Quantitation of G_0 and G_1 Phase Cells in Primary Carcinomas

Antibody to M_1 Subunit of Ribonucleotide Reductase Shows G_1 Phase Restriction Point Block

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Abstract

Human cancers have an apparent low growth fraction, the bulk of cells presumed to being out of cycle in a G_0 quiescent state due to the inability in the past to distinguish G_0 from G_1 cells. The allosteric M_1 subunit of ribonucleotide reductase (M_1-RR) is constitutively expressed by cycling cells (i.e., G_1, S, G_2-M). It is acquired during transition from G_0 to G_1, lost during exit to G_0 and thus distinguishes G_0 from G_1 cells.

To estimate the proportion of G_0 and G_1 cells in primary human breast (n = 5) and colorectal (n = 12) adenocarcinomas, we used both analytical DNA flow cytometry (ADFC) and immunoperoxidase staining of sections with the monoclonal antibody to M_1-RR (MAb M_1-RR). ADFC of fresh tumors revealed a low percentage of cells in the S phase (4.0±3.4%) but immunoperoxidase staining for M_1-RR revealed an unexpectedly high proportion of positive cells (52.4±12.7%) in the G_1, S, G_2-M phases indicating a high G_1 content of primary human tumors. Thus, human cancers are blocked in transition in G_1 and are not predominantly in a G_0 or quiescent differentiated state. This block was interpreted to mean that human cancers are responding to putative regulatory events at a restriction point in the G_1 phase, such as relative growth factor deficiency, density inhibition, antiproliferative cytokines, or gene products.

Using flow cytometry for both DNA and M_1-RR content we found that human colon cancer cell lines arrest in the G_1 but not G_0 phase upon serum deprivation or density inhibition. Similarly, human breast cancer cell lines are arrested in G_1 but not G_0 phase by medroxyprogesterone acetate (MPA) or tamoxifen exposure. These findings match our in situ observations, and support the concept of a restriction point block in primary human tumors. (*J. Clin. Invest.* 1991. 87:519–527.) Key words: tumor • growth • kinetics • differentiation • inhibition

Introduction

The cell cycle status of human cancers in vivo is poorly understood. Human cancers have been considered to have a low growth fraction and proliferation rate, with a relatively small percentage of cells in the S phase compared to cultured cells in log phase growth (1–6). The bulk of tumor cells have been presumed to be "quiescent", i.e., out-of-cycle or in a G_0 state (7), reflecting our inability in the past to distinguish G_1 from G_0 cells.

The division of the cell cycle into specific phases (e.g., G_1, S, G_2-M) to describe the temporal relationship between DNA replication and cell division remains a focus for research in cell kinetics. Control of cell proliferation is believed to be achieved by modulating the progress of cells through G_1 and hence into the S phase (8, 9). Various metabolic perturbations may inhibit passage through G_1 or produce entry into a "quiescent" or G_0 state, although no biochemical events have been identified that uniquely characterise the G_1 period, nor distinguish it from G_0 (10). The molecular nature of the switch(s) that regulate the transit of cells from either G_0 or previous mitosis (M), through G_1 to the initiation of DNA replication remain obscure. When a cell moves into a quiescent and/or terminally differentiated state from G_1, it clearly enters a different metabolic state. This state (G_0) has not yet been given a precise molecular, biochemical, or genetic definition. The roles of regulatory processes such as oncogenes, growth factors, and their receptors in cell growth and reproduction have been extensively studied. In the past responses to putative G_0 and G_1 regulatory events could only be assessed by measuring events in the S phase such as DNA replication itself or increases in enzymes associated with DNA replication but such events are considerably downstream in the cycle and separated from G_0-G_1 by at least several hours. This fact has bedeviled attempts to establish a nexus between cycle regulatory events, functional responses, and the mechanisms by which these are achieved.

As part of ongoing studies on cell cycle control we have developed methods to distinguish G_1 cells from those in the G_0 cell cycle phase, in primary human cancers and in tumor cell lines. Ribonucleotide reductase (RR)[1] catalyzes the first unique, rate-limiting step in DNA synthesis; both its large (M_1) allosteric and small (M_2) active site subunit are necessary for activity. Whereas direct studies of M_2 expression have previously shown a tight correlation with S phase, the kinetic features of M_1-RR expressing cells have remained ill defined (11). Therefore, using immunofluorescence flow cytometry and the MAb M_1-RR, we previously analysed changes in whole-cell M_1-RR levels and DNA content during various cell cycle and differentiation events (12). The M_1-RR, a protein of M_r 170,000 dimer binds nucleotide substrates and allosteric

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1. *Abbreviations used in this paper:* ADFC, analytical DNA flow cytometry; BUDR, 5-bromo-2'-deoxyuridine; IMPO, PAP 4-layer immunoperoxidase; MPA, medroxyprogesterone acetate; M_1-RR, M_1 subunit to the ribonucleotide reductase; PCNA, proliferating cell nuclear antigen.

effectors (deoxyribonucleoside triphosphates, dNTP) of the active site M_2 subunit of ribonucleotide reductase. In asynchronous exponential cultures M_1-RR levels are sustained throughout the cell cycle, including G_1 phase when M_2-RR levels and ribonucleotide reductase catalytic activity are known to be very low. By contrast M_1-RR is virtually absent from quiescent lymphocytes but is expressed following mitogenic stimulation, in G_1 phase before S phase cells appear. M_1-RR declines to low levels in "plateau phase" cultures, the major reduction in M_1-RR expression occurring in cells with 2N (G_0-G_1) DNA content. HL-60 promyelocytic leukemia cells, induced into either myeloid or monocyte-macrophage differentiation, show a similar marked decrease in M_1-RR levels concomitant with the cessation of cell division, i.e., on entering G_0. We concluded that the M_1-RR subunit of ribonucleotide reductase is constitutively expressed by cycling cells. It is synthesized during stimulated transition from G_0 to G_1 and is lost during exit to G_0 or terminal differentiation. This pattern of expression suggested that the determination of cellular M_1-RR content would be useful in distinguishing proliferating (including G_1) from quiescent (G_0) cells in vivo (13). In view of this we considered that quantitation of G_1 and G_0 in human tumors would yield information of considerable biologic and clinical value.

To differentiate cells in the G_0 from G_1 phase we studied fresh primary human colorectal and breast adenocarcinomas by immunoperoxidase staining of sections using MAb M_1-RR, combined with ADFC of dispersed nuclei. This revealed an unexpectedly high percentage of cells apparently in G_1 phase associated with a low S phase content suggesting that human cancers are blocked at a putative "restriction" point in G_1. We have interpreted this block to mean that human cancers in vivo may be responding to a series of (undefined) regulatory factors acting predominantly in the G_1 phase, e.g., contact inhibition, relative growth factor (or receptor) deficiency or antiproliferative cytokines or gene products. We have also studied in vitro, cell culture models to explore mechanisms of G_0-G_1 transit and control by metabolic modulation and the results support the above hypothesis.

Methods

Immunohistochemistry

(a) Fresh tissue specimens. Macroscopically viable colorectal (n = 12) and breast (n = 5) tumor tissue was obtained from fresh surgical specimens, including macroscopically normal colorectal mucosa, obtained from resection margins away from the tumor. Rectal mucosal biopsies were obtained at colonoscopy by Dr. F. Macrae from three healthy human volunteers as part of a separate but concurrent project (The Australian Polyp Prevention Project) and placed immediately in ice-cold 10% FCS (Flow Laboratories, Sydney, Australia) in RPMI 1640 culture medium (Flow Laboratories).

(b) Fixation for M_1 subunit of the ribonucleotide reductase (M_1-RR). For the detection of the M_1-RR antigen, 2-mm-thick tissue slices were fixed in 4% paraformaldehyde at room temperature for 2 h; left overnight in a solution of 7.5% sucrose in PBS at 4°C, and embedded in OCT (Miles Laboratories Inc., Naperville, IL) before freezing in isopentane chilled in liquid nitrogen. Cryostat sections of the tissue of 2–3 μm in thickness were cut at −20°C for immunoperoxidase studies.

(c) Incubation with 5-bromo-2'-deoxyuridine (BUDR). The mucosal biopsy specimens were placed on pieces of Millipore paper (Millipore Corp., Bedford, MA), mucosa side up, rinsed twice in 10 μM BUDR in 10% FCS in RPMI 1640 and incubated with 5 ml of 10 μM BUDR (Sigma Chemical Co., St. Louis, MO) in 35-mm petri dishes. The incubations for 2 h were performed in an anaerobic jar in 5% CO_2 and 95% O_2 at 1.6 atm pressure, at 37°C, protected from light. The mucosal strips were then rinsed in PBS, fixed in Carnoy's fixative for 2 h, and processed to paraffin blocks.

Paraffin sections 2–3 μm thick were cut serially, 20 μm apart and mounted onto slides. Before immunohistochemical staining, the sections were placed in a 60°C oven for 30 min. The sections were further dewaxed in two changes of xylene and rehydrated through two changes of absolute alcohol and one change of 70% (vol/vol) alcohol for 3–5 min in each solution. After a rinse in tap water, the sections were treated with 1 M HCl at 60°C for 8 min (14). The slides were rinsed well in tap water, before immunohistochemical staining.

(d) PAP four-layer immunoperoxidase (IMPO) method. The immunoperoxidase procedure used was based on a method developed by Sternberger (1979) (15). Sections were incubated with 10% noninactivated swine serum (Commonwealth Serum Laboratories, Melbourne, Australia) and 1% FCS in PBS (diluent 1) for 45 min to block nonspecific protein binding before incubation for 2 h with the primary antibody of interest. The MAb M_1-RR (In Ro Biomedtek, Umea, Sweden) was diluted to 50 μg/ml and the MAb BUDR (Becton Dickinson, CA), 1:5, in diluent 1. The slides were given 3 × 5-min washes in PBS followed by a 15-min incubation with a 1/100 dilution of rabbit anti-mouse antibody (Dakopatts A/S, Glostrup, Denmark), in 10% noninactivated swine serum, 1% FCS, 1% pooled human AB sera in PBS (diluent 2). Following 3 × 5-min rinses in PBS (pH 7.4), the sections were treated with 0.9% H_2O_2 (wt/vol) in PBS (pH 7.4) for 30 min, to block endogenous peroxidase activity. After 2 × 5-min rinses in PBS, the sections were incubated for 30 min in a 1/40 dilution of swine anti-rabbit antibody (Dakopatts A/S) in diluent 2. Following 3 × 5-min rinses in PBS (pH 7.4), the sections were incubated for 30 min in a 1/40 dilution of rabbit peroxidase-anti-peroxidase complex (Dakopatts A/S) in diluent 2. 3 × 5-min rinses followed, in PBS buffer. Demonstration of the peroxidase complex was achieved by incubating the slides for 5 min in 0.06% (wt/vol) 3,3'-diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) in PBS with 0.03% (wt/vol) of hydrogen peroxide (H_2O_2). The slides were counterstained in Lillie-Mayer's hematoxylin (16), cover slipped, and mounted in DePeX (BDH).

(e) Analysis of immunohistochemically stained sections. The percentage of positive cells stained with the M_1-RR antibody in the tumor sections was evaluated by manual counting of the cells from color photomicrographs projected onto a screen. The areas to be counted were photographed at 400× magnification as follows. Parallel lines 0.5 cm apart were marked on the underside of the histological glass slide over the tissue section. Another set of lines were then drawn perpendicular to the first set of lines, also 0.5 cm apart. Using these lines as a guide, every alternate square was photographed without any overlap between adjacent frames, to avoid counting cells twice. Once all the frames on a line were photographed, photography would commence on the next line, and similarly along the other set of perpendicular lines. Overlapping would occur again where the two perpendicular lines cross each other, and at areas where these perpendicular lines were encountered, this frame was ignored and photography was resumed only at the next frame.

Based on their staining intensity the cells were manually scored, as "++" (very positive), "+" (clearly positive but less intense), and "−" (negative) for the M_1-RR antigen. The total number of cells counted per sample for the M_1-RR antigen ranged from 1,725 to 11,702 cells, with an average of 5,625 cells.

Flow Cytometric DNA Analysis

(a) Preparation of archival material. A representative paraffin block was chosen from the tumors studied immunohistochemically to include the invasive margin of the tumor and the maximum amount of viable tumor tissue. For each case, a nuclear suspension was prepared from the block, their DNA content stained and analyzed, using the method developed by Hedley et al. (17). The pellet was resuspended and washed in PBS (pH 7.2) at 1,500 rpm, 20°C for 10 min before

staining with propidium iodide (PI; Calbiochem-Behring Corp., La Jolla, CA) at a concentration of 50 μg/ml in RPMI 1640 culture medium. Approximately 1×10^6 nuclei were stained per milliliter of dye solution for 30 min at room temperature before their fluorescence was analyzed.

(b) Preparation of cell culture samples. The portion of cells allocated for ADFC harvested from cell cultures were fixed in 25% (vol/vol) ethanol and stored at 4°C in the fixative until the commencement of staining with PI. After a wash in PBS, the cell pellet was incubated in 100 μl of 1 mg/ml ribonuclease 1A (Pharmacia LKB, Uppsala, Sweden) in PBS at 37°C for 20 min. Approximately 1×10^6 cells were stained with 50 μg/ml PI with 0.2% Triton X-100 (BDH) for at least 10 min before any flow cytometric analysis.

(c) Analytical DNA flow cytometric analysis (ADFC). Samples stained with PI were analyzed using a standard EPICS 751 Flow Cytometer (Coulter Electronics Inc., Hialeah, FL) equipped with an argon laser (Coherent Inc., Palo Alto, CA). Dye excitation was achieved at 488 nm wavelength with 500 mW power. Filter combinations for measuring red linear fluorescence were a 488-nm dichroic mirror and a 575-nm band pass filter. The fluorescence intensities of at least 10^5 nuclei per sample were measured on a linear scale of 256 channels and the data displayed and stored as a single parameter histogram, using a MDADS/86 V 2.0 software package (Coulter Electronics Inc.).

The cell cycle distribution based on DNA content derived from the archival material of the breast and colorectal carcinomas were analyzed on an IBM AT compatible computer using the Cytologic software package (Coulter Electronics Inc.). This allowed enumeration of cells in the G_0-G_1, S, and G_2-M compartments.

Culture and Treatment of Tumor Cell Lines

The human colonic adenocarcinoma HT-29 cell line (18), was a gift from Ms. Virginia Leopold (Peter MacCallum Cancer Institute, Melbourne, Australia), while the human breast adenocarcinoma cell lines, MCF-7 (19) and T-47D (20), were kindly given by Dr. R. Sutherland (Garvan Institute, Sydney, Australia).

All cell lines were cultured in 20 mM Hepes-buffered RPMI 1640 culture medium (pH 7.2) which was supplemented with 9.5 mM $NaHCO_3$ (BDH), 2 mM glutamine (BDH), 50 IU/ml penicillin, and 50 μg/ml of streptomycin (Flow Laboratories) and FCS. The HT-29 cell line was cultured in 15% (vol/vol) FCS in RPMI 1640 culture medium, whereas the other cell lines were grown in 10% (vol/vol) FCS in RPMI 1640; the T-47D and MCF-7 cells were further supplemented with 12.5 μg/ml bovine pancreatic insulin (Commonwealth Serum Laboratories). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

The population doubling times for the cell lines in log phase growth were ~ 20–24 h except for the MCF-7 cell line, which doubled every 40–48 h.

For the density inhibition and serum deprivation experiments, the HT-29 cells were subcultured at densities of $8 \times 10^4/cm^2$, and for the former, cells were allowed to grow to confluence. In this experiment, by 3 d, 75% confluence was reached and thereafter, the medium was renewed daily in these flasks; for the serum deprivation experiments, 24 h after subculturing, the 15% FCS in RPMI 1640 media was replaced with RPMI 1640 with all the supplements described above but omitting the FCS.

Tamoxifen (ICI Pty Ltd., Melbourne, Australia) was added to the MCF-7 cells at a concentration of 5 μM and MPA (Farmitalia Carlo-Erba Pty Ltd., Melbourne, Australia) to T-47D cells at concentration of 0.1 nM, 24 h after subculturing at densities of 5×10^5 cells/175 cm^2 flask. No further addition of the drugs to the cells occurred throughout the length of the 4-d experiment.

Flow Cytometric Immunofluorescence Labeling Using the Monoclonal Antibody to the M_1 Subunit of the Ribonucleotide Reductase

The portion of cells for flow cytometric immunofluorescence was washed in PBS and the cell pellet fixed in 4% paraformaldehyde for 5 min. The cells were permeabilized in 0.1% Triton X-100 (BDH) solution for 20 min, and resuspended in an ice-cold solution of 1% BSA (Sigma) and 1% Tween-20 (BDH) in PBS. The cells were stored in this solution at 4°C until the commencement of immunofluorescent staining.

The cell pellet with 100 μl of the MAb M_1-RR, diluted to 100 μg/ml in 1% BSA in PBS (diluent buffer) was incubated for 1 h at 37°C in a shaking water bath, set at 20 rpm. No more than 2×10^6 cells were stained per 100 μl of antibody. The cells were washed three times in 4 ml of 1% BSA and 1% Tween-20 in PBS (wash buffer) and incubated at 37°C in a shaking water-bath set at 20 rpm, for 1 h in 100 μl of FITC-conjugated sheep anti-mouse $F(ab)_2$ fragment (NEN Research Products, Du Pont Co., Wilmington DE) diluted 1:40 with the diluent buffer. The FITC-labeled cells were given three washes in buffer, and resuspended in 1 ml of PBS for flow cytometric analysis. The samples were analyzed on the EPICS 751 flow cytometer. Fluorescein excitation was achieved using an argon laser, at wavelengths of 488 nm, 500 mW power. The filter combinations used were a 488-nm dichroic mirror and a 525-nm bandpass filter. Single parameter log green fluorescence signals were measured and recorded over a three-log range at a resolution of 85 channels per decade. The software package used for the analyses was the MDADS/86 V 2.0 (Coulter Electronics Inc.).

Dissection of the Cell Cycle into Separate G_0, G_1, S, G_2-M Compartments

The M_1-RR antigen is present in cells that are in the G_1, S, G_2-M phases but not in cells that are in the G_0 phase of the cell cycle. Therefore, a differential cell count as described above permits the estimation of (*a*) the percentage of cells in the G_0 phase which stains negative for the M_1-RR antigen, and (*b*) the percentage of cells which stains positive for the M_1-RR antigen representing all the cells in G_1, S, G_2, and M phases. By combining the two different techniques of estimating the number of cells containing the M_1-RR antigen and ADFC on the same specimen, the percentage of cells in G_1 was obtained by

$$G_1 = G_0\text{-}G_{1(ADFC)} - G_{0(M_1\text{-}RR)},$$

where G_1 = derived percentage of cells in the G_1 compartment, G_0-$G_{1(ADFC)}$ = percentage of cells in the G_0-G_1 compartment, as derived from analytical DNA flow cytometry, and $G_{0(M_1\text{-}RR)}$ = percentage of cells in G_0, as derived from immunohistochemical methods, using the MAb M_1-RR.

Statistical Analyses

All data referring to the percentage of M_1-RR-positive, S phase and G_1 phase cells, and the G_1/S ratios of the breast and colorectal tumors are presented as mean±SD. The statistical significance of differences between groups was determined using the *t* test and Mann-Whitney test. $P > 0.05$ was considered not significant.

Results

Proliferation Kinetics of Normal Human Rectal Mucosa

(a) Use of monoclonal antibody to M_1 subunit of ribonucleotide reductase. The longitudinal section of a crypt from normal human rectal mucosa stained immunohistochemically with the MAb M_1-RR using the IMPO method is shown in Fig. 1. M_1-RR is clearly located in the cytoplasm of cells lining the crypt. A small cluster of cells at the base of the crypt which did not stain for the M_1-RR, appear to correspond to stem cells in G_0.

The staining intensity was most intense in the lower one-third of the crypt and gradually faded towards the upper portion of the crypt. The lower two-thirds of the crypt where staining for the M_1-RR occurred and the base of the crypt, comprised the "proliferative compartment" (Fig. 1 *A*), whereas the upper one-third of the crypt where no staining for the M_1-RR

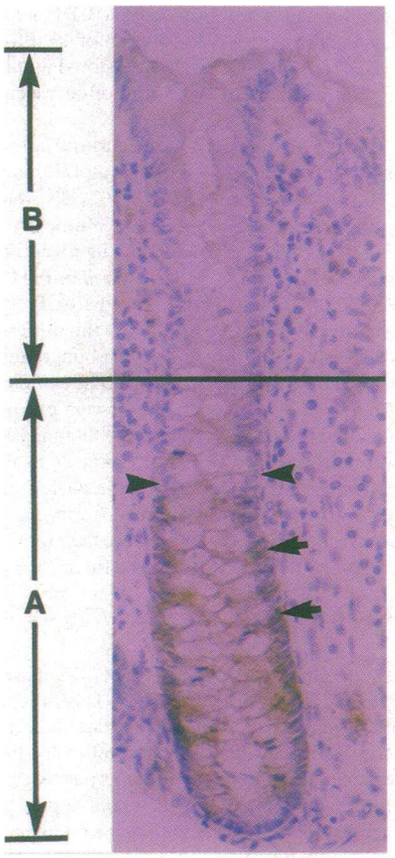

Figure 1. A full crypt from normal, human rectal mucosa, stained immunohistochemically with the MAb M_1-RR using the IMPO method. Region *A* represents the proliferative compartment, region *B*, the nonproliferative compartment. (*Arrows*) Cells staining positive for M_1-RR and (*arrowheads*) negative cells. Magnification, 256.

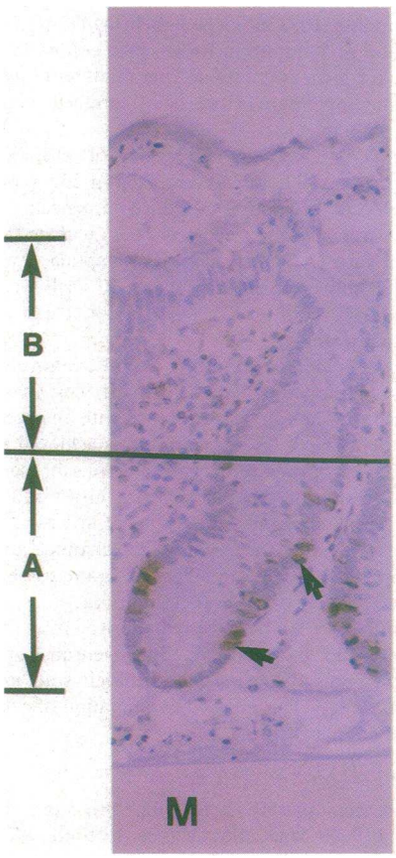

Figure 2. A full crypt from normal, rectal mucosa which has been labeled with BUDR, under hyperbaric conditions. Regions *A* and *B* are the proliferative and nonproliferative compartments, respectively. (*Arrows*) BUDR labeled cells. (*M*) Millipore paper. Magnification, 256.

occurred, comprised the "nonproliferative/differentiated" compartment region "B" (Fig. 1 *B*). These findings were made in normal colonic epithelium obtained from surgically resected specimens and confirmed in three endoscopic rectal biopsy specimens.

(b) Incorporation of 5-bromo-2′deoxyuridine (BUDR). The longitudinal section of a normal human rectal mucosal crypt, after BUDR labeling under hyperbaric oxygen conditions is shown in Fig. 2. BUDR labeling was observed only in the nuclei of cells which were distributed in the region confined to the lower two-thirds of the crypt, defined as the proliferative compartment. The location and area of the crypt occupied by the BUDR labeled cells or the proliferative compartment were very similar to that found by the M_1-RR, confirming the specificity of the MAb M_1-RR for cycling cells. Within this compartment, it was observed that the total number of cells labeled with BUDR was less than that labeled with M_1-RR. This was expected, because a 2-h pulse of BUDR would only label cells in the S phase at the time of labeling, which only comprises a fraction of the cycling cells found within the proliferative compartment. By contrast the MAb M_1-RR would be expected to label cells in the G_1 and G_2-M phases of the cell cycle, in addition to those in the S phase. Thus, the MAb M_1-RR elegantly dissects the proliferative-differentiation status of the colon and rectal mucosa.

Proliferation Kinetics of Primary Human Colorectal and Breast Adenocarcinomas

(a) Use of monoclonal antibody to M_1 subunit of the ribonucleotide reductase. An immunohistochemical study of a series of 17 primary human colorectal and breast adenocarcinomas was undertaken using the MAb M_1-RR. A section of primary breast tumor and colorectal tumor stained immunohistochemically with the MAb M_1-RR using the IMPO method, is shown in Fig. 3, *A* and *B*, respectively. In both sections, the cytoplasmic localization of the M_1-RR is clearly seen. In the section of breast tumor (Fig. 3 *A*), a strikingly high proportion of cells staining positive for M_1-RR is observed, whereas in the section of colorectal tumor, there is a much lower proportion of tumor cells staining positive for M_1-RR (Fig. 3 *B*).

The percentage of cells staining positive for M_1-RR, which varied from tumor to tumor, is shown in Fig. 4. The values for colorectal tumors, which ranged from 22 to 64% (48±14.4%) and for the breast, ranging from 46 to 77% (57.7±12.6%), did not differ significantly from each other.

In addition, in the colorectal tumors studied, the percentage of the M_1-RR positive cells did not correlate with the percentage of cells in the S phase, nor with tumor site, histological grade, or pathological staging (results not shown). For the breast tumors, a similar lack of correlation was found between the percentage of cells staining positive for M_1-RR, the percent-

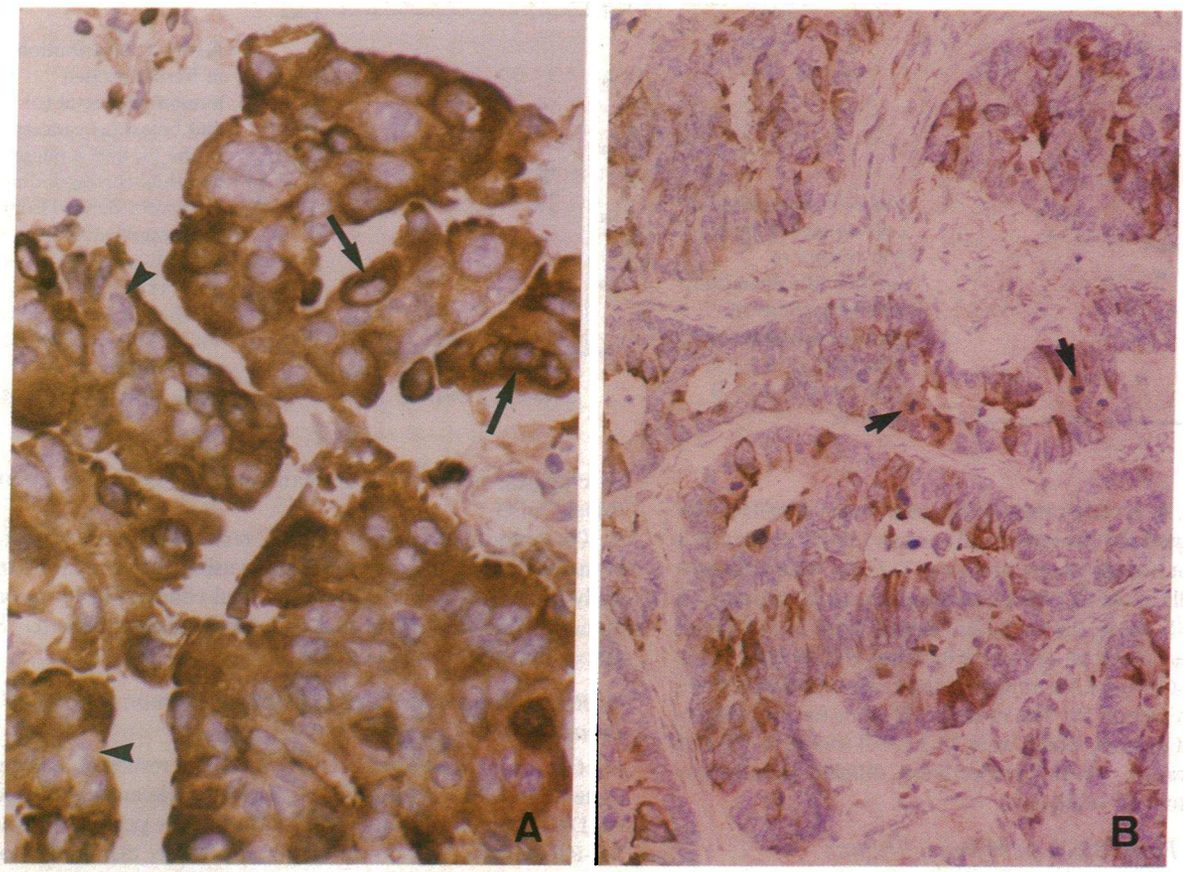

Figure 3. Breast (*A*) and colorectal (*B*) adenocarcinomas stained immunohistochemically with the MAb M_1-RR. In *A*, a high proportion of cells are positive for the M_1-RR (*large arrows*); there are only a few negative-staining cells, (*arrowheads*) magnification, 512. A tumor with a much lower proportion of cells stained with MAb M_1-RR is shown for contrast in *B*. (*Small arrows*) Cells in mitoses showing positive cytoplasmic staining. Magnification, 256.

age of cells in the S phase, tumor site, and histological grade (results not shown).

On the basis of previous analytical flow cytometry data using MAb M_1-RR (12, 21) and from the normal rectal mucosal studies above, we concluded that the cells staining positive for the M_1-RR represent cells in the G_1, S, and G_2-M phases collectively, and we therefore assigned negatively stained cells to the G_0 fraction of cells. The high percentages of breast and colorectal tumor cells apparently in the G_1, S, and G_2-M cell cycle phases were unexpected.

(b) Analytical DNA flow cytometric analysis (ADFC). When nuclei from this series of primary colorectal and breast tumors were analyzed for cell cycle phase distributions based on DNA content using flow cytometry, the percentages of cells in the G_0-G_1 phases were markedly higher than the percentages of cells in the S phase (Fig. 5). Although a significant difference ($P < 0.01$) was found between the mean percentage of cells in S phase for the colorectal versus breast tumors which was 6.2±3.7% and 2.1±0.9%, respectively, this may be a reflection of the relatively small numbers studied in this series. The range for the percentage of cells in the S phase for the colorectal tumors was from 1 to 14%, whereas that for the breast tumors was smaller, 1–3%.

No significant correlations were found between the percentage of cells in the S phase and DNA ploidy, nor with tumor site, histological grade, and pathological stage of the colorectal tumors; neither were any significant correlations found between the percentage of cells in the S phase and DNA ploidy, tumor site nor histological grade in the breast tumors (results not shown).

(c) Integrated DNA flow cytometric-M_1-RR cell cycle analysis. In view of the inherent problems involved in dissociating all the cells from the tumor stroma and obtaining an intact cell suspension, immunofluorescent flow cytometric measurements on these tumors using the MAb M_1-RR were not attempted; instead, M_1-RR was detected using IMPO on sections of the freshly excised human tumors. The immunohistochemical study of the primary tumors using the M_1-RR suggested an apparently high percentage of cells in cycle, i.e., in the G_1, S, and G_2-M phases. This appeared paradoxical in view of data from the ADFC studies demonstrating the expected low percentage of cells in the S phase, as previously reported in other studies (22–24). We therefore used data obtained from the M_1-RR studies to distinguish between the cells in the G_0 phase from the G_1 phase, which was not achievable by analytical DNA flow cytometry alone. Furthermore, this also enabled the derivation of a G_1/S ratio for each tumor, as explained in Methods.

The percentage of cells in the G_1 phase and their corresponding G_1/S ratio, derived from the integrated DNA flow cytometric-M_1-RR cell cycle analysis, compared to the percentage of cells in the S phase which was derived from ADFC, are

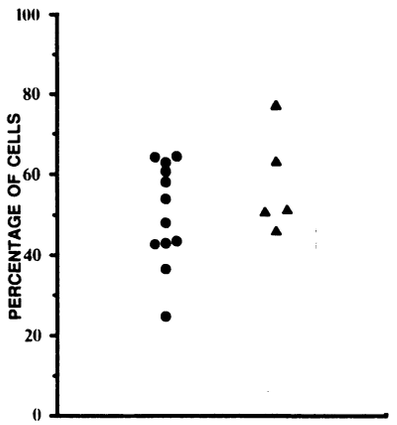

Figure 4. Percentage of cells expressing the M_1-RR antigen in immunohistochemically stained sections of freshly excised human colorectal (●; n = 12) and breast (▲; n = 5) cancers.

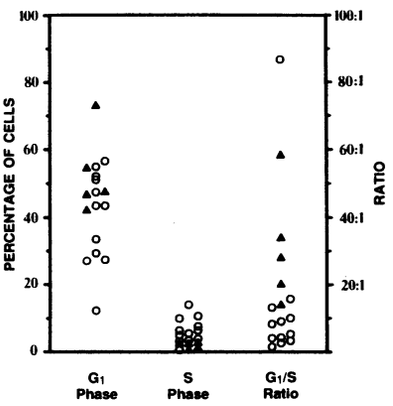

Figure 6. Distribution of freshly excised human colorectal (○) and breast (▲) cancers in the G_1 and S phases and their corresponding G_1/S ratio, derived from the integrated DNA-M_1-RR cell cycle analysis. The ordinate on the left hand side is for the G_1 and S phases; the ordinate on the right, for the G_1/S ratios.

shown in Fig. 6. A wide range for the percentage of cells in the G_1 phase existed for the colorectal tumors (12–57%) and to a smaller extent, for the breast tumors (42–73%). When the proportion of cells in the G_1 phase was compared to the proportion of cells in the S phase, the G_1/S ratio ranged from 2 to 18 for all but one of the colorectal tumors, which had a G_1/S ratio of 90. For the breast tumors, the range for the G_1/S ratio was distributed between 14 and 59. The mean G_1/S ratios for the colorectal and breast tumors, which were 13.3±23.4 (range, 2–90) and 33.1±17.7 (range, 14–58), respectively, differed significantly from each other ($P < 0.01$).

Restriction Point Block in Primary Human Adenocarcinomas

The integrated DNA flow cytometric-M_1-RR cell cycle analysis indicated that a high content of G_1 cells associated with a low percentage of cells in the S phase, exists in human primary cancers. This contrasts with the widely held view that primary human cancers are predominantly in a quiescent or G_0 state. Such a concept was based on the slow clinical growth and the low S phase content of tumors (and an inability to distinguish G_1 cells from G_0 cells). We have interpreted this high percentage of cells in G_1 to indicate a "block" or a marked slowing down in the transit of cells in or out of G_1, into the S phase. Thus, human cancers in situ may be responding to a series of regulatory events which could include intrinsic factors such as the products of tumor suppressor genes or microenvironmental changes, acting predominantly at a restriction point "block" in G_1.

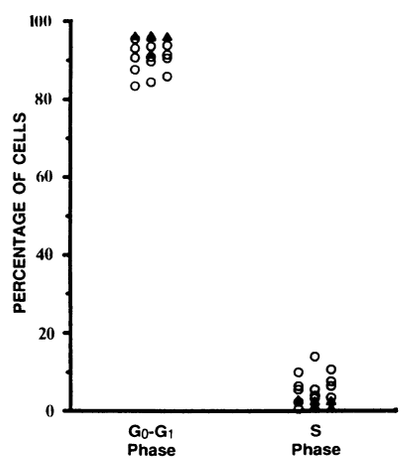

Figure 5. Percentage of cells in G_0-G_1 and S phases determined by DNA content in freshly excised human colorectal (○) and breast (▲) cancers.

To explore the concept of a G_1 restriction point we performed a series of in vitro experiments using tumor cell lines to study the effects of microenvironmental changes, likely to be found in solid tumors, on cell cycle phase distributions, G_1/S ratios, and expression of M_1-RR.

G_1 Restriction Point Block in Cultured Human Colon and Breast Cancer Cell Lines

(a) G_1/S ratio in unperturbed cell lines. The results of an integrated DNA flow cytometric-M_1-RR cell cycle analysis, based on DNA content and the proportion of cells positive for the M_1-RR detected via immunofluorescent flow cytometry, for the cultured cell lines (HT-29, MCF-7 and T-47D) at exponential growth are shown in Tables I and II. In these exponentially growing cells, < 3% of cells stained negatively for the M_1-RR antigen, and the G_1/S ratios obtained for these cells were all between 1 and 3.5.

(b) Density inhibition of HT-29 cells. HT-29 cells were allowed to grow to a maximal monolayer density with daily medium changes after cell density had reached 75% confluence at 3 d. Cell counts and viability, measured by the trypan blue dye exclusion method and cell cycle phase distribution and the expression of M_1-RR as measured by analytical flow cytometry, were performed daily. For these cells, a plateau phase-growth was achieved at 7 d, although at 2 d, there was an increase in the proportion of cells with a G_0-G_1 DNA content (results not shown), with a corresponding decline in the percentage of cells in the S phase. However, the percentage of cells staining positive for the M_1-RR did not fall even by 6 d (Table I) and indeed, through the whole period of observation of 9 d (results not shown), indicating that density inhibition induced a "restriction point block" with the cells accumulating in the G_1 phase, and that these blocked cells did not enter a metabolically quiescent phase, i.e., the G_0 phase.

(c) Serum deprivation of HT-29 cells. In a similar experiment where HT-29 cells were grown in serum-free medium, it was observed that after 2 d, cell growth had ceased; this cytostatic effect was maintained throughout the experiment until 9 d (results not shown). Data obtained from ADFC indicated a rise in the proportion of cells in the G_0-G_1 phase, commencing immediately after serum deprivation and becoming maximal by 2 d (results not shown). Representative results obtained at 6 d, are shown in Table I. In a manner analogous to the density inhibition study, the percentage of cells staining positively for

Table I. Cell Cycle Distribution of Density-inhibited, Serum-deprived HT-29 Cells in the G_1 and S Phases, their G_1/S Ratios and the Percentage of Cells Expressing the M_1-RR Antigen

	Exponential growth*	Density inhibition‡	Serum deprivation‡	Serum replenishment§
G_1 (%)	34.7±5.6	78.3±3.8	85.4±1.4	44.9±6.1
S (%)	35.5±5.2	14.5±2.0	4.5±0.1	39.2±3.9
M_1-RR positive (%)	99.5±0.2	99.6±0.2	97.0±2.1	97.7±1.4
G_1/S ratio	1.0±0.43	5.4±1.1	19.0±0.8	1.2±0.3

All values are derived from triplicate flasks.
* Doubling time, ~ 24 h.
‡ 6 d after treatment.
§ Cells were deprived of serum for 6 d before serum replenishment; data show results 24 h after serum replenishment.

the M_1-RR did not fall, even by 9 d (results not shown). This was interpreted to mean that the growth inhibition observed represented a block in G_1, and that these serum-deprived cells remained in the G_1 phase, without entering the G_0 phase.

This view was further reinforced by serum replenishment experiments. HT-29 cells which were deprived of serum for 6 d rapidly overcame their apparent G_1 block when serum was reintroduced into the culture medium. At the time of serum replenishment, the percentage of cells in the S phase was 5%; after a lag period of 12 h, the percentage of cells in the S phase began to increase, reaching 40% by 24 h, following serum replenishment (Table II).

(d) Hormone-induced growth inhibition of T-47D and MCF-7 cells. When the T-47D and MCF-7 breast adenocarcinoma cell lines were treated with MPA or tamoxifen, they ceased to proliferate. Analytical flow cytometric analysis of both DNA content and the percentage of cells expressing the M_1-RR demonstrated a "block" similar to that observed in the density-inhibited and serum-deprived HT-29 cells: a decrease in the percentage of S phase cells with a concomitant accumulation of cells with a G_0-G_1 DNA content, and the unabated expression of M_1-RR in virtually all of the cells (Table II). The

Table II. Cell Cycle Distribution of MCF-7 and T-47D Cells in the G_1 and S Phases, G_1/S Ratios and the Percentage of Cells Expressing the M_1-RR Antigen, after Treatment with Tamoxifen and MPA, Respectively

	MCF-7		T-47D	
	Exponential growth*	Tamoxifen treatment§	Exponential growth‡	MPA treatment§
G_1 (%)	62.6±2.7	78.1±0.6	66.9±0.54	77.2±0.5
S (%)	23.4±2.0	7.0±0.6	19.1±0.4	9.3±0.8
M_1-RR positive (%)	97.1±0.3	89.5±3.6	98.5±0.8	98.0±0.7
G_1/S ratio	2.7±0.3	11.2±1.1	3.5±0.1	8.6±0.6

All values are derived from triplicate flasks.
* Doubling time, 40–48 h.
‡ Doubling time, 24 h.
§ 3 d after drug treatment.

degree of block was not as complete as in the HT-29 cells, with the G_1/S ratio increasing by two- to fourfold. Therefore, growth factor deprivation or density inhibition or hormonal suppression can induce, albeit in different cell lines, a G_1 arrest, which has a kinetic state similar to that of primary human cancers.

Discussion

This study adequately demonstrates that in the normal human colorectal mucosa, the MAb M_1-RR specifically stains cycling cells in the proliferative compartment of the crypt. Cells stained by the MAb M_1-RR were in the same now well-established proliferative compartment (25–28) of the crypt, i.e., in the lower two-thirds of the crypt as cells pulse-labeled with BUDR. However, there were consistently more cells labeled with M_1-RR than BUDR, indicating that the former stained cells other than in the S phase, i.e., in the G_1, S, and G_2-M phases. The M_1-RR was absent in the mucosal cells lining the upper one-third of the crypt, which corresponds well with the fact that these are nondividing mature cells, which have ceased proliferative activity (25, 26, 29, 30) and therefore, are terminally differentiated. Hence, using the MAb M_1-RR, the proportion of cells in cycle, i.e., in the G_1, S, and G_2-M phases collectively can be estimated confirming our previously published results in another cell line using immunofluorescent analytical flow cytometry (12).

The integrated results of the MAb M_1-RR immunofluorescence and DNA flow cytometry on primary human tumors revealed an unexpectedly high proportion of G_1 cells. This was interpreted to mean that such tumors are blocked in transition in G_1 and are not predominantly in a G_0 or quiescent, differentiated state.

In most tumors it has been assumed that the greater majority of cells are nonreplicative (31). Estimates of a growth fraction (GF) in the order of 20–30%, supported the view that 70–80% of tumor cells are in the nonproliferative, or G_0 state. This was further reinforced by studies on several tumor types which compared the mean total cell cycle time (T_C) of approximately 2–4 d, with the typical mean tumor doubling time (V_D) of solid tumors, usually of 2–3 mo (7). The difference between the mean T_C and mean V_D was attributed to a high proportion of nonproliferating cells (31) and/or a high rate of cell loss (7). However, the methods used to approximate the proportion of cycling cells was by continuous labeling techniques, such as the percent labeled mitoses (PLM) based on the incorporation of nucleosides such as ^{3}H-thymidine (32) or more recently BUDR (33), of cells during the S phase. The accuracy of these methods in determining the GF are dependent on the continuous transit of cells throughout the cell cycle. Thus, cells blocked in the G_1 phase, which cannot transit into the S phase readily or are slowly cycling, will not be detected nor distinguishable from the noncycling fraction of cells in the G_0 phase. Also, using these methods, cells that are arrested in any of the other cell cycle phases will not be detected.

When the integrated DNA-M_1-RR cell cycle analysis, developed in the present study, was applied to freshly excised human primary breast and colorectal tumors, a low percentage of S phase cells was observed, ranging from 1 to 14%. However, the relatively high proportion of cells in the G_1 phase compared with the low percentage of S phase cells, suggests the presence of a G_1 block operating in these tumors. The situation was

more obvious in the breast tumors where the percentage of cells in the S phase was about threefold less than that found in the colorectal tumors while still maintaining similar percentages of cells in the G_1 phase. This was reflected in the G_1/S ratio of the breast tumors which was about threefold higher than that found in the colorectal tumors. Although our S phase values are lower than those found using ^{3}H-thymidine S phase labeling in tumor slices where the mean percentage of S phase cells for breast and colorectal carcinoma has been near 8 and 18%, respectively (1–6), these differences do not alter the conclusions made in this study. It is possible our lower S phase values using ADFC may reflect technical errors such as debris subtraction, host cell infiltrate, and the specific computer analysis program used (22, 34).

Immunohistochemical studies reported by others support our interpretation (although not made by those authors) of a high G_1 cell content (rather than G_0) in primary human adenocarcinomas (35, 36). In these studies of primary human cancers using antibodies to the *ras* oncogene products and to the proliferating cell nuclear antigen (PCNA or cyclin or DNA polymerase delta cofactor) the observed cell staining frequency (and variation) was almost identical to those for M_1-RR. Furthermore, other studies have found that the synthesis of *ras* mRNA occurs in mid-G_1 phase (13). Also, in work to be published separately, we have shown that both PCNA and M_1-RR mRNA synthesis occurs in the G_1 phase of PHA-activated peripheral blood lymphocytes (Parker, N., and R. M. Fox, manuscript in preparation). In addition, we note that the Ki-67 nuclear antigen has been used as a marker of the proliferative status of tumors (37, 38). We have shown, in a separate study, that the Ki-67 antigen is synthesized considerably later in G_1 or at the G_1/S interphase. The percentage of cells positive for Ki-67 in tumors has been markedly lower than that observed for the M_1-RR (Tay, D. L. M., manuscript in preparation), approximating that of the S phase fraction. Similarly, other workers have shown a low proportion of cells positive for the Ki-67 antigen (38–40).

We believe that the high percentage of G_1 cells in primary cancers reflects a major regulatory "block" or a marked slowing of transit in the G_1 phase. Such blocked cells are then apparently unable to enter either a G_0 phase or S phase. We have further hypothesized this G_1 block to be a reaction of the cells towards extrinsic microenvironmental phenomena and/or an inherent cancer cell property. In support of this, we have shown that tumor microenvironmental factors such as relative growth factor deficiency or density inhibition can produce a block in G_1, consistent with the concept of a "restriction point" in G_1, a concept which has been developed in some detail by Pardee and co-workers (9, 41, 42). According to them, the "restriction point" is a critical commitment event in G_1, which can only be overcome under the influence of appropriate growth factor and nutritional conditions. Pardee showed in several models, when cells were restimulated into the cycle after a block at the restriction point in G_1, DNA synthesis starts some 2 h after the restriction point, with increases in the enzymes essential for DNA replication. Thus, cells deprived of growth factor(s) would arrest in G_1, whereas cells beyond a certain critical point in G_1 would be committed to complete the rest of the cycle of DNA replication and arrest only upon reentering G_1 after mitosis.

In this study the G_1 block concept was modeled in vitro using serum-deprived or density-inhibited HT-29 cells, or MCF-7 and T-47D cells which had been subjected to tamoxifen and MPA treatment. The continued expression of M_1-RR after at least 6 d of serum deprivation and their rapid reentry into cycle (on refeeding) indicates that in certain cell systems at least, the transition from G_1 into a G_0 state is not an inevitable event upon growth factor deprivation, and as a result, the prolonged maintenance of a G_1 state can occur. An alternative to the prolonged G_1 state could be the relative failure of expression or synthesis of adequate growth factor receptor sites, or the effects of density inhibition. This was later tested in vitro.

Apart from the negative influences of growth factor deficiency we also considered the possibility of a positive metabolic perturbation being responsible for the G_1 arrested state. Antiproliferative cytokines such as interferons and tumor necrosis factor and in the case of breast cancer, hormones such as those of the estrogen/progesterone group could be appropriate mediators. Accordingly, when we treated human breast cancer cell lines with tamoxifen or the antiprogesterone MPA, a G_1 block, comparable to that obtained in the serum deprived HT-29 cells was observed. Previous studies by Osborne et al. (43, 44) using ADFC have also shown that tamoxifen induced a G_1 phase arrest in cultured cell lines. Our findings using MAb M_1-RR demonstrates that these cells maintain their G_1 status throughout the duration of the block and do not enter the G_0 phase. The concept of a hormonally induced and maintained G_1 arrest state in human breast cancer would accord well with the long and heterogeneous natural history of breast cancer.

An alternative (though not mutually exclusive) explanation for the apparent G_1 block would be an inherent genetic property of primary adenocarcinoma cells such as the recently recognised tumor suppressor genes in colorectal cancer (45). Such genes appear to operate by opposing or inhibiting "dominantly acting" activated cellular oncogenes and such a function in G_1 could in principle, block cell transit into the S phase. Some examples are the wild-type p53 gene product which can inhibit *myc* and *ras* functions, and transforming growth factor type-β which stimulates the synthesis of a protein inhibiting the transcriptional initiation of *c-myc* (46–48).

The apparent high content of cells blocked in the G_1 phase in primary human cancers implies a dynamic situation not previously realized. It poses fundamental questions which extend beyond the proliferation/suppressor balance in primary cancers, such as the mechanism(s) preventing cancers from entering G_0 and/or a terminally differentiated state.

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