Effects of Activated Macrophages on Tumor Target Cells in Discrete Phases of the Cell Cycle

James L. Krahenbuhl

Division of Allergy, Immunology, and Infectious Diseases, Palo Alto Medical Research Foundation, Palo Alto, California 94301, and Division of Infectious Diseases, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

Studies were performed in vitro to examine the cytostatic properties of activated macrophages on tumor cells in various phases of the cell cycle. EMT-6 target cells were synchronized in the M phase by mitotic selection techniques and placed in culture. As the population of target cells proceeded to various discrete phases of the cell cycle in a high degree of synchrony, these cells were challenged with normal macrophages or activated macrophages from Corynebacterium parvum-treated mice, and their subsequent ability to synthesize DNA and undergo mitosis was observed. Regardless of when normal macrophages were added, they had little effect on the target cells. In contrast, target cells in the M or G1 phase at the time of challenge with activated macrophages failed to undergo DNA synthesis (S phase), and their subsequent mitosis was inhibited. Similarly, when tumor cells were in the early or mid-S phase at the time of challenge, DNA synthesis was markedly inhibited, and mitosis was blocked. When activated macrophages were added to EMT-6 cells synchronized in the late S or G2 phase, many of the target cells failed to undergo the impending phase of mitosis. These results suggest that the inhibition of target cell DNA synthesis by activated macrophages is not a prerequisite for the ability of these effector cells to block target cell multiplication.

INTRODUCTION

Numerous reports in a variety of animal models have shown that activated macrophages are cytotoxic to tumor target cells in vitro (1, 2, 6, 9, 11, 19). As shown by Keller (7), whether an activated macrophage-mediated cytotoxic effect will be cytocidal or cytostatic appears to be determined by undefined characteristics of the tumor target cell line used. In the case of EMT-6 tumor cells (syngeneic for BALB/c mice), DNA synthesis is almost completely inhibited in vitro in the presence of activated peritoneal macrophages (10, 12), and subsequent mitosis is virtually abolished (13). These effects of activated macrophages on EMT-6 cells are solely cytostatic with no evidence of EMT-6 cell lysis or death (14). Important additional evidence (5) that the effects of activated macrophages on EMT-6 cells are solely cytostatic lies in the reversibility of their effects, as shown by renewed DNA synthesis (10) and multiplication (10, 14) by the tumor cells after their removal from the presence of the macrophages.

The present studies were performed to examine in greater detail the cytostatic effects of activated macrophages. EMT-6 cells, synchronized with respect to the cell cycle, when used, and the effects of activated macrophages on the 2 principal landmarks of the cell cycle (DNA synthesis and mitosis) were measured. To determine if certain regions of the cell cycle are more sensitive than others to macrophage-mediated cytostatic effects, experiments were performed in which populations of EMT-6 cells, synchronized in discrete phases of the cell cycle (M, G1, S, G2), were challenged with activated macrophages.

MATERIALS AND METHODS

Mice. Female Swiss Webster mice weighing 18 to 22 g were obtained from Simonsen Laboratories, Inc., Gilroy, Calif. To activate their macrophages, mice received a single i.p. injection of 1400 µg killed Corynebacterium parvum (Lot 582A; kindly provided by Dr. John Whisnant, Burroughs Wellcome Co., Research Triangle Park, N. C.) 7 days prior to macrophage harvest. Macrophages were harvested as described previously (12).

Target Cells. EMT-6 mouse adenosarcoma cells (22) syngeneic for BALB/c mice were obtained from Dr. Robert Kallman, Stanford University Medical Center, Stanford, Calif., and were maintained by twice-weekly passage in Waymouth's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 15% fetal calf serum (Way-15 FCS) and antibiotics.

Cell Synchronization. EMT-6 tumor target cells were synchronized in the M phase using a modification (13, 21) of the mitotic selection technique of Terasima and Tolmach (24). Briefly, 4 × 106 EMT-6 cells were suspended in 30 ml Ca2+-, Mg2+-free Joklik-modified minimum essential medium (Grand Island Biological Co.) containing 15% fetal calf serum in each of 10 plastic 150-sq cm culture flasks (Corning Glass Works, Corning, N. Y.). When the monolayer was almost confluent (24 to 30 hr), the more loosely attached mitotic cells were detached by shaking the flasks on a rotary shaker (2 revolutions/sec). The medium was removed and replaced with 13 ml of medium consisting of 50% fresh Way-15 FCS and 50% conditioned Way-15 FCS (previously used to culture EMT-6 cells). Harvests of mitotic cells were repeated every 15 min for 3 hr. Cells collected from the fifth through the 12th harvest were held on ice, concentrated by centrifugation, counted, and resuspended in cold Way-15 FCS. The degree of synchrony in the M phase was assessed by counting the percentage of cells in mitosis in cell preparations fixed with methanol:acetic acid (3:1) after hypotonic treatment with cold 0.1 M sucrose (21).

Timing of Macrophage Challenge. In all of the experiments

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involving synchronized target cells, 0 hr refers to the time of plating of synchronized mitotic EMT-6 cells. In order to determine the effects of normal and activated macrophages on tumor cells in different discrete phases of the cell cycle, 0.5 ml of a cell suspension consisting of $4 \times 10^6$ peritoneal cells from normal or C. parvum-treated mice was added to EMT-6 cells as their progress through the cell cycle reached the appropriate predetermined point. After 1 hr, nonadherent cells were removed from the wells by washing with 0.85% NaCl solution, and 1 ml of fresh medium was added.

$[^3H]dThd$ Incorporation and Mitotic Arrest. The position of the synchronized target cell population in the cell cycle was determined prior to, at the time of, and following macrophage challenge by quantitating the level of DNA synthesis (S phase) and/or mitosis (M phase) in the tumor cell population. DNA synthesis by EMT-6 cells, quantitated by measurement of $[^3H]dThd$ incorporation, was determined at intervals throughout the course of each experiment. At regular intervals, 5 $\mu$Ci of $[^3H]dThd$ (specific activity, 6 Ci/mmol; Schwarz/Mann, Orangeburg, N. Y.) were added to wells containing synchronized EMT-6 cells alone or EMT-6 cells challenged with normal or activated macrophages. After 30 min, the wells were washed, and $[^3H]dThd$ incorporation was quantitated by extraction of cellular DNA and liquid scintillation using methods described previously (12). In certain experiments, $[^3H]dThd$ incorporation was measured by autoradiography as described previously (20). In these experiments, growth and challenge of EMT-6 cells were carried out in 4-chamber Lab-Tek tissue culture slides (Lab-Tek Products, Naperville, Ill.). The presence of tumor cells in mitosis was quantitated by addition of 0.006% colchicine (Calbiochem, Los Angeles, Calif.) to the cultures during a 5- to 6-hr interval (13).

Retroactive Synchrony. In certain experiments in which synchronized EMT-6 cells were challenged with activated macrophages at points in the cell cycle estimated to be late S or G2 phase, the subsequent ability of the tumor cells to undergo the impending mitosis was quantitated using a modification of the retroactive synchrony technique described by Kimler et al. (8). Synchronized M-phase EMT-6 cells were seeded in parallel in 16-mm Linbro wells (2 sq cm) and tissue culture flasks (75 sq cm) to give an initial concentration of $1.25 \times 10^6$ cells/sq cm. After 12 hr of incubation at 37°C, the tumor cells were challenged with activated macrophages ($4 \times 10^6$ peritoneal cells/ml medium; 0.5 ml/Linbro well or 18.75 ml/flask). After 1 hr, nonadherent cells were removed by washing, fresh medium was added, and subsequent $[^3H]dThd$ incorporation was assessed in the Linbro wells as described above. Tumor cells entering mitosis in the presence or absence of macrophages were dislodged from the flasks by shaking at 15-min intervals from the 14th to 18th hr after plating. After each collection, the flasks were immediately replenished with warm (37°C) medium. Each of the cell harvests was chilled (4°C) immediately, the cells accumulated at the end of each hour were pooled and quantitated, and the number of mitotic cells was determined as described above.

RESULTS

Cell Cycle of Synchronized EMT-6 Cells. The progress of synchronized EMT-6 tumor cells through the cell cycle is shown in Chart 1. The initial mitosis was completed by the cell population within 40 min, resulting in an approximate doubling of cell numbers. The cells proceeded through a G1 phase of approximately 4 hr and underwent a synchronized wave of DNA synthesis (S phase), beginning at approximately 5 hr and peaking at 10 hr. After their initial mitosis, the number of cells in the population remained relatively constant between 2 and 11 hr. The detectable increase in number at 14 hr, culminated by the doubling in cell number seen at 16 hr, reveals the occurrence of a second synchronous wave of mitosis.

Effects of Activated Macrophages on Tumor Cells in the M or G1 Phase. As shown previously (12) and in Chart 2, when EMT-6 cells synchronized in the M phase were added to monolayers of normal macrophages, they were unaffected in their progress through the cell cycle. However, when they were added to activated macrophages, the tumor cells failed to proceed into the S phase and their subsequent mitosis was blocked. Not shown in Chart 2 is the observation that all of the M-phase tumor cells (>90% at the time of challenge) completed their initial synchronized phase of mitosis within 40 min, even in the presence of activated macrophages. However, entry into the S phase was blocked in the presence of activated macrophages, and the tumor cells appeared to have been arrested in G1.

The effects of challenge with activated macrophages on G1-phase tumor cells was determined by seeding the synchronized tumor cells 1.5 hr prior to challenge. As shown in Chart 3, challenge of G1-phase tumor cells with activated macrophages also appeared to result in their arrest in G1 (there was no evidence of their progress into the S phase).

Effects of Activated Macrophages on Tumor Cells in the S or G2 Phase. Charts 4 to 7 and Table 1 show the results of experiments in which populations of tumor cells synchronized in the early, mid-, or late S or G2 phase were challenged with normal or activated macrophages. Regardless of their position.

![Chart 1. Progress through the cell cycle of EMT-6 tumor cells synchronized by mitotic selection. Tumor cells were 92% in the M phase at 0 hr. Each point shows the mean of $[^3H]dThd$ ($[^3H]TdR$) incorporation (O) or cellular multiplicity (C) of triplicate cultures. Bars, S.D.](chart1.png)
in the cell cycle at the time of macrophage challenge, the progress of synchronized tumor cells through the cell cycle was not substantively altered by normal macrophages. The rate and level of DNA synthesis by the tumor cells, as well as their entry into mitosis, kept pace with that of the control group cultured in the absence of macrophages (Charts 4 to 6). In contrast, addition of activated macrophages to target cells that were engaged in or had already completed DNA synthesis resulted in marked alteration of their progress through the cell cycle.

As shown in Chart 4A, addition of activated macrophages to tumor cells in the early S phase (6 hr) permitted a short-lived low level of DNA synthesis to occur. Subsequent mitosis by these cells was almost totally inhibited (Table 1, Experiment 1). When activated macrophages were added at 10 hr, DNA synthesis by the tumor cell population was at its peak (Chart 4B), and the cytostatic effect of activated macrophages was only detectable by their ability to markedly inhibit the tumor cells from undergoing the subsequent wave of mitosis and by their ability to inhibit the entry of the tumor cells into a second wave of DNA synthesis (Table 1, Experiment 1). When activated macrophages were added at 14 hr (late S or G2 phase), [3H]-dThd incorporation by EMT-6 cells appeared to continue at the same rate as that in control cultures (Chart 4C) indicating that, in spite of the presence of activated macrophages, some of the cells in the tumor cell population had completed their preparations for the imminent mitosis, had divided, and had entered a second wave of DNA synthesis. However, 8 hr after challenge (i.e., at 22 hr), the cytostatic effects of activated macrophages...
Cytostatic Effects of Activated Macrophages were manifested by the abrupt and marked inhibition of further DNA synthesis by the tumor cells. Of particular interest was the observation that, even though activated macrophages were added to synchronized tumor target cells at a point in the cell cycle when DNA synthesis was essentially completed (i.e., 14 hr), many of the tumor target cells were inhibited from undergoing mitosis (Table 1, Experiment 1).

The observation that activated macrophages could inhibit the mitosis of tumor cells that had already completed DNA synthesis was also made in each of 2 additional experiments in which macrophages were added to cultures of synchronized tumor cells at discrete points in the cell cycle. Shown in Chart 5 and Table 1 (Experiment 2) are the results of a second experiment in which macrophage challenge was carried out when the tumor cells were in the late S or G₂ phase (13 hr). As above, normal macrophages did not appear to alter the progress of the tumor cells through the impending phase of mitosis and subsequent second wave of DNA synthesis (Chart 5). In contrast, as was the case in the experiment shown in Chart 4C, activated macrophages apparently permitted some of the target cells to undergo mitosis and embark upon a second wave of DNA synthesis (Table 1). In Chart 6, normal macrophages did not exert any marked effects. Activated macrophages totally inhibited DNA synthesis and subsequent mitosis of tumor cells challenged in the M phase (Chart 6; Table 1, Experiment 3); similar to results seen in Chart 2 and rapidly inhibited DNA synthesis and subsequent mitosis of tumor cells challenged in the early or mid-S phase (Chart 6B and C; Table 1). Challenge of late S-phase tumor cells with activated macrophages (Chart 6D; Table 1, Experiment 3) prevented their progress into a second wave of DNA synthesis and, in confirmation of the experiments described above, also showed that the addition of...
activated macrophages to a tumor cell population that has already completed DNA synthesis can inhibit the impending mitosis.

In the experiment shown in Chart 7, synchronized tumor cells were challenged with activated macrophages at 12 hr (late S phase), and their subsequent capacity for [3H]dThd incorporation and mitosis was measured. By the 18th hr, it was evident that entry into a second wave of DNA synthesis was being totally inhibited in the presence of activated macrophages. In addition, as shown by quantitation using the procedure of retroactive synchronization, the number of cells undergoing division in the impending phase of mitosis was markedly reduced in the presence of activated macrophages. These findings corroborate those presented above which suggest that activated macrophages can inhibit mitosis in tumor cells that have already undergone the DNA synthesis necessary for mitosis.

DISCUSSION

Two properties of the EMT-6 cell line facilitated these studies: (a) the effects of activated macrophages on EMT-6 cells are solely cytostatic. Activated macrophages do not lyse or kill EMT-6 cells (14), and the cytostatic effects are reversible (5, 10, 14); (b) large numbers of highly synchronized EMT-6 cells can be readily obtained by mitotic selection (13). This tedious but entirely physical selective detachment method, described originally by Terasima and Tolmach (24) for HeLa cells, was chosen as the means of synchronizing EMT-6 cells in order to avoid subjecting the target cells to the perturbations which may accompany synchronization procedures that require antimetabolic drugs or other biochemical means of temporary blockade (17).

This study presents considerable evidence that, regardless of the region of the cell cycle which EMT-6 target cells are in at the time of macrophage challenge, activated but not normal macrophages eventually block the multiplication of the target cells. Our inability to definitively demonstrate a discrete period in the cell cycle during which tumor target cells were particularly sensitive to the effects of activated macrophages was due largely to the 2 to 6 hr that elapsed between the time of macrophage challenge and measurable evidence of a cytostatic effect.

In the case of lymphocyte-mediated cytotoxicity, a rapid cytolytic effect (30 min) allowed Leneva and Svet-Moldavsky (15) to demonstrate that L-cells were most susceptible to lysis by immune lymph node lymphocytes during G1. However, in the case of macrophage-mediated cytotoxicity, a prolonged period of contact between populations of effector and target cells was necessary before a cytotoxic effect, whether cytoidal or cytostatic, could be manifested (12, 16). In the present study, when M-phase target cells were added to activated macrophages, they completed mitosis but failed to enter the S phase and thus were arrested in G1. Target cells in the G1 phase at the time of challenge entered the S phase in the presence of activated macrophages, but their ability to synthesize DNA was totally inhibited within 2 hr. Similarly, target cells in the early stages of the S phase at the time of challenge continued to synthesize DNA for 2 to 4 hr before the inhibitory effects of activated macrophages were expressed. Thus, the target cells could proceed through the cell cycle for several hr before the cumulative cytostatic effects of activated macrophages halted their progress.

Results of greater interest were obtained when activated macrophages were added to synchronized cultures of EMT-6 cells that were in the late S or early G2 phase at the time of challenge. Two types of cytostatic effects were observed. In the first, tumor cells completed their preparation for the impending phase of mitosis and divided in the presence of activated macrophages, but cytostasis was ultimately manifested within a few hr as shown by inhibition of the second synchronous wave of [3H]dThd incorporation. These results are consistent with the inhibition of tumor cell DNA synthesis by activated macrophages after a 4- to 6-hr delay. The second type of macrophage-mediated cytostatic effect was manifested to a varying degree in each of the experiments performed in which challenge took place while the tumor cells were in the late S or early G2 phase. A significant number of target cells failed to undergo the imminent mitotic phase. As shown by the symmetrical shape of the curves representing [3H]dThd incorporation by control cultures of EMT-6 cells in each of these experiments, the cell population remained in a high degree of synchrony through at least one cell cycle and most probably precedes the possibility that the inhibited target cells were only in the early S phase at the time of challenge. One interpretation of these results is that an unknown event which occurs in the target cells in the late S or early G2 phase and is critical to mitosis is blocked by activated macrophages. Thus, the cytostatic effects of activated macrophages on EMT-6 cells can be expressed even if the EMT-6 cells have already completed DNA synthesis.

The mechanisms underlying these cytostatic effects are only conjecture at this time. Several reports have shown that cultures of macrophages may secrete thymidine into their supernatant media and that the presence of this DNA precursor may account for apparent macrophage-mediated cytostatic effects on tumor target cells by competitively inhibiting the uptake or incorporation of [3H]dThd (3, 18). If the target cell line is highly sensitive to thymidine blockade, the level of thymidine reacted by macrophages may be sufficient to actually inhibit the multiplication of the target cells (23). Clearly, when activated macrophages were added to synchronized EMT-6 cells while the latter were anywhere between the M and early S phase, tumor cell DNA synthesis was sufficiently inhibited to explain the prevention of subsequent mitosis. However, in a recent report from our laboratory in which synchronized as well as unsynchronized EMT-6 target cells were used, the cytostatic effects of activated macrophages could not be attributed to supernatant factors (14).

In addition to the readily identifiable cell cycle events of DNA synthesis and mitosis, a large number of other metabolic processes must certainly take place in a precise sequence before cell division occurs. Blockage of any one of several of these processes by activated macrophages could be sufficient to induce the reversible state of cytostasis which was observed. For example, Granger et al. (4) recently reported that activated macrophage-mediated cytostatic effects on L1210 leukemia cells occurred in the absence of cytoidal effects. The mechanism(s) of these cytostatic effects appeared to lie in the ability of the activated macrophages to inhibit cellular respiration and consequently mitochondrial ATP production, forcing the cells to be dependent upon glycolytic pathways. Whether these
events are linked to phases of the cell cycle is presently unknown, but such a mechanism of macrophage-mediated cytostasis could be operating separately or in combination with the inhibition of DNA synthesis or another mechanism and could underlie the cytostatic effects of activated macrophages on EMT-6 cells.

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James L. Krahenbuhl


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