A Method for the Establishment and Long-Term Maintenance of In Vitro Monocytic Cultures With Functional and Morphological Homogeneity

By Beverly S. Packard, Mehdi Tavassoli, George L. Dale, and Ernest Beutler

The tendency of monocytes to develop into morphologically heterogeneous and often multinucleated populations is a source of difficulty in studying and particularly of quantitating monocytic functions in vitro. By testing various combinations of media and sera used in previous studies for the culture of monocytes from blood and marrow, we have established and maintained morphologically and functionally homogeneous cultures of monocytes derived from human peripheral blood in vitro for up to 3 mo.

Methods

The procedure used for separation of monocytes from peripheral blood was a modification of the Boyum method. Peripheral blood, drawn from normal healthy volunteers with heparin at a final concentration of 15 U/ml, was diluted 1:2 with 0.154 M sodium chloride. This suspension was divided into 25-ml portions, each of which was pipetted over 10 ml of Hypaque-Ficoll and centrifuged at 1000 g for 12 min at room temperature. The cells at the Hypaque-Ficoll/plasma interface were collected, resuspended in HBSS, and washed 3 times, centrifuging at 450 g for 10 min. They were then resuspended at a concentration of approximately 10^7/ml in the medium and the serum to be studied. Starting with 200 cc peripheral blood, 20-26 x 10^6 cells were isolated after the Hypaque-Ficoll gradient and 3 washes. After a cell count, 0.25 x 10^7 mononuclear cells were added to each 17-mm diameter well containing a 12-mm diameter round coverslip; a differential count showed on average that this cell population was 30% monocytes. The dishes were allowed to stand at 37°C for 2 hr in an atmosphere of 5% CO2/95% air to allow monocytes to attach (adherence phase). During this phase, cells were cultured in combinations of RPMI 1640 or Fischer's medium with 10% heat-inactivated autologous or heterologous serum or 25% horse serum (Table I). The plates were then washed 3 times with HBSS and the adherent cells overlaid with 0.5 ml of medium-containing serum (maintenance phase). Media were changed twice per week. Combinations of Neuman-Tytell or Fischer's medium with autologous, heterologous, or horse serum were used for this phase (Table I). Additionally, fetal calf serum was used with several media in pilot experiments and found to give very poor cell viability.

The number of cell nuclei was quantitated by the addition of 2 drops of Zap-o-globin/17-mm well at the end of the incubation period (3 wk in experiments reported in Table I) to release nuclei, which were then counted in a Coulter counter. The fraction of cells containing 2 or more nuclei, i.e., percent multinucleated cells, was determined by direct count at the light microscopic level.

The DNA content of the cell cultures was determined by the method of Brunk et al. A coverslip with attached cells was washed in saline and then placed in a 15-ml conical centrifuge tube along with

Materials and Methods

Materials

Fischer's medium (F) in powdered form, McCoy's medium in powdered form, serumless (Neuman-Tytell [N-T]) medium in liquid form, RPMI 1640 in liquid form, Hanks' balanced salt solution (HBSS), and penicillin/streptomycin were purchased from Grand Island Biological Company (Grand Island, N.Y.). Horse serum (HS) (lot no. R592519) from GibCO was used principally; six other lots of HS from GibCO, Flow Laboratories (Rockville, Md.), and Irvine Scientific (Irvine, Calif.) were tried and found suitable. Different lots of fetal calf serum (FCS) were obtained from GibCO and Microbiological Associates (Bethesda, Md.).

Hypaque-Ficoll and reagents for nonspecific esterase strain, α-naphthyl acetate esterase (kit nos. 90-A1), were purchased from Sigma Chemical Company (St. Louis, Mo.), preservative-free heparin from Biochemical, Inc. (Hawthorne, Calif.), Wright-Giemsa stain from Harleco (Gibbstown, N.J.), polystyrene latex particles (10%) (1.091 μ±.0082) (lot 1A39) from Dow Chemical Company (Indianapolis, Ind.), plastic 35-mm diameter Petri dishes (Falcon no. 3001) and 17-mm multwell culture dishes (Falcon no. 3008) from Scientific Products (Sunnyvale, Calif.) 12-mm diameter round glass coverslips from Belco Glass, Inc. (Vineland, N.J.), Zap-o-globin H from Coulter Electronics (Hialeah, Fla.), and Hoechst Dye 33258 from Calbiochem (La Jolla, Calif.).

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300 μl of 1% (w/v) taurocholate in phosphate-buffered saline. The coverslip was pulverized with a glass stirring rod, and the final suspension was frozen and thawed 3 times. After thorough mixing, aliquots were taken for DNA determination with the fluorogenic dye Hoescht H33258. Calf thymus DNA was used as a standard; the assay was linear from 0 to 5 μg of DNA.

Incorporation of 3H-thymidine was monitored in a separate experiment. Cells were attached for 2 hr as outlined above, the nonadherent cells washed off, and fresh media (25% HS in Fischer’s) applied. One microcurie of 3H-thymidine was added to each 500 μl of media. The cells were incubated with this media, or a fresh change, for up to 60 min. Aliquots were taken for DNA determination with the fluorogenic dye Hoescht H33258. Calf thymus DNA was used as a standard; the assay was linear from 0 to 5 μg of DNA.

To study the phagocytic function, coverslips were removed and placed in 35-mm diameter Petri dishes. One milliliter of a solution containing 0.05% latex particles in medium (without serum) was added to each Petri dish, which was then incubated at 37°C for 30 min. Medium was removed, plates were washed 3 times, air-dried, and stained. The cells were stained for nonspecific esterase (NSE) using the procedure of Yam et al.

RESULTS

A variety of media and sera were studied to determine conditions optimal for maintenance of peripheral blood monocytes/macrophages in culture. The number of nuclei and degree of polynucleation per culture for various conditions are shown in Table 1. Conditions yielding the highest numbers of cells and lowest degrees of polynucleation were considered optimal. Using these two criteria we ranked the cultures in order; Table 2 shows the eight best culture conditions. It can be seen that a characteristic common to all is some exposure to horse serum, with three of the four best culture conditions including horse serum in the maintenance phase medium. In these cultures, more than 99% of the cells were mononucleated and esterase positive, with cell diameters ranging between 30 and 60 μm. As a measure of phagocytic function, each cell in culture no. 36 endocytized 33.3 ± 5.1 latex particles.

In contrast to the cultures listed in Table 2, many other cultures contained cells with two or more nuclei, i.e., polynucleation. This was particularly true when the cell density was very high: all cultures with greater than 3 x 10⁵ cells (nos. 1, 4, 7, 19, 22, and 28) had at least 10% polynucleated cells; there was a substantial heterogeneity in the number of nuclei per polynucleated cell, ranging from 2 to 30.

There were striking morphological differences between cultures without polynucleation and those containing polynucleated cells. In the latter, giant cells displayed multiple nuclei all contained within a large mass of cytoplasm surrounded by one membrane as
LONG-TERM MONOCYTE CULTURE

Table 2. Optimum Culture Conditions

<table>
<thead>
<tr>
<th>Culture No.</th>
<th>No. Cells (x 10^-4)/Culture</th>
<th>Adherence Phase Medium</th>
<th>Growth Phase Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>2.37 ± .86</td>
<td>F + 10% AHS (*)</td>
<td>F + 25% HS</td>
</tr>
<tr>
<td>6</td>
<td>2.20 ± .29</td>
<td>RPMI + 10% AHS (*)</td>
<td>F + 25% HS</td>
</tr>
<tr>
<td>34</td>
<td>1.98 ± .72</td>
<td>F + 25% HS</td>
<td>F + 10% AHS</td>
</tr>
<tr>
<td>13</td>
<td>1.47 ± .86</td>
<td>RPMI + 25% HS</td>
<td>NT + 10% AHS</td>
</tr>
<tr>
<td>36</td>
<td>1.37 ± .26</td>
<td>F + 25% HS</td>
<td>F + 25% HS</td>
</tr>
<tr>
<td>18</td>
<td>1.25 ± .19</td>
<td>RPMI + 25% HS</td>
<td>F + 25% HS</td>
</tr>
<tr>
<td>35</td>
<td>0.657 ± .40</td>
<td>F + 25% HS</td>
<td>F + 25% HS</td>
</tr>
<tr>
<td>32</td>
<td>0.546 ± .15</td>
<td>NT + 10% HHS</td>
<td>F + 10% AHS</td>
</tr>
</tbody>
</table>

The starting number of monocytes for each well was approximately 7.5 x 10^4.

F, Fischer's medium; RPMI, RPMI 1640 medium; NT, serumless (Neuman-Tytell) medium; AHS, autologous serum; HHS, heterologous serum; HS, horse serum.

*Heat-inactivated (56°C, 30 min).

contrasted with the more homogenous mononucleated cells observed in the former.

To determine if the attached monocytes in these cultures divided or were end-stage cells, we carried out two types of experiments. In the first, we monitored the total DNA content of a number of identical culture wells as a function of time. The data from two of three separate experiments are shown in Fig. 1; it is clear that after the first 5–10 days, the DNA content of the cultures is relatively stable. The fluctuations in the first 10 days may be due to detachment of some weakly bound cells; however, such a loss of cells might also mask the division of healthy attached cells. To eliminate the possibility that the cells may be dividing, cultures were incubated with 3H-thymidine. No 3H-thymidine incorporation into DNA was observed in experiments that could detect division of 0.1% of the cells.

Cultures were established from six different individuals using the conditions of culture no. 36, in which both the adherence and maintenance phases used Fischer's medium containing 25% horse serum. These cultures were all maintained satisfactorily for at least 3 mo, retaining a uniform morphological appearance and developing no multinucleated cells.

DISCUSSION

We have developed a method to establish and maintain populations of monocytes in vitro that are morphologically and functionally homogeneous for up to 3 mo. By using various combinations of media and sera we have identified several combinations that permit effective maintenance. Common to all is the presence of horse serum somewhere in the culture history, suggesting that this exposure is essential for optimal development. Such cultures contain greater than 99% mononucleated cells, all positive for nonspecific esterase and

![Fig. 1. DNA content of monocytic cultures. Monocytes from two individuals were established in culture and the DNA content of duplicate wells determined as a function of time. In another study (not shown) no significant change in DNA content was observed during the first week of culture.](image-url)
phagocytic function. Horse sera from seven different lots were all comparably effective.

In addition to providing optimal culture conditions, horse serum has the advantage over autologous human serum in that it permits a comparison among cultured cells from different individuals without the influence of serum factors that may vary. Therefore, we believe the conditions of culture no. 36, Fischer’s medium with 25% horse serum in both the adherence and maintenance phases, represents the method of choice for comparative human studies.

Consistent with the observations of others,4,7 many of our cultures contain giant and epithelioid cells, each possessing from 2 to 30 nuclei. Such morphological heterogeneity is unacceptable in trying to quantitate monocytic functions in vitro. Table 2 lists eight combinations of media and sera that produce homogeneous cultures.

We believe our method, facile and reproducible and resulting in stable homogeneous cell populations, may be useful in the evaluation of basic functions of cells of the monocytic lineage.

REFERENCES

A method for the establishment and long-term maintenance of in vitro monocytic cultures with functional and morphological homogeneity

BS Packard, M Tavassoli, GL Dale and E Beutler