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.

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## 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-

maining serum. For prompt culture, cell suspensions from 4 to 6 donors were pooled and centrifuged to remove erythrocytes. The leukocytes were withdrawn and mixed with culture medium. Cells to be cultured after 2 weeks were stored at 4°C in their corresponding, individual sera. Before culture, these suspensions were pooled and centrifuged. The serum was removed and cells suspended in culture medium. Cell counts were unsatisfactory because of the number of clumped or degenerated cells. However, it was estimated that the inocula contained either approximately $10^5$ day-old cells or $10^4$ two-week cells per ml of medium. The first 7 pools of cells were cultured in Leighton tubes by placing 1 ml of cell suspension on the surface of fibrin clots prepared from frozen and thawed chicken plasma and chick embryo extract.* Transferred cultures were grown directly on glass without fibrin. Plastic flasks† without fibrin were used both to initiate and transfer cultures from the subsequent 3 pools of leukocytes, using 5 ml of culture medium per flask. Homologous pooled serum was used for the first few feedings. Cultures were usually incubated at 36.5°C and fed every 2 or 3 days by replacing half the medium with fresh nutrient. Transfers were made at one- or 2-week intervals following release of the cells by trypsin or scraping. Cultures were occasionally maintained with slow growth at 32°C, requiring feeding at 2-week intervals. Permanent records of cells as they appeared in culture were prepared by inserting 8 × 35 mm strips of DuPont leader film(2) in Leighton tubes before adding cells and medium. After growth had occurred, the strips were removed and the cells were fixed and stained. For preservation, cells were suspended in medium containing 10% glycerol and frozen at —60° C. On thawing, they were sedimented, resuspended in culture medium, placed on fibrin clots on glass or directly on plastic and incubated at 36.5°C.

To determine the effect of heat on early cultures, cells after 2 transfers were released from the culture surface with trypsin, washed, resuspended in Puck's medium N16 in saline F(3), and sealed in glass ampules. These were immersed for 15 or 30 minutes in water at temperatures ranging from 46° to 60°C. The heated cells were added to nearly confluent cultures that had been exposed to 2000 r x-radiation to prevent multiplication. These feeder cells with the test cells were incubated at 36.5°C in an atmosphere containing 5% carbon dioxide and high relative humidity. Cultures were considered negative only after failure to observe increase in cells on 2 subsequent transfers.

*Results.* A few cells multiplied in each of the 10 attempted cultures of day-old cells and in each of the 4 instances where cells stored 2 weeks in serum were used. These growing cells produced widely scattered, small, hemispherical colonies composed of nearly round, granular cells that varied from about 10 to 16 $\mu$ in diameter (Fig. 1). The colonies increased in size until they contained 25 to 35 cells. Then flattened cells grew from the edges and spread as an epithelial sheet. Other areas contained spindle-shaped and a few giant cells. Usually after the second transfer, the cells grew well in a monolayer that covered most of the culture surface (Fig. 2). In early transfers comparatively large cells were present in small numbers. Cinemicrographs showed most of these to have motion characteristic(4,5) of monocytes. A few resembled large lymphocytes. In established lines most of the cells were flat and angular. They were motile and exhibited active pinocytosis. The cytoplasm was clear with small bodies that appeared as dark areas under dark contrast phase illumination. The nuclei were round and most contained well-defined nucleoli. A few had narrow cytoplasmic protuberances where the cells had been attached to the culture surface and then contracted. Occasional multinucleated cells occurred in early and late transfers (Fig. 2 and 3). The cultural characteristics of cells cultured promptly and those cultured 2 weeks after bleeding were apparently identical.

These cell lines, derived from normal adult human blood at Kennedy Hospital, were designated KeNAHB. Some lines were terminated after brief periods of culture, 24 to 164

* Obtained from Microbiological Associates, Bethesda, Md.

† Tissue culture flasks, 3x5 cm, Falcon Plastics Co., Los Angeles, Calif.

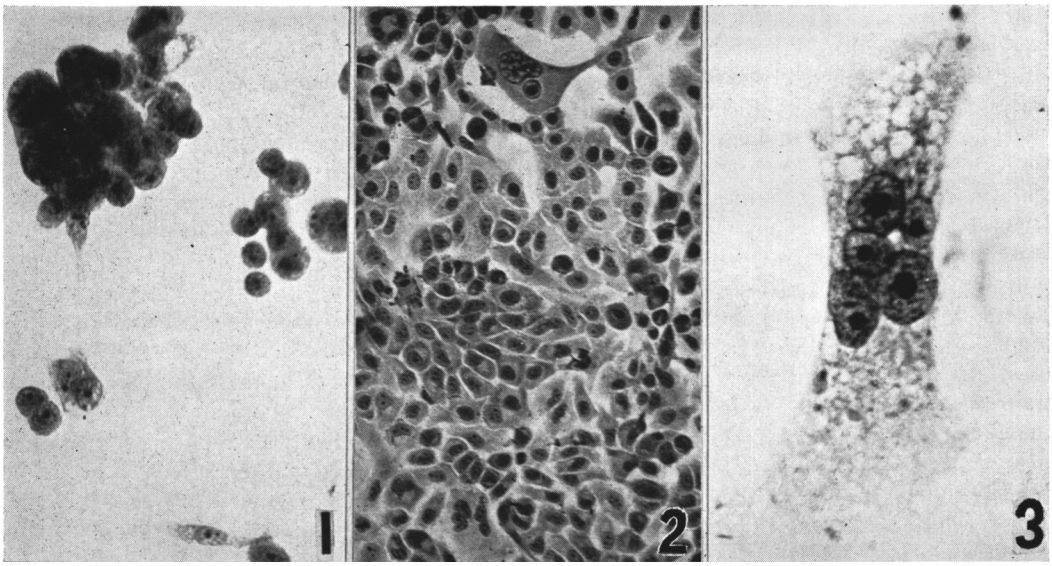


FIG. 1. Leukocyte colony in culture 4 days after planting pooled cells from normal blood. Papanicolaou stain. 300×.
FIG. 2. Established line after culture for 14 mo. Papanicolaou stain. 150×.
FIG. 3. Multinucleated cell in culture after 3rd transfer of line. Papanicolaou stain. 600×.

days, involving 2 to 19 transfers. Continuous culture of line KeNAHB-1, started with day-old cells from 6 donors, was deliberately discontinued when the line had been grown for 615 days and was in the 61st transfer. Another line started with fresh cells, KeNAHB-18 derived from a pool of cells from 4 donors, was grown continuously until contaminated in the 50th transfer after culture for 460 days. Numerous samples of cells after various periods of cultivation were frozen for preservation. On thawing, a sufficient number of cells multiplied to produce transferable cultures. The longest periods of storage and culture were in the instance of a line started from cells stored 2 weeks, KeNAHB-9. This was placed in culture on 3 occasions separated by periods of 7 and 11 months in the freezer. The periods of culture have totalled 28 months, comprising 97 transfers, the longest period of continuous culture being 18 months.

To determine the period of time refrigerated cells would survive, aliquots of leukocyte suspensions that gave growth in cultures started at 1 and 14 days were pooled and cultured after storage for 4 weeks at 4°C. No motile cells were observed in cinemicrographs made after incubation and no evidence of multiplication was obtained by microscopic examination of the cultures and stained films.

The donors were identical for only 2 of the 10 pools. All 21 donors were still in apparent good health and their total and differential leukocyte counts were within normal limits 20 to 41 months after they were bled for culture of cells.

No evidence of multiplication was observed after cells had been heated for 15 or 30 minutes at 56°C. In one of 2 similar experiments, cells multiplied after they were maintained at 48°C for 30 minutes. Unheated cells and cells heated at 40°C for 30 minutes gave good growth. There were no indications of multiplication of irradiated feeder cell controls either directly or on subculture.

*Discussion.* Methods employed for processing serum do not always assure the elimination of leukocytes. In one technique that has been widely used to prepare serum for use in media, it is removed from the contracted clot and centrifuged to clarify without subsequent heating or filtration before use. The heavy centrifugation recommended by Syverton, Scherer and Elwood(6), 30 minutes at 3,000 rpm, is not always performed. If the rate of centrifugation and the time period are not sufficient to form a firm pellet of sediment, the slight currents engendered as the cen-

trifuge slows down and the tubes are handled may raise some of the leukocytes from the surface of the sediment to a point where they are picked up by the aspiration tube. Occasionally, among such cells would be some of those shown by the above experiments to be capable of multiplication in series after refrigeration for 2 weeks, a longer period than is usually required for sterility tests and other procedures before serum is used. The indications are, therefore, that viable cells capable of culture in series are occasionally present in some tissue culture media, but in concentrations far less than were used in establishing the KeNAHB lines. Puck and his associates (7) have shown that single HeLa cells are capable of proliferation in the presence of actively metabolizing cells. Similar cultural conditions would occur when appropriate leukocytes are added in small numbers to an established culture of another line of cells that would act as supportive, feeder cells. Such contamination of cell lines by cells from the serum used in the culture media has been suggested as a possibility by Coriell, Tall and Gaskill(8).

Risk of this type of contamination of cell lines can be eliminated by suitable filtration or heating of serum before use in culture media. In our experiments, heating to 56°C for 15 minutes destroyed the ability of KeNAHB cells to multiply even under specially favorable conditions.

Others have established cell lines under various circumstances from human peripheral blood. Leukemic blood elements were cultured continuously for 6 months or 1 year by Osgood and Brooke(9) using the gradient technique. Berman and Stulberg(10) developed strains of cells from healthy individuals following administration of estrogenic material either to the donor before bleeding or to the culture medium. Paul(11) established strains from buffy coat in 3 of 4 attempts. The identity of the cells that initiated our cultures has not been determined. The cinemicrographic evidence in favor of monocytes is strengthened by the finding of Bond, Cronkite, Fliedner and Schork(12) that monocytes in the peripheral blood synthesize DNA and so are presumably capable of multiplication.

*Summary.* Cell lines were established from leukocytes stored in homologous serum for 2 weeks at 4°C. Similar viable cells may be present occasionally in serum used as a nutrient for tissue cultures and so may initiate mixed cultures. This can be prevented, however, by heating the serum for 15 minutes at 56°C to destroy any leukocytes present.

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