Simultaneous Measurements of Macrophage-induced Cytostasis and Cytotoxicity of EMT6 Cells by Flow Cytometry

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ABSTRACT

Cytostasis and cytotoxicity as well as survival and growth of EMT6 cells following exposure to tumoricidal macrophages have been examined using multiparameter flow cytometry. Macrophages were isolated from tumor cells, and the number of surviving EMT6 cells at different interaction times was determined by adding, to each cell suspension, fluorescent latex particles the concentration of which was known and counting them along with the cells. The percentages of EMT6 cells in S phase were determined by analysis of DNA histograms and compared to the percentages as determined by autoradiography. The progression of cells through the cell cycle was also examined during exposure to macrophages by dual parameter analysis of DNA content and bromodeoxyuridine incorporated into DNA prior to exposure to macrophages. The results showed that, by 4 h of interaction with tumoricidal macrophages, EMT6 cells stopped progressing; tumor cell progression was inhibited in all phases of the cell cycle. By 24 h, however, surviving tumor cells which escaped the tumoricidal activity of macrophages exhibited a population doubling rate similar to control cells.

INTRODUCTION

It is well established that, when tumoricidal macrophages are coincubated with tumor target cells, tumor cell growth is inhibited (cytostasis), and tumor cells are killed (cytotoxicity). This phenomenon is independent of species and strain (1–4). Macrophages acquire tumoricidal activity by coming in contact with a lymphokine (MAF) which may be γ-interferon (5). Macrophages can be activated in vivo (by BCG) (6) or in vitro (by MAF produced as a result of culturing lymphocytes with mitogens) (7).

Macrophage tumoricidal activity has been studied extensively using a variety of approaches. In the simplest assay, the phenomenon is visualized qualitatively in a “spot” test developed by Hibbs et al. (1). Other assays depend on the release by tumor cells of incorporated radiolabels including 51Cr (8), [3H]dThd (6), or [125I]dUrd (9, 10). Thomasson and Stewart (11) developed a method for evaluating the survival of the clonogenic fraction of tumor cells by comparison to control cultures. The major difficulty with all of these approaches is that they do not allow quantitative retrieval of surviving tumor cells as a function of time so that cytostasis, cytotoxicity, and regrowth kinetics can all be measured simultaneously.

The purpose of this study was to measure cytostasis, cytotoxicity, and regrowth of surviving tumor cells as a function of time using flow cytometry, by combining the methods of Kaplan et al. (12) and Haskill (13) for resolving macrophages and tumor cells with a flow technique of Stewart and Steinkamp (14) for counting cells. The kinetics of tumor cell growth following exposure to tumoricidal macrophages has been evaluated in relation to changes in survival as well as to changes in the cell cycle distribution as determined (a) by [3H]dThd labeling, (b) by the use of anti-BrdUrd to detect BrdUrd incorporation, and (c) by the analysis of histograms of relative DNA content.

MATERIALS AND METHODS

Mice. Adult (6- to 12-wk-old) female C57BL/6 mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

Target Cell Culture. EMT6 cells (a gift from Dr. S. Rockwell) were grown as monolayers in Falcon T-75 flasks in αMEM (Grand Island Biological Co., Grand Island, NY) supplemented with 10% FCS (Reheis Chemical Co., Kankakee, IL), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were maintained free of contamination with Mycoplasma. The cultures were incubated at 37°C in a humidified 5% CO2 atmosphere.

Effector Cells. Peritoneal exudate cells were collected from mice given injections i.p. of 1.5 ml of Brewer’s thioglycollate medium (Difco Laboratories, Detroit, MI) 3 days prior to harvest. The macrophages obtained in this manner were subsequently activated in vitro. In vivo-activated macrophages were collected from mice given injections i.p. of 1.0 ml of BCG (106 viable organisms/ml) (Trudeau Institute, Saranac Lake, NY) 3 days prior to harvest. Peritoneal cells were collected using 5 ml of αMEM (prewarmed to 37°C) containing 20 mm (N-morpholino)-propanesulfonic acid (Calbiochem, La Jolla, CA) and heparin (5 units/ml) (Liquaemin Sodium; Organon, W. Orange, NJ), and the cells were recovered as previously described (15). Cell suspensions were pooled and centrifuged at 200 x g for 10 min. The pellet was resuspended in minimal essential α-medium containing 20 mm (N-morpholino)propanesulfonic acid (without FCS) at 2-4 x 105 cells/ml. Cell concentrations were determined in an electronic particle counter using the cetrimide technique as described elsewhere (15).

Macrophage Adherence. One ml of cell suspension was plated in each 35-mm culture dish (Coming), and the dishes were incubated at 37°C without CO2 for 45 min. Each dish was rinsed with culture medium (αMEM with bicarbonate buffer and containing 10% FCS) to remove nonadherent cells, and fresh culture medium was added to each dish. Adherent cells on replicate plates were removed with cetrimide and counted so the number of effector cells was known.

In Vitro Activation of Macrophages with MAF. MAF was produced by rat spleen cells incubated with concanavalin A (Sigma, St. Louis, MO). Briefly, spleens from 12- to 20-wk-old female Fischer 344 rats (Charles River Laboratories, Inc., Wilmington, MA) were obtained aseptically and dispersed using a 380-μm stainless mesh and the rubber tip on the plunger of a 1-ml syringe. Cell concentration was adjusted to 5 x 106 cells/ml in culture medium containing concanavalin A (5 μg/ml) and incubated at 37°C for 44-48 h in a T-150 culture flask (Coming). The...
supernatant fluid was collected, centrifuged at 700 × g for 30 min, aliquoted, and stored at −20°C. The fluid was filtered immediately prior to use using a 0.22-μm filter unit (Millex; Millipore, Bedford, MA). Adherent thioglycollate-elicited macrophages were incubated for 6–18 h with 10% MAF in culture medium. After incubation at 37°C with 7% CO2, these plates were washed twice with culture medium before adding 2 ml of fresh culture medium.

Incubation of EMT6 Cells with Macrophages. A 1-ml suspension of EMT6 cells (at 1.0 × 10^6/ml) was added to each dish containing adherent, activated (in vitro or in vivo) or nonactivated macrophages or no macrophages. The dishes were placed in the incubator at time zero, and at various time intervals (from 2–72 h), cells were removed from duplicate dishes by treatment with trypsin. The supernatant medium was removed, and 0.5 ml of 0.125% trypsin/EDTA (0.5 mm) was added. After 5 min of incubation at 37°C, 1.5 ml of culture medium was added, and the cells were pipeted and removed into a 15-ml centrifuge tube; the dish was rinsed with 2 ml of culture medium. The cell suspensions were then either stained immediately or fixed before staining. For fixation, cells were centrifuged, and the pellet was resuspended in 0.5 ml of (g/liter) glucose (1.1):NaCl (8.0):KCl (0.4):Na2HPO4•12H2O (0.39):KH2PO4 (0.15) containing 0.5 mm EDTA. While vortexing, 5 ml of cold 70% ethanol were added. Using this procedure, samples can be collected at various times, stored in ethanol, and analyzed together at some later time.

Staining Cells for Flow Analysis. Fixed cell suspensions were centrifuged, and the pellets were resuspended in 2 ml of staining solution containing M1 (100 μg/ml) [generously provided by Pfizer Co. (Croton, CT)] in 50 mM Trizma (pH 7.3) (Sigma, St. Louis, MO) containing 15 mM MgCl2 using the technique described by Crissman and Steinkamp (16).

For some experiments, the vital DNA-specific dye HO (Cabilochem) (17) was used. Staining cells under viable conditions had the advantage that no centrifugations, with accompanying potential cell loss, were required. For staining with HO, the trypsinized cells were incubated for 45 min at 37°C with HO (2.0 μg/ml) in αMEM with bicarbonate buffer and containing 10% FCS. Cell staining was accomplished in closed 50-ml polyethylene tubes to which CO2 had been added to maintain appropriate pH conditions, and the cells were incubated during staining in a shaking water bath (Model G76 gyratory water bath shaker; New Brunswick Scientific Co., Edison, NJ).

To the cell suspensions (fixed or viable), 10^6 fluorescent latex particles (9.45-μm beads; Particle Technology, Inc., Couler Electronics, Los Alamos, NM) in 50 μl were added to each tube containing the stained cell suspensions. The concentration of the beads had been determined previously by triplicate counts using the electronic particle counter (after sonication and frequent vortexing to eliminate aggregates). The samples were then filtered through a 44-μm nylon mesh (Cistron Corp., Lebanon, PA). Uniform mixing of the sample suspension was maintained during flow analysis by use of a stir bar and magnetic stirrer.

Flow System Analysis. Single-laser analysis of Mi-stained cells was performed using an argon-ion laser (Spectra Physics Model 164) operating at 457 nm with emission measured from 495-520 nm. Since the fluorescence emission of the latex beads overlapped the DNA distribution of Mi-stained cells, a second photomultiplier tube was used to collect only the emission of the beads (i.e., emission > 520 nm).

When HO-stained viable cells were analyzed, simultaneous dual-laser analysis of the blue fluorescence intensity of HO staining was performed using an argon-ion laser (Spectra Physics Model 164-05) operating in the UV (351.1 and 363.8 nm) with emission measured from 420–490 nm; a second argon-ion laser operating at 488 nm was used to excite the green fluorescence intensity of the beads, with emission measured above 550 nm. Cell volume was also measured.

Data were acquired in correlated list mode and stored, as described previously, by a PDP-11/20 computer (18). Subsequent reprocessing of the data allowed complete resolution of beads, macrophages, EMT6 cells, and dead cells. [EMT6 cells were discriminated from macrophages on the basis of their DNA content (near tetraploid)].

Pulse Labeling With [3H]Thymidine and Autoradiography. At each time point, cells were rinsed and pulsed for 15 min with [methyl-3H]dThd (1 μCi/ml) (New England Nuclear), specific activity, 6.7 Ci/mm. Using a cytocentrifuge (Shandon Southern Instruments, Inc., Pittsburgh, PA), microscope slides were prepared from each ethanol-fixed cell suspension (the same cell suspension used for flow analysis). The slides were then dipped into NTB-2 liquid photographic emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 5 days before developing. After staining with May-Grunwald/Giemsa, we determined the fraction of labeled cells by counting a minimum of 100 cells per slide. Duplicate slides were counted for each time point, and the average of the two counts was obtained.

BrdUrd Incorporation and Labeling Cells with Anti-BrdUrd:FITC and PI for Flow Analysis. The flow cytometry technique of Dolbeare et al. (19) was used to measure simultaneously DNA content and BrdUrd incorporation by cells in S phase at 0-h incubation. Briefly, monolayer EMT6 cells in exponential growth were incubated with 10 μM BrdUrd for 30 min at 37°C with 5% CO2, then washed with warm culture medium, removed by trypsin, and plated, as described above, onto macrophage monolayers. At each time of assay, cells were removed by treatment with trypsin and fixed in 5 ml of 10% ethanol at 0°C for 24 h. To each sample, 5 ml of 4 N HCl were added and mixed, and the suspensions were incubated at room temperature for 30 min. The cells were washed (200 × g, 10 min), resuspended in 2.5 ml of 0.1 M Na2B4O7, washed again, and resuspended in cold 70% ethanol before storage at 0°C for 24 h.

Before flow analysis, the cell suspensions were centrifuged, and the pellet was resuspended in 50 μl of 0.5% Tween 20 in phosphate-buffered saline. Finally, 5 μl of anti-BrdUrd:FITC (20) (Becton Dickinson Monoclonal Center, Mountain View, CA) were added to each tube. The suspensions were mixed by tapping and incubated for 30 min at room temperature. The cells were washed and resuspended in 2 ml of phosphate-buffered saline containing 20 μg of PI per ml. It was not necessary to use RNase before PI staining because the hydrolysis procedure denatures double-stranded RNA.

Single-laser flow analysis was performed using an argon-ion laser operating at 488 nm. Two-color fluorescence emission (corresponding to FITC and PI emission) was measured using a 530-nm band pass filter in combination with a 580-nm dichroic mirror for FITC, and PI emission was measured above 610 nm. Data acquisition and storage were accomplished by a PDP-11/23 computer (21, 22).

Data Analysis to Determine EMT6 Cell Number. To obtain the number of surviving tumor cells and their cell cycle distribution, analysis was performed as illustrated in Chart 1. The top row in Chart 1 shows relative cell volume, green fluorescence (from beads and cells), and blue fluorescence (from cells only). The difference between the relative volumes of beads and cells was used to verify their resolution. Fluorescence emission in the blue region (corresponding to HO staining) was detected only from macrophages and EMT6 cells and not beads. When excited at 488 nm, both the HO emission from cells and the fluorescence emission of the beads appear in the green channel; however, by collecting emission above 550 nm, the emission from the beads was completely resolved from that of cells as seen in the histogram for total green fluorescence (Chart 1).

The number of beads analyzed was determined by reprocessing on green fluorescence (Channels 190–255) and blue fluorescence (Channels 0–10) as indicated by the dotted lines in the middle row of Chart 1, and the integrated area equals the number of beads analyzed. In a similar way, a window was set on the original histogram of blue fluorescence events (Channels 60–255 to exclude macrophages) so that, following repro cessing, the corresponding distribution (and number) of EMT6 cells was obtained as shown in the bottom row of Chart 1.

The total number of EMT6 cells (Nc) at each time point was calculated from

\[ N_c = \frac{N_{TC} \times V_2 \times C_{B0}}{N_{BA}} \]
MACROPHAGE-INDUCED CYTOSTASIS AND CYTOTOXICITY OF EMT6 CELLS

Chart 1. Computer analysis and reprocessing of data to generate new histograms corresponding either to beads alone or to EMT6 cells alone. Following incubation with BCG-activated macrophages, cells were removed, stained viability for DNA content with HO, and analyzed by flow cytometry. A known number of fluorescent beads were added to each sample immediately prior to flow analysis. Top, original histograms of total events for the three parameters: relative volume; green fluorescence (beads and cells); and blue fluorescence (cells only). Middle, new histograms resulting when appropriate windows (indicated by arrows) are selected on the original histograms to include events corresponding to beads only. Bottom, a lower window set on the original histogram of blue fluorescence events so that the resulting new histograms correspond to EMT6 cells only. By this technique, the integrated area under the curve for the distribution of beads only (middle) reflects the number of beads analyzed, and the integrated area under the curve for the distribution of EMT6 cells only (bottom) reflects the number of EMT6 cells accumulated during analysis. Knowing the number of beads added to the original sample and the number of beads and EMT6 cells analyzed, the number of EMT6 cells present at each time point can be calculated.

where \( N_{db} \) is the number of beads accumulated during analysis, \( C_{bc} \) is the concentration of the beads in the sample, \( N_{te} \) is the number of tumor cells analyzed, and \( V_s \) is the original volume of the suspension. This calculation was performed for each of two replicate dishes at each time point.

The fractions of EMT6 cells in \( G_1, \) \( S, \) and \( G_2 + M \) were determined using the procedure described by Dean and Jett (23).

RESULTS

The effect of in vivo-activated macrophages and nonactivated macrophages on the growth of EMT6 cells is shown in Chart 2. When EMT6 cells were cultured alone or with macrophages from mice not given injections, no significant difference in the growth rate was observed. However, when EMT6 cells were incubated with activated macrophages, at two different effector:target ratios, a significant decrease in the number of EMT6 cells is apparent by 24 h. Regrowth, however, begins to occur sometime after 24 h, and after 48 h, the population doubling time of surviving EMT6 cells is nearly the same as for control cells. These data indicate that some EMT6 cells are not killed by macrophages, and these cells can eventually repopulate the culture.

The initial decreases in the observed growth rate may reflect the net result of macrophage-induced target cell cytosis and cytostasis of some tumor cells, as well as proliferation of others which are replacing those that are dying. In order to shed some light on the mechanism involved, the cell cycle distributions associated with each time point were analyzed to determine the cell cycle status of the tumor cells. Chart 3 shows the DNA histograms corresponding to the time points shown in Chart 2 for the EMT6 control and the EMT6 cells incubated with the higher concentration of activated macrophages. Control cells in exponential growth have a high proportion of S-phase cells on Days 1 and 2, but by Day 3, there is a lower proportion of cells in S phase as the culture becomes confluent and cells enter plateau phase. EMT6 cells in the presence of tumoricidal macrophages also have DNA distributions similar to those of the control cultures on Days 1 and 2. For the treated cells, the apparent increase in the fraction of cells in early S phase has been found by labeling with MAC1 (a macrophage-specific surface marker) to be due to macrophage doublets which are seen in the channel number corresponding to exactly twice that of macrophages having a 2C DNA content (data not shown). On Day 3 there is a high fraction of S-phase cells present in the cultures with activated macrophages. This is probably due to the fact that these cells showed an early drop in cell number, so that by Day 3, although they have returned to the growth rate of the control, the cell number is not sufficient for them to have entered plateau phase and, unlike the control cultures, they are still in exponential growth. The most significant observation, however, is that, on Days 1 and 2 (when cell number and growth rate were diminished), the cell cycle distributions show that tumor cells were present in each phase of the cycle.

Since the cell number was decreasing during the first 24 h without a large change in the S-phase fraction, we wondered whether surviving cells were actually progressing through the cell cycle. Accordingly, the percentage of cells in S phase was determined both by cell-cycle analysis of DNA histograms using flow cytometry and by determining the labeling index after incubation of cells with \(^{3}H\)dThd. In order to examine closely the

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Chart 3. Cell cycle distributions of EMT6 cells alone and EMT6 cells in the presence of BCG-activated macrophages (Mφ). Following incubation with macrophages, cells were removed, stained viably for DNA content with Ho, and analyzed by flow cytometry. Cell cycle distributions are shown for Days 1, 2, and 3 following coincubation with activated macrophages. Contaminating macrophages appear in the distribution but are seen to be resolved from tumor cells because of their lower DNA content.

Events occurring during the early stages of contact and cytolysis, samples were taken every 2 h for the first 24 h and subsequently at 36 and 48 h. The cell cycle distributions for 10, 14, 20, and 24 h are shown in Chart 4. At 0–10 h (not shown), cells from both cultures exhibited similar distributions. By 14 h, the control EMT6 cells showed an increase in the percentage of cells in S phase, and at 20 and 24 h, these cells exhibit cell cycle distributions expected for exponentially growing cultures. (The increase in S phase at 14 h was probably due to recovery from trypsinization effects.) For the EMT6 cells incubated with activated macrophages, however, the distributions throughout the 24-h period were essentially the same. Thus, the cells appear to have been arrested in every phase of the cell cycle.

To substantiate this possibility, we determined the percentage of cells in S phase by computer analysis of the DNA distributions obtained by flow cytometry and compared the results to those obtained by autoradiography of [3H]dThd-pulsed cells. These comparisons are shown in Chart 5. For control cells, the fraction of S-phase cells is similar at all time points whether measured by flow analysis or by [3H]dThd incorporation. In contrast, the frequency of cells having an S-phase DNA content as measured by flow analysis remains high at all time points while, by 24 h, virtually no cells were labeled with [3H]dThd. These results suggest that cells are arrested in all phases of cell cycle as a result of interaction with activated macrophages. Further, calculations (not shown) of the number of cells present in each phase of cell cycle as a function of time suggest that cells are not selectively killed in one phase of the cell cycle, but they may be killed in all phases beginning at about 18 h. As shown in Chart 2, the nadir in cell number is at 24 h, and at some time between 24 and 48 h, cells begin to increase in number, suggesting that for cells that escape killing, the cytostatic effect is lost after 24 h so that cells are able to repopulate the cultures.

In order to examine more closely the apparent phenomenon of cytostasis, suggested by the above data, we allowed EMT6 cells to grow to confluency and then used these nonexponentially growing cells in the same assay. The rationale was that cells in stationary phase, when replated at a low density, would have to reinitiate DNA synthesis. If macrophages indeed "freeze" cells wherever they are in cycle, EMT6 cells cocultured with activated macrophages would not be able to reenter S phase. When the distributions were compared (primary data not shown), the cells from the two culture conditions again exhibited similar distributions through the first 10 h. These DNA distributions revealed a significantly reduced frequency of cells in DNA synthesis. By 14 h, however, the cell-cycle distribution of control cells showed a large increase in cells entering S phase, and by 20 and 24 h, the distributions were those expected of cells in exponential growth. In contrast, the distributions of macrophage-treated cells did not change much between 10 and 24 h. Analysis
of these cell cycle distributions (Table 1) revealed that the percentage of cells in S phase for the control cultures increased from 16% at time zero to 66% by 14 h and was 64% at 24 h; however, for the macrophage-treated cultures, the percentage of cells in S phase ranged only from 16 to 21% over the entire 24-h period. Thus, these results also provided strong evidence that macrophages induce cytostasis of tumor cells randomly in cell cycle and not at some specific point. When tumor cells were treated with unactivated macrophages, the percentage of cells in S phase changed from 13% at 0 h to 82% at 14 h and returned to 54% by 24 h. Therefore, this effect of macrophages on cell cycle progression could not be attributed either to depletion of nutrients (due to high cell concentration in the dishes containing macrophages) or to a thymidine block caused by high levels of macrophage-secreted thymidine. Rather, it was a specific effect induced by tumoricidal macrophages.

DISCUSSION

The capability of simultaneously examining cell killing and cytostasis as well as escape and repopulation was possible because of multiparameter flow cytometry in combination with a unique technique for determining cell concentration. In addition, the use of the relatively new technique for following the progression of cells through the cell cycle using anti-BrdUrd to identify those cells synthesizing DNA at the time of the pulse, and when PI was used to label all cells for relative DNA content at the times of assay, the results (Chart 6) show that the BrdUrd-labeled cohort in the control culture progressed around the cell cycle during the first 24 h of culture. The position of the labeled cohort became somewhat randomized by 24 h. In contrast, when EMT6 cells were incubated with activated macrophages, the BrdUrd-labeled cells remained fixed in the S phase of cell cycle throughout the 24-h culture period. Further, there was no evidence of progression of unlabeled cells out of G1 or the G2 + M phase.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>EMT6 + macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16 ± 5</td>
<td>16 ± 5</td>
</tr>
<tr>
<td>8</td>
<td>25 ± 11</td>
<td>23 ± 9</td>
</tr>
<tr>
<td>14</td>
<td>66 ± 10</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>24</td>
<td>64 ± 7</td>
<td>21 ± 10</td>
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* The percentage of cells in S phase was determined by computer analysis as described in "Materials and Methods." In the case of three distributions for which computer analysis was not possible, the areas corresponding to G1, S, and G2 + M were estimated graphically.
* EMT6 cells were grown to confluency, and 24 h later, the cells were removed by trypsinization and replated at 100,000 cells in 35-mm culture dishes at the indicated times, duplicate dishes were removed and analyzed by flow analysis of DNA content.
* EMT6 cells grown 24 h past confluency were replated at 100,000 cells in 35-mm culture dishes already containing adhered activated macrophages. The cultures were then treated exactly as the control cultures above.
* Mean ± SD for three separate experiments.

The low [3H]dThd labeling index for macrophage-treated tumor cells might have been due to a lack of [3H]dThd uptake caused by increased thymidine pool sizes as a result of macrophage secretion of thymidine (10, 24) and not due to cytostasis and concomitant progression arrest even though the medium had been changed during the pulse to reduce the possibility that exogenous unlabeled dThd might alter [3H]dThd uptake. However, this did not eliminate the possibility that intracellular pools remained large and so might have competed with exogenous [3H]dThd. To circumvent this problem, S-phase tumor cells were labeled with BrdUrd prior to incubation with macrophages so that their progression through the cell cycle could be followed by flow cytometry using a fluoresceinconelated monoclonal antibody specific for BrdUrd (19, 20).

When this antibody was used to identify those cells synthesizing DNA at the time of the pulse, and when PI was used to label all cells for relative DNA content at the times of assay, the results (Chart 6) show that the BrdUrd-labeled cohort in the control culture progressed around the cell cycle during the first 24 h of culture. The position of the labeled cohort became somewhat randomized by 24 h. In contrast, when EMT6 cells were incubated with activated macrophages, the BrdUrd-labeled cells remained fixed in the S phase of cell cycle throughout the 24-h culture period. Further, there was no evidence of progression of unlabeled cells out of G1 or the G2 + M phase.

The most important finding in this study was that EMT6 cells arrested by macrophages appear to be blocked everywhere in the cell cycle and that it takes 4–10 h for the full effect of

<chart>Chart 5. Comparisons of the percentage of cells in S phase as determined by autoradiography of [3H]dThd-labeled cells and by computer analysis of cell cycle distributions obtained by flow analysis of mithramycin-stained cells (shown in Chart 4). Percentages are shown for both EMT6 cells alone (CONTROL) and EMT6 cells + macrophages (+Mφ) at 10, 14, 20, and 24 h following coincubation of EMT cells with macrophages. The most important finding in this study was that EMT6 cells arrested by macrophages appear to be blocked everywhere in the cell cycle and that it takes 4–10 h for the full effect of</chart>
cytostasis to occur. Other investigators have also observed cytostatic effects. Our data are consistent with those of Krahnbuhl (1980) who showed that synchronized EMT6 cells stopped progressing 2–6 h after contact with cytolytic macrophages. In contrast, Haskill (13), who used two-parameter flow cytometry to evaluate cytostasis and cytotoxicity, suggested that macrophages block the progression of HeLa cells near the G1-S interface. There are at least two possible explanations for our differing results. (a) EMT6 cells may behave differently from HeLa cells.

We have studied six other established cell lines and found that the cells are blocked everywhere in the cell cycle (26). The phenomenon therefore appears to be a universal one. (b) We know that the action of macrophages on target cells is not instantaneous but occurs over a period of time, which has not been well characterized and may depend on target cell sensitivity and the degree of macrophage activation. For example, from time-lapse movies, we showed (27) that EMT6 cells continued to divide for 6 h after contact with cytotoxic macrophages. Since S-phase-enriched cells were used by Haskill, they may have progressed out of S phase and even divided by the time the macrophages exerted their effect, resulting in the appearance of a sensitive phase in G1. Since our system allowed us to explicitly follow a BrdUrd-labeled cohort as well as to follow the progression of unlabeled G1 and G2 + M cells as they progressed (or failed to progress) through the cell cycle, this problem in interpretation was eliminated. In addition, because we evaluated cytostasis beginning at 4 h after coinoculation with macrophages, we were able to detect inhibition in all phases of the cell cycle occurring several hours before cytotoxicity was observed. We conclude, therefore, that there is no unique place in the cell cycle where cell progression is held up after interaction with macrophages. Rather, there is a differing sensitivity of cells to the mechanism by which macrophages exert their effect. This differing sensitivity will allow some cells to progress further and others to progress less throughout the cell cycle after they have been added to macrophages.

There have been several reports recently which show that there were cells with an S-phase DNA content but which did not take up [*]dThd when progressing tumors from animals were analyzed (28, 29). Like the results described above, there appears to be a population of tumor cells in situ that are not progressing or are progressing very slowly through the cell cycle. Virtually all solid tumors contain some macrophages, even though their tumoricidal state may not be known. Their presence, coupled with our finding that they arrest tumor cells throughout the cell cycle, provides a basis to understand the existence of this population of nonprogressing tumor cells in solid tumors.

One possible mechanism for growth inhibition has been suggested by Hibbs et al. (30), who have shown that release of iron from tumor cells occurs at 4–6 h after incubation with tumoricidal content) and with FITC-anti-BrdUrd to identify the BrdUrd-labeled cohort. Each individual dot represents a single cell whose position relative to the ordinate and abscissa depicts the relative amount of green (FITC) and red (PI) fluorescence, respectively, associated with each cell. The distributions each contain the same number of dots. At zero h, the BrdUrd-labeled cohort is seen to be distributed throughout S phase (indicated by the area enclosed in the box). For the control cultures, this cohort can be seen to progress to late S by 4 h and to both G1 and G2 by 8 h; by 11 h, the cohort is predominantly seen in G1, and by 13 h, some portion of the cohort is seen to reenter S phase. By 24 h, the BrdUrd-labeled cohort is seen to be distributed throughout all phases of the cell cycle. In contrast, for EMT6 cultures in the presence of macrophages, the BrdUrd-labeled cohort is seen to remain distributed primarily throughout the S phase for all times shown.
macrophages. They suggest that the loss of iron from enzymes crucial in metabolic pathways could result in inhibition of mitochondrial respiration and DNA replication. This, in turn, would deprive the cell of the energy required to progress through the cell cycle.

The important questions now center on the mechanism by which growth inhibition occurs and whether cytostasis (or the biochemical pathways involved) is a prerequisite for cytolysis. Normal cells may resist killing by macrophages because they are not sensitive to the interrupted biochemical pathway, while tumor cells represent a spectrum from nonsensitivity to exquisite sensitivity. The most exciting challenge that faces us is to relate these findings to the mechanism(s) by which macrophages exert their effect.

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