REPETITIVE MATURATION CYCLES IN A CULTURED MOUSE MYELOMA

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Recently, several murine myelomas have been adapted to continuous in vitro culture (1, 2). In general, these cell lines grow as stationary suspension cultures with few cells attaching to the growing surface. Exponential growth in these cultures continues indefinitely as long as cell density is kept below $1–2 \times 10^6$ per milliliter. Cultures with more than 2×10^6 cells per milliliter have been reported to undergo rapid deterioration. Considerable morphologic variation, especially in nuclear-cytoplasmic ratios, among the various cell lines has been observed. However, within a given cell line little morphologic variation in regard to the stage of plasma cell maturation has been described. We report here the establishment of a mouse myeloma cell line which undergoes typical plasma cell morphological differentiation. Shortly after the fully mature stage is reached, greater than 99% of the cells die; the few viable cells remaining initiate a new cycle of replication and maturation. At the time of this writing, these cells have continued to cycle in this fashion for over 1 yr. The underlying nature of the observed cycling does not appear to be related to cell density or to depletion of medium components.

MATERIALS AND METHODS

Animals

6–8–wk old female Balb/C mice were obtained from Jackson Laboratories, Bar Harbor, Maine.

Myeloma

Initially, mice bearing generation 117 of the plasma cell neoplasm ADJ PC-5 were obtained from Dr. M. Potter by Dr. D. Talmage who kindly supplied us with a recent transplant. We have maintained the tumor in vivo in both the ascites and subcutaneous forms by normal transplantation techniques.

Initiation of the Cell Line

1 million viable dispersed myeloma cells were injected intravenously into syngenic mice. 3 wk later, the animals were sacrificed, and their spleens were removed and teased into a single cell suspension. $10^4–10^5$ cells per milliliter were placed in a modified Eagle's minimal essential medium (3) containing 10% fetal calf serum (FCS) and incubated at 37°C in Falcon tissue culture flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Oxnard, Calif.) in an atmosphere of 83% N, 10% CO_2, 7% O_2. At first, the cells grew as a stationary suspension. After several passages, some of the tumor cells began to adhere to the bottom of the flasks. These cells were selected for propagation by pouring off nonadherent cells. The cyclic growth characteristics to be reported below were first observed after the adherent cell line was established. Stock cells have been maintained by media changes every 4–5 days accompanied by passage when necessary.

Experimental Procedure

A series of flasks was seeded at a density of 10^4 cells per milliliter with cells obtained from a culture which had recently passed from the death phase of the cycle into the replicative phase. One flask was chosen for light microscope observation. Sequential light micrographs of developing colonies were taken with a Leitz ortholux microscope. The remaining flasks were harvested one at a time at 2-day intervals for a period of 2 wk. The cells obtained from these flasks were prepared for electron microscopy. Cells were fixed for 30 min in 2.5% cacodylate-buffered (0.1 M, pH 7.3) glutaraldehyde, washed in buffer several times, postfixed for 1 hr in 1% OsO_4, dehydrated in a series of graded alcohol washes, and embedded in Epon. Ultrathin sections were stained with lead citrate and uranyl acetate and were examined with a Siemens Elmiskop I electron microscope at an acceleration voltage of 60 kv.

RESULTS

The adherent cell line established by us has the following growth characteristics. When $10^4–10^5$ cells per milliliter are plated into plastic tissue culture flasks, rapid attachment ($\sim$ 1 hr) of the cells to the plastic surface occurs. However, the adhesive bond between the cells and the plastic is quite weak and remains so during the entire maturation cycle; that is, the cells can be easily dislodged from the growing surface by gentle agitation. As replication progresses, discrete circular colonies are formed. The morphology of the

cells generally is spherical but extensive ameboid movements of individual cells can be observed under the microscope. Careful microscopic observation of the living cells reveals extensive cytoplasmic budding and release of these buds into the medium. The significance of this budding in relation to immunoglobulin release is under investigation.[1] Preliminary studies using enzyme-labeled antiimmunoglobulins (4) demonstrate that these cells continue to synthesize immunoglobulin but the class has as yet not been definitely determined. The parent tumor is an IgG_{2a} producer.

Shortly after establishment of the adherent cell line, we observed that the cultured cells undergo a very unusual growth cycle. The cells grow exponentially (doubling time 24–26 hr) until the density of the colony reaches 250–1000 cells. At this time the cells cease dividing and become granular in appearance. Shortly afterward death (observed by cytoplasmic lysing) of more than 99% of the cells in the colony occurs. For some as yet unexplained reason most of the colonies in a given bottle will degenerate within a period of 48 hr. The few remaining cells then re-enter an exponential growth phase to repeat the cycle. This process is illustrated in Fig. 1.

In an attempt to define a cause for this very unusual observation, we did the following experiments. First, we examined the possibility that the phenomenon was due to cell density within the colonies. To do this, we took a bottle of cells which was in an early recovery phase and continuously passed the cells such that the density of the individual colonies was never greater than 4–16 cells. In spite of this treatment, after 8–10 doublings cessation of cell division was followed by death and recovery as before. However, possibly due to lack of progenitor cells, surviving cells were not seen in every colony. This experiment also tended to rule out depletion of essential medium components since fresh medium was added with each passage. Moreover, results of additional experiments make us certain that depletion of medium components is not a factor in the observed cycling. When complete changes of medium were made every 12 hr during an entire cycle, the average doubling time decreased somewhat but a death phase still resulted after nine doublings. In addition, placing medium from cells which had just undergone the death phase onto cells in earlier phases of the cycle did not cause them to die. Here, no other medium was present. Perhaps even more significant was the observation that, after the death phase, when as few as 10–20 surviving cells were plated into small tissue culture flasks, the colonies which resulted continued to cycle. In this experiment the total number of cells per bottle containing 5 ml of medium never exceeded 20,000 cells at any time during the cycle.

We then decided that this phenomenon may represent a maturation process, especially since the number of doublings for one cycle ranged between 8 and 10, a number which is in close agreement to that reported by Nossal and Mäkelä for the maturation of normal plasma cells (5). At various times during the growth cycle, cells were harvested and prepared for electron microscopy. Upon observation we did indeed see morphologic evidence of plasma cell maturation (Fig. 2). The appearance of the cells ranged from blast or very immature plasma cells early in the recovery period to full maturation just before the death phase occurred. At any given time 70–90% of the cells appeared morphologically similar.

The degree of maturation was determined by both nuclear and cytoplasmic parameters. As maturation progressed there was an increased amount of heterochromatin at the periphery of the nucleus. More reliable, however, since bizarre nuclei were frequently encountered in these cells, was the progression of cisternal development of the granular endoplasmic reticulum. Early in the maturation cycle, cisternae were few in number and quite contracted. As maturation progressed cisternae became more apparent and progressively more dilated until, a day or two before the onset of the death phase, there was a preponderance of morphologically mature plasma cells. The maturation that we observed paralleled closely that reported for normal plasma cells by Leduc et al. (6).

DISCUSSION

The observations reported here are unique both in that apparent survival of progenitor cells after the death phase allows repetitive maturation cycles to occur and in that progeny of individual surviving cells reach maturation synchronously. However, in vitro differentiation of bone marrow–derived normal and leukemic cells has been reported in animal and human experiments (7–9). In these experiments the colonies that develop contain differentiating granulocytes but all stages

[1] Saunders, G. C., and W. S. Hammond. 1971. In preparation.

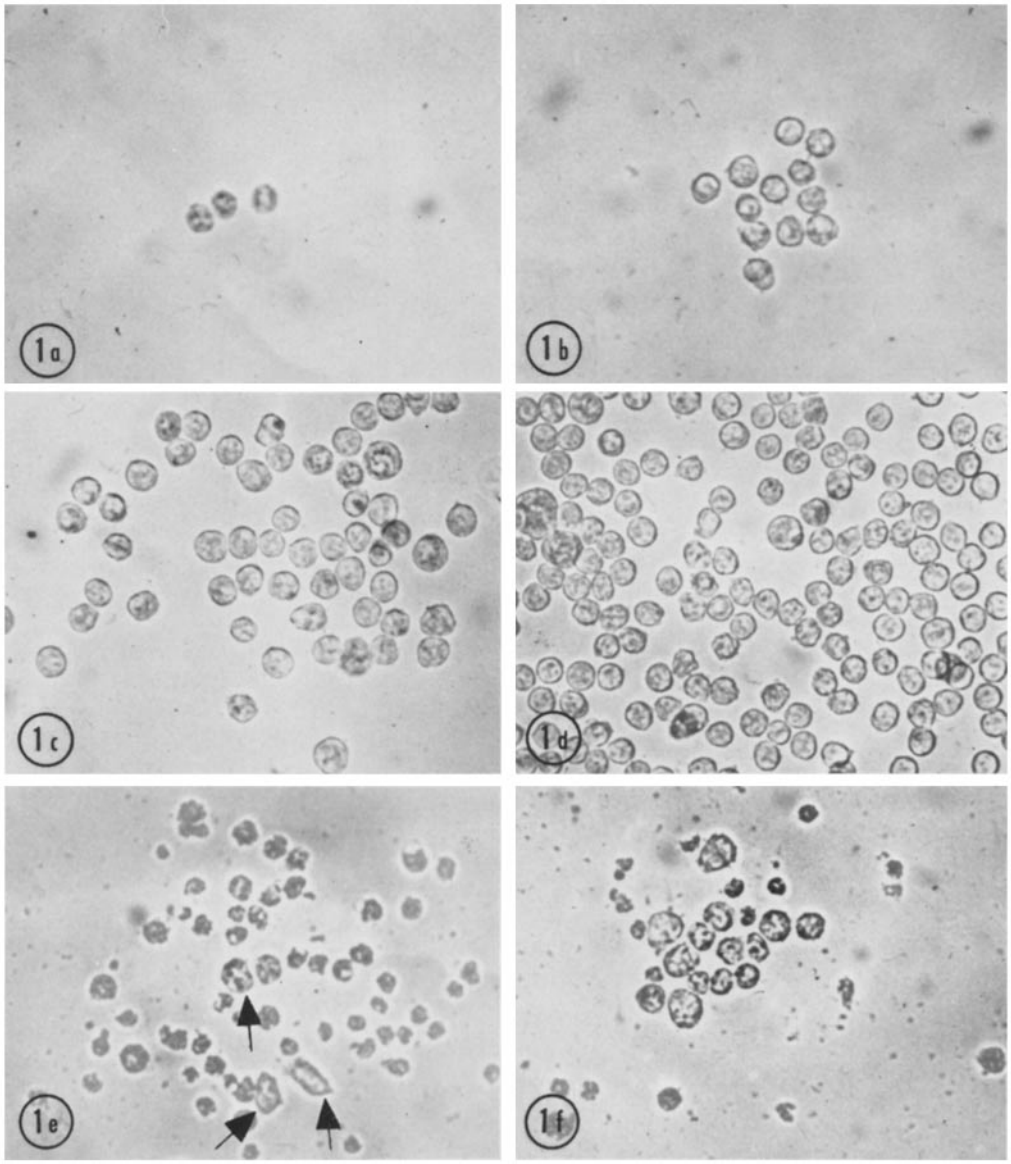

FIGURE 1 One complete maturation cycle. (*a*) 2 days after initiation of culture (see text). (*b*) 4 days. (*c*) 6 days. (*d*) 8 days. (*e*) 10 days: most of the cells are dead. The small dark dots represent the former point of attachment of the dead cells. The arrows indicate viable cells. (*f*) 14 days: the surviving cells have entered into a new replicative phase. (*a–f*) × 200.

may be present at any given time. This is in contrast to the data reported here, in that at any point in time most cells in developing myeloma colonies have similar morphology. In addition, the cycling reported in our experiments has not been reported to occur in granulocyte colonies.

The control mechanism responsible for the events which occur in this cell line have not been determined; however, several possibilities exist. First, we must not overlook the fact that these cells harbor a cytoplasmic virus-like particle (Fig. 2), and its relation to the growth of these cells must be determined. We do know that the average number of virus particles per plane of section of cell does not significantly change during the course of the cycle. At any point in time some cells will contain

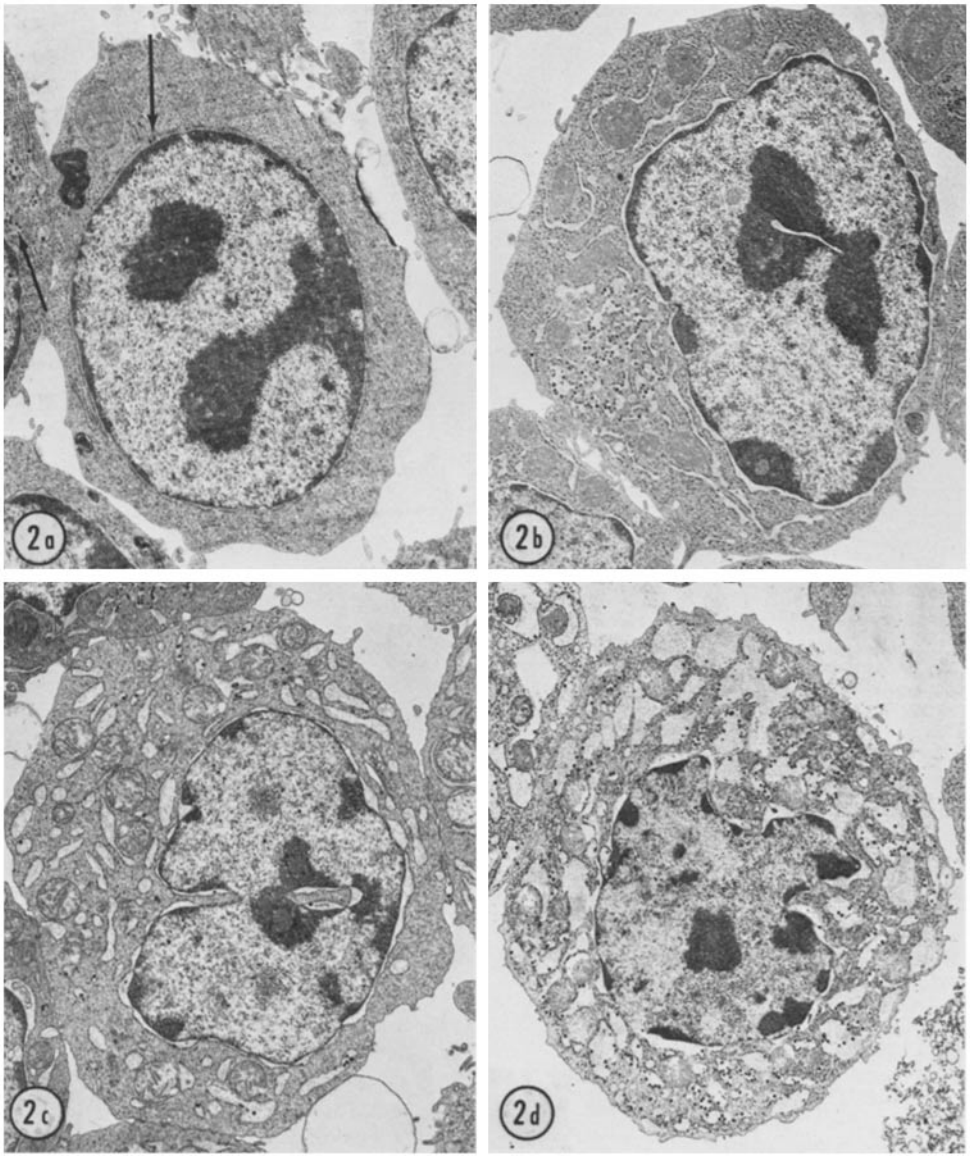

FIGURE 2 Representative cells at different points in the maturation cycle. (*a*) 2 days after initiation of culture (see text). At this stage, blast and very immature forms predominate. Virus-like particles (arrow) are seen to a greater or lesser extent in all cells. × 6500. (*b*) 4 days: cisternal development has begun. × 5500. (*c*) 6 days: cisternal development continues; nucleus is more typical of that of plasma cell. × 5500. (*d*) 8 days: appearance of cells just before death phase. Except for the presence of virus-like particles, this cell closely resembles a normal mature plasma cell. × 5000.

large numbers of these particles while other morphologically similar cells contain very few. Other myeloma cell lines are reported to have similar particles (10) but apparently do not undergo the maturation process reported here.

A second possibility is an intrinsic or extrinsic genetic mechanism. Nossal and Mäkelä have shown that in a normal immune response the number of plasma cell doublings is approximately 9; that is, essentially the same as we observe. In normal plasma cell maturation it may be that, once stem cells are derepressed, they are intrinsi-

cally triggered to undergo a series of doublings destined to end in suicidal maturation of most cells. In this case, any surviving cells are quiescent until restimulated by antigen. However, in our system there is a continued source of progenitor cells available which, by a yet unknown mechanism, are cyclically derepressed. The number of progenitor cells generated per cycle is greater than one since these cells do produce tumors when injected into Balb/C mice. Generally, three to five cells out of a colony of 500 cells will survive the death phase.

Extrinsic genetic control is also possible, in that a maturation factor or inducer, e.g. immunoglobulin, is produced by these cells which, through a feedback mechanism, causes maturation. It has been established that an inducer is present in the splenic conditioned medium which is responsible for the differentiation of both normal and leukemic cells in the human granulocyte colonies described above (8). Indeed, a defect in the production of this factor is postulated to be the cellular lesion in acute granulocytic leukemia.

Other mechanisms are possible but in any event the cell line we have isolated should provide a vehicle heretofore unavailable for the study of differentiation and maturation of cells.

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