Long-Term Tissue Culture of Human Bone Marrow

I. Report of Isolation of a Strain of Cells Resembling Epithelial Cells from Bone Marrow of a Patient with Carcinoma of the Lung

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Tissue culture of human bone marrow has been concerned mainly with the growth, maturation and behavior of the specific myeloid and erythroid elements, with only occasional references to the activity of the other cells. Osgood's numerous contributions have dealt with marrow cells suspended in a fluid medium; Fieschi and Astaldi have used plasma clot cultures with and without an overlayer of nutrient fluid; and Plum has made use of an elaborate perfusion apparatus. The particular interests of these investigators were studies of the specific parenchymal or hemic cells. On the other hand, Pomerat and his co-workers have used a plasma clot method on coverslips in roller tubes containing fluid medium for making observations of the total behavior of explants of human bone marrow, giving attention to the organization of the stromal tissue and capillaries as well as hemic cells in the outgrowths. The types of materials selected by different investigators were determined by their aims and, to some extent, their aims were limited by the types of material at hand. For example, actual fragments of marrow tissue were used by some, whereas others cultured only the cells suspended in the fluid part of aspirated bone marrow, the bulk of the tissue fragments having been allowed to separate out of the specimen by sedimentation or centrifugation.

At Detroit Receiving Hospital the fragments of organized marrow tissue are separated from the disorganized suspension of free cells in the fluid portion of aspirated marrow. Both types of material are examined and tissue culture procedures have been adjusted to accommodate each (figs. 1 and 2). For short-term studies of such matters as leukocyte and erythroblast survival, mitosis and maturation, the plasma clot methods developed by Fieschi and Astaldi and fluid suspension methods based on Osgood's ideas are quite adequate. However, they are not as well adapted to the study of long-term cultures for the purpose of investigating the reactions of the stromal elements whose behavior is also of

* Because common morphologic terms have different meanings among anatomists, pathologists, hematologists and tissue culture workers it is necessary to indicate the meanings implied by our usage. It is difficult to find a satisfactory term to embrace the various kinds
Fig. 1. Migration area of a culture of an explanted fragment of bone marrow from a patient with pulmonary tuberculosis and macrocytic anemia, at 29 hours. Most of the cells, which are migrating directly on the glass surface of a coverslip, are polymorphonuclear neutrophils. 69X. May-Grünwald-Giemsa stain.

Fig. 2. Cells on glass in the original explant area from the same culture illustrated in figure 1, at 122 hours. Fibroblastic cells are intermingled with some persisting myeloid cells. In the lower left hand corner of the field a mitotic figure is visible. 69X. May-Grünwald-Giemsa stain.

interest to hematologists, especially with respect to the myeloproliferative syndromes in which fibrosis may occur as an end stage, or to the myelophthisic conditions in which the marrow is affected by the presence of metastatic tumors.

In the course of devising methods for long-term studies of human bone marrow it was observed that our cultures passed through three phases similar to those reported by others who used classic plasma clot techniques. In the first phase the myeloid cells remain identifiable (figs. 3 and 4) and, while there is mitotic activity (fig. 5), especially among progranulocytes, myelocytes and erythroblasts, there is a continuous decline in their number.* The second phase is characterized by increasing numbers of large round cells, often of monocytoid appearance, which become attached to glass (figs. 6 and 7). These include the histioid cells and macrophages mentioned by others in plasma clot cultures.† The third phase, often overlapping the second for one or more days, begins with the appearance of cells other than hemic cells which may be present in marrow. The specific parenchymal cells of marrow are considered to include those which are specific for myeloid tissue, namely, erythroblasts, granular leukocytes and their precursors, as well as megakaryocytes. Other hemic elements present in marrow include lymphocytes and monocytes and their precursors, but these are not peculiar to bone marrow, as they are also formed in extramedullary tissues. The non-hemic cells include osteoblasts, osteoclasts, plasma cells, fat cells, tissue mast cells, vascular endothelial cells, fibrocytes and fibroblasts, muscle cells of blood vessels, reticulum cells and probably even some elements of nervous system origin. For want of a better general designation we have called this large group of non-hemic cells the stromal elements of bone marrow.

* Recently Osgood has described a method of tissue culture of human bone marrow in a fluid medium, with conditions arranged so that there is a significant increase of myeloid cells.
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Fig. 3

Fig. 4

Fig. 5

Fig. 6

networks and palisades of spindle-shaped or fibroblastic cells* which soon cover the culture fields and can be propagated indefinitely, as is characteristic of cul-

* The term “fibroblastic” used herein refers to the morphologic appearance of the cells of the third phase. They consist of elongated spindle-shaped cells resembling the familiar fibroblasts of connective tissues. Their orientation is usually in the form of palisades but often individual cells have cytoplasmic extensions which create a stellate appearance and a crisscrossing network. It is recognized that morphologic criteria for absolute identification of fibroblasts are inadequate, and we do not imply that the fibroblastic cells in our cultures are identical with the fibroblasts of collagenous connective tissue. There are probably different kinds of fibroblastic cells in marrow cultures, and we use the term in the sense
tures of many different tissues. The sequence is typical of cultures of marrow from normal persons and from our patients with various types of anemia, but the duration of the different phases is quite variable.

The specific purposes of this communication are to describe our methods and to report the development of a strain of cells in a culture from the marrow of a man with carcinoma of the lung and which, for reasons to be stated, we believe may be derived from a metastasis in the marrow.

METHODS

The methods used in connection with the event to be described were designed to permit cultivation of human cells suspended in the fluid part of aspirated sternal and iliac bone marrow. Details are given in the Appendix. In brief, suspensions of marrow cells in a fluid medium consisting of balanced salt solution, human cord serum, embryonic tissue extract and a pH indicator are placed in 3-ounce medicine bottles (fig. 8) lying on their flat sides.* Medium is refreshed when the pH falls below an optimum range. Daily observations are made through the flat sides of the bottles with an ordinary light microscope at 60X or 100X magnification. The optical quality of the glass of these bottles is not adequate for detailed study but it suffices for determining the general condition of the cultures and serial photomicrographs can be made (figs. 9, 10, 11). At intervals the supernatant fluid containing unattached cells is sampled and sometimes subcultured. The cells attached to glass are removed by addition of trypsin solution. The suspended trypsinized cells are concentrated by centrifugation for 10 minutes at 750 G. Smears of the concentrated suspensions of cells are stained by the May-Grünwald-Giemsa method. The suspensions, brought to a standard volume, are mixed in a small flask on a magnetic stirrer. Aliquots are removed for the purposes of making cell counts and subcultures. When preparations of cells attached to glass are needed for study at higher magnification some of the subcultures are made in Porter flasks (fig. 8). For repeated sampling of cultures for morphologic study, when trypsinization must be avoided, the marrow specimens (fragments as well as suspensions) are cultivated of Wilmer’s “mechanocytes,” namely, a group of cells termed “fibroblasts” not because the cells necessarily have any connection with the formation of fibers, but because they are similar in appearance to the cells in the body which are believed to function in this manner.

in 5 ml. Beckman beakers which accommodate 18 mm. round coverslips on their bottoms (fig. 12). This provides for coverslip preparations with cells attached directly to glass.

**Pertinent Observations**

In a culture of bone marrow from a man with carcinoma of the lung several opalescent plaques became visible on the culture area amongst the usual proliferations of fibroblastic cells. This occurred on the 51st day of continuous culture. The plaques consisted of cellular material from which emerged cords and sheets
of cells differing from the networks of fibroblastic cells which are characteristic of cultures of this age. The cells assumed polygonal shapes and formed colonies which reached diameters of 3 to 6 mm, within several days. These colonies were then removed by scraping and transplanted into other flasks and beakers for
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Fig. 12.—Rubber-stoppered 5 ml. Beckman beakers containing 18 mm. coverlips

Fig. 13.—Coverslip-beaker culture of an explanted fragment of Detroit-6 tissue picked off the glass of a prescription bottle culture from the marrow of a patient with carcinoma of the lung. 23X. May-Grünwald-Giemsa stain after alcohol fixation.

Further propagation, where they grew outward on the glass and formed sheets (figs. 13 and 14), Trypsin was then used to break up the cell layer, producing a suspension of individual cells which were then subcultured. The appearance of these cells should be compared with that of an explanted fragment of fibroblastic tissue from a culture of similar age (figs. 15 and 16). Since its isolation in November 1954 this strain has been maintained through periodic subcultures without discernible change of morphologic characteristics or growth pattern. These cells have been designated the Detroit-6 strain.

Stable, long-term cultures of epithelial cells have been maintained in the past only with great difficulty and usually exhibit either slow growth or self-destroying keratinizing characteristics. A notable exception was the isolation of a strain of human malignant epithelial cells (strain HeLa) derived from a carcinoma of the cervix by Gey and co-workers. The HeLa strain has been used in the study of
viruses since it is the only stable strain of epithelial cells of human origin thus far used successfully on a large scale. There are significant similarities as well as some differences between the Detroit-6 and HeLa cells. The chief morphologic similarity is cytologic, as observed in living unstained cells and in fixed stained preparations with ordinary light microscopy. Individual cells of the Detroit-6 strain appear indistinguishable from HeLa cells, except that the former more often include cells with multiple irregular nucleoli whereas the latter more often include cells with single large round nucleoli (figs. 17, 18, 19, 20). On the other hand, the topographic appearances of the cellular proliferations on glass are sufficiently dissimilar to enable us to identify the strains by observing the entire culture areas
in most, but not all, instances. For example, the Detroit-6 strain has a greater tendency for cohesive growth and, if cultures are allowed to age, these cells have a greater tendency to grow in a three-dimensional manner in compact masses, whereas the HeLa cells remain as a monacellular layer on glass for longer periods of growth.

The metabolic activity, as indicated by the rate of fall of pH values during growth of cells is considerably greater in the Detroit-6 cultures than in HeLa cultures. Apart from this, the two strains appear to grow at approximately the same rates. An inoculum of 700,000 cells in 8 ml. of medium per bottle will usually yield a three-fold or greater increase in five days with either strain.

The effectiveness of trypsin for making cell suspensions is greater for the HeLa cells than for the Detroit-6 cells. With our standard procedure the cells of the HeLa type are separated into single cells after 75 minutes as compared with 110 minutes for Detroit-6 cells.
Fig. 21.—Prescription bottle culture showing effect of herpes simplex virus on Detroit-6 cells four days after infection. Cytopathic effect is indicated by rounded cells bordering the clear space (left) where affected cells have sloughed off the glass and adjacent to a sheet of unaffected cells (right). 135X. Unstained.

Fig. 22.—Prescription bottle culture showing effect of Type I poliomyelitis virus on Detroit-6 cells 17 hours after infection. Only small islands of degenerated cells remain on the glass. 135X. Unstained.
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It is of interest to compare the Detroit-6 cells and HeLa cells with respect to their ability to support the growth of certain viruses. A series of studies in progress is concerned with the susceptibility of the Detroit-6 strain to viruses of human origin. Preliminary results have been obtained with strains of poliomyelitis, Coxsackie, herpes simplex, and several influenza viruses. Detroit-6 cultures inoculated with a Type I poliomyelitis virus, a Group B Coxsackie virus, and a herpes simplex virus underwent degeneration in a manner similar to that shown by the HeLa strain of epithelial cells inoculated with the same agents (figs. 21 and 22). The propagation of these viruses was evident not only by their cytopathogenicity but also by their ability to multiply to high titers analogous to titers obtained in HeLa cell cultures. On the other hand, A, A', and B strains of influenza viruses failed to exhibit either ability to destroy Detroit-6 cells or to multiply within them. These results paralleled the effects of the same quantities of influenza viruses inoculated into HeLa cells. These observations will be reported in greater detail elsewhere.

DISCUSSION

Although readers of this journal are interested chiefly in the hematologic aspects of this subject, the isolation of a strain of cells which are presumably epithelial suggests the possible usefulness of long-term culture of human bone marrow for other purposes. Virologists may find human bone marrow a readily available source of human cells suitable for their studies. If morphologic and functional similarities between Detroit-6 and HeLa strains can be accepted as evidence of the malignant epithelial nature of Detroit-6 cells*, marrow culture of the type described may provide opportunities for detecting metastasis in patients with occult tumors.

We know that success in maintaining a strain of cells since November 1954 does not mean that we have obtained a stable strain. Gey et al.4 have stated that they were able to maintain a strain of normal epithelial cells for 400 days, after which it rapidly keratinized itself out of existence. It is possible that additional studies with other viruses may yield evidence of differing susceptibilities of the Detroit-6 strain as compared with the HeLa strain. If not, a similarity of susceptibilities may be an indication of a fundamental unity in the behavior of human epithelial cells of different origins.

The usual fibroblastic proliferations which are easily obtained by the methods described may also be worth studying. Fibroblastic cells from different marrows have different morphologic characteristics if, indeed, they are all really fibroblasts and not fibroblastoid reticulum cells or modified cells of other types. Although the speed of appearance and growth of fibroblastic cells may vary greatly perhaps even among different samples of marrow due to the inherent lack of homogeneity

* Although similarities between Detroit-6 and HeLa cells are impressive, our explanation of the occurrence of epithelial cells in a marrow culture is not the only one to be considered. Hematologists experienced in the study of aspirated marrow expect the occasional inclusion of fragments of skin or its appendages in marrow specimens. The cells resembling epithelium in the culture discussed may have had their origin from such an inclusion. It would be worthwhile to investigate the behavior of fragments of skin or its appendages placed in marrow cultures.
of the marrow, particularly in myelophthisic lesions, it is possible that by obtaining multiple samples from different sites and at different times from a single patient, the problem of studying factors influencing such variations may become feasible. Investigation of the stromal fraction of human bone marrow seems to have been neglected because of the intense interest in the short-lived parenchymal fraction. Furthermore, the organization of outgrowths of stromal tissue from marrow explants may have some influence on the behavior of the myeloid cells.

Finally, with the methods which have been described, the same cultures which have use in short-term investigations often can be extended into the long-term range.

**SUMMARY**

Using methods described, long-term tissue cultures of human bone marrow pass through three typical growth phases: (1) a myeloid phase during which myeloid cells can be recognized; (2) a round-cell phase during which histioid and monocytoid cells predominate; and (3) a fibroblastic phase which can be maintained thereafter. The three phases are of variable onset and duration.

In a culture from a patient with carcinoma of the lung, isolated colonies of polygonal cells appeared on the 51st day of continuous cultivation. These were removed and subcultured. By means of trypsinization, the sheets of polygonal cells were transferred and subcultured in bulk and in a manner making it possible to obtain replicate cultures. The morphologic characteristics and growth behavior of the strain of cells (Detroit-6 strain) are similar to those observed for HeLa cells. The pattern of growth of the Detroit-6 strain is that of epithelial cells, presumably of metastatic origin from a carcinoma of the lung.

The susceptibilities of the Detroit-6 strain to infection with various viruses (poliomyelitis, Coxsackie, herpes simplex, and influenza) are similar to those observed in the HeLa strain of human malignant epithelial cells originating from a carcinoma of the cervix.

The possible values of long-term tissue culture of human bone marrow have been discussed.

**Summario in Interlingua**

Esseva elaborate methodos permitente le cultivation de cellulas human suspendite in le portion fluide del aspirate medulla ossee sternal e iliac. (Iste methodos es describite detaliatemente in le appendice del presente reporto.) Lor uso resultava in le constatation que histoculturas a longe durantia de medulla ossee human passa per tres typic phases de crescentia: (1) Un phase myeloide durante le qual cellulas myeloide es recognosibile. (2) Un phase a cellulas rotunde durante le qual cellulas histioide e monocytoid es predominante. (3) Un phase fibroplastic que postea pote esser mantenite. Le tres phases varia in le tempore de lor declaration e in lor durantia.

In un cultura ab un patiente con carcinoma del pulmone, isolate colonias de cellulas polygon appareva le 51me die de continue cultivation. Iste colonias eseva removite e perpetuate in subculturas. Per medio de trypsinisation, le folios de cellulas polygon eseva transferite e subcultivate in mass in un maniera que rendeva possibile le obtention de culturas replicate. Le caracteristicas
morphologic e le conducta crescential de iste racia de cellulas-designate como Detroit-6—es simile a illos observate in cellulas HeLa. Le conducta crescential de Detroit-6 es illo de cellulas epithelial, presumite de origine metastatic ab un carcinoma del pulmone.

Le susceptibilitates de Detroit-6 a infection per varie viruses—poliomyelitis, Coxsackie, herpete simplice, e influenza—es simile a illos observate in le racia HeLa de maligne cellulas epithelial human originari ab un carcinoma del cervice.

Es discutite le possibile valor de histocultururas a longe durantia de medulla ossee ab humanos.

APPENDIX

Preparation of Glassware and Other Equipment

* Bottles, beakers, petri dishes, teflon-covered stirrer rods, rubber goods, metalware. Used equipment is placed in cold tap water until ready for washing in a solution of Calgonite*, containing 8 ounces of the detergent for each 25 gallons of tap water. The equipment is brushed and boiled in the solution for 20 minutes, then rinsed under a tap with hot running water followed by two rinses in distilled water and two rinses in double distilled water.

* Coverslips. These are cleansed in boiling metasilicate solution and rinsed in tap water, distilled water and absolute alcohol according to the method described by White15 after which final drying is accomplished by placing the coverslips on stainless steel wire racks in a drying oven.

* New rubber goods. Stoppers and rubber tubing are boiled in 0.5 N NaOH solution for several hours, rinsed, and then boiled in 0.5 N HCl for 30 minutes. After rinsing in tap water they are handled in the same manner as glassware.

* Pipettes. After thorough rinsing in cold tap water, the pipettes are soaked in Haemo-Sol solution† according to the manufacturer's instructions. Then they are rinsed in an automatic pipette washer in tap water, after which they are transferred to another automatic pipette washed for at least three rinses in distilled water.

Preparation of Media

* Medium for cell cultivation. The nutrient medium consists of 40 per cent human cord serum, 2 per cent chicken embryonic extract and 58 per cent Hanks' balanced salt solution (CSaEE2BSSa). The pH is adjusted to 7.6 with 1.4 per cent sodium bicarbonate solution. Phenol red† in a final concentration of 0.002 per cent is used as an indicator. When the medium is used for mass cultivation of epithelial cells, penicillin and streptomycin are included in final concentrations of 100 units and 100 μg/ml., respectively.

* Human cord serum is prepared from free-flowing cord blood collected in screw-capped tubes. After clotting and centrifugation the individual sera are removed, pooled and sterilized by filtration through a Seitz-type filter.§ The filtered serum is stored at −20 C. until used.

* Hanks' balanced salt solution and chicken embryonic extract are prepared according to the methods described by Syvertor, Scherer and Elwood.14

* Maintenance medium. The maintenance solution, composed of amino acids, purines, vitamins and other substances in balanced salt solution, was proposed by Scherer11 for maintenance of cells undergoing viral infection. It is prepared according to the methods described by Syvertor, Scherer and Elwood.14 For the virus studies, the medium is composed of maintenance solution 90 per cent, inactivated rabbit serum 10 per cent (MSaRS10).

* Calgonite, Commercial Formula, Calgon Corp., Pittsburgh, Pa.
† Meinecke & Co., New York City.
‡ TC Phenol Red 1%, Difco Co., Detroit, Michigan.
§ Hercules asbestos filter pad, ST type, Hercules Filter Corporation, Paterson, New Jersey.
Trypsin solution. This is a 0.5 per cent suspension of trypsin* sterilized by filtration through a Seitz filter and stored at -20 C.

Aspiration and Preparation of Bone Marrow

Methods used are based on a modification of those described by Berman.† Marrow is aspirated into a syringe which has been moistened with heparin solution.§ Ten ml. of marrow are transferred into a cooled conical pyrex centrifuge tube previously evacuated and autoclaved and capped with a sleeve-type rubber stopper. All transfers of fluids into and from the tube are made through the stopper with syringes supplied with 18-gauge spinal needles. The marrow specimen is centrifuged at 4-10 C. for 15 minutes at 40 G. The supernatant layer of plasma containing the suspended marrow and blood cells is placed in a 3-ounce prescription bottle containing enough nutritive medium to make a final volume of 8 ml. Counts are made from a portion of the cell-plasma suspension. When the cell count and total volume of cell-plasma suspension are known, the material is distributed among prescription bottles so that each contains from one to two million cells. We have not completed studies of the effects of varying the initial cell concentrations. However, cultures appear healthier within the range indicated than with smaller inoculums. Tentatively it would seem desirable to adjust the cell concentrations to some standard range.

After the cell-plasma supernatant layer is removed from the centrifuge tube, Hank's* salt solution is added and the sediment containing erythrocytes and fragments of marrow tissue is resuspended. The mixture is then dumped into a large petri dish so that the material is spread out in a thin layer from which the fragments of marrow tissue can be removed with an opsonic pipette. The particles of marrow are placed in fresh Hank's solution in an embryological staining dish and washed free of blood by changing the fluid. The marrow particles are used for cultures in various ways, including the placing of the fragments on coverslips in 5 ml. Beckman beakers containing fluid nutritive medium. The beaker technique is particularly useful for obtaining coverslip preparations of suspensions of cells from cultures of stabilized growths of fibroblastic or epithelial cells. In the case of Detroit-6 and HeLa cells or trypsinized suspensions of fibroblastic cells, an inoculum of approximately 100,000 cells in one ml. of medium per beaker will provide a growth covering the entire area of the coverslip within one to three days. When fragments of marrow tissue are placed on coverslips, the washed piece of tissue (or several small pieces to provide a mass about 1 x 2 x 2 mm.) is placed in the center of the coverslip. Excess fluid is removed with a fine pipette. The mounted specimen is placed in an empty beaker which is immediately stoppered. After an hour the fragment is adherent to the glass and then one ml. of the fluid medium is added. Aluminum caps are placed over the stoppers to keep the rims of the beakers free of contamination from air. It is possible to continue beaker cultures for long periods by removal of old medium and replacement with new (1 part used medium to 2 parts fresh medium). After rinsing in warmed balanced salt solution the preparations can be air-dried and stained with hematological stains or they may be fixed in Bouin's fluid for subsequent staining. If air-dried preparations are made, the original explant is removed by blotting with dry fine filter paper. This procedure also removes any fluid on the coverslip which dries very rapidly in air. The explant is picked off the paper onto the side of a wooden cylindrical applicator stick. The stick bearing the explant is rolled on clean dry slides to obtain "roller smears" which exhibit the cells in a single layer. In this manner it is possible to obtain dry films of the cells on the coverslip as well as those remaining within the original explant.

Transfers, Subcultures and Trypsinization

When the bottoms of the culture bottles are well covered with cells culture medium is removed and replaced with 8 ml. of trypsin solution. The bottles are incubated at 36 C.

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* BSA trypsin 1:250, Difco Co., Detroit, Michigan.
† Heparin Sodium for parenteral use, 1,000 U.S.P. units per cc., Testagar & Co., Inc., Detroit, Michigan.
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(marrow cultures 20 minutes; HeLa cultures 75 minutes; Detroit-6 cultures 110 minutes) until individual cells or sheets of cells are loosened from the glass. The detached cell suspensions are drawn into serologic pipettes or syringes several times to break up clumps of more than a few cells. The cell suspensions are sedimented by light centrifugation, the supernatant trypsin solution is discarded and the cells are resuspended in the desired volume of fresh nutritive medium. The resuspended cells are then transferred to a flask containing a teflon covered metal bar and placed on a magnetic stirrer. Aliquots may be removed for making cell counts in a hemocytometer. When the number of cells per ml. has been determined, the suspension is adjusted so that it contains approximately 100,000 cells per ml. Eight ml. of the suspension are then introduced into 3-ounce prescription bottles and incubated at 36 C. in a stationary position. At times, spent (conditioned) medium from the parent culture is used in a ratio of 6 parts fresh medium and 2 parts old medium to initiate growth or to feed cultures. Cultures are re-fed, either by complete replacement with fresh medium or by replacement of 2/5 of the old medium with fresh medium. Feeding times vary with the type of cell under cultivation. The rate of growth of cells can be gauged roughly by the rate of fall of pH. Although the cultures appear to remain in good condition over a wide range of pH values, it is advisable to re-feed the cultures when the indicator reaches an orange color (pH 7.0). Usually the Detroit-6 strain requires a feeding at least every 48 hours until transferred.

Virus Studies

Sources of viruses. The viruses employed were: 1. poliomyelitis virus, Type I (Mahoney) obtained from Dr. I. W. McLean, Jr., Parke, Davis & Co.; 2. Coxsackie virus, Group B, Type 1 (Conn.-5) obtained through the courtesy of Dr. J. L. Melnick, Department of Preventive Medicine, Yale University Medical School; 3. herpes simplex virus recently isolated in this laboratory; 4. influenza A virus (PR8 strain); 5. influenza A' virus (FM1 strain); and 6. influenza B virus (Lee strain). The influenza viruses were obtained from the Viral and Rickettsial Registry of the American Type Culture Collection.

Inoculation of Detroit-6 cells with viruses. Poliomyelitis and Coxsackie viruses were grown and titrated in cultures of HeLa cells prior to inoculation of the Detroit-6 strain. Herpes simplex virus and the influenza virus strains were grown and titrated in chick embryos for subsequent infectivity studies in Detroit-6 cultures. The seed suspensions of the various viruses were diluted with maintenance medium to contain the desired number of HeLa cell infective doses (TCIDmo) of poliomyelitis and Coxsackie viruses, or the number of chick embryo ID50 of herpes simplex or influenza viruses, per ml. of inoculum. One ml. of the desired concentration of the various viruses was introduced into individual flasks containing well-formed sheets of Detroit-6 cells. Following incubation of the infected cultures at 36 C., 0.25 ml. aliquots of the culture fluids were removed at intervals, placed in ampules and stored at -20 C. until titrated. The cultures were observed regularly for evidence of viral cytopathogenicity. Details of these methods based on those described by Stulberg et al.13 will be reported elsewhere.

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