Differentiation of Mouse Plasmocytomas \textit{In Vitro}: Two Phenotypically Stabilized Variants of the Same Cell


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\textbf{Abstract.} Mouse myelomas from Balb/c and C3H mice were established in tissue culture for more than 200 passages. When allowed to attach to a surface, they differentiated into two stabilized forms, one of which was fibroblast-shaped, grew without contact inhibition, and could be transplanted back to mice. The other had the morphology of epithelial cells, showed contact inhibition, and was not transplantable back to mice. For one strain (MOPC 173) it was demonstrated that both types of cells synthesize molecules with idiotypic determinants of the original myeloma protein. The relationship between the host cells and a leukemia-type virus present in the original tumor cells has been studied during different stages of cellular differentiation.

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Myeloma cells in culture have been used, until now, in experiments to define the mechanisms of synthesis of immunoglobulins\textsuperscript{1–4} or to study the genetic control of immunoglobulin synthesis.\textsuperscript{5–7} The establishment of clones of immunocompetent cells, able to express their differentiated phenotypes in tissue culture, is necessary for further understanding of the role of the various cell populations that are involved in the immune response. This communication deals with myeloma cells growing permanently in tissue culture, which can be induced to differentiate \textit{in vitro} into cells keeping some of their original properties, while expressing new functions characteristic of the differentiated state.

\textbf{Materials and Methods. Tumor lines:} Mouse plasmocytomas MOPC 173 and 5563 were repeatedly adapted to tissue-culture conditions by dispersing the cells or by placing very small pieces of tissue in Earle’s medium with Tris-HCl buffer, lactalbumin, yeast extract, vitamins, 20\% calf serum and 5\% mouse embryo extract, in disposable plastic bottles (Falcon Plastics). After a few weeks the attached cells were trypsinized, washed, and put in new plastic bottles with the same medium, except that no mouse embryo extract was included and the serum concentration was lowered to 10\% (for the fibroblast-type differentiation) or 2\% (for the epithelial type differentiation). In addition, to favor fibroblastic differentiation, the cells were trypsinized twice a week and reseeded at a lower cell concentration (50,000 cells per 30-ml bottle). The epithelioid state was obtained by dispersing the cells with EDTA once every 10 days and reseeding at a high cell concentration (500,000 cells per 30-ml bottle). In this paper we shall report only the results obtained with the MOPC 173 myeloma cells.

\textbf{Animals:} Balb/c mice were obtained from the Centre National de la Recherche Scientifique, Centre des Petits Animaux de Laboratoire, Gif-sur-Yvette.

\textbf{Staining and electron microscopy:} For light microscopy, cells were grown in

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Leighton tubes with glass coverslips. Attached cells were observed directly by phase-contrast microscopy or after staining by the Giemsa technique. For electron microscopy, cells were fixed with 1.6% glutaraldehyde and post-fixed with 2% osmium tetroxide. They were then dehydrated in acetone and embedded in Epon.

**Transplantable tumor assays:** Balb/c mice (2 months old) were injected with various doses of cell suspensions by the subcutaneous route and animals were observed during 3 months for tumor growth at the site of inoculation.

**Immune response assays:** Mice showing no tumor growth were challenged according to different schedules, either by injecting small pieces of the original tumor or by injecting a known amount of transplantable fibroblastic cells. Mice were kept under observation for 3 months for tumor growth at the site of inoculation.

**Immunoglobulin production:** A radiolabeled anti-idiotype antibody against the original myeloma immunoglobulin MOPC 173 was prepared in rabbits. Extracts were prepared from tumors induced either by the fibroblasts or by epithelioid cells which had been reverted to the fibroblastic state. The presence of the idiotypic determinants was determined by the Ouchterlony technique.

**Results.** MOPC 173 tumor cells grew immediately and differentiated to round floating cells (which were all discarded after a few weeks when the medium was changed) and to attached fibroblastic cells. Between the 2nd and the 4th month of culture, these attached cells started to differentiate again, mainly when the medium contained 2% calf serum. Round areas of contact-inhibited cells were formed; these were surrounded by a large reticulum of fibroblast-like cells which did not show contact inhibition (Fig. 1). By differential trypsinization (contact-inhibited cells are resistant to trypsin treatment) and repeated clonal selection, two stabilized "clones" of cells were isolated: one fibroblastic without contact inhibition (Fig. 2) and the other epithelioid showing marked contact inhibition (Fig. 3).

**Oncogenic properties (Table 1):** Between the 30th and the 60th passages,
the two cell phenotypes were not yet clearly differentiated and in most cases injection of $10^6$–$10^7$ cells induced tumors in Balb/c mice. After 100 passages in culture the fibroblastic cells induced a tumor in 100% of the mice if at least $10^6$ cells were inoculated, whereas after 150 passages as less as $10^3$ cells induced a tumor in almost all injected mice.

The epithelioid cells induced tumors after 60 passages in 25% of the mice at a dose of $10^3$ cells or more, but the growth of these tumors was much slower than those induced by the fibroblast cells. After 90 passages, the epithelioid cells were completely devoid of oncogenic capacity, even when as many as $8 \times 10^8$ cells were injected into each mouse. If the epithelioid cells were trypsinized twice a week for a month and cultivated in a medium containing 10% calf serum, they reverted to a fibroblastic type of cell and again became oncogenic. We have also obtained a fibroblastic revertant without oncogenic properties. Until now, we have failed to obtain a complete reversion from the fibroblastic to the epithelioid form; the fibroblastic cells seem to be more stable than the epithelioid cells.

Table 1. Oncogenic and immunogenic properties of fibroblastic and epithelioid cells at different stages of differentiation.

<table>
<thead>
<tr>
<th>No. of passages</th>
<th>30–60</th>
<th>100–150</th>
<th>150–200</th>
<th>60–90</th>
<th>90–130</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oncogenesis</td>
<td>20/24</td>
<td>10/10</td>
<td>11/15</td>
<td>10/41</td>
<td>0/41</td>
</tr>
<tr>
<td>Immunity</td>
<td>5/10</td>
<td></td>
<td></td>
<td>16/22</td>
<td>9/26</td>
</tr>
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</table>
Immunogenic properties (Table 1): One half of the mice inoculated once with $10^8$-$10^9$ cells of the 30th to the 60th passage were resistant to challenge with the original MOPC 173, while 80% of the control animals exhibited tumor growth. A very strong immunity (16/22) developed in mice injected with $10^7$-$10^8$ epithelioid cells from the 60th to the 90th passage. Few mice (9/26) injected with epithelioid cells from the 90th to the 130th passage were immune to the same challenge with MOPC 173. In another type of experiment, eight mice injected 3 weeks apart with $2 \times 10^7$ epithelioid cells and then challenged 1 week later with $10^6$ fibroblasts demonstrated tumors 5 days later, and four animals died in 5 weeks. In the control group only six of 10 mice demonstrated tumors after 2 weeks and two mice died after 2 months. In this case epithelioid cells had induced either immune facilitation to the fibroblastic cells or tolerance.

Immunoglobulin synthesis: Immunoglobulin synthesis was directly demonstrable during the first few passages, the cells being strongly radiolabeled. Moreover, extracts prepared from tumors induced by the fibroblastic cells or by the reverted form of the epithelioid cells both gave a reaction of partial identity with an anti-idiotypic serum prepared against the MOPC 173 myeloma globulin. High concentrations of extracts, however, had to be used in order to obtain a visible line of precipitation by the Ouchterlony technique.

Virion synthesis: The production of viral particles was studied in 2-day-old cells cultivated either in monolayers or in suspension. The presence of incomplete particles of the mouse leukemia virus group has been already described.9,10 We were able to find definite relationships between the host cell type and the number and/or the stage of maturation of these viral particles (Table 2).

Table 2. Relationship between host cells and virus particles.

<table>
<thead>
<tr>
<th></th>
<th>No. of A particles</th>
<th>No. of C particles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original tumor cells</td>
<td>9000*</td>
<td>10</td>
</tr>
<tr>
<td>Tumor cells induced by the fibroblastic cells</td>
<td>300</td>
<td>60</td>
</tr>
<tr>
<td>Tumors cells induced by the reverted epithelioid cells</td>
<td>&lt;10</td>
<td>100</td>
</tr>
<tr>
<td>Fibroblastic cells in monolayers†</td>
<td>2000</td>
<td>20</td>
</tr>
<tr>
<td>Fibroblastic cells in suspension†</td>
<td>700</td>
<td>500</td>
</tr>
<tr>
<td>Epithelioid cells in monolayers†</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Epithelioid cells in suspension†</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Average particle count in sections of 100 cells.
† 2-day-old cells from tissue culture.

Particles were extremely numerous in the original tumor MOPC 173, most of them being incomplete, i.e. of the A type11 without envelopes. In tumors induced by fibroblastic cells, A type virions were still numerous, while in tumors derived from the reverted epithelioid cells, particles were rare, most of them being enveloped virions of the C type.

In the fibroblastic cells cultivated in monolayers, numerous virions were seen, most of them being of the A type (Fig. 4), but 1% being complete virions of the C type (Fig. 5). The same cells cultivated in suspension gave about half of the total number of particles seen in the cells cultivated in monolayers; 40% of the particles were of the C type. With the epithelioid cells, virions were only rarely detected, irrespective of the culture system used.
FIG. 4. (a) "A" type particles (arrows) in the cytoplasm of a fibroblastic cultured cell. (b) Higher magnification of "A" particles budding into a cisterna of endoplasmic reticulum. Fig. 5. (a) and (b) "C" type virions in the extracellular space between fibroblastic cultured cells.
Discussion. Myeloma tumor cells which were allowed to attach to plastic or glass bottles differentiated into two different, phenotypically stabilized cells. The following considerations suggest that the two cell lines obtained represent two phenotypic expressions of the same cell.

(1) The differentiation occurred equally well for cells from either induced or spontaneous murine myelomas.

(2) Although we could not definitely establish the development of single cell clones, repeated clonings were performed by growing one colony from disperse plated cells or by the cluster-pick method, and in no instance did we observe an atypical clone—that is, cloning fibroblastic cells in a medium supplemented with 10% serum produced only fibroblastic cell colonies and cloning epithelioid cells in a medium supplemented with 2% serum gave rise only to epithelioid cell colonies. Moreover, when we reverted epithelioid cells to fibroblasts, in every case but one, the total population reverted and no isolated fibroblast colonies appeared.

(3) Cross-immune reactions, characterized by tumor immunity between the epithelioid line and the original tumor or by immune facilitation between both phenotypically stabilized cell lines, can be explained only by assuming that they share a common antigen which is immunogenic in Balb/c mice.

(4) Both cells have at least one idiotypic marker of the original MOPC 173 myeloma protein.

(5) If the fibroblastic and the epithelioid cells had a different origin, we would have to assume that these two different cell populations can grow together during hundreds of passages in vivo and in vitro without preferential selection of one of them. We would also be forced to assume that a myeloma tumor consists of a mixed cell population.

Rabinowitz and Sachs\textsuperscript{12} have observed that hamster cells transformed by polyoma virus showed differentiation from elongated fibroblastic cells into flattish epithelioid cells, which were unstable and had a different modal number of chromosomes. The reversible differentiation between myeloma cells described in this paper appears very similar. We have isolated reverted cells from the 130th passage of the epithelioid cells that had a fibroblastic morphology and lacked oncogenic properties even when 10^7 cells were inoculated to Balb/c mice. Thus it seems possible to isolate intermediate stages of differentiation between the epithelioid and the fibroblastic forms. A number of different factors seem to induce this reversion.\textsuperscript{13}

The relationship between the host cell and the synthesis of viral particles is of particular interest. Work dealing with cell transformation induced by viruses has focused attention on the role of the virus in modifying cell physiology. Fogel and Sachs\textsuperscript{14} have shown that irradiation of polyoma-transformed nonvirus-producing cells can induce virus production and inhibits cell growth, which led them to assume that the inhibition of cell multiplication in the polyoma-transformed cells is associated with the induction of virus synthesis. In the plasmocytoma cells, where the detected virus particles might or might not be concerned with the transformation, it seems that the opposite relationship could exist: in contact-inhibited cells, which have a long life-cycle, few virus particles (A and
C types) are synthesized while in the fibroblasts, which have a short life cycle many virions are detectable.

Cell differentiation has been induced in vitro mainly by DNA or RNA viruses. However, such differentiation without the help of a virus has also been reported for a mouse neuroblastoma,16 for Balb 3T3 cells transformed by SV4018 or by the murine sarcoma virus,14 and for myoblasts,17 chondrocytes,18 retina pigment cells,19 and pancreatic cells.20

All these results lead us to propose that, in vitro, one cell has the capability to express different linked phenotypic functions, depending on the milieu extérieur (which can be compared with the "milieu intérieur" in vivo). We emphasize that some of these functions, such as absence of contact inhibition, oncogenic properties, and the ability to synthesize viral particles for the fibroblast cells on the one hand and contact inhibition, absence of oncogenic properties, and inability to synthesize the virions for the epithelioid cells on the other hand are expressed in common.21

Biochemically "differentiated" cells have been characterized as cells committed to the synthesis of inessential or luxury molecules.22 Many modern embryologists agree that dedifferentiation is characterized by the loss of this specific function. As we have seen, plasmocytoma cells are able to express different phenotypes; a similar diversity might be expected for the cells involved in the immune response which share the same origin. Such a phenomenon could be invoked to explain the apparent necessity of cellular cooperation in the immune system23-26—that is, one might assume that a common cell precursor which bears the antibody site is induced to differentiate into different cells such as those of the bone marrow or thymus or even to the macrophage; because of this common antibody site these cells would now have increased probability to cooperate.

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