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Drug-Induced Alterations of Tumor Necrosis Factor-Mediated Cytotoxicity: Discrimination of Early Versus Late Stage Action

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The killing of L-M cells by murine tumor necrosis factor (mTNF) was investigated by a combination of drug, antiserum neutralization, and kinetic studies. Kinetic studies with ^{125}I-mTNF showed that the bulk of association of ligand with L-M cells peaked within 2 hr and the ligand was not degraded. Cell surface receptors were depleted (down regulated) by 6 hr when death commenced. The off-rates of acid-dissociable (surface bound) and acid-indissociable (internalized) compartments were found to be 9 min and 35 hr, respectively. Nevertheless, complete cell killing required the persistent presence of mTNF for up to 20 hr. This requirement was ablated by the concomitant addition of cycloheximide. Antiserum completely inhibited cytotoxicity when it was applied up to 4 hr after mTNF, but antiserum added 1 hr after mTNF was not neutralizing in the presence of cycloheximide. Thus, the inclusion of cycloheximide temporally dissociated early events (internalization and signal transduction) from lysis. Other drugs with and without cycloheximide were found to preferentially affect either early or later aspects of cell death. Phorbol myristate acetate and the ionophore A23187 were potent inhibitors of cytotoxicity, and staurosporine was a potent enhancer. These agents were more effective when added 1 hr before mTNF and cycloheximide than when added 1 hr after and likely affected early events in the cytolytic program. In contrast, chloroquine and cAMP likely affect more terminal aspects of cytotoxicity. Dibutyrylcyclic AMP, cholera, and pertussis toxins enhanced cytotoxicity. They were equipotent when added either before or after mTNF regardless of the presence of cycloheximide. Likewise, chloroquine was an equipotent inhibitor when added either before or after mTNF regardless of the presence of cycloheximide. Agents that primarily affect association events may be more likely to impinge on other TNF-mediated activities than agents that primarily affect lysis.

Key words: ligand-binding kinetics, receptor down-regulation, sensitivity, signal transduction, internalization

Abbreviations used: TNF, tumor necrosis factor; PMA, phorbol myristate acetate.

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Tumor necrosis factor (TNF) is an acute phase response protein that is produced principally by activated macrophages. Its role in injury, inflammation, and infection [reviewed in 1,2] and clinical applications are under investigation. It produces the symptoms of septic shock and the hemorragic necrosis of tissues when it is injected into rodents [3–5], and it is cachectic in mice bearing transplanted tumors that produce TNF [6]. TNF produces a plethora of effects on cultured cells. It shares a number of properties with other "catabolic" polypeptide hormones (interferon, interleukin-1, beta-transforming growth factor): it is lipomodulatory [7,8], growth-promoting for fibroblasts [9,10], surface antigen modulatory [11,12], and anti-viral [13,14]. Notably, it is cytotoxic to a variety of tumor cell lines [10,15].

The expression of these properties likely begins with the binding of TNF to cell surface receptor(s). Studies with ^{125}I-TNF demonstrate specific and high-affinity binding to approximately 1,000 cell surface receptors on cultured cells [16–21]. Binding appears to be an obligate but insufficient requirement at least for the manifestation of cytotoxicity [16,17]. Furthermore, agents that reduce receptor number [22–25] or affinity [26] reduce the cytoxic response, and molecular modifications that alter binding characteristics commensurately change the cytotoxic response [27]. Once bound by receptor, a large proportion of the ^{125}I-TNF is internalized [17,18].

The manner in which these events trigger the functional responses of target cells is also under study. G proteins may be involved in signal transduction or at least in some functional aspect of TNF-mediated cytotoxicity [28–30]. Protein synthesis is altered after treatment of target cells with TNF [11,12,31,32]. With respect to cytotoxicity, internalization and lysosomal activity may be required [33,34]; additionally, the persistant presence of TNF throughout prolonged culture periods is required for complete cytotoxicity [34].

While a variety of agents have been used to alter the expression of cytotoxicity, these agents may affect cytotoxicity in multiple ways. Herein, kinetic and antiserum neutralization studies were performed in order to establish the time course of association of TNF with a sensitive target cell. Furthermore, an attempt was made to discriminate the influence of drug treatment on "early" effects (such as binding, internalization, or signal transduction) versus lysis to help clarify their influence on the mechanism of cytotoxicity and to help define the mechanism of TNF-mediated cytotoxicity.

MATERIALS AND METHODS

Murine TNF (mTNF), also called necrosin, was isolated from J774.1 cell line supernatants as described [35]. Standard recombinant human TNF (lot 86/659) and recombinant human lymphotoxin were obtained from the National Institute for Biological Standards and Control, Hertfordshire, UK. Relative to the standards, the mTNF preparation used herein had a specific activity of 8×10^5 U/μg as measured on L-M cells in the presence of 4 μM cycloheximide [34]. Antisera to mTNF were generated in New Zealand white rabbits by conventional methods. The antisera were judged to be monospecific with respect to other J774.1 products as assessed by $NaDodSO_4$ polyacrylamide gel electrophoresis of crude supernatants followed by immunoblotting and also by specific immunoprecipitation from crude supernatants. Anti-mTNF neutralized mTNF and human TNF but not lymphotoxin. TNF was radioiodinated to a specific activity of 0.4 mCi/nmole monomer. Iodination and equilibrium-specific binding were performed

as described previously [35]. Off-rates were analized similarly to those described for pinocytosed sucrose in cultured cells [36].

L-M (CCL 1.2), a murine tumorigenic fibroblast, was obtained from the American Type Culture Collection. It was cultured at 38°C in a humidified atmosphere of 95% air/5% CO_2 in medium (Eagle's minimum essential medium with Earle's salts), 5% iron supplemented calf serum (Hyclone). Survival titrations were performed similarly to those previously described [34]. Survival and binding studies were performed with growth medium that contained 1% calf serum and 10 mM Hepes. Cholera and pertussis toxins were obtained from List Biological Laboratories. Staurosporine and A23187 were obtained from Calbiochem. Other agents were obtained from Sigma.

RESULTS

When L-M cells were treated with highly purified preparations of mTNF, the time course of cytotoxicity was similar to that which had been observed with more crude TNF preparations [34]. Cells were killed during an 18–20 hr exposure. They began to die after about 6 hr, and complete killing required the persistant presence of mTNF throughout the duration. This requirement would have rendered the interpretation of drug effects complex, since, while effects on binding characteristics could be readily measured, other effects (signal transduction, transcription, lysis) may have contributed to the influence of a drug on cytotoxicity. However, the concomitant use of cycloheximide was found to greatly reduce the amount of exposure time required to kill L-M cells. Thus, a system was devised that temporally and operationally dissociated the cytotoxic program into an initial "association" stage and a latter stage, the onset of cell death or "lysis." Neutralization and kinetic studies illustrate this dissociation.

Neutralization and Cycloheximide

Anti-mTNF antiserum completely neutralized cytotoxicity when added simultaneously with mTNF. The antiserum was nearly as effective when added 4 hr after mTNF (Fig. 1A). Later additions were increasingly less neutralizing (Table I). These results suggested that the initial effects of mTNF were insufficient for cell death and confirmed the requirement for the persistant presence of TNF for complete cell killing. When mTNF-mediated cytotoxicity was carried out in the presence of cycloheximide, 10-fold less mTNF was required to kill all the cells (Fig. 1B). Cycloheximide alone also killed the cells, but after a much more prolonged exposure (>30 hr, not shown). The simultaneous addition of antiserum neutralized the killing, but antiserum failed to neutralize when added only 1 hr after mTNF (Fig. 1B). Thus, in the presence of cycloheximide, only a brief exposure to mTNF was required for complete cell killing, although death did not commence until 3–4 hr later.

Association

The kinetics of association of biologically active ^{125}I-mTNF with L-M cells were examined in some detail, and the results indicated that the association of ^{125}I-mTNF was diminishing prior to the onset of lysis. The time course of association of biologically active ^{125}I-mTNF is shown in Figure 2. Association was defined as that radioactivity that was not washed away by vigorous rinsings at 4°C. Association accounted for ligand both on

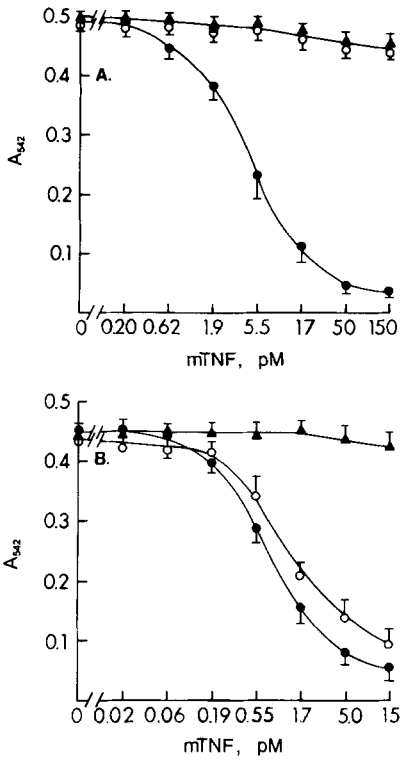

Fig. 1. The influence of time and cycloheximide on neutralization. **A:** mTNF was titered on 3×10^4 growing L-M cells/0.3 cm^2 well/200 μl assay medium. Serum (final dilution 1:500) was added at the indicated times: ○, preimmune serum at t = 0; ▲, immune serum at t = 0; ●, immune serum at t = 4 hr. The cultures were incubated at 38°C in a humidified atmosphere of 95% air/5% CO_2 for 20 hr. Wells were treated with 0.006% neutral red for 1 hr, washed, lysed, and A_{542} was determined. Points show the average of triplicate wells; bars indicate the SEM. **B:** The effect of serum on mTNF titrations was determined as described above, except twice the number of cells were used, and cycloheximide (final concentration = 4 μM) was added simultaneously with mTNF. ○, immune serum at t = 60 min.

TABLE I. Time Course of Antiserum Neutralization*

	% neutralization	
Time (hours)	Cycloheximide	No cycloheximide
0	100 ± 0	100 ± 0
1	19 ± 12	ND
2	0 ± 0	100 ± 0
4	0 ± 0	100 ± 0
8	ND	89 ± 2
12	ND	62 ± 3
16	ND	36 ± 5

*Neutralization was performed as described in the legend to Figure 1 except that antiserum or control serum was added at the indicated time after mTNF. Trays were scored at 20 hr. Neutralization was calculated based on the amount of mTNF giving 50% survival. Nos. are the average of 3 determinations ± the SEM.

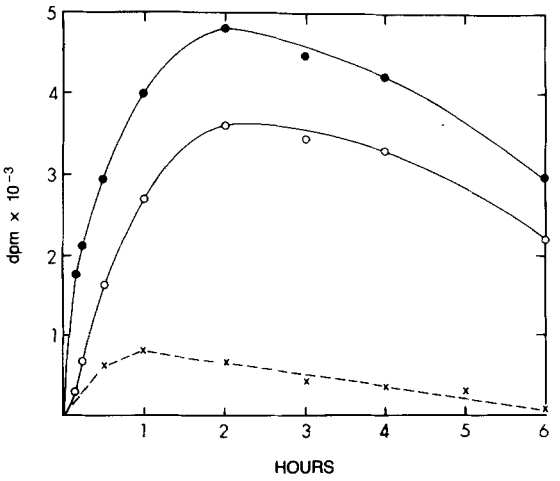

Fig. 2. Time course of specific association. 100 pM ^{125}I-mTNF (0.3 mCi/nmol) was added to 10^6 L-M cells/10 cm^2 well in 1.5 ml binding medium at 38°C. At the indicated times, wells were washed 3 times with 4°C binding medium. The wells were treated twice for 1 min with cold 140 mM NaCl, 0.05 N HCl. The cells were solubilized with 1% $NaDodSO_4$, then acidic eluates and detergent solubilizates (O) were counted in a gamma counter. ● = total specific association (eluate + solubilizate). x = total specific association on cells treated with 10 pM ^{125}I-mTNF and 4 μM cycloheximide. Points are the mean of triplicate wells subtracted from a companion well that contained a 100-fold excess of unlabeled mTNF (nonspecific association). The SEM for each measurement was less than 10% of the mean. The specific association accounted for >95% of the total radioactivity.

and in the cells. Two concentrations were examined: 100 pM, which would have killed all the cells in a 20 hr period, and 10 pM mTNF plus 4 μM cycloheximide, which would also have killed all the cells in 20 hr. Association was followed for 6 hrs; thereafter, cell death became apparent by microscopic examination. Specific association at 10 pM mTNF and in the presence of cycloheximide peaked at 1 hr and declined to one-half that amount in 4 hr (Fig. 2). Association at 100 pM peaked at 2 hr and declined by one-third that amount at 6 hr (Fig. 2). The specific association at 100 pM was resolved into acid-dissociable and acid-indissociable compartments (representing surface and internalized pools, respectively, Fig. 2). At 10 min, the acid-indissociable radioactivity comprised 20% of the total, and the percentage increased with time: 33% at 15 min, 56% at 30 min, 68% at 1 hr, and approximately 77% for up to 6 hr. The increase in acid-indissociable radioactivity was consistant with the notion of receptor-mediated internalization.

Off-rates were determined for the surface-bound and internalized compartments (Fig. 3A). The initial rate, assumed to represent the surface compartment, followed first-order kinetics (Fig. 3A) and had a half-time of 9 min (Fig. 3A, inset). The off-rate for the internalized compartment was non-linear (Fig. 3B). Curve peeling [36] was used to delineate two compartments (Fig. 3B, inset) with apparent half-lives of 2 hr and 35 hr. The acid-indissociable radioactivity that was released by the cells was compared to control ^{125}I-mTNF by $NaDodSO_4$ polyacrylamide gel electrophoresis and autoradiography (Fig. 4). The released ligand comigrated with the control ligand; that is, released ligand that had been previously internalized was apparently not degraded.

The decline in specific association appeared to have been due in part to down-regulation of surface receptors. Exposure to 100 pM mTNF resulted in a biphasic loss in

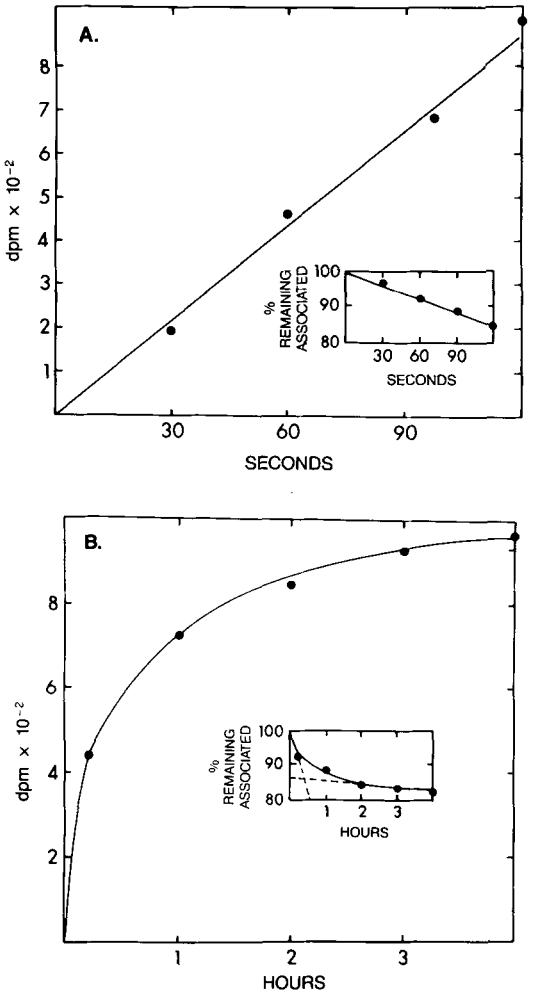

Fig. 3. Off-rates. **A:** Initial off-rate. ^{125}I-mTNF (50 pM) was specifically prebound to 2 x 10^6 L-M cells/10 cm^2 for 2 hr at 4°C. The wells were washed 3 times with cold binding medium. Then they were floated on a 38°C bath and simultaneously filled with 38°C binding medium that was sampled for radioactivity counting at the indicated times. Points show the mean of triplicate wells subtracted from a companion well that contained a 100-fold excess of unlabeled mTNF (nonspecific release). The SEM was <40% of the mean. **Inset:** Mean specific release subtracted from the total specific associated radioactivity (4,920 dpm) and expressed as a percentage of the total remaining. Note log ordinate scale. Half-time was computed from the slope in the inset. **B:** Acid-indissociable off-rate. Points were determined as described above, except binding proceeded overnight and was followed by 2 x 1 min exposures to cold acidic saline and 3 washes with cold binding medium. The SEM was <20% of the mean. The total specific associated radioactivity was 10,490 dpm. Half-times were computed from the extrapolated slopes (dashed lines) in the **inset.** Graphs are representative of duplicate trials.

surface binding capacity (Fig. 5). An initial transient loss in binding capacity (40% loss after 2 hr) coincided with the peak of association. This initial loss was followed by a replenishment in the surface binding capacity (95% of the initial capacity after 3 hr), followed by a more dramatic and irreversable loss of surface binding capacity (20% of the initial capacity after 8 hr) that coincided with the onset of cell death. The replenish-

Fig. 4. $NaDodSO_4$ polyacrylamide gel electrophoresis of shed ligand. L-M cell supernatants were harvested as described in the legend to Figure 3B. Approximately 500 dpm was applied to a 15% gel/lane. **Lanes left to right:** ^{125}I-mTNF in binding medium; 0–1 hr shed; 0–3 hr shed; 0–6 hr shed.

ment of surface receptors between 2 and 4 hr appeared to require new protein synthesis, as surface binding capacity was much reduced by cycloheximide treatment (Fig. 5).

Down-regulation was also examined in the presence of 4 μM cycloheximide, which was the concentration that made a brief exposure to mTNF sufficient to kill all the cells. Cycloheximide alone caused a decline in the binding capacity. The half-time for the initial loss of surface binding with cycloheximide alone (an indication of receptor turnover without ligand) was 1.5 hr, which was slightly faster than the times observed for human receptor [37,38]. Concomitant exposure to mTNF accelerated the decline (Fig. 5). A shoulder was seen at 3 hr with cycloheximide/mTNF treatment. The shoulder coincided with the period of receptor replenishment seen in the absence of cycloheximide. Regardless of the presence of cycloheximide, very little binding was observed at 6 hrs. Thus, the surface expression of mTNF receptors was diminished prior to the onset of cell death.

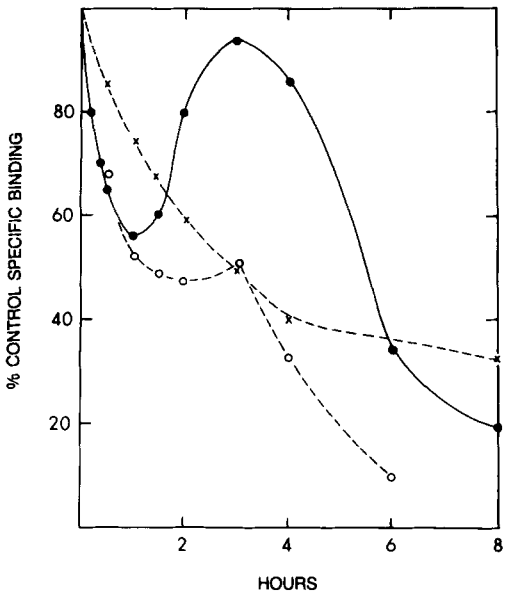

Fig. 5. Down-regulation of specific binding. L-M cells $10^6/10\ cm^2$ well) were treated at 38°C with 100 pM mTNF (●), 4 μm cycloheximide (x), or mTNF plus cycloheximide (○). At the indicated times, the wells were washed 3 times with cold binding medium, exposed for 2 × 1 min cold acidic saline washes, and washed 3 times with cold binding medium. Saturation specific binding was determined for each point (the mean of triplicate wells subtracted from a nonspecific well) using 100 pM ^{125}I-mTNF at 4°C for 18 hr. The SEM was <10% of the mean. Points relate treated to no prior exposure to mTNF (control). Graphs are representative of duplicate trials.

Drug Effects

The antiserum neutralization and receptor kinetic studies provided a rationale for discriminating between those agents that may effect cellular events mainly during the association stage versus later events such as lysis. A number of agents known to influence cytotoxicity were added either before or after TNF in both the absence and presence of cycloheximide. Figure 6 provides a partial example of these analyses. It shows the influence of pre- and post-treatment with dibutyrlcyclic AMP on the TNF titration curve in the absence of cycloheximide. The relative amount of TNF that was required to give 50% survival was converted to the fraction that has been previously referred to as "sensitivity" [34]. Pretreatment with dibutyrylcyclic AMP increased sensitivity by 1 $\log_3$ (a 3-fold increase in sensitivity). Treatment was equally effective when given either 1 hr before or 4 hr after TNF.

Repetitions of these results and examples of others similarly obtained are summarized in Table II. Dibutyrylcyclic AMP was also found to increase sensitivity when titrations were performed in the presence of cycloheximide. Dibutyrylcyclic AMP was also equally effective when given either 1 hr before or 1 hr after mTNF and cycloheximide. Since dibutyrylcyclic AMP was effective when added at a time when extracellular TNF was not required for the cytotoxic reaction, dibutyrylcyclic AMP primarily influenced later stage events in the cytotoxic program. Cholera toxin (10 ng/ml) and pertussis toxin (10 ng/ml) were also found to increase L-M sensitivity, and as with dibutyrylcyclic AMP, these agents were effective when given after TNF.

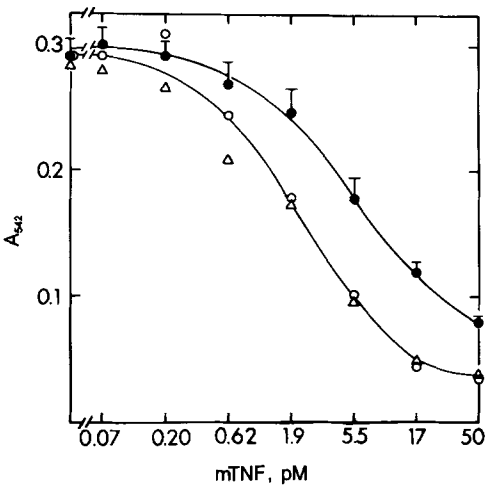

Fig. 6. The effect of dibutyrlycyclic AMP on mTNF survival. mTNF titrations were carried out on L-M cells as described in the legend to Figure 1A, except without antiserum. One millmolar dibutyrylcyclic AMP was added either 1 hr before (○) or 4 hr after (△) mTNF. Bars indicate the SEM and are representative of all groups. ●, control (no dibutyrylcyclic AMP).

TABLE II. The Influence of Drugs on Sensitivity*

	Sensitivity		Sensitivity + cycloheximide	
Agent[a]	1 hr pre	4 hrs post	1 hr pre	1 hr post
dbcAMP, 1 mM	4.0 ± 1.0	4.3 ± 0.6	2.7 ± 0.6	2.1 ± 0.5
Cholera tx, 10 ng/ml	3.4 ± 0.8	2.8 ± 0.6	1.8 ± 0.6	2.0 ± 0.4
Pertus. tx, 10 ng/ml	2.7 ± 0.7	2.0 ± 0.8	1.0 ± 0.2	1.0 ± 0.2
Chloroquine, 20 μM	0.1 ± 0.07	0.2 ± 0.1	0.5 ± 0.1	0.6 ± 0.2
A23187, 250 nM	0.1 ± 0.04	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.1
PMA, 30 nM	0.9 ± 0.6	1.0 ± 0.1	0.02 ± 0.02	0.9 ± 0.2
Staurosporine, 1 nM	1.4 ± 0.1	1.1 ± 0.2	11 ± 2.5	5.0 ± 0.8

*TNF survival titrations were carried out in triplicate as described in the legend to Figure 1A, B, except agents or diluent (control) were added at the indicated times. Titrations were related in the following way: The concentrations of mTNF at the midpoints were converted to a fraction, i.e., sensitivity, by dividing the control value by the treated value. Nos. are the average of 4 separate determinations ± the SEM.
[a]Abbreviations: dbcAMP, dibutyrylcyclic AMP; pertus., pertussis.

Chloroquine rendered cells resistant to cytotoxicity. It reduced sensitivity 10-fold. Like dibutyrylcyclic AMP, chloroquine was found to be as effective when given either before or after mTNF. Cycloheximide reduced the effectiveness of chloroquine; nevertheless, in the presence of cycloheximide, chloroquine was equipotent when given before or after mTNF. Thus, chloroquine also appeared to primarily influence later-stage events in the cytotoxic process.

The Ca^{+2} ionophore A23187 decreased sensitivity, but in contrast to the agents discussed above, ionophore was substantially less inhibitory when given after TNF. The effectiveness of ionophore was diminished in the presence of cycloheximide, which suggests that the protective effect required protein synthesis.

In the presence of cycloheximide, PMA reduced sensitivity 50-fold. PMA was much less effective when added after TNF and cycloheximide, and PMA had marginal effects on L-M cell sensitivity in the absence of cycloheximide. Thus, PMA and A23187 primarily influenced early association events. The capacity of PMA to diminish cytotoxicity and, perhaps unrelatedly, to down-regulate TNF receptors is well established [22–25]. In support of the hypothesis that phosphorylation influences cytotoxicity, staurosporine, a kinase inhibitor [39,40], was a potent enhancer of cytotoxicity. Like PMA, staurosporine was much more effective in the presence of cycloheximide, and it was more potent when added before TNF and cycloheximide than when added 1 hr later.

DISCUSSION

The persistent presence of TNF was required over a 20 hr period to kill L-M cells. Ligand-binding kinetics revealed that total specific association as well as the cell surface binding capacity had declined substantially by the time lysis began to become microscopically apparent. The half-times of the acid-indissociable (internalized) compartment were long (1.5–35 hr), and a relatively small number of molecules (4,400 monomers) was associated at peak time per cell. At 10 pM, a concentration giving 50% survival, only 700 monomers were specifically associated at peak time per cell. In contrast to the degradation seen using recombinant TNF on L929 cells [17] and HeLa cells [18], mTNF was apparently not degraded by L-M cells. A lack of degradation has been seen with other native catabolic hormones in other cell types [41,42]. Degradation likely reflects cell line metabolism rather than the source of the purified ligand [41]. The off-rate for acid-indissociable (internalized) ^{125}I-mTNF was composed of at least two compartments. The 2 hr half-time was consistent with a lysosomal compartment [36], whereas the 35 hr half-time showed that some mTNF was tightly bound in an intracellular compartment (perhaps membranes, since the acid-indissociable ligand fractionated with membranes in broken cell preparations, unpublished). The relationship of these associations with cytotoxicity remains controversial. Regardless, mTNF initiated a program of events that led to cell death.

Neutralization studies with and without cycloheximide showed that it ablated the requirement for the persistant presence of mTNF and hastened cell death. Thus, the use of cycloheximide helped to distinguish agents that affected TNF-mediated cytotoxicity.

Agents that were most effective when added before TNF primarily affected association events, whereas agents that were equipotent when added late affected distal events. For example, the TNF receptor is regulated by phosphorylation [22,23]. Our results support the notion that a kinase affected an early event. PMA, staurosporine, and the Ca^{+2} ionophore A23187 were most inhibitory when applied early. Curiously, neither PMA nor staurosporine was very effective in the absence of cycloheximide. One possible explanation is that up-regulated receptors, which likely contribute to cytotoxicity in the absence of cycloheximide, are refractory to kinase regulation. A23187 was more effective without cycloheximide and was partially inhibitory when added late. Thus, our results do not preclude the potential role of other Ca^{+2}-dependent processes [43].

We examined reagents that are reputed to affect adenylate cyclase and other G protein second messenger systems. Some have been previously reported to enhance sensitivity to TNF [28,29]. TNF increased a pertussis toxin-sensitive GTPase activity [29] and increased ADP-ribosylation [30]. Dibutyrylcyclic AMP was equipotent at

increasing cytotoxicity when added late. Thus, it affected more terminal events and did not impinge on receptor regulation.

Chloroquine was a potent inhibitor when added late. Our results challenge the notion that chloroquine (and other amines) reduce cytotoxicity by impairing aspects of internalization such as receptor recycling or ligand degradation [34]. In L-M cells, degradation is not likely related to cytotoxicity since L-M did not degrade the ligand. Our results suggest the potential involvement of lysosomal function in more terminal aspects of cytotoxicity. Chloroquine is also an inhibitor of phopholipase A_2, which has been recently implicated in the mechanism of cytotoxicity [44]. However, chloroquine is a relatively weak inhibitor of phospholipase A_2, and more potent inhibitors of phospholipase A_2 were less effective at reducing cytotoxicity (unpublished).

Our studies temporally relate binding to cytotoxicity; however, we wish to emphasize the necessary but insufficient aspect of binding as it relates to cytotoxicity. Cycloheximide treatment lowers binding substantially yet markedly increases sensitivity. A further example is seen in the synergistic effects of subtypes of interferons that may or may not influence receptor number but increase sensitivity to TNF [38].

In conclusion, our results suggest that agents that affect the association of TNF with target cells are more likely to influence the responses of many cell types to TNF. Lysis is restricted to selected cell lines, and agents that affect the lysis stage seem less likely to influence other TNF responses. Some recent evidence supports this notion. An elevation of cyclic AMP levels in synovial cells did not affect an increase in phospholipase activity, an early TNF-mediated response in those cells [45].

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