Effect of Aging and Rotation on Human Amnion Cell Response to Polio and Sindbis Viruses.* (24677)

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Enders(1) noted that the strain of Type II poliovirus adapted to the chick embryo by Roca-Garcia and co-workers(2) was, like other strains, regularly and markedly cytopathogenic for human amnion cells. However, in contrast to polioviruses so far examined, it failed to induce obvious changes in human renal cells. In our initial attempts to repeat Enders' observations, the response of amnion cells to infection proved highly variable. Frequently the cells remained unaffected even when large amounts of this virus were added, although occasionally they were partially or completely destroyed. Comparable irregularities were also seen in cultures of human amnion cells exposed to Sindbis virus, an agent that is highly destructive to chick embryo cells(3). Consideration of differences in techniques employed suggested that rotation of the culture and its age might be important factors in determining response of human amnion cells to these agents. Accordingly, the effect of agitation and aging has been investigated and the results are here summarized.

Materials and methods. Cultures. Primary cultures of human amnion and chick embryo cells were prepared from trypsinized tissues(4,5). The cells were grown on glass in pyrex-brand, stoppered 15 × 150 mm tubes or 160 ml Breed milk dilution bottles and incubated at 35-37°C either in stationary position or in roller wheel operating at approximately 20 rph. Amnion cell cultures were at first nourished with bovine amniotic fluid medium containing 20% horse serum. After inoculation with virus they were maintained wtih bovine amniotic medium (BAF) containing 5% horse serum(4). To establish sheets of chick cells suitable for plaquing (6) the yield from five 10-day embryos was suspended in 200 ml BAF medium and dispensed in 10 ml aliquots into bottles. Fluid was changed 1 or 2 days later and virus added on the 3rd day. At beginning of investigations, Dr. T. W. Chang of New England Medical Center informed us of a possible correlation between sex of conceptus and quality of amnion cultures and cell-reaction to infection. With this in mind the following observations were made. Cultures were prepared with cells from either 1 or 2 membranes. Thirty-eight consecutive lots of cells were used over 9-month period and the cultures were rated as "excellent", "fair", "poor", or "unusable", according to degree of cellular granularity, non-specific degeneration, and presence or absence of extracellular precipitate. Thirteen of 21 lots from female membranes and 2 of 17 lots from male membranes were rated "excellent". Two male lots were unusable and the remaining 21, although rated "fair" to "poor", were nevertheless adequate for work with viruses of known, pronounced cytopathogenicity. Response to viral infection of amnion cell cultures prepared from conceptus of different sex was also investigated. In this respect no consistent differences were observed. *Viruses.* Brunhilde and Lansing strains of poliovirus(7) were derived from 3rd and 4th human tissue culture passages. Rogers strain of Type II poliovirus was isolated by Dr. Sidney Kibrick from stool obtained at post mortem examination of patient with fulminating bulbo-spinal poliomyelitis. Material from third human tissue culture passage was used. Chick embryo adapted Type II MEF_1 poliovirus(2) was obtained through kindness of Dr. Herald Cox of Lederle Labs. in the form of material from 140th egg passage. It was passed once in eggs in this laboratory and once in human amnion cell cultures. Materials from both the egg passage and tissue culture passage were used. Types I, II, and III attenuated polioviruses currently under test as vaccines

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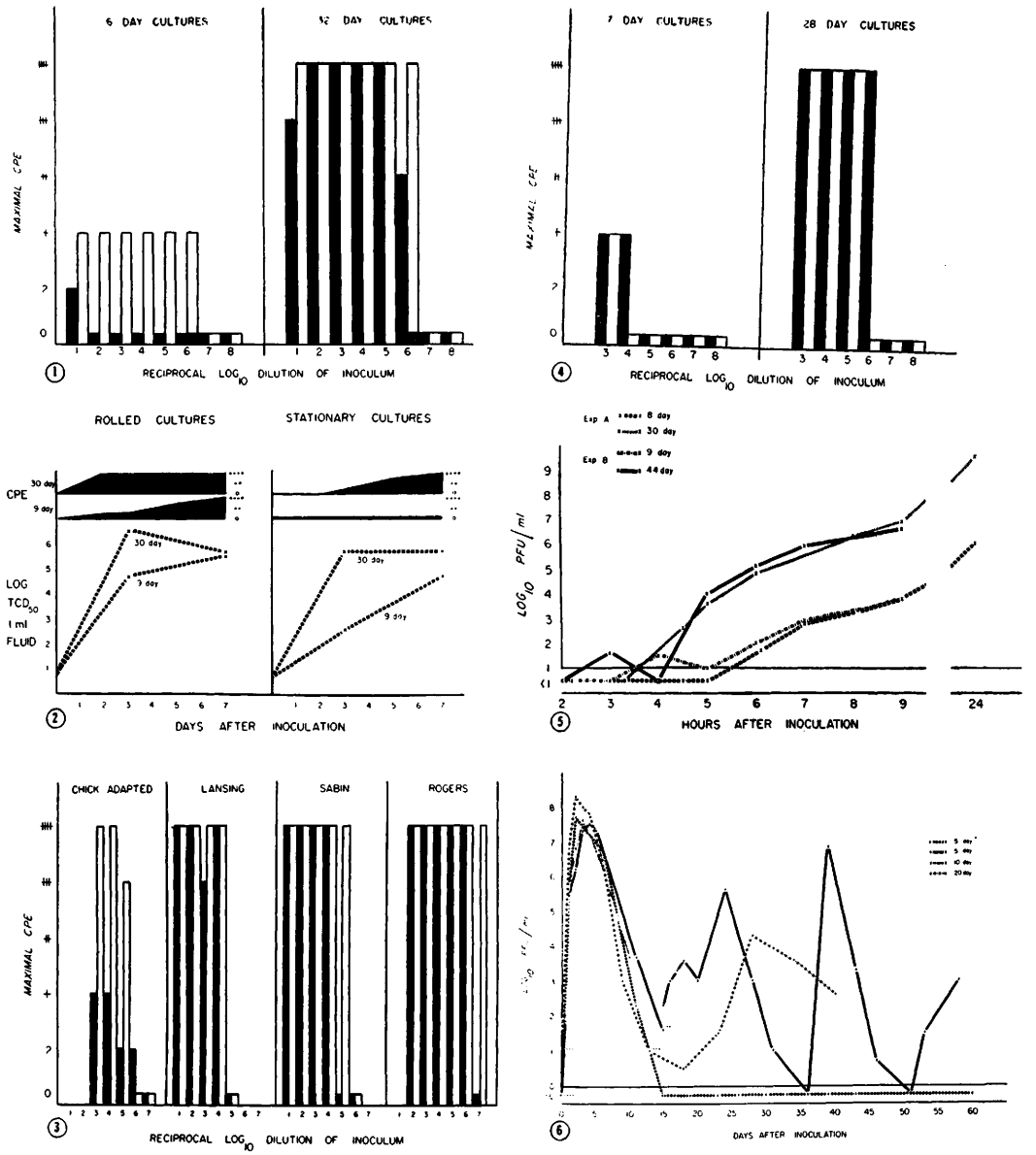

FIG. 1. Cytopathic response to chick-adapted poliovirus in stationary and rolled human amnion cell cultures of different ages.

CPE–0 — No CPE
? — Minimal degeneration of questionable specificity
\+ — Definite foci of degeneration
++ — Degeneration of about 50% cell population
+++ — Degeneration almost complete
++++ — Complete degeneration

Each bar indicates CPE in 3 cultures; black bars, stationary cultures; white bars, rolled cultures; cross-hatching, CPE in 1 of the cultures.

FIG. 2. Cytopathogenicity and multiplication of chick-adapted poliovirus in stationary and rolled human amnion cell cultures of different ages.

FIG. 3. Effect of rolling on the cytopathic response of 14 day human amnion cell cultures to strains of Type II poliovirus. Each bar indicates CPE in 2 cultures. See Fig. 1 for other notation.

FIG. 4. Cytopathic response to Sindbis virus in stationary and rolled human amnion cell cultures of different ages. Each bar indicates CPE in 2 cultures. See Fig. 1 for other notation.

by Dr. Albert B. Sabin, were obtained from him and designated as "Sabin strains"(8). Sindbis virus(9) strain AR339 was furnished by Dr. Richard M. Taylor in the form of 10% suspension of mouse brain. Throughout, material from 10th chick embryo passage was used. Stock viruses were kept in CO_2 cabinet with the exception of Sabin's strains which were stored at −20°C. *Antiserum.* Anti-Sindbis virus rabbit serum was prepared essentially by the method described by Ramos-Alvarez and Sabin for ECHO viruses(10). Virus employed as antigen was grown in chick embryo cultures maintained in medium 199. *Assay of virus.* Serial 10-fold dilutions were prepared in BAF medium. Three tube cultures were inoculated with 0.1 ml of each dilution tested. Titers are expressed as log TCD_{50}/ml of culture fluid. Plaque assay of Sindbis virus in chick embryo bottle cultures was performed essentially by the method of Dulbecco and Vogt as adapted to bottles by Hsiung and Melnick(11). Plaques appeared within 24 hours and attained maximal numbers by 4 days. Titer was expressed as plaque-forming units (PFU)/ml. Assays of stock virus performed on 12 separate occasions during 6 months gave a range of 15-46 $\times 10^8$ PFU/ml with mean of 30×10^8 PFU/ml. *Age of cultures.* This was expressed in days from time of dispensing the cells into culture vessels. Between this procedure and obstetrical delivery 2-5 additional days elapsed. Cultures 5-10 days old were designated as "young," those 11-21 days as "intermediate" and those over 21 days old as "old". *Cytopathogenic effect (CPE).* Unless otherwise specified this was evaluated by frequent low power microscopic inspection during at least 2 weeks after inoculation for poliovirus and 10 days for Sindbis virus.

Experimental. Chick embryo-adapted poliovirus. Cultures were divided into 2 groups. Fluids of the first, designated "young" cultures, were changed on days 5 and 6. After change on day 6, 0.1 ml of a wide range of 10-fold dilutions of virus (10^{-1}-10^{-8}) were added to 6 cultures/dilution. Three of these cultures were then rolled and 3 left stationary. Estimations of CPE were made every 1 to 5 days during following 19 days. Fluids of second group, designated "old" cultures, were changed on days 5, 11, 19, 26 and 32. After last change, cultures were inoculated with dilutions of virus and maintained under same conditions as those in Group 1. Fig. 1 graphically records the occurrence of CPE. It is evident that CPE was minimal or absent in young cultures depending upon whether they were rolled or kept stationary. In old cultures irrespective of motion, the virus rapidly destroyed all or nearly all cells. This experiment was repeated with cells from different amnions, once with the same range of viral inocula and once with single small inoculum. Comparable results were obtained. It seemed possible that the greater number of fluid changes undergone by second group of cultures might be responsible for the difference in response. Accordingly, fluids of 6 cultures of intermediate age prepared from a single lot of cells were changed on days 6 and 13. In addition, fluids of 3 cultures were changed daily on days 8 through 12. Each culture was inoculated with approximately 100 TCD_{50}/ml of virus on day 13 and subsequently incubated in stationary position. No significant difference was noted in CPE which was recorded as "1+" in all.

The effect of age was also studied in cultures prepared with different cell lots but nourished with same lot of medium. Fluids of 8-day and 29-day-old cultures were replaced with the same lot of BAF medium and inoculated with approximately 1000 TCD_{50}/ml of virus. All cultures were incubated in stationary position. The 8-day cultures showed no definite degeneration during ensuing 25 days. In contrast, the 29-day cultures degenerated within 4 days.

Increase of virus and metabolic activity of cells as indicated by rate of pH fall in young and old cultures, were compared as follows. Two groups of cultures derived from a single

FIG. 5. Increase of Sindbis virus in fluid phase of amnion cell cultures of different ages.

FIG. 6. Concentration of Sindbis virus in fluids of 4 amnion cultures in the absence of CPE. +, Age of culture at inoc. ++, This culture reinoc. and washed on day 15. +++, Less than 10 PFU/ml. ++++, No PFU/ml undiluted fluid.

the kidney of living monkeys without causing manifest injury. When cultured, however, cells from the infected kidney are subsequently destroyed(17).

From a practical point of view, these results indicate that in the study of viral agents whose behavior in tissue culture is uncertain, disregard of the effects of cultural age and agitation may be misleading.

Summary. 1) The cytopathic response of primary human amnion cell cultures to chick embryo adapted Type II poliovirus was absent in stationary cultures inoculated at 5-10 days of age. In stationary cultures inoculated when 30 or more days of age, complete destruction of cells followed. Multiplication of virus in young cultures occurred in the absence of recognizable cytopathic change. Cytopathic response of cultures of approximately 14 days was markedly enhanced by rolling after inoculation. The effect of rolling was not clearly apparent when other strains of poliovirus including attenuated strains were tested. 2) Frequently the cytopathic response of amnion cultures to Sindbis virus was reduced or absent in young as compared with old cultures, but response of young cultures was variable. No effect of rolling on cytopathic response could be demonstrated. Viral multiplication was greater in older cultures.

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A Micrometer Plunger Adaptation to the Stern-Kirk Microrespirometer. (24678)

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The Stern-Kirk microrespirometer(1) has a number of features which make it well suited for studies of respiration in small samples of tissue. It is sturdy, easy to operate and sensitive. The respiration chamber and thermobarometer compensation chamber consist of 2 recesses drilled in a metal block base. The metal quickly equilibrates temperature differences between the 2 chambers, so that accuracy of the apparatus is not affected by small changes in surrounding temperature. Since the chambers are closed during experiments, repeated thermobarometer corrections are unnecessary. In this respect, the Stern-Kirk respirometer resembles volumetric respirometers of similar construction(2-5). Unlike them, however, it requires time-consuming calibration procedures. To overcome this