A New System of Hemopoietic Colony Formation for Permanent Slides and Medium Changes: Use of Glass-Fiber Filters

By F. Kodama, A. Maruta, S. Motomura, K. Fukushima, and M. Umeda

A new method for hemopoietic colony formation that allows the preparation of permanent slides and medium changes during the incubation period was developed in vitro. Bone marrow cells from mice were spread over glass-fiber filters, which were placed on agar medium and cultivated for 7 days. Hemopoietic colonies appeared on the glass-fiber filters. The glass-fiber filters with colonies were stained by a peroxidase and nonspecific esterase double-staining method and mounted as permanent slides. Each colony could be clearly identified and easily counted after the staining. The dose–response relationship between the number of seeded cells and the colony counts was a linear one, with the line very nearly passing through the origin on extrapolation. The colonies were classified into three types by the staining results: granuloid type, monocyte-macrophage type, and mixed type. The last containing both granuloid and monocyte-macrophage cells. Medium change during the incubation period was attempted in the experiment for phagocytic activity of the cultured cells and proved to be useful. This system appears to be useful and convenient in the study of hemopoietic colony formation.

IN VITRO METHODS of hemopoietic colony formation have been reported in the past. Most of them utilized semisolid media. It is, however, difficult to change medium during the cultivation or to prepare permanent specimens of the formed colonies by the use of such media. In order to cover these disadvantages, several attempts have been made until now. None of them, however, could provide a system in which both medium change and the preparation of permanent slides became feasible in vitro.

Recently, Enaka and Umeda have devised a new culture system that consists of the cultivation of cells on glass-fiber(GF) filters placed on solid agar plates. In this method, the cells can be inoculated on the filters and cultured to form colonies. The filters can be transferred to new agar plates for the purpose of medium change during the cultivation, and fixed and stained for permanent preparations.

Application of this system to hemopoietic colony formation was made, and cell types of the formed colonies were identified using a peroxidase and nonspecific esterase double-staining method or by tests for phagocytic activity. Here we report our method and discuss the advantages and disadvantages.

MATERIALS AND METHODS

Cell Preparation

C57BL/6N male mice, 6-8 wk old, were obtained from Japan Clea Co., Ltd., Tokyo. They were killed by cervical dislocation, and the femurs were removed under sterile condition. Bone marrow cells were isolated by flushing the medullary cavity of the femurs with Hanks' balanced salt solution (BSS). The cells were pipetted into a single cell suspension, washed once with BSS, and then suspended in BSS for seeding at a cell density of 2 x 10^6 cells/ml.

Culture Medium

The constituents of culture medium were 65% (v/v) Ham's F12 (Nissui Seiyaku Co., Ltd., Tokyo), 20% (v/v) unoinactivated horse serum (Flow Laboratory), 15% (v/v) conditioned medium, and 0.5% (w/v) Bacto agar (DIFCO). Conditioned medium was prepared by the incubation of the peritoneal membranes of C57BL/6N mice in 80% Ham's F12 and 20% horse serum as described by Horiiuchi and Ichikawa. It was kept at -20°C until ready for use. The same batch of conditioned medium and the same lot of horse serum were used in the present experiments.

Culture Method

The culture method used in this study was described previously. Briefly, glass-fiber filters (Whatman, GF/A type, 24 mm in diameter, England) were sterilized by autoclaving and dried beforehand. After the agar culture medium was poured into 35-mm glass Petri dishes at a volume of 2.0 ml/dish and solidified, GF filters were placed on the agar plates. A 25-μ1 aliquot of cell suspension containing 5 x 10^6 cells was evenly distributed on each filter using a micropipette. The dishes were incubated at 37°C in a humidified 5% CO₂-95% air incubator for 7 days. Each experiment was performed using quadruplicate cultures.

Staining

The procedure for staining was as follows: the GF filters on which colonies were generated were placed on a Buchner-type funnel with a sheet of cellulose filter paper and washed gently with physiologic saline. The filters were fixed in 0.5% cold copper sulfate for 5 sec and then placed on tissue-paper for a few seconds in order to absorb the excess fixative. Subsequently, peroxidase staining and nonspecific esterase staining were performed on the filters. Peroxidase staining by the method of Inagaki was slightly modified. The substrate solution contained 10 mg of 2,7-fluorescin dihydrochloride (Sigma) in 10 ml of 0.1 M Tris buffer. One drop of 1% hydrogen peroxide solution and 100 μg of ZnSO₄·7H₂O were added to 10 ml of the...
substrate solution just prior to use, and the mixture was filtered through cellulose filter paper. The GF filters with colonies were incubated in the mixture for 30 min at room temperature, followed by a rinse with physiologic saline using the Buchner-type funnel. Nonspecific esterase staining was performed for 15 min at room temperature according to method-1 of Leder with the use of α-naphthyl acetate (Tokyo Kasei Co., Ltd., Japan) as a substrate. After this double-staining, the counterstaining was carried out with Myer's hematoxylin solution for 10 min, followed by a rinse to remove the excess staining solution. The GF filters were then dehydrated through the ethanol series, immersed in xylene for about 10 min, and mounted on glass slides with H.S.R. solution (Harleco Co., Ltd., U.S.A. and Green Cross Co., Ltd., Japan) which is composed of 62.5% (w/w) Pycolite (Harleco Co., Ltd., U.S.A.) in xylene. The preparations were examined microscopically using low magnification (×100) for colony count and a high magnification (×1000) for the observation of morphological and cytochemical characteristics of cells. Colonies consisting of more than 50 cells were counted.

Tests for Phagocytic Activity

One drop of Pelikan India-ink (Günther Wagner, Germany) was diluted with 4 ml of BSS and the diluted solution was filtered through two sheets of cellulose filter paper. Two tests for phagocytic activity were carried out. In the first test (test 1), the diluted and filtered India-ink solution was spread directly over the cultured GF filters at a volume of 0.3 ml/dish and the dishes were incubated for an additional 3 hr. After rinsed with physiologic saline, the GF filters were stained for nonspecific esterase activity described before. In the second test (test 2), the cultured GF filters were rinsed aseptically on the sterilized Buchner-type funnel, transferred to agar-medium plates containing 30 μg/ml cytochalasin-B (Sigma), an inhibitor of phagocytosis, and incubated for 3 hr. Thereafter, they were treated with the India-ink solution as in the case of test 1.

RESULTS

Colony Formation on Glass-Fiber Filters

Concentrations of horse serum and conditioned medium suitable for the cultivation of hemopoietic cells on the GF filters were determined. Several experiments were performed and similar results were obtained in each experiment. A typical result is shown in Table 1. The maximum colony number was obtained using a concentration of 20% horse serum and 15% conditioned medium. Therefore, subsequent experiments were performed under this condition.

Number of colonies was determined by plating different number of bone marrow cells. A linear dose-

![Fig. 1. The relationship between the number of 7-wk-old C57BL mouse bone marrow cells seeded on the GF filters and the number of colonies developed with the addition of C57BL mouse peritoneal conditioned medium in the agar medium. The incubation period was 7 days. Each circle with vertical bars is the mean and standard deviation of the results from the quadruplicate culture.](image)

Table 1. Effect of Concentration of Horse Serum and Conditioned Medium on Colony Formation

<table>
<thead>
<tr>
<th>Horse Serum (%)</th>
<th>Conditioned Medium (%)</th>
<th>10</th>
<th>15</th>
<th>20</th>
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<tr>
<td>10</td>
<td>147 ± 14*</td>
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<td>168 ± 14</td>
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<tr>
<td>20</td>
<td>179 ± 10</td>
<td>191 ± 23</td>
<td>176 ± 9.7</td>
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</table>

Each value represents the mean ± SD of four dishes.

*Results are expressed as the number of hemopoietic colonies per 5 x 10⁶ cells plated.

response relationship was observed and the line passed very nearly through the origin on extrapolation (Fig. 1).

Staining of the Colonies

The peroxidase and nonspecific esterase double-staining was performed on the GF filters as described in Materials and Methods. Peroxidase-positive cells were recognized as having dark-green granules in the cytoplasm. Nonspecific esterase-positive cells were identified as cells with diffuse brown or red-brown stains in the cytoplasm. Granulocytes were easily recognized as positive cells in peroxidase activity, but negative in nonspecific esterase reaction. They were smaller in size than monocyte-macrophage cells, and the shape of their nuclei was polymorphous: round, indented, band-shaped, and segmented (Fig. 2A). Monocyte-macrophage cells were identified as mononuclear cells with nonspecific esterase-positive reaction and exhibited large spindle-shaped or polygonal appearance, frequently accompanied with several cytoplasmic processes (Fig. 2B).

The dark-green granular stain in the peroxidase-positive cells was retained for 3 wk after the mounting, but, thereafter, its color began to fade gradually and disappeared almost completely within 2 mo. In contrast, the diffuse brown stain in the nonspecific esterase-positive cells was retained for more than 6 mo after the mounting.

Colony Count

The colonies were classified into three types by the results of the staining: “granulocytic colonies” consist-
small number of mononuclear cells, both negative in the double staining (Fig. 2C).

Morphologically, colonies were also classified into three types: "compact type" (Fig. 3 A, B, C), "dispersed type" (Fig. 3 D and E), and "intermediate type" (Fig. 3F). Table 2 shows a result of differential counts of the colonies that were formed on GF filters, stained, and classified from the cytochemical and morphological viewpoints described above. The percentages of granulocytic, monocyte-macrophage, and mixed-type colonies were 38, 36, and 26, respectively.

Tests for Phagocytic Activity

As India-ink particles could be washed out easily from the filter by rinsing, it was not difficult to determine the phagocytic activity of cells under a microscope. Two types of experiments were performed to examine the phagocytic activity of the cells in colonies. In test 1, India-ink particles were applied directly on the cultured filters. Ninety-six percent of positive cells in nonspecific esterase staining were shown to phagocytose a large or moderate number of India-ink particles in the cytoplasmic areas (Fig. 4 A and B). On the other hand, in test 2, India-ink particles were applied on the filters after the treatment with cytochalasin-B, which is an inhibitor of phagocytosis, and a few or no ink particles were found in the nonspecific esterase-positive cells under a microscope. From these results, it was concluded that almost all the nonspecific esterase-positive cells generated on the GF filters possessed phagocytic activity and were consistent with monocyte-macrophage cells.

DISCUSSION

The present study demonstrated that mouse hematopoietic cells could form colonies on GF filters that were placed on the agar medium containing conditioned medium. The data of dose–response relationship were compatible with the assumption that the colonies arise from single cells. Because the filters become transparent in xylene, permanent preparations of the filters can be made after treatment with ethanol-xylene series and mounting by the use of a suitable mounting medium. Several mounting media were examined, but some of them were not suitable because air entered into the filters a few days after the mounting. H.S.R. solution was very suitable as a mounting medium. Thus, stainings with hematoxylin-eosin, PAS, and so on are applicable to cells on GF filters.

The double-staining method by Litt is very useful, because it can discriminate granulocytes from monocytes, simultaneously. The method, however, does not use xylene but glycerol for mounting. As an alternative
Fig. 3. Various kinds of colonies generated on the GF filters. (A, B, and C) Three kinds of compact-type colony: compact granulocytic, compact monocyte-macrophage, and compact mixed, respectively (×25). (D and E) Two kinds of dispersed-type colony: dispersed granulocytic and dispersed monocyte-macrophage, respectively (D, ×33, E, ×25). (F) An intermediate mixed-type colony (×25). All the colonies were stained in situ by peroxidase and nonspecific esterase double-staining.

Mixed-type colonies, which contained both granulocytes and monocyte-macrophage cells, were demonstrated by the present method and counted as 26% as shown in Table 2. These facts might support the ideas that granulocytes and monocyte-macrophage cells originate from the same progenitor cells.

A small number of mononuclear cells negative in both peroxidase and nonspecific esterase reaction were observed occasionally in each kind of colony. The
### Table 2. Cytochemical and Morphological Classification of Mouse Hemopoietic Colonies Generated on Glass-Fiber Filters

<table>
<thead>
<tr>
<th>Filter No.</th>
<th>Number of Colonies</th>
<th>Type of Colonies</th>
<th>Number of Colonies</th>
<th>Type of Colonies</th>
<th>Number of Colonies</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Granulocytic</td>
<td>C</td>
<td>D§</td>
<td>I</td>
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<td>11</td>
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<td>10</td>
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<tr>
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<td>13</td>
<td>43</td>
<td>6</td>
<td>7</td>
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<td>3</td>
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<td>14</td>
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<td>24</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>4</td>
<td>170</td>
<td>19</td>
<td>50</td>
<td>8</td>
<td>6</td>
<td>11</td>
</tr>
</tbody>
</table>

Average number (± SD) 154 ± 12 14 ± 3.0 42 ± 3.0 2.8 ± 1.9 37 ± 6.0 12 ± 3.8 6.0 ± 1.4 8.5 ± 2.6 8.8 ± 3.5 23 ± 5.4
Average percentage of each type 9.1 27 1.8 24 7.8 3.9 5.5 5.7 15

*Bone marrow cells obtained from a C57BL mouse were seeded at 5 × 10⁶ cells/filter and cultured for 7 days.
†Colonies that contain both granulocytes and monocyte-macrophage cells.
‡Compact-type colonies.
§Dispersed-type colonies.
|Intermediate-type colonies.

exact nature of these cells, however, remains unclarified.

Phagocytic activity of monocyte-macrophage cells could be demonstrated by incubating the cells in the presence of India-ink. After the treatment with cytochalasin-B, India-ink particles were scarcely observed in the cytoplasmic area. Because cytochalasin-B inhibits the phagocytic activity and does not decrease the adhesiveness of cells, ⁰ the presence of India-ink particles in the cytoplasmic area in the former experiment can be assumed as the result of phagocytosis by the cells.

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Fig. 4. Phagocytic activity of cells generated on the GF filters with or without the pretreatment with cytochalasin-B. The colony in A and the cells in B, which were observed in the experiment without cytochalasin-B pretreatment, are positive in nonspecific esterase staining and positive in phagocytosis (A, ×33; B, ×670). The black India-ink particles phagocyted are observed in the cytoplasm. The colony in C and the cell in D, which were observed in the experiment with cytochalasin-B pretreatment, are positive in nonspecific esterase staining but negative in phagocytosis (C, ×33; D, ×670).
Our results in Table 2 demonstrate that most granulocytic colonies showed a dispersed type and most monocyte-macrophage colonies exhibited a compact type. This relationship is reversed from that reported by others using soft-agar. This reverse relationship would be derived from the difference of cultural environment between the present method and the other method.

In the present culture system, the medium permeates up into the GF filters after they are placed on agar plates. Therefore, minute spaces among the glass-fibers seem to be filled with the medium and to become a microenvironment for cell proliferation. In addition, the glass fibers serve as a substrate for cell attachment.

One of the advantages of this culture system is the ease of medium change during the cultivation. Since this merit was introduced and put to practical use in the test 2 experiment of phagocytosis, its beneficial effects were confirmed. On the other hand, it should be very difficult that medium change would be practiced by the conventional method using semisolid medium. Besides our method, the method used by Yoshida has been reported to be able to prepare permanent slides and to change medium during the cultivation. By her method, however, cellulose acetate membranes were primarily implanted into a mouse peritoneal cavity for a few days in order to produce macrophage-fibroblast layers as feeder cells on them and it can not utilize a conditioned medium in place of the macrophage-fibroblast layers. In contrast, the method presented here is an entirely in vitro system for hemopoietic colony formation.

Contrary to the above advantages, our method contains a disadvantage. The colonies growing on these GF filters can not be followed under an ordinary inverted light microscope, because the filters during the incubation are opaque. Further investigation is necessary to improve this weak point.

This culture system appears to be useful and convenient for the studies on hemopoietic colony formation.

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REFERENCES

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