NOTES

Separation of Serum Bactericidal and Opsonizing Activities for *Haemophilus influenzae* Type b

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When assay conditions permitted bacterial growth, immune serum had no bactericidal activity for *Haemophilus influenzae* but promoted rapid phagocytic killing of this organism by human leukocytes.

In an attempt to elucidate the biology of host defense against *Haemophilus influenzae* type b, we previously studied the ability of serum to enhance the phagocytosis of this organism by human leukocytes (1, 2, 5, 6). Serum from normal adults containing its own complement activity exhibited significant phagocytosis-promoting (opsonizing) activity, whereas serum from four of five children with congenital agammaglobulinemia and one patient with C3 deficiency lacked such activity (1, 5). Serum from adults vaccinated with the capsular polysaccharide of type b *H. influenzae* showed a prompt and sustained rise in opsonizing activity after vaccination (2). Specific immunoglobulin (Ig)G, anticapsular antibody purified by affinity chromatography from postvaccination serum had opsonizing, as well as bactericidal and hemagglutinating activity in the presence of added complement (6).

Since almost all sera with opsonizing activity also had bactericidal activity, the question arose as to whether or not phagocytosis is necessary for defense against this organism. In this regard, Ward and Wright had shown in 1932 that serum alone had as much bactericidal activity for *H. influenzae* as whole blood did (11). In addition, Fothergill et al. reported that virulent *H. influenzae* could survive inside phagocytes from the cerebrospinal fluid (4). We present here evidence to indicate that circulating human phagocytes kill type b *H. influenzae* rapidly. Moreover, under conditions which permit bacterial growth, serum may lack effective

bactericidal activity yet efficiently promote phagocytic killing of the organism.

The bacterium (ATCC strain 9795) was maintained as previously described (1). A log-phase culture was centrifuged at $5,000 \times g$, resuspended in Levinthals broth to an optical density of 0.070 at 590 nm (3), and then diluted with 20 volumes of broth. A previously described modification of the method of Quie (10) was used to study phagocytic bactericidal activity (3), except as noted here. The reaction mixture consisted of 2.5×10^6 peripheral blood phagocytes (primarily neutrophils) in 0.6 ml of Krebs-Ringer phosphate buffer, approximately 4×10^6 bacteria in 0.3 ml of broth, and 0.1 ml of high-titered immune serum from an adult vaccinated with capsular polysaccharide (2) or serum from a pool of nine normal adults. Sera were processed to preserve complement activity (8) and thus served as their own complement source. The immune serum had a hemagglutination titer of 1:256 and a bactericidal titer of 1:128, and the normal serum had a hemagglutination titer of 1:8; all assays were performed by Porter Anderson, using published techniques (1). Enrichment medium (Isovitalex, BBL Division) (1% vol/vol) was added to the Krebs-Ringer phosphate buffer, which also contained 0.2% glucose and 0.2% bovine serum albumin. Dilutions of the reaction mixture (1:180 and then 1:18) were made in 9 ml of 0.4% bovine serum albumin, and leukocytes were disrupted at both dilutions by sonic disruption in a "cup horn" (Heat Systems-Ultrasonics, Inc., Plain-

view, N.Y.) for 1 min at 60 W. The second dilution (0.1 ml) delivered by micropipet (Biolab, Inc., Derry, N.H.) was spread on the surface of chocolate agar with enrichment medium, and colonies were counted the next day.

Two modifications of the original assay (10) require explanation. The addition of albumin to the water for dilution was necessary because *H. influenzae* did not survive well in water. In turn, the presence of albumin necessitated that the leukocytes be disrupted (for release of viable bacteria) with sonic disruption rather than agitation. To demonstrate that phagocytes are, in fact, disrupted by this technique, 2.5×10^6 phagocytes were suspended in 9 ml of 0.4% bovine serum albumin, sonically treated, centrifuged, and resuspended in buffer. The sonically treated phagocytes lost all ability to ingest *Latex* particles or pneumococci, as indicated by reduction of nitroblue tetrazolium dye (8), or to kill *H. influenzae*, whereas control phagocytes which were not sonically treated performed normally in both assays. Stained smears made of centrifuged leukocyte preparations after sonic disruption showed no intact phagocytic cells.

As shown in Fig. 1A, the number of viable bacteria in the reaction mixture with immune serum and phagocytes was rapidly reduced, more than one-half the bacteria being killed in 20 min and more than 95% being killed by 35 min. In a second experiment summarized in

Fig. 1B, there was no phagocytic killing in the absence of serum as a source of opsonins, and there was no effective bactericidal activity by immune serum alone in the absence of phagocytes. When the immune serum present with phagocytes had been heated to 56 C for 30 min, bacterial killing was insignificant; yet killing was greater than that achieved in the absence of serum (Fig. 1B), in agreement with previous studies showing decreased but definite opsonization by heated immune serum (5). When leukocytes and bacteria were suspended in buffer from which growth factors for *H. influenzae* had been deleted, the number of viable bacteria remained approximately constant for 60 min in the presence of phagocytes alone or in the absence of serum and phagocytes, but the bactericidal effect of pooled normal serum was marked (Fig. 1C). In the presence of 0.05 ml of immune serum, bacterial killing was almost identical to that shown here with 0.1 ml of normal serum (data not plotted).

Rapid killing of bacteria by phagocytes, reported here for type b *H. influenzae*, has been described with other extracellular bacteria (8-10). When the in vitro milieu was favorable for growth of *H. influenzae*, the bactericidal effect of immune serum alone was negligible, whereas that of serum and phagocytes was marked. This is in contrast to the efficient bacteriolysis achieved by immune or normal serum when we used a buffer which did not

FIG. 1. Killing of *H. influenzae*, type b, by serum and phagocytes or serum alone. The number of viable bacteria per 1 ml of reaction mixture is plotted as a function of time. In the experiments represented by graphs A and B, growth factors for *H. influenzae* were present in the reaction medium; these factors were omitted in experiment C.

permit bacterial growth, and to the findings of Ward and Wright (11), in which serum bactericidal activity was so effective that the killing achieved by phagocytes could not be clearly determined. These differences demonstrate the importance of the suspending medium to results obtained, using *H. influenzae* in phagocytic bactericidal assays, and emphasize that caution must be exercised in extrapolating from in vitro results to the in vivo situation. In this regard, the ability of *H. influenzae* to multiply in the suspending medium of an assay would seem to approximate the in vivo situation more closely than an inability to do so. For this reason, it would perhaps be advisable that the bacteria be able to multiply in the absence of the test serum in any assay of serum or phagocytic bactericidal activity designed to estimate the status of immunity in the patient.

Considerable indirect evidence has accumulated in recent years to indicate that extracellular pathogens (pyogenic and most enteric bacteria) must be cleared from the body primarily by the process of phagocytosis. Our results do not prove that the role of serum in host defense against *H. influenzae* is mediated through opsonization rather than bacteriolysis, but they do remove the theoretical objection to this viewpoint posed by the inability to separate serum bactericidal from opsonizing activity in vitro. In agreement with the concept that bacteriolysis plays a minor role in host defense are the findings that patients with a leukocyte defect of phagocytic killing often die from infections due to enteric bacteria, which are easily killed by serum in vitro (7), and that a patient with absence of the sixth complement component (and thus absence of bacteriolysis but not opsonization) had had no difficulty with infections (J. P. Leddy, M. M. Frank, T. Gaither, R. S. Heuskinveld, R. T. Breckinridge, and M. R. Klemperer, *J. Clin. Invest.* 52:50a, 1972).

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