Establishment of a Human Plasma Cell Line in Vitro

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ABSTRACT

A human plasma cell line designated ARH-77 has been established and propagated in culture for the past 2 years. The cells exhibited morphological characteristics of plasma cells under light and electron microscopic examination. An average of 40% cells are positive for immunoglobulin G by direct immunofluorescence, while an immunoglobulin G-specific radioimmunoassay reveals the production of \(1.21 \times 10^8\) ng/10\(^6\) plasma cells. The karyotype is aneuploid with a modal chromosome number of 45 to 46 and no marker chromosome. Growth kinetics characteristics are: doubling time, 110.4 hr; generation time, 56.4 hr; G\(_1\) + G\(_{0}\)-phase transit time, 45.5 hr; S-phase transit time, 10.9 hr; growth fraction, 74%; mitotic index, 1.5%; labeling index, 14.3%; and cell loss, 31.0%. Some of the growth kinetics characteristics were markedly similar to the properties displayed in vivo by plasma cells of patients with multiple myeloma and suggest that the cell line might be a useful in vitro model for the study of human myeloma.

INTRODUCTION

Development of in vitro PC lines would be useful for comprehensive studies to determine the biological properties of MM. Numerous attempts have been made to culture human malignant PC obtained from MM patients (4, 10-14). However, these attempts have been fraught with difficulty, and few established cell lines have been derived. Of the resultant established lines, 4 were established from peripheral blood samples [lines RPMI-8226 (12), 226 B1 (13), IM-10 (14), and RPMI-5286 (10)], 3 were established from bone marrow aspirates [lines 225 Bm (13), 268 Bm (13), and IM-9 (14)], and 1 was taken from a thoracocentesis sample [line LA-49 (11)].

The cell line described in this report was derived from the peripheral blood of a 33-year-old female with IgG PC leukemia. The patient was previously untreated prior to sampling. The cells were originally maintained in spinner culture. However, after 18 months of continuous propagation, this system was found to be detrimental to the structural and functional integrity of the cells. Cultures were then implanted as monolayer cultures. After 6 months the cells adapted to monolayer conditions and presently achieve near confluency in approximately 7 days. Morphological, cytogenetic, ultrastructural, and cell kinetics parameters were evaluated and were found to correlate with similar properties of in vivo PC.

MATERIALS AND METHODS

Tissue Culture. Plasma from 2 units of blood was centrifuged, and the cell concentration was adjusted to \(2.2 \times 10^7\) cells/ml of plasma. The cell suspension was split in equal volumes to 2 spinner flasks containing 100 ml of Ham’s F-10 medium supplemented with 10% fetal calf serum, vitamins (1% solution; Grand Island Biological Co., Grand Island, N. Y.; 100 x minimal essential medium), glutamine (200 mm; Grand Island Biological Co.), and antibiotics [penicillin (100 units/ml) and Tyloclin (60 \(\mu\)g/ml; Grand Island Biological Co.)]. Cultures were fed twice weekly by partial replacement of spent medium with fresh medium. After 18 months of continuous propagation, cells were transferred to monolayer culture in 18-oz Owen’s bottles. Cells were initially seeded in a concentration of \(1 \times 10^7\) cells/bottle, suspended in 50 ml of the nutrient medium described previously, and grown in a humid, 5% CO\(_2\) atmosphere maintained at 37°. The cultures were left undisturbed for approximately 2 months with the exception of infrequent microscopic monitoring for attachment and visible growth. Upon achievement of a near-confluent monolayer, serial passage was accomplished by agitation the culture bottle and pouring off the cell suspension into 50-ml centrifuge tubes. Cells were centrifuged at 2500 rpm for 5 min, resuspended in fresh medium, and aliquoted into new culture vessels. Cultures were routinely monitored for Mycoplasma contamination by biochemical (2) and electron microscopic means.

Morphological Studies. Morphological observations of live cultured cells were made with a Zeiss inverted microscope. Multicellular clumps observed floating free in the supernatant medium were carefully aspirated with a disposable long-stemmed pipet and incubated with an equal volume of 0.4% trypan blue in Hank’s balanced salt solution. Smear preparations of these cells were examined by light microscopy for cell viability and cell morphology. For additional morphological analysis cells were grown on sterile slides placed in 150-mm Petri dishes containing nutrient medium. After 7 days slides were removed, washed once in 0.9% NaCl solution, and fixed for 10 min in 95% methanol or air-dried. Slides were rinsed twice in 0.9% NaCl solution and stained with hematoxylin-eosin, colloidal iron, Sudan black, oil red, Naphthol AS-D chloroacetate esterase, periodic acid-Schiff, methyl green-pyronin, and May-Grünewald’s according to routine staining protocol (3).

Ultrastructural Studies. Cells harvested from a single culture were centrifuged at 3000 rpm for 5 min, fixed in 2.5% glutaraldehyde for 45 min, postfixed in 1% osmium tetroxide for 1 hr, and embedded in Epon 812. Sections were stained with uranyl acetate and lead citrate and examined in a Zeiss EM 10C transmission electron microscope.
were cut on an LKB Ultratome III ultramicrotome, (LKB Instruments, Inc., Rockville, Md.), stained with 1% uranyl acetate and Reynold’s lead citrate, and examined in a Siemens 102 electron microscope.

**Cytogenetic Studies.** Chromosomal analyses were performed on exponentially growing monolayer cultures seeded in 60-mm Petri dishes by incubating the cells with colchicine (0.04 µg/ml) for 6 hr. Cells were aspirated from the dishes, washed in 0.9% NaCl solution, and treated in a hypotonic solution (0.075 M KCl). Cells were fixed with Carnoy’s fixative glacial acetic acid:methanol, (1:3) for 30 min. Air-dried preparations were made and stained according to Giemsa banding protocol (1). Well-spread metaphases were photographed with a Polaroid Aristophot attachment, and the total chromosome number was recorded. Karyotypes were constructed from photographic enlargements according to the Paris Conference classification scheme.

**Production of Immunoglobulin.** Cells were examined for immunoglobulin production by 2 methods: (a) direct immunofluorescence (6); and (b) measurement of IgG content by a soluble-phase radioimmunoassay for human IgG developed by us (5). For immunofluorescent studies, cells were grown on microscope slides for 7 days as described previously. Slides were rinsed twice in 0.9% NaCl solution, fixed for 10 min in reagent grade acetone, and washed twice in 0.9% NaCl solution. The cells were partially air-dried and incubated with fluorescein isothiocyanate-labeled polyvalent goat anti-human immunoglobulin or monospecific goat anti-human IgG, IgA, IgM, anti-κ or anti-λ (Behring Diagnostics, Somerville, N. J.), for 30 min at 37°. (The monospecificity of these antisera had been confirmed by microimmunoelectrophoresis.) Slides were then washed twice in 0.9% NaCl solution, counterstained with Evan’s blue dye (0.006%), washed twice, coverslipped in 1% Sorbock blue dye (0.006%), washed twice, coverslipped in 1% Sorbock solution, and examined with a UV Zeiss research microscope with a UV Osram HBO-200 lamp, UV 38 and UV 1 exciting filters, and 41 barrier filter. Specificity was demonstrated by competitive blocking of immunofluorescent reactivity with equimolar amounts of antigens (human IgG) and antibody (fluorescein isothiocyanate goat anti-human IgG).

For quantitative IgG determinations; aliquots of 2.5 × 10^6 cells/dish were incubated in replicate dishes for 6 days at 37°. The supernatant medium from triplicate dishes was assayed for IgG content by double antibody radioimmunoassay (5). Briefly, 100-µl aliquots of supernatant medium were incubated simultaneously with ^125^I-labeled IgG in competition for goat anti-human IgG (Immunoagents, Seguin, Texas). Controls consisted of fresh culture medium; fetal calf serum, bovine serum albumin; and purified IgA, IgM, and Bence Jones protein. Immune complexes were precipitated with the addition of rabbit anti-goat IgG (Immunoagents), and quantitative estimates of supernatant IgG content were read from standard IgG inhibition curves generated in parallel with these experiments. Results were expressed in ng of IgG per 10^6 cells.

**Growth Kinetics Studies.** DT determinations were performed by seeding 2.2, 3.5, and 7.2 × 10^6 cells in 60-mm Petri dishes and allowing the attainment of exponential growth (about 24 hr). Every 24 hr thereafter, duplicate plates were harvested by collecting the supernatant medium in appropriately labeled centrifuge tubes and incubating the remaining cells attached to the dish with 0.5 ml of 0.2% EDTA for 1 min at room temperature. The contents of the EDTA harvest were added to the initial cell suspension, monodispersion was ensured by gentle agitation with a disposable pipet, and the cells were counted with a Model ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Estimates of DT were made from In 2/slope. Slopes were calculated by linear regression through data points covering the initial 10 days.

LI determinations were performed by pulse-labeling cells in exponential growth with [^3H]dThd (1 µCi/ml; specific activity, 20 Ci/mmol) for 30 min. Cells were rinsed, harvested with 0.2% EDTA for 1 min at room temperature. The contents of the EDTA harvest were added to the initial cell suspension, monodispersion was ensured by gentle agitation with a disposable pipet, and the cells were counted with a Model ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Estimates of DT were made from In 2/slope. Slopes were calculated by linear regression through data points covering the initial 10 days.

RESULTS

**Tissue Culture.** Approximately 2 to 3 months after transfer from spinner flask cultures, the cells consistently proliferated as a monolayer culture and reached a state of near confluency in approximately 7 days although the monolayer never reached saturation density. The cultures are easily passaged nonenzymatically by jarring the loosely attached cells from the vessel surface and dividing the resultant cell suspension into 2 equal aliquots. Cultures are free from *Mycoplasma* contamination.

**Morphology.** Monolayer cultures display an admixture of cell morphologies including numerous round cells, some elongated cells, and occasional polygonal cells (Fig. 1). Round cells tend to grow as large clumps that float freely in the supernatant medium and contain 95% viable cells as assessed by trypan blue exclusion. Although the cells displayed considerable variation in size and shape, most elements are morphologically identifiable as PC characterized by eccentric nuclei, basophilic cytoplasm (Fig. 2), intense pyronin and periodic acid-Schiff staining (Figs. 3 and 4). The cells were routinely negative for acid mucopolysaccharide, lipids, fats, and esterase activity when assayed cytochemically with colloidal iron, Sudan black, oil red, and Naphthol AS-D chloroacetate esterase, respectively. Electron microscopic observations revealed the presence of PC-like elements with distinct ultrastructural character-
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The nuclei were somewhat eccentrically located and displayed substantial hetero- and euchromatization and prominent nucleoli. A number of cytoplasmic organelles were observed, including mitochondria, rough endoplasmic reticulum, numerous polyribosomes, and occasional inclusion bodies. Evidence for active cellular synthesis and secretion of IgG was substantiated indirectly by the presence of the aforementioned polyribosomes, the rough endoplasmic reticulum, and the abundant extracellular material associated with the external plasma membrane. The differences in cell size and shape noted previously were not easily defined at the ultrastructural level.

Cytogenetics. The karyotype of this line was aneuploid with a modal chromosome number of 45 to 46 (range, 44 to 47) (Fig. 6). No marker chromosome was noted, however, some cells displayed a long submetacentric chromosome 2.

Production of IgG. Immunofluorescence examination showed an average of 40% cells (range, 10 to 50%) that were positive for intracytoplasmic IgG with lambda type light chain. Typical fluorescent patterns are depicted in Fig. 7. Most cells exhibiting intracytoplasmic fluorescence had eccentrically located nuclei. The fluorescent activity was routinely blocked with the addition of equimolar unlabeled IgG.

Quantitative radioimmunoassay determinations of the cumulative amounts of IgG released into the supernatant medium by 6-day-old cultures yielded an average of \(1.21 \times 10^4\) ng/10^6 PC with a range of 1.13 to 1.28 \(\times 10^4\) ng/10^6 PC. These results compare favorably with our data from quantitative IgG analysis in other myeloma cell cultures with identical methods (5). The range of IgG content in 10 such cultures was 1.43 to 6.75 \(\times 10^4\) ng IgG per 10^6 PC with a mean value of 3.86 \(\times 10^4\) ng IgG per 10^6 PC. All controls were essentially negative.

Growth Kinetics. Doubling times analyzed as a function of initial cell inocula (i.e., 2.2, 3.5, and 7.2 \(\times 10^5\) cells/dish) were derived from In 2/slope where slopes were calculated by linear regression through the initial 10 sampling days (Chart 1). The average observed doubling time (\(T_{do}\)) analyzed from these growth curves was 4.6 days (110.4 hr) with a range of 3.8 to 4.7 days. The DT was independent of the initial cell concentrations assayed by us. However, after cells reached a density of approximately \(1.5 \times 10^6\) cells/dish, a slower rate of growth reflected by a shallower slope was observed.

The M1 ranged from 1.0 to 2.0 with a mean value of 1.5, whereas the flash LI ranged from 8.6 to 20.0 with a mean of 14.3. Growth kinetics parameters were measured by continuous exposure of cultures to \(^{3}H\)dThd. Cultures showed an increase in the LI at the rate of 1.4/hr to a plateau of approximately 74% labeled cells (range, 71 to 76%) attained at 46 hr (Chart 2). This plateau was considered a reflection of the proportion of proliferating cells (growth fraction) within the constraints of the experimental interval (8). Thus, cell kinetics parameters were calculated from the following equations:

\[
T_C = T_{ci} + T_{ci} + T_M = \frac{GF}{Li} \times T_S;
\]

\[
T_S = \frac{T_{ci} + T_{ci}}{GF - 1};
\]

\[
T_M = \frac{ML \times T_C}{In 2};
\]

\[
T_{DP} = \frac{T_C}{GF};
\]

\[
\phi = 1 - \frac{T_{DP}}{T_{DO}}.
\]

where \(T_{DP}\) is the potential doubling time, \(\phi\) is the intrinsic cell loss, and \(T_{DO}\) is the observed doubling time.

These calculations resulted in the following values: \(T_C = 56.4\) hr; \(T_{ci} + T_{ci} = 45.5\) hr; \(T_S = 10.9\) hr; \(T_M = 1.2\) hr; \(T_{DP} = 76.2\) hr; and \(\phi = 31.0\%\).

DISCUSSION

The cell line described in this report, henceforth known as cell line ARH-77, displayed a number of features characteristic of PC. Morphologically, the cell showed identifiable markers of PC at the light microscope level. Ultrastructurally, this was confirmed by substantial (although atypical) endoplasmic reticulum and numerous polyribosomes.
In addition, electron microscopy revealed abundant macromolecular products secreted to the extracellular milieu associated with the external plasma membrane; although its nature was not determined, it is probable that this material is IgG in view of the large amounts of secreted IgG demonstrated by our radioimmunoassay.

The fact that this line proliferated loosely attached to the surface of the vessel but never achieved confluence and yielded freely floating cell clumps is indicative of a cell suspension type of in vitro propagation rather than of classical monolayer growth. This observation was further supported by the routine, nonenzymatic technique used to subculture these cells. Such a suspension culture pattern of growth would not seem unlikely or improbable for these cells in view of their hemopoietic origin.

ARH-77 cells were found to synthesize human IgG when assayed by monospecific immunofluorescence and IgG-specific radioimmunoassay. The cells synthesize in vitro the same class of immunoglobulin molecule (IgG, \(\lambda\)-type light chain) produced by the donor MM patient, although idiotypic specificities were not defined. The observed intracytoplasmic immunofluorescence of cells with eccentric nuclei lends further credence to the presence of PC-like elements in culture. The high percentage of cells positive for IgG by immunofluorescence (40%) suggested a very active macromolecular synthetic machinery. Detection of substantial IgG in the supernatant medium (1.21 \(\times\) 10\(^4\) ng/10\(^6\) PC) confirmed this hypothesis. Our results are similar to the values determined in other primary cultures of MM cells (5).

The growth kinetics features of line ARH-77 are particularly interesting. The relatively long \(T_C\) (56.4 hr) is not unlike other human malignant lines studied in this laboratory, but the GF is among the lowest ever recorded for in vitro cell lines (9, 15).

Drewinko and Alexanian (7) recently proposed a cell renewal model for the growth kinetics of MM where PC mature and die spontaneously and suggested that a high rate of cell loss might be an obligatory event for an unperturbed myeloma cell mass. The 31.0% cell loss calculated for ARH-77 cells may reflect an analogous situation in which a fraction of the in vitro cell population spontaneously undergoes maturation and eventual death. Such a phenomenon could be the end point of a continuous spectrum of in vitro PC development. In this context all cells comprising the population would arise from a common clone to embark on a course of in vitro maturation and eventual senescence resulting in the relatively high cell loss and the disparate spectrum of morphological entities. If this hypothesis is confirmed by further studies, line ARH-77 may provide the tool for studies concerning the regulatory mechanism of PC growth.

In summary line ARH-77 appears as an excellent in vitro model for study of MM. The cells display morphological features characteristic of PC and retain the capacity to synthesize the macromolecule (IgG, \(\lambda\)-type light chain) associated with the donor patient even after 2 years in culture.

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REFERENCES

Fig. 1. Typical morphology of ARH-77 cells after 3 days in tissue culture. Variations in shape and size, the rounded cells being the predominant cell type. Upper left of center, clump of rounded cells, floating freely in the supernatant medium. × 60.

Fig. 2. Morphological characteristics of line ARH-77 after 6 days in culture. May-Grunwald’s, × 350.

Fig. 3. Line ARH-77 after 6 days in culture. Intense pyronin staining. Methyl green-pyronin, × 350.

Fig. 4. High-power details of PC from line ARH-77 6 days after subculture. Periodic acid-Schiff, × 440.
Fig. 5. Electron micrograph of 6-day-old cultured PC from line ARH-77 showing a substantial rough endoplasmic reticulum and particulate surface material indicative of secretory activity. Glutaraldehyde fixation. Lead citrate and uranyl acetate. \( \times 875 \).

Fig. 6. Representative karyotype of line ARH-77 arranged according to Paris Conference classification scheme following Giemsa banding. Arrows, additions of chromatin material.

Fig. 7. Immunofluorescent preparation of cultured PC in exponential growth. Fluorescein-conjugated, monospecific antisera. \( \times 175 \).
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