Intratumor Maturational Heterogeneity within the Murine Myeloma MOPC-315

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ABSTRACT

The murine myeloma MOPC-315 secretes a paraprotein which binds dinitrophenylated compounds. Maturational subsets which mimic normal B-cell differentiation were shown to exist within this monoclonal neoplasm. The maturational subsets were defined by an in vivo stem cell activity (plasmacytoma colony-forming unit-spleen), DNA synthesis ([3H]thymidine incorporation), membrane-bound paraprotein, and cells secreting the MOPC-315 paraprotein. Velocity sedimentation at unit gravity separated cells enriched for secreting the MOPC-315 paraprotein. An in vivo sequential analysis of the appearance of tumor (i.v. challenge) in the spleen of BALB/c hosts did not reveal any discordant appearance of these same maturational subsets with respect to time. The MOPC-315 myeloma contains maturational subsets that mimic normal B-cell differentiation and apparently differentiate during a very early stage of its evolution within the host.

INTRODUCTION

The maturation and expansion of normal B-cell clones have been examined by a variety of morphological (18, 19) and immunological (4, 10, 25) techniques. This maturation includes cells from the pre-B-cell, to a B-cell with antigen-sensitive immunoglobulin receptors, and finally to a plasma cell which synthesizes and secretes immunoglobulin. Similarly, the maturational state of a variety of B-cell neoplasms has been examined by a number of investigators (1, 2, 9, 16, 26). The results of these studies indicate that there is a wide spectrum of B-cell tumors which represent an arrest in various stages of normal B-cell differentiation. Plasmacytomas are representatives of a late stage in this maturational lineage.

There are at least 2 possibilities to be considered when dealing with the tumor biology and therapy of plasmacytomas. (a) Plasmacytomas may represent a single and terminal maturational state in which an oncogenic transformation has occurred. (b) Although not generally accepted, these neoplasms may contain a maturational spectrum of B-cell differentiation in which the majority of the tumor population matures to immunoglobulin-secreting cells. The induction of this maturational event could be either intrinsic in the evolution of the myeloma stem cell or possibly host driven.

In this investigation, I have examined the in vivo growth kinetics of possible maturational subsets within the spleen of the host after i.v. challenge with MOPC-315 cells. These subsets were defined by various stages of paraprotein expression, DNA synthesis and stem cell activity. If maturation was indeed occurring, one might expect to see a discordant appearance with respect to time after challenge or a difference in the respective growth kinetics of the various maturational subsets. In addition, maturation is also expressed by changes not only in biological function but in the physical characteristics of volume (7, 8, 12), density (7, 20), surface membrane charge (20, 28), and membrane fluidity (17). Various maturational subsets have been separated from an established MOPC-315 population on the basis of their cell diameters.

MATERIALS AND METHODS

Mice. Eight-week-old male BALB/cAnN mice were obtained from Dr. Ralph Graff, Department of Surgery, Jewish Hospital of St. Louis, Mo.

MOPC-315 Myeloma. The characteristics and growth properties of the MOPC-315 myeloma that is maintained i.v. have been described previously (3). The separation studies used an ascites preparation of this MOPC-315 i.v. line. This ascites was obtained from animals that were given i.p. injections of 1 × 10^6 MOPC-315 cells from the i.v. line 12 days previously. The ascites cells were passed only one generation i.p. from the i.v. line. This protocol was especially useful because it yielded a tumor preparation with 90 to 100% viable cells, few contaminating cells of the host (80% or greater large tumor cells), few clumped tumor cells, and no detectable alteration in the sensitivity of the plaque assay or the in vivo stem cell assay for PCFU-s. Such a population increased the effective sensitivity of the velocity sedimentation technique since the total (tumor cells, host cells, dead cells, clumps, etc.) number of cells to be loaded is limiting. Ascites cells were washed twice with Medium L-15 + 10% fetal calf serum before use.

Assays for Paraprotein Expression. The assays used to detect mlg^* and sec.Ig^* were specific immunocytoadherence with TNP-SRBC (rosette-forming cells) and a modified Jerne plaque assay with TNP-SRBC (plaque-forming cells). These techniques have been described in detail elsewhere (3, 6).

PCFU-s Assay. The PCFU-s assay measures the fraction of cells in the myeloma population which lodge in the spleen and have sufficient growth potential to form macroscopic tumor foci within 14 days. These nodules have been termed stem cell colonies and probably arise from a single cell. This assay does not consider those cells of limited growth potential that may have sufficient growth potential to form macroscopic tumor foci within 14 days. These nodules have been termed stem cell colonies and probably arise from a single cell. This assay does not consider those cells of limited growth potential that may have sufficient growth potential to form macroscopic tumor foci within 14 days.

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Received November 20, 1979; accepted October 8, 1980.
undergo several cell divisions. This assay has been described in detail elsewhere (14, 16, 18, 23).

**Labeling of MOPC-315 Cells with [3H]Thymidine.** Myeloma cells were suspended in 20 ml of medium L-15 at a concentration of 2 to 4 x 10^6 viable cells/ml. This suspension was placed in a T-3024 flask (Falcon Plastics, Oxnard, Calif.) and incubated for 30 min at 37° in a 5% CO₂ atmosphere. A total of 40 μCi of [3H]thymidine (20 Ci/mm; New England Nuclear, Boston, Mass.) was added to the culture and then incubated for 1 hr at 37°. The cells were pelleted at 700 x g for 7 min and washed twice with medium L-15. Pelleted cells were resuspended in 0.5% BSA and diluted in PBS at pH 7.4 for velocity sedimentation.

**Quantitation of Incorporated [3H]Thymidine.** The total amount of [3H]thymidine incorporated per cell was quantitated by precipitation of incorporated material with trichloroacetic acid (24). The precipitates were collected on Whatman GF-A filters and counted in a liquid scintillation counter. The fractions, which contained less than 1 x 10^6 MOPC-315 cells, were corrected to the number of cpm of ³H per 10^6 cells. A direct linear relationship was shown to exist for a labeled MOPC-315 population between the number of cpm and the number of cells. This relationship was linear over the range of 10^4 to 10^6 labeled MOPC-315 cells.

**Autoradiography of Labeled MOPC-315 Cells.** A qualitative analysis of the percentage of MOPC-315 cells that had incorporated [3H]thymidine was achieved by autoradiography (21, 24). Separated and unseparated fractions of MOPC-315 cells that had been labeled with [3H]thymidine, as described previously, were pelleted on 1% BSA-coated slides by means of a Cyto-Spin centrifuge (Shandon Southern, England). Slides were overlaid with NTB-3 emulsion (Eastman Kodak Co.) and incubated in a light-proof box for 14 days at 4° (optimal developing time). A total of 200 to 300 cells was examined from each slide, and the percentage of positive cells was recorded. Nuclei were very heavily labeled (necessitated by the concomitant quantitation of total incorporated counts) and were considered positive if they were completely obliterated by the silver grains >50 grains. Negative cells contained background number of silver grains.

**Velocity Sedimentation at Unit Gravity.** The velocity sedimentation procedure described by Worton et al. (27) was used for all separations. Briefly, tumor cells (25 to 50 ml at 2 to 5 x 10^6/ml) in PBS containing 0.5% BSA were loaded into a conical separation chamber. A linear gradient 1 to 2% BSA in PBS was then pumped into the chamber. MOPC-315 cells were allowed to sediment through the gradient for 1.5 hr at 4° (optimal separation time). Fractions (25 ml) were collected and evaluated by the biological and immunological techniques described above. Cell recovery ranged between 60 to 80% of the total cells loaded. Ten to 20% of the cells loaded could be washed from the chamber walls after collection. These cells appeared to adhere nonspecifically to the wall since all assays performed on these cells could not distinguish them from the starting population. In all experiments, 90 to 95% of the cells were accountable with >95% viability by trypan blue.

**Protocol for the Appearance of MOPC-315 Cells in the Host Spleen.** Sixty-five 8-week-old female BALB/c mice were given i.v. injections of 1 x 10^6 MOPC-315 cells on Day 0. On Days 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 18, and 22, 5 mice were randomly selected, and their spleens were removed. Each spleen was analyzed separately for the total number of nucleated cells, viability, percentage of rosette-forming cells (mlg+ cells), percentage of plaque-forming cells (secre.lg+ cells), and frequency of stem cells (PCFU-s cells).

**RESULTS**

**Sequential Analysis of MOPC-315 Appearance in the Spleen of the Host.** None of the assay markers was able to detect any tumor prior to Day 8 (Chart 1). There was a 3- to 4-fold increase in the number of direct (IgM) anti-DNP plaques on Days 3 and 4. The significance of such an increase is uncertain. However, no increase in direct plaques to unconjugated SRBC was seen in the controls. This suggested that this phenomenon was associated with the specific stimulation of DNP clones (same idiotype as MOPC-315?) and was not due to polyclonal B-cell activation.

The total number of secre.lg+ cells/spleen and the total number of recoverable PCFU-s+ cells/spleen rose to detectable levels on Day 8. These subpopulations then showed parallel growth kinetics up to Day 22 with an average doubling time of 12 hr. The mlg+ cells also showed a significant increase on Day 8 but remained at a plateau until Day 12, at which time they began a growth phase much akin to the other 2 subsets. The increase in mlg+ cells on Days 8 to 12 was specific since anti-idiotypic antisera to the MOPC-315 paraprotein inhibited the rosette formation to the background of normal BALB/c spleen cells (data not shown). The background rosettes were due to cytoadherence of cells with unconjugated SRBC which were included as controls in all rosette assays. Inhibition of TNP-SRBC rosettes on Days 0 to 6 was not seen when either

![Chart 1](cancerres.aacrjournals.org)
anti-idiotypic antiserum or free hapten, DNP-lysine, was added (data not shown). These reagents routinely inhibit MOPC-315-TNP-SRBC rosettes by 95 to 100%. This high background of SRBC rosettes made it difficult to quantitate precisely the specific anti-DNP MOPC-315 rosettes until Day 12.

Separation of Subpopulations within the MOPC-315 Tumor by Velocity Sedimentation. A representative velocity sedimentation separation is shown in Chart 2, A to D. The peak sedimentation velocity for the intact cell populations was 12.5 mm/hr. The mlg* total population was not enriched in any one given fraction, but there was a sharp drop in activity in the slower sedimenting population (Chart 2B). This was probably due to a small contamination in the ascites preparation by lymphocytes of the host. A peak of mlg* secr.lg~ cells was observed at a sedimentation velocity of 11.3 mm/hr (Chart 2B). This population cosedimented with the PCFU-s* population.

The secr.lg* cells were characterized as the fastest sedimenting population, 19.5 mm/hr. This population was clearly separated from the PCFU-s* population (Chart 2, A and C). The PCFU-s* population had a peak enrichment at 11.3 mm/hr. However, even though this represented a 5-fold enrichment over the unseparated population, the percentage of PCFU-s* cells was only 0.4%. A peak activity of [3H]thymidine incorporation per cell was noted at 13.8 mm/hr (Chart 2D). Distribution of the percentage of cells that incorporated [3H]thymidine, as determined by autoradiography, remained constant between 60 to 70% throughout most of the separated fractions (Chart 2D). This demonstrated clearly that the majority of the MOPC-315 cells are in cycle. It suggests further that, even though the fractions enriched in secr.lg* cells are depleted in stem cells, they do undergo some limited cell divisions since they label with [3H]thymidine.

The mean sedimentation velocities of the various subpopulations ± S.E. for 5 separate velocity sedimentation separations are shown in Chart 3. The slight variation that was noted was due to the differences between ascites populations. However, the secr.lg* population was the fastest sedimenting population, always representing at least a 2-fold enrichment over the unseparated population, and clearly separable from the PCFU-s* population.

DISCUSSION

Differentiation can be defined as the process of acquiring new or specialized characteristics that differ from those of the progenitor cell. Clearly, the data in this paper show that subset populations (PCFU-s*, mlg* secr.lg~, mlg* secr.lg*) exist.
Within the intact MOPC-315 population. These subsets differ in their mean cell diameter and the tumor progenitor and secretory population can be separated by velocity sedimentation at unit gravity. Whether these subsets represent various maturation stages that are identical to normal B-cell differentiation cannot be totally resolved from this study. A more detailed analysis of various normal B-cell differentiation antigens that may be present on the MOPC-315 subpopulations is necessary.

The sequential examination of the spleen of the host for the appearance of PCFU-s*, mg*, and secr.lg* subpopulations of MOPC-315 failed to provide any direct evidence for in vivo maturation. Unlike the report of Rohrer et al. (15, 16), the PCFU-s* population was not the first to increase, followed by a rise in mg* cells and finally by an increase in the secr.lg* cells. Within the limits of detection, all these subsets were shown to be detectable at precisely the same time. This discrepancy between the 2 findings may represent a difference in the behavior of MOPC-315 cells during the in vivo growth in Millipore diffusion chambers versus the spleen of the host. The inability to detect a distinct and discordant appearance of maturation subsets after i.v. challenge with tumor more probably stems from the lack of sensitivity of the assays does not permit a phenotypic characteristic to be assigned to this subset. The subpopulations are ordered according to their proposed maturational lineage. The variation between separations was due to differences between MOPC-315 ascites preparations. The stem cell-enriched population, PCFU-s*, and the secretory cell-enriched population, secr.lg*, were always clearly dissociable.

The studies with [3H]thymidine incorporation showed that a peak of [3H]thymidine incorporation per cell occurred in a fraction characterized as a slightly faster sedimenting population, Chart 2D. The lack of direct correlation between this peak and the peak enrichment for PCFU-s is not particularly surprising since the PCFU-s peak only represents 0.4% of the total cells versus 60% of the cells incorporating [3H]thymidine. Since within this peak the percentage of cells incorporating [3H]-thymidine (Chart 2D) remained constant, this peak was also not explicable on the basis of cells in cycle. Although the separation of G2 cells from other cells in the cell cycle has been reported (5, 11, 13, 22), this in itself would not account for a 10- to 20-fold increase in [3H]thymidine incorporation per cell. This peak probably represents a marked heterogeneity within the MOPC-315 myeloma with respect to endogenous thymidine pools, thymidine kinase activity, and/or the activity of various catabolic or metabolic pathways of thymidine.

Separated maturational subpopulations within a monoclonal tumor provide the means to study the regulation of well-defined maturational events. Whether these maturational events are inherent within the evolution of the myeloma or whether they are host driven remains to be answered. The existence of a spectrum of B cell maturational stages within a murine myeloma suggests that the clonal origin of some myelomas may be an early B cell. Monoclonal populations that differentiate are valuable tools to study the gene regulation and expression asso-
associated with differentiation. In addition, clinical manipulation of the maturation state of neoplasms may prove to be useful in their direct treatment or in conjunction with other therapeutic modalities.

ACKNOWLEDGMENTS

The author wishes to thank Dr. F. Valeriote, Dr. C. Stewart, and Dr. A. Nakoff for their suggestions and assistance. I thank Dr. Richard G. Lynch for the laboratory space. I also gratefully acknowledge the secretarial skills of M. E. Daley and K. Aragon in the preparation of this manuscript.

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