Direct Hemagglutination by *Hemophilus influenzae*.* (28637)

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(Introduced by Stanley Levey)
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Hemagglutination is a well-established property of certain viruses, notably the myxovirus group. Certain bacteria possess the property of agglutinating erythrocytes(1) and this ability has been suggested as a differential criterion(2) for separating *Hemophilus aegyptius* (Koch-Weeks bacillus) from *H. influenzae*.

From a teleological point of view, it would seem odd that two organisms so closely related (if not identical) in all their basic properties could be separated on the basis of erythrocyte agglutination. Consequently, a reinvestigation of the capacity of *H. influenzae* to directly hemagglutinate mammalian erythrocytes was conducted. Data to be presented demonstrate that this organism is capable of directly agglutinating mammalian erythrocytes under proper conditions.

Materials and methods. Organisms and culture conditions. Thirty-five organisms isolated from patients admitted to the Communicable Disease Service of the Los Angeles County General Hospital over a period of 1½ years were employed in this study. Thirty-three isolates of *H. influenzae* type b and one of type f were obtained from spinal fluid of patients with purulent meningitis. One strain identified as *H. aegyptius* was obtained from a patient with acute conjunctivitis.

Organisms were grown in Eagle's minimum essential medium (MEM)(3) containing 1% Supplement B (Difco) or nicotinamide adenine dinucleotide (NAD) (1.0 mg/liter) and hemin (10 mg/liter) in stationary culture at 37°C. Turbidity of bacterial cell suspensions was measured at 660 mμ employing a Coleman Spectrophotometer (Model 6A).

Hemagglutination (HA) test. Red blood cell suspensions were prepared daily from sheep erythrocytes stored in Alsever's solution (Hyland Laboratories), freshly drawn guinea pig blood or "outdated" human type "O" blood. Test incubation times were varied from one to 24 hours at 37°C (water bath), room temperature and 4°C while erythrocyte concentrations were varied from 0.1% to 1.0%. The final choice of test conditions was based upon reproducibility, clarity of end points, the magnitude of HA titer and ease of preparation.

Results. Hemagglutination test: Hemagglutinin titrations were finally standardized as follows: equal volumes (0.5 ml) of serial 2-fold dilutions of modified MEM grown cultures and 0.5% suspensions of washed sheep erythrocytes were shaken and incubated for 2 hours in a 37°C water bath followed by 2 hours incubation in the cold (4°C). Though comparable titers were obtained by overnight incubation at room temperature or at 4°C, the preliminary 37°C water bath incubation accelerated the reaction and permitted earlier reading of the test. Guinea pig erythrocytes consistently gave titers which were one tube higher than that obtained with sheep red blood cells or human "O" cells. However, the final choice of sheep erythrocytes was based more on their commercial availability rather than on titers obtained since the significance of a one-tube differential is to be questioned. The choice of the 0.5% suspension was based on the magnitude of HA titer and the clarity of the end points produced.

Hemagglutination by Hemophilus isolates. Preliminary experiments were carried out with 24-hour, unconcentrated cultures of all the Hemophilus strains available. Only 2, *H. aegyptius* and H-86 (*H. influenzae* type b), were capable of causing hemagglutination under the conditions described above. *H. aegyptius* agglutinated to a titer of 1:4 while H-86 strongly agglutinated only as an undiluted suspension. Since the turbidity of the latter culture appeared intermediate between that of *H. aegyptius* and all other iso-

* This study was supported by grants from Nat. Inst. Health and Division of Air Polution, P.H.S.

TABLE I. Influence of Culture Age and Cell Concentrations on HA Titers of *H. influenzae* and *H. aegyptius*.

Isolate	Type	Days incubation	Culture conc	Titer: Undil	1:2	1:4	1:8	1:16	1:32	1:64	1:128
H-118	b	1	1×	—	—	—	—	—	—	—	—
			10×	+	+	±	—	—	—	—	—
		5	1×	+	—	—	—	—	—	—	—
			10×	+	+	+	—	—	—	—	—
H-86	b	1	1×	+	±	—	—	—	—	—	—
			10×	+	+	±	—	—	—	—	—
		3	1×	+	+	—	—	—	—	—	—
			10×	+	+	+	+	+	—	—	—
		5	1×	+	+	+	—	—	—	—	—
			10×	+	+	+	+	+	—	—	—
		9	1×	+	+	+	—	—	—	—	—
H-125	f	3	1×	+	+	—	—	—	—	—	—
			10×	+	+	+	+	+	±	—	—
H-5	b	1	1×	—	—	—	—	—	—	—	—
		4	1×	+	+	±	—	—	—	—	—
			10×	+	+	+	+	+	—	—	—
H-70	b	1	1×	—	—	—	—	—	—	—	—
		5	1×	+	+	+	+	—	—	—	—
			10×	+	+	+	+	+	+	+	±
H. aegyptius		1	1×	+	+	+	—	—	—	—	—
			10×	+	+	+	+	+	+	—	—

lates, it was reasoned that the presence or absence of hemagglutination by these organisms was directly dependent upon the concentration rather than the absolute presence or absence of an hemagglutinin.

To demonstrate that all isolates produced an hemagglutinin, cells were grown in stationary culture for 14 days and aliquots were tested daily for hemagglutination directly (1X) or as concentrated cell suspensions (10X). For concentration, 40 ml samples were centrifuged at 2500 RPM for 30 minutes, 36 ml of supernatant medium was discarded and the cell button resuspended in the remaining 4 ml of medium.

With the 1X cell suspensions, the hemagglutinin concentration increased with increasing age of the culture. In all cases the peak HA titer was achieved by the fifth day and remained constant for an additional 2 to 7 days. When tested as 10X suspensions of 24-hour cultures, positive HA tests were obtained with the 33 isolates of *H. influenzae* that failed to show hemagglutination as 1X suspensions. Representative data obtained with 6 of the isolates employed is recorded in Table I.

Isolate H-86 was employed to quantitate the relationship of culture turbidity to HA titer. Stationary cultures of H-86 incubated for 3 days were centrifuged and adjusted with sterile medium or saline to various cell concentrations as measured by optical density. The HA titer was directly related to the concentration of organisms and each titer was restricted to a relatively narrow range of optical density values (Table II). No

TABLE II. Relationship of Culture Turbidity to HA Titer with Isolate H-86.

Range of optical density	Titer: Undil	1:2	1:4	1:8	1:16	1.32
008-.020	+	—	—	—	—	—
022-.081	+	+	—	—	—	—
097-.143	+	+	+	—	—	—
.143-.300	+	+	+	+	—	—
.310-.569	+	+	+	+	+	—
620-.866	+	+	+	+	+	+

hemagglutination was observed if the optical density of the cell suspension was less than 0.008.

The ability of the isolates to hemagglutinate is not lost by continued daily passage of the organisms for a 2-week period. When in-

cubated in shake culture, the hemagglutinin appeared in 1-3 days and, with the majority of isolates tested, the maximum titers were not significantly altered. Similar results were obtained when cells were grown in Brain Heart Infusion Broth, (Difco), GC Medium (Difco) or Eugon Broth (BBL) containing 1% Supplement B.

Type-specific animal antisera and human convalescent sera were found to inhibit the direct bacterial hemagglutination. An hemagglutination-inhibition test employing a standardized hemagglutinin for determination of antibody titer is currently under study.

Discussion. Direct bacterial hemagglutination has been demonstrated with *H. pertussis*† (4,5,6), *H. parapertussis*†(4), *H. bronchiseptica*†(4), and *H. aegyptius*(2). In contrast to these species it has been reported that *H. influenzae* although capable of indirect hemagglutination(7,8) does not, with the exception of a few strains(2,9), directly agglutinate mammalian erythrocytes.

From the data obtained in this study it is evident that *H. influenzae* does produce an hemagglutinin but in low concentration and/or of weak reactive ability, and that hemagglutination is a normal property of these species. It is therefore suggested that hemagglutination is not a valid criterion for separating *H. influenzae* and *H. aegyptius*.

Summary. Thirty-four spinal fluid isolates of *Hemophilus influenzae* were found capable of directly agglutinating sheep, guinea pig and human "O" erythrocytes. The hemagglutination titer is directly related to concentration of the organisms employed in the HA test.

The authors wish to express their appreciation to Mrs. Margaret Parrish for typing the isolates employed in this study.

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† Currently placed in the Genus *Bordetella*.

Received July 15, 1963. P.S.E.B.M., 1963, v114.

Culture of Leukocytes after Storage in Serum. (28638)

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Serum is sometimes used in media for tissue cultures without heating or filtering(1), procedures that would destroy or eliminate leukocytes that might be present. Studies were undertaken to determine whether any such cells might be capable of continuous cultivation and so could contaminate tissue cultures. A few cells among relatively large numbers of leukocytes stored for 2 weeks in homologous serum possessed ability to multiply and to initiate serial cultures. One line has been cultured continuously for more than a year.

Materials and methods. Culture medium consisted of 80% solution 199 (Difco) and 20% pooled human serum that had been heated for 30 minutes at 56°C. Leukocytes were derived from laboratory workers who donated 200 ml blood after fasting 12 hours. The blood was allowed to clot at room temperature for 2-4 hours. After overnight refrigeration, most of the serum was removed and centrifuged. The sedimented cells were mixed with cells that were collected by gently rinsing the surface of the clots with the re-