below) as to whether the antibodies detected by the IFA technique are responsible for the bactericidal activity of serum. Nonetheless, the heat lability of immunofluorescent antibodies to an unencapsulated *H. influenza* implies that bactericidal antibody might also be heat labile under the conditions used to inactivate complement.

It would thus appear that in any studies of bactericidal antibody serum should not be heated to inactivate complement and most investigators performing such studies have avoided heating sera used in such studies (7–10). Furthermore, there seems to be no particular necessity for destroying the complement contained in the serum to be assayed for bactericidal activity. Some workers have utilized the complement contained in the serum sample to be assayed without adding additional complement (7, 9), others have added additional complement (8) and still others have performed the assay either with or without added complement (10). In any case, it is deficiency of complement, rather than a complement excess, which must be avoided in carrying out bactericidal studies.

The far more frequent lack of bactericidal antibodies to the unencapsulated *H. influenzae* isolate which we observed in 21 (16%) of 130 sera is difficult to explain. Fothergill and Wright (1) noted that all the sera which they tested from each age group were bactericidal for an unencapsulated *H. influenzae* (1). It might be suggested that these 21 sera lacked sufficient complement to mediate the bactericidal reaction, but this is obviously incorrect since 20 of these 21 sera were bactericidal for the *H. influenzae*, Type B, organism. One possible explanation is that these sera lacked antibody to cell-wall antigens which are specific for the unencapsulated organism. However, the fluorescent antibody studies do not seem to bear this explanation out, since five of the sera which lacked bactericidal activity against the unencapsulated *H. influenzae* demonstrated IgG antibody to this organism in titers of 1:20 to 1:40, quite comparable to the titers of 3 sera which demonstrated bactericidal activity. The question then arises as to how well do the immunofluorescent antibodies correlate with antibodies as measured by the bactericidal technique?

Goldschneider and co-workers (10), in a study of both fluorescent and bactericidal antibodies to meningococci, found a high de-

gree of correlation between these two methods of antibody measurement. However, three sera which were bactericidal to meningococci failed to show any IgG, IgM, or IgA meningococcal antibodies as measured by immunofluorescence. However, they did not find any sera containing antibodies as measured by the IFA technique, which did not contain bactericidal antibodies. Norden *et al.* (3) found four of eight sera which lacked bactericidal activity to the Type B *H influenzae*, to contain antibodies to this organism as measured by the IFA technique. This discrepancy could be explained by the fact that they heated the sera used in the bactericidal test as already commented upon. It appears as if it is IgG which is the bactericidal antibody, at least in the case of *H. influenzae* (3). Thus, we have no explanation for the lack of correlation we observed between the IgG titers we measured to the unencapsulated *H. influenzae* and the bactericidal activity.

The lack of bactericidal activity against unencapsulated *H. influenzae* in 16% of normal sera is probably of little clinical significance since this organism is relatively nonpathogenic in man (5). The only disease with which it has been prominently associated is chronic bronchitis (11). In this disorder, the bronchial tree, normally a sterile area, often contains these organisms (12). Because a lack of bactericidal antibodies against this organism might be one explanation for the susceptibility of chronic bronchitics to the long-term bronchial infection with unencapsulated *H. influenzae*, we studied sera collected from 19 such patients with chronic bronchitis in order to measure bactericidal antibodies to both the unencapsulated and the Type B organism. All of these patients demonstrated bactericidal antibodies to both hemophilus isolates and, therefore, any lack of bactericidal activity is unlikely to be an explanation for the chronic carriage of *H. influenzae* by patients with chronic bronchitis.

The bactericidal activity demonstrated by the agammaglobulinemic sera is not surprising considering that the IgG levels, 440 and 390 mg/100 ml in the two sera in which they were measured, were still quite adequate 30 days after the last gamma globulin therapy. Gamma globulin levels of 200 mg/100 ml are considered sufficient to prevent invasive bacterial infection (13). The fact that two of these sera had easily detectable levels of IgG and yet undetectable levels of IgM and IgA, underlines the apparent critical role of IgG in the bactericidal reaction.

Summary. Studies on the incidence of bactericidal antibodies to a *Hemophilus influenzae*, Type B, and an unencapsulated *H. influenzae* were carried out on sera from 130 healthy individuals, ranging in age from 13 to 78, and from 19 chronic bronchitics. Of the 130 sera from normal people only 2 (1.5%) lacked bactericidal antibodies to the *H. influenzae*, Type B, whereas 21 (16%) lacked bactericidal antibodies to the unencapsulated *H. influenzae*. All of the 19 sera from the chronic bronchitics were bactericidal for both *Hemophilus* isolates. All five sera from agammaglobulinemic patients who were on gamma globulin replacement therapy demonstrated bactericidal activity against the *H. influenzae*, Type B isolate.

Utilizing the indirect fluorescent antibody technique, no correlation was apparent between the presence or absence of bactericidal antibodies and antibody levels as measured by immunofluorescence.

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