ABSTRACT

The immediate effect of tumor necrosis factor (TNF) added to cultures of L-cells is cytostasis, manifested as cell arrest in G₂. This effect prevails during the initial 4 hr when the number of G₂ cells increases markedly in the absence of any significant cell death in the culture. Shortly thereafter, the cytolytic effect becomes apparent; extensive cell lysis can be detected after 7 hr of exposure to TNF. After 24 hr nearly all cells are lysed. Results of experiments in which the effects of TNF on the kinetics of cell progression through various phases of the cell cycle were studied indicate that in the presence of TNF cells do progress through G₂, although with considerable delay, and reach mitosis. Most cells die (undergo lysis) especially at late stages of mitosis (telophase) or soon after cytokinesis. Sensitivity of cells to the lytic effect of TNF is increased by cell arrest in mitosis with Colcemid or vinblastine. TNF exerts little effect on rates of cell progression through G₁ or S phases of the cell cycle, although few cells die during S phase. Nonlysed cells from cultures treated with TNF do not show any changes in nuclear chromatin, suggesting that neither DNA nor nuclear proteins are the primary targets of the TNF. The present data implicate metabolic changes which occur during mitosis (cytokinesis), perhaps associated with synthesis or assembly of cell membrane components, as being responsible for increased cell sensitivity towards the cytolytic effect of TNF. The mechanism of the early cytostatic effect of the factor is unknown.

INTRODUCTION

TNF is a protein found in the serum of mice, rats, or rabbits presensitized with Bacillus Calmette-Guérin or Corynebacterium parvum and challenged with bacterial endotoxin (4, 16, 19, 20, 22). Its name derives from the hemorrhagic necrosis that is observed in certain transplantable tumors of the mouse after systemic administration of the TNF. Because bacterial endotoxin elicits a similar antitumor reaction, it had to be proved that the activity of TNF was not due to the presence of residual or modified endotoxin. Evidence accumulated over the past several years indicates that TNF and endotoxin are separate factors and that TNF is involved in mediating the hemorrhagic necrotic effect of endotoxin (for reviews, see Refs. 17, 18, 23, and 27). A major distinction between endotoxin and TNF is that TNF has a direct cytotoxic or cytostatic effect on a variety of different tumor cell types in vitro, whereas endotoxin does not. Cloned lines of mouse histiocytomas produce a factor with TNF activity, suggesting macrophages as at least one of the normal cellular sources of TNF in vivo (21, 23). Although it was observed that cell sensitivity to TNF correlates with rate of proliferation (27), mechanisms by which TNF exerts its effect on the target cells, especially as related to the cell cycle position, are unknown.

Flow cytometric techniques recently have been developed to investigate the cell cycle distribution and kinetics in more detail than was possible previously (for review, see Ref. 6). These techniques, based on simultaneous differential staining of DNA versus RNA (9, 10) or analysis of nuclear chromatin as reflected by sensitivity of DNA in situ to denaturation (8, 11), were extended to studies on the mechanism of drug effects in relation to the cell cycle (12, 13). Utilizing these methods, we have investigated the cell cycle-specific effects of TNF. Staining of DNA alone or simultaneous staining of DNA versus RNA enabled us to observe 2 distinct effects of TNF, cytostasis followed by cytosis, as well as to measure the extent of RNA accumulation during cytosis. Effects of TNF on cell progression through G₁, S, G₂, and transition to M were studied in stathmokinetic experiments, in which recognition of cells at different phases of the cycle, as well as living versus dead cells, was based on differences in both DNA content and chromatin structure (11–13).

MATERIALS AND METHODS

TNF Preparation. A highly active TNF fraction was prepared from the serum of C. parvum-primed (1 mg i.p.; Burroughs Wellcome Laboratories, Triangle Park, N. C.) female CD-1 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.), which were given injections of endotoxin on Day 10 (10 μg i.v., lipopolysaccharide W from Escherichia coli 0127:B8; Difco Laboratories, Detroit, Mich.) and exsanguinated 1.5 hr later (4, 16). After lipid was removed by differential centrifugation (16 hr at 110,000 × g), the serum was fractionated by batch method DEAE-ion exchange using a ratio of 10 g dry gel to 1 g protein and was further purified by Sephadex G-100 and G-200 (Pharmacia Fine Chemicals, Piscataway, N. J.) column chromatography and Affi-Gel Blue (Bio-Rad Laboratories, Richmond, Calif.) affinity chromatography. The final preparation of TNF contained 355 μg protein per ml and was further diluted for the experiments described in the text.

Cells. Mouse L-M cells derived from the NCTC clone 929 (L) line were obtained from the American Type Culture Collection and grown in Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with nonessential amino acids, penicillin (100 units/ml), streptomycin (100 μg/ml), and 5% heat-inactivated fetal calf serum at 37°C. A TNF-resistant L-cell line was derived from sensitive L-cells by repeated passages in TNF-containing medium (4, 16). The activity of TNF was assayed (4) by adding 0.5 ml of medium containing serial dilutions of TNF to wells (24-well Costar plates) seeded 3 hr previously with 50,000 trypan-positive L-cells (0.5 ml of medium), and determining the amount of protein that resulted in 50% cell kill estimated at 48 hr by phase microscopy. The TNF preparation used in this study had an activity of 3.2 ng (50% cell kill) on the sensitive cell line but showed no cytotoxicity on the resistant L-cell line up to 7 μg (the highest concentration tested). Cells for cell cycle experiments were trypan-positive and inoculated into T-35 flasks (Falcon) 12 hr before addition of various concentrations of TNF. During exponential growth,
the doubling time of these cells was 18 to 20 hr.

Stathmokinetic Experiments. Two sets of parallel cultures of exponentially growing cells were prepared in T-35 flasks (500,000 cells/flask). At 0 time, all cultures received vinblastine (0.1 µg/ml, final concentration; Eli Lilly, Indianapolis, Ind.). At the same time, one set of cultures was treated with 0.88 µg TNF per flask. Beginning from 0 time, individual cultures were harvested by trypsinization at hourly intervals up to the 12th hr. Care was taken to collect both cells that were attached to dishes (removed by trypsinization) and cells floating in the medium. The latter consisted of either living cells detached during mitosis or dead cells. All cells from each culture were pooled, rinsed with Hanks' salt solution, and fixed as described previously (11–13). The stathmokinetic experiment was repeated several times using different mitotic blockers (Colcemid, 0.05 µg/ml, and vincristine, 0.01 µg/ml) and different concentrations of vinblastine, with essentially similar results as reported presently. Different concentrations of TNF (0.3 to 2.2 µg) were tested, and the experiments were also performed on the TNF-resistant subline of L-cells.

Cell Staining. Several staining techniques were used for cell analysis. To stain DNA in intact cells, 0.2-ml aliquots of cell suspensions (containing approximately 2 to 5 x 10^5 cells in tissue culture medium) were admixed with 2.0 ml of a solution containing DAPI (2 µg/ml, 0.1% (v/v) Triton X-100 (Sigma Chemical Co., St. Louis, Mo.), 0.1 µg piperoxazine-N,N'-bis(2-ethanesulfonic acid buffer (Calbiochem, La Jolla, Calif.) and 2 mM MgCl2, pH 6.4 and 0.05% DAPI was synthesized and kindly provided by Dr. Jan Kapuscinski of the Sloan-Kettering Institute. At 0.1% Triton X-100 and in the presence of serum proteins (from the original suspension), the cells do not lyse (unless subjected to mechanical stresses), but they do become permeable and, thus, may be stained with DAPI. In some experiments, only viable cells were stained. To this end, prior to staining, cells were preincubated in the presence of a freshly prepared mixture of enzymes containing DNase I (100 µg/ml; Worthington Biochemical Corp., Freehold, N. J.) and trypsin (2.5 mg/ml; Grand Island Biological Co.) in Hanks' salt solution, at 37°C for 30 min. As described before (7, 25), such pretreatment removes from the suspension all dead cells that cannot exclude the enzymes, leaving viable cells with unchanged DNA content. Nearly 10^6 cells were counted per DNA channel at maximal cell frequency (Chart 1), and approximately 2 x 10^5 cells were counted for each sample.

Simultaneous staining of DNA and RNA by AO (Polysciences, Inc., Warrington, Pa.) was described previously (9, 10). The staining of permeable cells was done at an AO concentration of 13 µM and dye/DNA-P molar ratio >2.0 in the presence of 1 mM sodium EDTA. Under these conditions, interaction of AO with DNA results in green fluorescence, with maximum emission at 530 nm (F530) whereas binding to RNA gives red metachromasia with maximum emission at 640 (F640). The intensity of green fluorescence is proportional to DNA content of the cell, and the intensity of red fluorescence correlates with RNA content (2). The specificity of DNA and RNA staining was evaluated by preincubation of permeable cells with DNase or RNase as described previously (9, 10).

Denaturation of DNA in situ by acid was described previously (9–11). Briefly, cells, after fixation, were resuspended in 1 ml of Hanks' solution containing 1000 units of RNase A (Worthington) and incubated at 37°C for 1 hr. Then, 0.2-ml aliquots (~4 x 10^5 cells) were admixed with 0.5 ml of 0.2 M KCl/HCl buffer, pH 1.35, at 20°C. Thirty sec later, the cells were stained with AO solution (4 µg/ml) in 0.1 M citric acid/0.2 M NaHPO4 buffer, pH 2.6. The red and green fluorescence of cells thus stained represents the stainability of denatured and double-stranded DNA, respectively (8, 13). In this technique, the distinction between cells at various phases of the cell cycle is based both on differences in DNA content and in chromatin structure. Changes in chromatin structure, especially during progression through G1 or transition between G2 and M, are reflected in the extent of DNA that is sensitive to denaturation (11–13).

Fluorescence Measurements. Fluorescence of individual cells stained with DAPI was measured with an ICP-22 flow cytometer (Ortho Diagnostics, Westwood, Mass.) using the UG 1 excitation filter and emission filter transmitting between 450 and 510 nm. The coefficient of variation of the mean value of the G1 peak was approximately 2.0%. Fluorescence of cells stained with AO was measured in an FC 200 flow cytometer (Ortho Diagnostics) as described in detail previously (8, 29). The red (F<sub>440</sub> 600 to 640 nm) and green (F<sub>530</sub> 515 to 575 nm) fluorescence emission from each cell was measured simultaneously by separate photomultipliers, and the data were stored in the computer. The pulse width values of nuclei fluorescence were recorded to determine nuclear diameter and to eliminate cell doublets from the measurements (29). The experiments were repeated at least twice, and 5,000 or 10,000 cells were measured per sample.

The number of cells in G1, S, and G2 (or G2 + M) compartments was obtained using a modified graphical method of Baisch et al. (1) by folding the left half of the G1 peak and the right half of the G2 + M peak around their modes and interpreting areas under the new, symmetrical G1 and G2 (or G2 + M) peaks as representing G1 and G2 (or G2 + M) cells, respectively, and the cells in between these peaks as being in the S phase. Following acid denaturation of DNA, cells in mitosis could be distinguished as described previously (7) based either on single-parameter (F<sub>440</sub>) histogram analysis or by the gating analysis of the 2-parameter (F<sub>440</sub> versus F<sub>530</sub>) histograms, inasmuch as the M-cell clusters were totally separated from the interphase cells.

In all experiments, parallel to the cultures exposed to TNF, the time-matched untreated control cultures were analyzed to assess whether or not changes in cell cycle distribution or viability occurred throughout the experiment. In none of the time-matched control cultures was there ever observed a higher than 2% change in the proportion of cells in any particular phase of the cell cycle or in the proportion of nonviable cells when these cells were stained with DAPI. In AO-stained samples, the viability was higher but did not exceed 3.5%. Thus, the experiments were performed on cells during the steady-state exponential phase of their growth. The straight line representing accumulation of cells in mitosis during the stathmokinetic experiment (Chart 5) provides additional evidence that the cells studied grow exponentially.

The reproducibility of the staining and analysis steps, tested by staining 10 separate samples from a single culture, and expressed as maximal variability in the percentage of cells in any particular phase of the cell cycle from the mean value, was below 1% for DAPI-stained cells and below 2.5% for cells stained with AO.

A technical note should be added on frequency histograms as shown in Charts 1 and 3. In Chart 1, for comparison of the relative heights of G1 versus G2 + M peaks within and between the samples, the vertical scale is constant (10^5 cells/channel), and G1 peaks are of the same height, with approximately 10^5 cells/channel at maximum. Consequently, when cells are arrested in G2 + M, the total number of counted cells per sample is proportionately higher because there are proportionately fewer G1 cells. Therefore, although the percentage of cells in S phase may be the same or even lower (e.g., Chart 1, A versus B or D), the outline level of the S-phase distribution on the histograms may be elevated because more S-phase cells have to be counted for each channel (between 2C and 4C), and more cells have to be counted per sample. In contrast to Chart 1, the frequency histograms generated by the computer in Chart 3 (insets) have a different vertical scale; thus, the relative heights of G1 versus G2 peaks can be compared only within the same sample.

RESULTS

Effects of TNF on Exponentially Growing Unperturbed Cultures

Staining of Cellular DNA with DAPI. Effects of TNF on exponentially growing L-cells were analyzed by studying the distribution of cells in the cell cycle and cell viability by 3 different staining techniques. The representative data from one of the experiments in which the cultures were treated with TNF for up to 8 hr and the cells harvested at hourly intervals and their DNA content analyzed after staining with DAPI are shown in Chart 1.
Effects of TNF on the Cell Cycle

In this experiment, the cells were stained either directly after harvesting or following incubation with a freshly prepared mixture of trypsin (2.5 mg/ml) and DNase I (100 μg/ml). The enzymatic treatment, as reported previously (7, 25), results in hydrolysis of DNA in dead cells, which, having lost their membrane integrity, cannot exclude the enzymes, leaving the viable cells unchanged for DNA analysis. A similar procedure was recently proposed by Frankfurt (15) as a viability test for flow cytometry. The data in Chart 1 clearly indicate that, in the presence of TNF, the proportion of cells with a G2 + M DNA content is increased. Thus, during the 6-hr exposure to 4.1 μg of TNF, there was an increase from 21 to 33% of G2 + M cells and a decrease in the proportion of G1 and S-phase cells, indicating that TNF arrests cells in G2 + M.

In all cultures, both control and TNF-treated ones, the relative proportion of cells in G1, S, and G2 + M was measured before and after incubation with trypsin plus DNase. Differences no larger than up to 2% in cell cycle distribution, as a result of the enzymatic treatment, were seen in all control cultures and up to 4 hr of exposure to TNF in TNF-treated cultures (not shown). After 4 hr, however, the proportions changed, and as is evident in Chart 1E, in TNF-treated and in trypsin-plus-DNase-incubated cultures, a significant decrease in percentage of G1 and an increase in percentage of S-phase cells were observed. These data indicate that, among nonviable cells removed by the enzymatic treatments, there was a higher proportion of cells with a G1 DNA content than among viable cells. When only viable cells are compared during the experiment (Chart 1, A versus E), the specific cytostatic effect of TNF in arresting cells in G2 + M is even more pronounced, inasmuch as the respective heights of the G1 versus G2 + M peaks at 0 time and after 6 hr of treatment with TNF show an even greater difference. The experiment reported in Chart 1 was run at hourly intervals. The results not included in Chart 1, i.e., at 1, 3, and 5 hr of treatment with TNF, agree with the included data, showing, e.g., an increase in the percentage of G2 + M cells from 22 to 25 and 29% at 1, 3, and 5 hr, respectively. In this experiment, the parallel, time-matched control cultures were analyzed and were found not to vary by more than 2% in DNA distribution throughout the experiment (not shown).

Simultaneous Staining of RNA and DNA. In this set of experiments, the cultures were treated with different concentrations of TNF for various periods of time up to 24 hr, and the cells were then stained with the metachromatic fluorochrome AO under conditions in which cellular RNA and DNA stain differentially (9, 10). Description of the method, its applicability, and specificity of staining are given elsewhere (6, 7, 9, 10). With this technique, it is possible not only to discriminate cells at different phases of the cell cycle (based on DNA stainability) and to estimate their relative RNA content but also to distinguish nonviable, lysed cells which have lost most of their cytoplasmic constituents, including RNA (7). Results of these experiments confirming the data already described are shown in Chart 2 and Table 1. Namely, the effects of TNF were visible shortly after addition of TNF to cultures; initially (up to 7 hr), the cytostatic effect was most evident and was seen as an arrest of cells in G2 + M. With time, the cytolitic effect became predominant, and the lysed cells could be recognized easily on the scattergram representing DNA versus RNA values (Chart 2). Their number was markedly increased in cultures exposed to TNF for 7 hr or longer. The population of lysed cells had a disproportionately higher number of cells with a G2 DNA content in comparison with nonlysed cells. Nonlysed cells from TNF-treated cultures had increased RNA content, which was especially evident for the G2 + M population.

Table 1 lists quantitative changes in the cell cycle distribution,
proportion of dead (lysed) cells, and RNA content as a result of exposure to 2.2 and 8.8 μg of TNF. The cytostatic effect is most evident at 4 hr in culture with 8.8 μg TNF. At that time, the number of cells in G₂ + M is increased from 23.4 to 34.9%, concomitant with a decrease in G₁ from 49.4 to 39.4%. Thus, nearly total cell arrest in G₂ + M is observed under these conditions at a time when there are only few (8.6%) dead cells. After 7 hr, the number of cells arrested in G₂ + M is similar at both concentrations of TNF and is not significantly different from that seen at 4 hr. Cell lysis, however, is already extensive. At 7 hr, one-fourth and one-third of the total cell population is lysed in cultures with 2.2 or 8.8 μg TNF, respectively. Surviving cells arrested in G₂ + M have their RNA content increased by 14 to 18%, while cells in G₁ or S phase show only a 5% increase in RNA. Parallel, time-matched, untreated control cultures did not show a change greater than 3.5% in cell viability or in the percentage of cells in G₁ versus S versus G₂ + M greater than 3.5%; the variability in mean values of F₈₀₀ in these controls was below 3%.

### Distinction between Cell Arrest in G₂ versus M. It is clear from the data obtained by cell staining with DAPI (Chart 1) and AO (Table 1) that the TNF induces a transient cytostatic effect, arresting cells in G₂ + M. An increase in RNA content of the viable G₂ + M population suggests that the arrest is in G₂ rather than in M because during mitosis cells do not synthesize RNA. To obtain direct evidence, however, as to whether the arrest is in G₂ or in M, experiments were done in which the TNF-treated cultures were stained according to another technique utilizing AO, which makes it possible to discriminate between G₂ and M (7, 8, 11). The results shown in Chart 3 indicate that among viable cells there is an increased proportion of cells in G₂.

The results of several experiments in which 3 different techniques of cell staining were used all agree that TNF induces a transient cell arrest in G₂ + M, lasting for approximately 4 to 6 hr, which is followed by cell death (lysis). Among dead cells, there is a disproportionately high percentage of cells with a G₁ DNA content in comparison to that of viable cells. In these studies, 3 different techniques were used to distinguish nonviable cells, namely, cell ability to exclude the enzymes, loss of the cytoplasmic constituents, and high denaturability of DNA: The first technique detects the loss of cell membrane integrity; the second, cell lysis; and the third, nuclear chromatin changes, perhaps leading to pyknosis (11-13). Thus, the population of nonviable cells detected by each of these methods may not be identical and may represent different stages of cellular changes associated with cell death.

### Stathmokinetic Experiment

The experiments described above, in which exponentially
Growing cells were exposed to TNF, could detect major effects of TNF such as cell arrest in G2 or cell death but could not reveal minor changes in the cell cycle (if they did occur) such as a slowdown in cell progression through G1 or S phase, for instance. To this end, the next series of experiments were done in order to estimate in more detail the points in the cell cycle that may be specifically sensitive to the action of TNF. The recently introduced method of cell cycle analysis based on the stathmokinetic experiment (12, 13) was used. In this technique, the investigated drug and the stathmokinetic agent (vinblastine) are added to cultures of exponentially growing cells at the same time; cell samples are collected at hourly intervals and stained with AO in such a way that individual phases of the cell cycle, including G2 versus M, may be distinguished (13). In pilot experiments, it was observed that the cytotoxicity of TNF is increased in the presence of vinblastine. Therefore, to investigate a similar range of TNF-induced cytostatic and cytotoxic effects as shown in Charts 1 or 2 and Table 1, a lower concentration of TNF (0.88 μg) was used in stathmokinetic experiments. Histograms representing cell distribution in cultures treated with vinblastine alone or with vinblastine and TNF are shown in Chart 4.

As cell progression through the cycle is halted in M during stathmokinosis, several kinetic parameters may be estimated by analyzing changes in cell number in G1, S, G2, and M (12, 13). It is already evident from the raw data (Chart 4) that vinblastine alone induces a transient (up to 4 hr) arrest of cells in G2 prior to blocking them in M. This was observed in numerous experiments using different concentrations (0.05 to 0.5 μg/ml) of vinblastine as well as of Colcemid and vincristine. Thus, the subline of L-cell used in this study, after addition of the mitotic blockers, exhibits an unusually long delay before cells begin to accumulate in M; during the lag phase the cells are blocked in G2. This peculiarity of the cells does not interfere with the stathmokinetic experiment, because, although transiently arrested in G2 instead of in M, the cells do not "leak" through the mitotic block (see below), and thus the transit rates through earlier phases can be estimated.

Several conclusions may be drawn concerning the effect of TNF on the cell cycle based on visual inspection of the histograms obtained from stathmokinetic experiments (Chart 4). It is evident that TNF does not markedly affect the kinetics of cell progression through the early phases of the cycle; the rates of emptying of G1 and early S-phase compartments are quite similar, with only a few cells remaining in G1 after 12 hr in both sets of cultures. Likewise, the proportion of cells in S phase (among viable cells) is similar in both the control and TNF-treated cultures throughout the experiment. The main effects of TNF are manifested as a deficit in M-cell population and in the appearance of dead cells, both changes already clearly seen after 8 hr and very

Chart 4. Progression of cells through various phases of the cell cycle and their accumulation in mitosis, in vinblastine (left column)- and vinblastine plus TNF (right column)-treated cultures. Vinblastine and TNF (0.88 μg) were added at 0 time, and cells were sampled hourly up to 12 hr; cell histograms after 0, 4, 8, and 12 hr are shown. Cells at different phases of the cell cycle were distinguished as described previously (12, 13). In addition, dead cells with low FMO values (increased DNA denaturation) could be discriminated and quantified.
pronounced after 12 hr. This observation alone implies that in
TNF-treated cultures cell death occurs quite specifically, either
in G₂, during transition from G₂ to M, or in mitosis. If cell death
were to occur at random during the cycle, this would result in
preservation of identical proportions of cells in all phases, includ-
ing M, among surviving cells in treated cultures as compared
with the control. This, however, was not observed.

Quantitative analysis of changes in cell number in G₁, G₂ + M,
and M during the stathmokinetic experiment, allowing estimation
of the rates of cell progression through various points of the
cycle (12) is provided in Chart 5. A slowdown in the rate of cell
exit from G₁ is evident after 5 hr of exposure to TNF. The effect
is rather minor and therefore escapes detection by visual inspec-
tion of the histograms (Chart 4). The slope representing cell
accumulation in G₂ + M or M during stathmokinesis permits one
to estimate the duration of the cell cycle (12, 26); based on this
slope, the duration of the cycle in L-cells is 18 hr. This value is
in agreement with the doubling time of these cells (18 to 20 hr)
as estimated from the growth curves. In the presence of TNF, a
decrease in the rate of cell entrance into G₂ + M is already
observed by 3 hr. The most pronounced differences between
control and TNF-treated cultures is expressed in the rate of cell
accumulation in M (Chart 5). In the presence of TNF, fewer cells
are arrested in mitosis between 4 and 12 hr. The difference
increases progressively with time, and after 12 hr there are more
than twice as many mitotic cells in the control cultures than are
in the TNF-treated cultures.

Comparison of the slopes representing accumulation of cells
in M versus G₂ + M in the TNF-treated cultures indicates that at
later stages (6 to 12 hr) the deficit in cell number in M is higher
than in G₂ + M, with respect to control values. This indicates
that there is a higher proportion of G₂ cells in TNF-treated than
in control cultures, compensating for the deficit in M cells. As a
consequence, the change in G₂ + M attributed to TNF (in
absolute cell number) is less pronounced than the change in M.
At the end of the experiment (12 hr), among viable cells there
were 64.4% G₂ cells in TNF-treated cultures, compared with
48.9% in controls (not shown). This finding is consistent with the
observation that TNF induces cell arrest in G₂ + M (see Charts
1 and 3 and Table 1). However, the staining method used in the
stathmokinetic experiment, which discriminates G₂ versus M
cells, makes it possible to localize the point of cell arrest in the
cell cycle with greater accuracy than does single-parameter DNA
analysis (Chart 1) or simultaneous staining of RNA and DNA
(Table 1). These data also confirm the observation on exponen-
tially growing cells, as shown in Chart 3.

Changes in proportions of all cells, including dead (lysed) cells,
are shown in Chart 6. In control cultures, a continuous decrease
in cell number in G₁ is paralleled at first by an increase in G₂ (up
to the 4th hr) and then by an increase in M, with cells in G₂
remaining at a plateau during the later stage. A decrease in S-
phase cells becomes evident after 8 hr, and there are few dead
cells throughout the experiment. In cultures treated with TNF, a
continuous decrease in cell number in G₁ is also observed.
However, there is a progressive increase in the G₂ cell population
without reaching a clear plateau. The increase in M-cell popula-
tion is minor, and the number of dead cells begins to increase at
4 hr and shows a dramatic rise after 9 hr, reaching over 30% by
12 hr.

The cytostatic and cytolytic effects of TNF, although less
pronounced than described above, were also observed at a
lower concentration of TNF (0.03 μg/culture). No perturbations
of the cell cycle were observed when the TNF-resistant subtype
of L-cells was treated with TNF (up to 8.8 μg/culture) under
similar conditions. The control, time-matched untreated cultures did not show changes greater than 3.5% in the cell cycle distribution throughout the experiment, up to 12 hr.

Although it is rather difficult to estimate distribution of the cell cycle among dead cells based on chromatin staining (Charts 3 and 4), it appears that in the stathmokinetic experiment most dead cells are characterized by a G2 + M DNA content, as their position on the histograms is close to G2 and M clusters (Chart 4; 8 and 12 hr). By contrast, in the absence of vinblastine, dead cells in TNF cultures have a predominantly G1 DNA content (Chart 1; Table 1). These data, together with other evidence (see “Discussion”), indicate that in the absence of vinblastine the TNF-treated cells die preferentially either late in M (telophase) or in G1; vinblastine, however, arrests them in metaphase, precluding transit to G1. In the presence of vinblastine, therefore, cells die preferentially either in metaphase or earlier in M during transition from G2. It should be noted that in the presence of vinblastine (or other mitotic inhibitors), the cells become more sensitive to the cytotoxic action of TNF.

**DISCUSSION**

Based on the results of experiments described above, the following sequence of events leading to death of cells exposed to TNF can be constructed. The first effect is a transient cell arrest in G2. The arrested cells show signs of unbalanced growth, characterized by elevated RNA content. Some increase in RNA also occurs in cells that progress through S or G1. Few cells, however, die in G1, S, or G2 during the first 0 to 8 hr following addition of TNF. Although delayed, the cells enter and progress through mitosis. Cell death (lysis) occurs specifically at the late stages of mitosis (telophase) or shortly following cytokinesis. As a consequence, most lysed cells have a G0 content of DNA. In summary, either telophase or some very early postmitotic stage appears to be the specific point in the cell cycle at which TNF treated cells undergo lysis. This conclusion is also supported by the earlier phase-contrast and time-lapse cinematography observations of cells in culture, indicating that in the presence of TNF many cells enter mitosis and lyse.5

The stathmokinetic experiment was designed to analyze in more detail the cell cycle-specific effects of TNF, as was done previously for several antitumor drugs (13, 29). The initial experiments brought to light 2 findings that require discussion. The first was the observation that the subline of L-cells used in this study responded to mitotic inhibitors such as Colcemid, vincristine, or vinblastine by a long delay prior to entering M phase. These agents induced a prolongation of G2 lasting approximately 4 hr. Following this delay, cells entered M and became arrested in M as expected. Other sublines of L-cells do not show this effect (3). The second finding was an increased sensitivity to TNF in the presence of the mitotic inhibitors. Thus, in stathmokinetic experiments utilizing vinblastine, the concentration of TNF had to be lowered 10-fold to obtain a range of cytotoxicity comparable to the experiment in which vinblastine was not used. The mechanism of the synergistic action of the mitotic blockers and TNF is unknown. One may assume, however, that if M is the most sensitive portion of the cell cycle to TNF action, prolongation of M by the blockers could enhance the cytotoxic effect of TNF.

The stathmokinetic experiment also provided evidence that cells in G1 are resistant to TNF. Cell exit from G1 was affected very little by TNF, and also, few G1 cells died during exposure to TNF. Likewise, TNF exerted little effect on cell transit through S phase, although some cell death occurred during S. Analysis of the relative proportions of cells at various cell cycle phases during the stathmokinetic experiment and the kinetics of progression suggests that the observed deficit in M-cell population in TNF-treated cultures is a result of both cell death and their transient arrest in G2. Two mechanisms may account for this observation: (a) TNF retards cell progression through G2 and selectively kills cells that advance to, and progress through, M; (b) TNF arrests cells in G2, and some cells die during arrest while others progress to M. In light of the data discussed above, the first mechanism is more likely.

The method of cell staining used in the stathmokinetic experiment (8, 11–13) makes it possible to observe changes in chromatin structure. Recognition of the cell cycle phases is based both on differences in DNA content and chromatin structure; the latter is reflected by in situ DNA sensitivity to denaturation. Dead cells that have markedly increased DNA sensitivity to denaturation could be recognized and quantified as described previously (11, 13). Viable cells from TNF-treated cultures did not show any differences in chromatin structure in comparison with control

---

5 J. Hlinka, B. Williamson, S. Green, and L. J. Old. Tumor necrosis factor-cycle dependent cytotoxicity by TNF on L929 cells, unpublished observations.
cells. Since drug-induced changes in DNA or nuclear proteins are immediately reflected in altered sensitivity of DNA to denaturation (12, 13, 30), this observation suggests that neither DNA nor nuclear proteins are the primary targets of TNF.

The increased sensitivity of mitotic cells to the cytotoxic action of TNF bears certain implications. Because the cytotoxicity of TNF is enhanced in combination with the mitotic blockers, perhaps as a result of prolongation of the TNF-sensitive phase of the cell cycle, further enhancement might be expected if highly synchronized mitotic populations (e.g., obtained by sequential synchronization by hydroxyurea or thymidine, followed by vinblastine) were used. On the other hand, noncycling cells should be resistant to TNF and their recruitment, and progression through the cycle would increase sensitivity. Indeed, decreased sensitivity to TNF was observed in L-929 cultures proliferating at lower rates as a consequence of reduced serum concentration (28).

Similar to many antitumor agents, especially those affecting DNA structure (e.g., intercalating drugs), TNF induces cell arrest in G$_2$. In contrast to these drugs, however, the arrest is of rather short duration and is followed by cell lysis occurring at quite specific point of the cell cycle, i.e., during or shortly after cytokinesis. The exact point(s) of the cell cycle at which other natural antitumor substances produced by eukaryotic cells (lymphokines, interferon) affect the cells are not well established. In the case of interferon, transition of cells from quiescence into the cell cycle was reported to be the most sensitive phase (5). Thus, combination of TNF and interferon with TNF affecting cells in the cycle and interferon affecting quiescent cells which undergo recruitment to the cycle appears to offer, at least in theory, a very attractive approach to tumor cell killing. The presence of quiescent cells resistant to most radio- or chemotherapeutic regimens is believed to be responsible for common failures in tumor therapy (14).

The mechanisms by which TNF exerts it cytostatic or cytolytic effects are unknown. TNF causes an early increase in RNA synthesis as measured by the rate of $[^{3}H]$deoxyuridine incorporation (24) and accumulation of RNA above the control levels has been observed in the present study (Chart 2; Table 1). In view of these observations, and the evidence that actinomycin D or cycloheximide markedly enhances sensitivity to TNF (21, 24), the role of ongoing or even accelerated protein synthesis as a mechanism repairing the potentially lethal effects of TNF has been postulated (24, 27). The maximal sensitivity to TNF coincides in time with maximal expansion of cell surface area (cytokinesis), i.e., with the highest rates of production and assembly of the cell membrane components. Thus, the possibility that components of cell membrane may be primary targets of TNF, as suggested by Ruff and Gifford (27, 28), should be considered.

ACKNOWLEDGMENTS

The authors wish to thank Robin Nager for her assistance in the preparation of the manuscript.

REFERENCES

Cell Cycle-specific Effects of Tumor Necrosis Factor
