Survival of Animal Tissue Cells in Primary Culture in the Absence of Serum

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The ability of cells from tissues of several species of animals to survive in primary culture without serum was tested. Of the species tested, cells from the kidneys of Macaca mulatta (rhesus) and Cercopithecus aethiops (vervet) monkeys and chicken embryo cells not only survived under these conditions, but indeed developed into confluent monolayer cultures. The addition of either serum or its globulin or albumin fraction enhanced the development of cell monolayers and permitted those cells unable to survive in the absence of serum to do so. Certain specific protein trypsin-inhibitors not of serum origin were unable to provide conditions necessary for cell survival or growth when used in place of serum proteins.

In most mammalian cell culture systems, serum protein is incorporated into the medium to insure optimal cell growth. The protein may be in the form of either whole serum or serum components (e.g., protein growth factor [13], alpha globulins [11], fetuin [20], and albumin [15, 25]), or both. Although continuous cell lines have been used extensively in establishing nutritional requirements, little use has been made of primary cell cultures in this regard. Healy and Parker (10) were able to grow newly explanted mouse embryo cells in a serumless chemically defined basal medium. Rubin and Hattie (23) and Levinthal and Rubin (12) noted that chicken embryo cells grown without serum did not exhibit the increase in cell size nor the formation of numerous polyribosemes and differential cytoplasmic structures, fibrils, and microtubules as did serum-stimulated cells. Rappaport (21) and Wallis et al. (26) have reported the successful cultivation of primary cultures of monkey kidney cells in the absence of serum. The latter investigators (26) suggest that one role of serum proteins in cell culture medium is that of inhibiting the action of trypsin enzymes, synthesized by the cells themselves and released into the culture medium.

Because of these and other observations, it became of interest to examine more closely the ability of cells to survive at 37 C in the absence of serum. This communication describes our results in subjecting primary cells from a number of animal species to cultural conditions devoid of serum.

MATERIALS AND METHODS

Preparation of serum fractions and growth media. A fetal calf serum with demonstrated growth-promoting properties was used as the control serum. A portion of the serum was separated into its albumin and globulin fractions by two successive precipitations with 50% saturated ammonium sulfate. The salt was removed from the fractions by dialysis against Hanks balanced salt solution (HBSS). The protein solutions were either concentrated by evaporation or diluted with HBSS back to the original volume of the serum. All operations were performed aseptically at 4 C.

The serum protein fractions and 1% (w/v) solutions of egg-white albumin and soybean trypsin inhibitors in HBSS were used as substitutes for 10% whole serum in Eagle minimum essential medium (MEME), except as noted.

The undiluted solutions of fetal calf serum, globulin, albumin, soybean trypsin inhibitor, and egg-white albumin contained 34.49, 9.13, 15.75, 6.56, and 6.19 mg of protein per ml, respectively, by Kjeldahl determinations.

Preparation of primary cell cultures. Cells were dispensed from the kidneys of guinea pigs, young rabbits and hamsters, juvenile Macaca mulatta (rhesus) and Cercopithecus aethiops (vervet) monkeys, and from whole embryos of chickens, mice, and hamsters by a method previously described (17). The cells were washed several times with Dulbecco saline after removal from the trypsin solution by centrifugation. Washed cells were counted and diluted appropriately in MEME or saline without serum and
inoculated into 2-oz (ca 0.06 liter) prescription bottles containing the particular fluid under study.

Inoculated cell cultures were incubated at 37 C for 2 to 8 days as indicated in the different experiments. After removal of the fluids, the cultures were rinsed briefly with saline, and the cells were dispersed with 0.25% trypsin. Viable cells were counted in a hemocytometer using the trypan blue dye-exclusion method. In certain experiments, samples of incubated cultures were taken for determining the number of viable cells every 2nd day as described above. In other experiments, cells were allowed to remain undisturbed before they were dispersed, counted, and subcultured at a split ratio of 1:2.

In addition, rhesus and vervet monkey kidney cells were seeded in several synthetic or chemically defined media (Table 1) both with and without the addition of serum for 5 days. Finally rabbit kidney and chicken embryo cells were used to examine the influence of inoculum size on their ability to be sustained in serum-free medium.

RESULTS

Primary cells from the several species of animals tested varied in their capacity to survive in the absence of serum (Fig. 1). It appeared that cultures of chicken embryo and both species of monkey kidney cells developed to about the same degree in Eagle medium with or without serum. Most of the cells from the other species of animals were less consistent in their ability to grow in the absence of serum. Hamster embryo, hamster kidney, and guinea pig kidney cells were never successfully maintained in serumless medium, and maintenance of rabbit kidney cells under such conditions was poor and erratic. Medium containing either whole serum or its globulin or albumin fraction benefited about equally well all of the primary cells. However, trypsin inhibitors not of serum origin, when used in place of serum proteins, failed to provide any beneficial effects for cells unable to survive in serumless medium.

The observation that certain primary cells appeared capable of being propagated in MEME without serum (Fig. 1) led us to examine further this growth potential by experiments designed to separate cell attachment and spreading from actual cell multiplication. Trypsinized primary cells were inoculated as previously described into culture vessels containing saline as well as MEME. Some of the vessels in each group contained 2% serum. Inoculated cells were allowed 48 hr to settle and adhere to the vessel surface before being removed by decanting the fluids. Those cells that had attached themselves to the vessels were rinsed with saline, and some cultures were used for determining the number of attached viable cells. Photomicrographs were taken also at this time. The remaining cultures were refed with the respective fluids and further incubated for an additional 6-day period. Photomicrographs again were taken and compared with those taken earlier. In addition, the number of viable cells in the cultures were counted.

Representative results from these experiments are shown in Fig. 2–4. In all instances, under the most optimal conditions employed, more than 50% of the primary cells failed to attach to the vessel surface in 48 hr. Moreover, it is apparent that not only does MEME without serum enhance cell survival over that

| TABLE 1. Percent of inoculated cells attached and viable after 5 days incubation in various synthetic media* |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| Medium                                           | Reference | Vervet monkey kidney cells | Rhesus monkey kidney cells |
|                                                  |           | With 2% fetal calf serum | Without serum | With 2% fetal calf serum | Without serum |
| MEME                                             | 3         | 77 ± 1.4                 | 69 ± 3.0      | 99 ± 4.1                 | 59 ± 1.5     |
| Waymouth MAB87/3 without insulin                 | 6         | 86 ± 3.0                 | 24 ± 0.9      | 88 ± 3.8                 | 24 ± 0.5     |
| Waymouth MAB87/3 with insulin                    | 6         | 83 ± 2.6                 | 25 ± 0.7      | 46 ± 2.5                 | 18 ± 0.3     |
| Medium 199                                       | 19        | 71 ± 1.7                 | 26 ± 1.0      | 37 ± 1.3                 | 15 ± 0.3     |
| RPMI 1640                                        | 18        | 59 ± 1.7                 | 27 ± 1.0      | 45 ± 2.5                 | 16 ± 0.2     |
| NCTC 109                                         | 4         | 48 ± 0.4                 | 19 ± 0.9      | 67 ± 4.1                 | 13 ± 0.1     |
| Ham’s F-12                                       | 8         | 56 ± 1.5                 | 18 ± 0.7      | 41 ± 1.9                 | 10 ± 0.1     |
| HEPES* Hanks BME                                 | 27        | 53 ± 2.6                 | 12 ± 0.1      | 16 ± 1.0                 | 5 ± <0.1     |
| NCTC 135                                         | 5         | 24 ± 1.0                 | 18 ± 0.4      | 18 ± 1.0                 | 13 ± 0.1     |
| Ham’s F-10                                       | 7         | 20 ± 1.2                 | 7 ± 0.1       | 17 ± 1.5                 | 4 ± <0.1     |

* Averages calculated from quadruplicate cultures ± mean standard error. Each bottle was inoculated with 7 ml of a suspension containing 3 × 10⁶ cells/ml.

*N-2-hydroxyethylpiperazine-N'-2'-ethanesulfonic acid.
SURVIVAL OF CELLS WITHOUT SERUM

Fig. 1. Inoculum multiplication indexes of primary cell cultures from seven species of animals after 7 days in MEME without serum, with 10% serum, or with trypsin inhibitors. Quadruplicate bottles were each inoculated with 7 ml of suspension containing $3 \times 10^6$ cells/ml. Indexes of inoculum multiplication were determined from the expression $C_f/C_i$; where $C_f$ = final cell count; $C_i$ = initial cell count.

Fig. 2. Survival of cells in medium and in saline with and without serum. On day 2, all cells which had not adhered were removed by decanting the fluids, after which fresh fluids of the respective type were added to the cultures. Numbers represent averages from three cultures. Maintenance of mouse embryo cells. Triplicate bottles were each inoculated with 7 ml of cell suspension containing a total of $18 \times 10^6$ cells.

Fig. 3. Survival of cells in medium and in saline with and without serum. Maintenance of vervet monkey kidney cells. See legend to Fig. 2.

found in saline, but indeed allowed some cell multiplication to occur even in the absence of serum.

The growth potential of all primary cells kept in serum-free conditions for 7 days was examined further by transferring the same num-
ber of cells recovered from each fluid into MEME now containing serum for 5 additional days. Cells kept in MEME retained their growth potential, whereas those kept in saline did not survive.

Table 2 presents data showing the yield of viable chicken embryo and rabbit kidney cells at 5 days from cultures initiated with various cell concentrations. In the absence of serum, an inoculum of $3.0 \times 10^6$ cells per ml produced a monolayer culture of chicken embryo cells but not of rabbit kidney cells. Likewise, monolayer cultures of monkey kidney cells were obtained from an inoculum of $3.5 \times 10^6$ cells per ml in 7 to 8 days even though inoculated into serum-free medium. The mean population doubling time for the exponentially growing monkey kidney cells was calculated to be between 40 and 60 hr when grown with serum and 70 and 90 hr when serum was omitted from the medium.

Primary monkey kidney cells were inoculated into several synthetic or chemically defined media in the absence of serum (Table 1). The data show the percent viable cells recovered after 5 days relative to the number inoculated. Some of the chemically defined media appeared to be better than others for maintenance of primary monkey kidney cells. Although monolayer cultures of primary cells from the kidneys of rhesus and vervet monkeys and from mouse and chicken embryos could be obtained in the absence of serum, at no point could cells which had been cultured previously with serum be transferred to serum-free medium and survive. Cells either initially or subsequently grown in the presence of serum, however, could be transferred some 12 or more times before the culture degenerated.

Despite the fact that undiluted spent fluids from the serumless-grown monkey kidney cells cleared solutions of powdered milk, supposedly indicative of tryptic action (26), these fluids failed to remove monolayers of cultured cells from glass or plastic surfaces after exposure at 37 C for several days. The osmolarity of unused and depleted culture fluids with and without the protein supplements was found to be within acceptable limits of 273.8 to 283.5 milliosmols.

**DISCUSSION**

Added credence to certain long-held tenets of workers in tissue culture was provided by the results of our study. We observed (i) the variation in the nutritional needs of cultured cells from various species of animals, (ii) the variation in the growth potential of different lots of cells from the same species, (iii) that the final cell population in a confluent primary culture is obtained from only a fraction of the inoculated viable cells, (iv) that the addition of serum to the medium accelerates the growth of cells from each species in vitro.

Rhesus and vervet monkey kidney, mouse embryo, and chicken embryo cells, but not those from other animals tested, could consistently be kept for a time in serumless medium. This we take as indicating differences in the nutritional or other needs of primary cells. However, we are unable to explain the variation observed among different cell lots of rabbit kidney.

**TABLE 2. Relationship between inoculum size and survival of primary chicken embryo and rabbit kidney cells in medium with or without serum**

| No. of viable cells inoculated $\times 10^6$/ml | No. of viable cells recovered after 5 days growth in medium with: |
|--------------------------------------------|----------------------------------------------------------|
|                                           | 5% Fetal calf serum | No serum |
| Rabbit kidney                              | Chicken embryo    | Rabbit kidney | Chicken embryo |
| 7.0                                       | 9.5 ± 1.1          | 7.7 ± 1.3     | 0.9 ± <0.1   | 6.2 ± 1.0     |
| 6.0                                       | 6.3 ± 1.3          | 7.6 ± 2.4     | 0.7 ± 0.1   | 5.8 ± 0.9     |
| 5.0                                       | 6.0 ± 1.2          | 6.8 ± 2.2     | 0.6 ± <0.1  | 5.0 ± 1.2     |
| 4.0                                       | 4.8 ± 1.7          | 6.3 ± 1.1     | 0.3 ± <0.1  | 4.4 ± 1.1     |
| 3.0                                       | 4.3 ± 0.9          | 6.0 ± 2.0     | 0.2 ± <0.1  | 3.5 ± 0.8     |

*Average cell count $\times 10^6$ per ml ± mean standard error. Triplicate bottles were each inoculated with 7 ml of cell suspension at the level shown.

Fig. 4. Survival of cells in medium and in saline with and without serum. Maintenance of rabbit kidney cells. See legend to Fig. 2.
Some earlier findings on the role of serum in tissue culture systems have been reviewed (14). Recently, Wallis and co-workers (26) postulated that one role of serum in the growth of monkey kidney cell cultures is to inhibit proteolytic enzymes synthesized by the cells themselves. Shodell and Rubin (24) found that serum was needed to stimulate mitotic activity in chicken embryo cells. Our results partially confirm and extend the observations of Wallis et al. and may disagree with those of Shodell and Rubin in that we found that primary cells of monkey kidneys and chicken embryos do not require serum for initial growth in vitro. However, the protease claimed by Wallis and co-workers to be responsible for cell sloughing and autolysis could not be inhibited by certain known specific trypsin inhibitors of nonsemen origin.

In addition to whole serum, either the globulin or albumin fraction was sufficient to facilitate accelerated cell growth and to allow primary monkey kidney cells to be transferred serially for at least 12 passages. Although serum in cell culture medium is thought to contribute to its osmolarity and buffering systems, in our experience the absence of serum did not adversely affect these parameters.

Birch and Pirt (2), working with continuous cell lines, have demonstrated that serum provides additional amounts of choline above that normally included in basal media. Perhaps the intracellular nutrient pool of some primary cells contains a sufficient concentration of choline for which a time precludes the need for the additional amount supplied by serum. Conversely, it may be that certain primary cells require less choline than do established cell lines.

It has been our experience that primary cells unable to adhere to glass die, i.e., they would not grow in suspension. The lytic enzyme responsible for the clearing of milk may well have come from released lysosomal material of dead or dying cells. In some cases, the dying process of cells grown in serum-less medium may even be increased by latent agents no longer under the specific or nonspecific inhibition provided by serum (unpublished observations).

Results from preliminary studies on the use of primary cells grown in serumless medium as substrates for virus replication indeed suggest an enhanced infection by certain viruses. This is in agreement with the findings of Rappaport (21). It is thus suggested that primary cells grown without serum may provide a more rapid and clearer demonstration of the presence of adventitious agents.

In addition, the omission of serum from cell culture medium denies cholesterol-requiring mycoplasmas their optimal growth conditions (22) and eliminates one source of contaminating mycoplasmas (1) and viruses (16) from tissue culture systems.

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