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Research Article

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Inhibition of Tumor Cell Glutamine Uptake by Isolated Neutrophils

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Abstract

Antitumor activity of phorbol myristate acetate- (PMA) stimulated neutrophils was measured against CCRF-CEM cells. Neutrophils and tumor cells were incubated (a) as a suspension with continuous mixing to maximize the availability of oxygen or (b) after centrifugation as a pellet to maximize cell-cell contact. The cells were then incubated briefly as a suspension with [^{14}C]glutamine under conditions that blocked further damage to the tumor cells. When cells were incubated as a suspension, inhibition of tumor-cell glutamine uptake was mediated by the myeloperoxidase/hydrogen peroxide/chloride system of stimulated neutrophils. Inhibition was blocked by adding catalase, an inhibitor of myeloperoxidase, or compounds that scavenge hypochlorous acid or chloramines. When cells were incubated as a pellet, a portion of the inhibition could not be blocked in this way, indicating that a nonoxidative mechanism contributed to inhibition. In both systems, inhibition of glutamine uptake was rapid and was obtained at effector-cell/target-cell ratios as low as 0.5:1. This inhibition was obtained under conditions that did not result in ^{51}Cr release from cells labeled with [^{51}Cr]chromate, indicating that inhibition of glutamine uptake measured cytotoxicity rather than cytolysis. ^{51}Cr release was observed only when cells were incubated together for an hour or more as a pellet at high E/T ratios. This cytolysis was mediated by the myeloperoxidase system, and a nonoxidative contribution to cytolysis was not observed. The results indicate that stimulated neutrophils are potent antitumor effector cells when cytotoxicity rather than cytolysis is the measure of activity. Because glutamine is required for growth of many tumor cells, inhibition of glutamine uptake may represent a significant tumoristatic or tumoricidal effect.

Introduction

Leukocyte antitumor activities contribute to natural resistance to tumor growth and provide a basis for efforts to treat cancer by immunization or with immunoregulatory substances. Certain activated macrophages and lymphocytes recognize and selectively kill tumor cells. Neutrophils apparently do not recognize tumor cells as targets in the absence of anti-tumor

antibodies, but can kill many different types of cells when appropriately stimulated. For example, contact with opsonized cells stimulates neutrophil secretion of toxic proteins and peptides and the respiratory burst, which produces toxic oxidizing agents. Neutrophil toxins may contribute to antitumor activity when resistance is promoted by immunization against tumor-cell antigens or when tumor cells are damaged in ways that create signals for neutrophil binding and stimulation.

Many studies have examined the toxicity of stimulated neutrophils to tumor cells in vitro (1-12). These studies have concluded that antitumor activity is due to the production of toxic oxidizing agents by the myeloperoxidase (MPO)¹/hydrogen peroxide (H_2O_2)/chloride (Cl^-) system, though proteins (13) and peptides (14) isolated from neutrophil secretory granules were also shown to be toxic. High ratios of neutrophils to tumor cells were required for detectable toxicity, which raised questions about the physiologic significance of neutrophil antitumor activity.

These studies evaluated antitumor activity by measuring the release of radiolabeled intracellular macromolecules from tumor cells labeled with [^{51}Cr]chromate. The ^{51}Cr -release method is rapid, simple, and applicable to many different target cells. Only the target cells are labeled, so that results are not complicated by the release of materials from effector cells, and it is not necessary to separate effector and target cells before measuring ^{51}Cr release. However, this method also has important disadvantages. Stimulated leukocytes can ingest or precipitate labeled target cell components, resulting in an underestimate of cell damage. Moreover, ^{51}Cr release measures cytolysis rather than cytotoxicity. This assay may overlook subtle effects on target cell structure, metabolism, and growth that would be important in vivo. Using a physiologic process as the indicator of cytotoxicity might permit studies at effector-cell:target-cell (E/T) ratios closer to those achieved in vivo. Such an assay might also help to identify the cell functions that are sensitive to leukocyte toxins and important targets for cytotoxic activities.

Glutamine transport and metabolism are physiologic processes that have been well studied in a number of cells and tissues. Glutamine is the predominant amino acid in human plasma (15), and many mammalian cells have a transport system for glutamine (16). Cells of lymphoid origin and certain tissues take up glutamine rapidly and may use glutamine rather than glucose as their major energy source (16-20). Growth of most mammalian cells in vitro requires high levels of glutamine (21, 22), although this requirement may be an artifact of the tissue-culture system (23). High rates of glutamine use may be characteristic of tumor cells both in vitro and in vivo (24-26), and experimental cancer therapies have been developed based on depriving tumor cells of glutamine (27-30). Therefore, most tumor cell lines used in studies of

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1. Abbreviations used in this paper: AIB, α -aminoisobutyric acid; MeAIB, N-methyl α -aminoisobutyric acid; MPO, myeloperoxidase.

leukocyte antitumor activity probably express high rates of glutamine uptake and require glutamine for growth. In comparison, neutrophils have very low levels of transport systems for amino acids (31). Glutamine uptake was chosen as an indicator to investigate mechanisms of cytotoxicity of stimulated neutrophils toward CEM tumor cells.

Methods

[⁵¹Cr]Chromate (1 mCi/ml) and U-[¹⁴C]glutamine (20–50 Ci/mol) were purchased from Amersham Corp., Arlington Heights, IL. Amino acids, antioxidants, choline chloride, 4,4'-diaminodiphenyl sulfone (dapson), and SOD were obtained from Sigma Chemical Co., St. Louis, MO, as were the reagents for spectrophotometric determination of ammonia (NH₄⁺) based on NH₄⁺-dependent consumption of NADH catalyzed by glutamate dehydrogenase in the presence of α -ketoglutarate. Catalase was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. FCS and RPMI 1640 medium were obtained from Gibco Laboratories, Grand Island, NY. PMA was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Reagents for chemiluminescent determination of ATP were obtained from Turner Designs, Mountain View, CA. MPO was purified (32, 33) from human leukemic leukocytes. Catalase crystals were washed by centrifugation in water and dissolved in a Cl⁻ medium containing 0.14 M NaCl with 15 mM potassium phosphate, pH 7.4, or in a Cl⁻-free medium with 67 mM Na₂SO₄ in place of NaCl. Na⁺-free media were also prepared in which 0.14 M choline chloride or LiCl replaced NaCl. Monochloramine (NH₂Cl) was prepared by reacting sodium hypochlorite with NH₄⁺ in Cl⁻ medium (34).

Cells. Neutrophils were isolated from human blood by density-gradient centrifugation (35). Neutrophil preparations were free of mononuclear cells and erythrocytes, but contained an average of 6% eosinophils. CCRF-CEM cells (ATCC CCL 119; American Type Culture Collection, Rockville, MD), a line of human lymphoblast tumor cells (36), were cultured as a suspension in RPMI 1640 medium with 10% FCS and 4 mM glutamine. Cells were harvested by centrifugation and washed in Cl⁻ medium or the other isotonic media. ⁵¹Cr-labeled cells were prepared by incubating cells at 2 × 10⁷/ml with 10 mM glucose and 0.1 mCi/ml [⁵¹Cr]chromate for 1 h at 37°C. The cells then were washed three times in cold Cl⁻ medium.

Incubations. Neutrophils and CEM cells were combined in 0.5 ml total volume containing cold Cl⁻ or Cl⁻-free medium with 5 mM glucose, 1 mM MgSO₄, and 80 nM PMA added as a 1:1 molar complex with BSA (37). To maximize the availability of O₂, suspensions in 15-ml siliconized glass tubes were incubated at 37°C with continuous mixing. To maximize cell-cell contact, suspensions in flat-bottomed siliconized glass vials were centrifuged in the cold for 1 min at 900 g into a pellet consisting of a cell layer of uniform depth and 1.2 cm² area, and then incubated at 37°C without mixing.

Glutamine uptake. Incubation mixtures were placed on ice, cell pellets were resuspended, and the mixtures were supplemented with 30 μ g/ml catalase, 0.1 mg/ml SOD, 50 μ M DTT, 0.5 mM unlabeled glutamine, and 22 nCi/ml [¹⁴C]glutamine. The mixtures were incubated for 15 min at 37°C, placed on ice, diluted with 3 ml cold medium, and centrifuged 10 min at 14,000 g in the cold. Cell pellets were extracted with 0.5 ml of 10% (wt/vol) sulfosalicylic acid for 5 min at 100°C and then centrifuged. Radioactivity in the extracts was measured in a liquid scintillation spectrometer. Percent inhibition of uptake was calculated as 100 (1 - (A - C)/(B - C)), where A and B are uptake with and without neutrophils. C is the radiolabel associated with cells held for 15 min on ice.

⁵¹Cr release. Incubation mixtures with ⁵¹Cr-labeled CEM cells were placed on ice and then centrifuged in the cold. Radioactivity in the supernatant was measured with a gamma spectrometer. When cells were treated with oxidants in the absence of neutrophils, percent specific ⁵¹Cr release (6) was calculated as 100 (A - B)/(C - B), where A is the experimental value, B is spontaneous release from cells incubated

without oxidants, and C is label released by cells incubated with 1% Triton X-100. When cells were incubated with PMA-stimulated neutrophils, percent PMA-dependent specific ⁵¹Cr release was calculated by substituting D for B, where D is label released with neutrophils but without PMA. Unstimulated neutrophils suppressed the spontaneous release of label from CEM cells, which was 20–30% of total label.

Results

Inhibition of uptake. Incubating CEM cells with neutrophils and PMA resulted in a rapid loss of the ability of CEM cells to take up glutamine during a subsequent incubation with [¹⁴C]-glutamine (Fig. 1 A). During the first 20 min, inhibition was greater when the cells were incubated in suspension, but within 30 min, greater inhibition was obtained when the cells were incubated as a pellet. In both systems, strong inhibition was observed within 10 min. After 1 h of incubation, inhibition was observed at E/T ratios as low as 0.5:1 (Fig. 1B). Greater inhibition was obtained at all E/T ratios when cells were incubated as a pellet.

Inhibition of CEM cell glutamine uptake required the addition of an agent to stimulate neutrophil secretion and the respiratory burst. In these experiments and those described below, controls were included in which PMA or neutrophils were omitted. Little or no inhibition was observed without PMA, and PMA had no effect on glutamine uptake by CEM cells in the absence of neutrophils.

Stimulation of neutrophils with PMA resulted in cell-cell aggregation, and separating neutrophils and CEM cells from these aggregates was not possible. Therefore, measurements of glutamine uptake were performed using the mixtures of cells. Experiments with neutrophils alone indicated that these cells did not take up glutamine in the presence or absence of PMA, so that all the uptake by cell mixtures was due to the CEM cells.

Antioxidants were added along with the [¹⁴C]glutamine to prevent oxidation of the [¹⁴C]glutamine and to prevent further oxidative damage to the CEM cells during the 15-min incubation required for the uptake assay. Catalase was added to eliminate H₂O₂, DTT to act as a scavenger for long-lived oxidants such as chloramines (12), and SOD to prevent superoxide (O₂⁻)-dependent oxidation of DTT (37). Fig. 1 A shows that this combination of antioxidants was sufficient to block inhibition. There was no inhibition of uptake during the 15-min

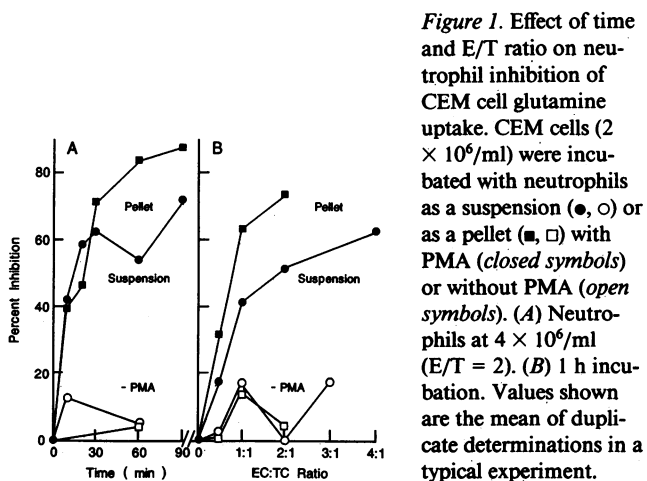


Figure 1. Effect of time and E/T ratio on neutrophil inhibition of CEM cell glutamine uptake. CEM cells (2 × 10⁶/ml) were incubated with neutrophils as a suspension (●, ○) or as a pellet (■, □) with PMA (closed symbols) or without PMA (open symbols). (A) Neutrophils at 4 × 10⁶/ml (E/T = 2). (B) 1 h incubation. Values shown are the mean of duplicate determinations in a typical experiment.

incubation with [^{14}C]glutamine unless the cells were incubated at 37°C before the addition of antioxidants and [^{14}C]glutamine. As described below, antioxidants did not completely block inhibition when cells were incubated as a pellet. However, the cells were resuspended before incubation with [^{14}C]glutamine, and antioxidants were effective under these conditions.

Experiments were performed in which CEM cells and stimulated neutrophils were incubated together, washed by centrifugation in cold buffer, and then incubated with antioxidants and [^{14}C]glutamine. Inhibition of uptake did not differ from that seen without washing. These results rule out many possible explanations for the decrease in uptake, such as neutrophil secretion of a glutaminase enzyme, release of unlabeled amino acids that compete with [^{14}C]glutamine for transport, acidification of the medium, or accumulation of a reversible inhibitor in the medium. Instead, the results indicate a cytotoxic interaction of neutrophils with CEM cells that inhibited CEM cell functions, and which was not reversed by washing.

Experiments were also performed in which CEM cells were incubated 30 min with [^{14}C]glutamine to load them with radiolabel, washed, and then incubated alone or with neutrophils as a suspension or pellet. Loss of label from the cells was linear with time, and ~ 15% of the label was lost in 15 min at 37°C. Stimulated neutrophils did not cause an increase in the rate of loss of label. Therefore, inhibition of glutamine uptake was not due to nonspecific permeabilization of the CEM cell membrane and loss of the ability to retain [^{14}C]glutamine or its metabolites.

Mechanisms of inhibition. Cytotoxicity depended at least in part on the generation of oxidants by the MPO/H₂O₂/Cl⁻ system of stimulated neutrophils (Table I). The addition of MPO increased toxicity, indicating that secretion of neutrophil MPO was a limiting factor. SOD increased cytotoxicity by promoting the dismutation of O₂⁻ to O₂ and H₂O₂, whereas catalase decreased toxicity by promoting the dismutation of H₂O₂ to O₂ and water. Cytotoxicity was also decreased by omitting

Cl⁻ from the medium or by adding dapsone (38) to inhibit MPO-catalyzed oxidation of Cl⁻.

Cytotoxicity was also decreased by adding agents that detoxify oxidants produced by the MPO/H₂O₂/Cl⁻ system. The amine compound taurine, ⁻SO₂CH₂CH₂NH₃⁺, reacts with hypochlorous acid (HOCl), monochloramine (NH₂Cl), and dichloramine derivatives (RNCl₂) to yield taurine-monochloramine, which is a relatively nontoxic oxidant (12, 39–42). Hypotaurine, ⁻SO₂CH₂CH₂NH₃⁺, contains an amino group like taurine and also a sulfinate group that reduces the oxidized chlorine moieties of HOCl and chloramines to Cl⁻ (34). Thiodipropionate, S(CH₂CH₂CO₂⁻)₂, contains a thioether group like that of methionine, which has been used in many studies to block toxicity of the MPO system. Thioethers also reduce oxidized chlorine moieties to Cl⁻ (34, 43).

Taurine, hypotaurine, or thiodipropionate at 0.1 mM did not react with 10 mM H₂O₂ during a 1-h incubation at 37°C. Nevertheless, these compounds were as effective as catalase in blocking toxicity, indicating that toxicity was mediated by the MPO system and that H₂O₂ did not contribute directly to toxicity. Adding 3 mM H₂O₂ to CEM cells and incubating for 1 h at 37°C did not inhibit glutamine uptake, indicating that these cells were highly resistant to the toxicity of H₂O₂.

Thiodipropionate and hypotaurine have advantages as agents for blocking cytotoxicity in this and other studies. Unlike taurine, they inhibit the accumulation of long-lived oxidants such as chloramines (34, 43). In addition, these compounds did not inhibit neutrophil O₂⁻ uptake, interfere with measurements of neutrophil O₂⁻ or H₂O₂ production, or inhibit CEM cell glutamine uptake. Therefore, the compounds and their oxidation products were nontoxic and did not interfere in assays for neutrophil or CEM cell functions. Thiodipropionate is not a close structural analogue of amino acids and would not be expected to inhibit glutamine uptake. Hypotaurine is oxidized to taurine, which is taken up by a transport system specific for β -amino acids (16). Taurine does not inhibit uptake of α -amino acids such as glutamine. In comparison, methionine competes with other α -amino acids for transport, and oxidized forms of methionine are toxic (44, 45).

Ammonia (NH₄⁺) increases the toxicity of the MPO system to target cells such as bacteria (39) and erythrocytes (46), as well as to neutrophils (40). The reaction of NH₄⁺ with HOCl produces the highly toxic oxidizing agent NH₂Cl. However, NH₄⁺ at concentrations up to 1 mM had little effect on neutrophil inhibition of CEM cell glutamine uptake, and had a small protective effect at higher concentrations (Fig. 2). This observation may indicate that sufficient NH₄⁺ was present for optimal NH₂Cl production in the absence of added NH₄⁺. As described below, CEM cells released large amounts of NH₄⁺ into the medium, and neutrophils also release NH₄⁺ (12). High concentrations of added taurine were required to block cytotoxicity (Fig. 2), indicating that taurine was competing with a high endogenous concentration of NH₄⁺.

Results in Table I indicate that cytotoxicity was due primarily to the MPO system when the cells were incubated as a suspension. On the other hand, agents that almost completely blocked cytotoxicity in suspension were only partly effective when the cells were incubated as a pellet. Each of the agents lowered toxicity to about the same level. These results suggest that part of the higher level of toxicity obtained in the pellet system was due to an additional nonoxidative mechanism that

Table I. Inhibition of CEM Cell Glutamine Uptake by Stimulated Neutrophils

	Percent inhibition of uptake	
	Suspension	Pellet
Complete	51±9 (9)*	75±10 (7)
+ MPO	77±14 (8)	91±9 (3)
+ SOD	61±4 (4)	86±6 (3)
+ Catalase	13±11 (7)	51±2 (3)
- Cl ⁻	13±12 (4)	55±3 (2)
+ Dapsone	8±10 (4)	58±13 (3)
+ Taurine	16±10 (4)	57±7 (3)
+ Hypotaurine	16±13 (5)	51±9 (5)
+ Thiodipropionate	14±8 (3)	53±8 (3)

The complete system consisted of neutrophils (4 × 10⁶/ml) and CEM cells (2 × 10⁶/ml) incubated with PMA for 1 h at 37°C as a suspension or a pellet. When added, MPO was 50 nM (6 µg/ml), SOD was 50 µg/ml, catalase was 30 µg/ml, dapsone was 0.1 mM, and taurine, hypotaurine, or thiodipropionate was 1 mM.

* Mean±SD from the number of experiments in parentheses. Duplicate determinations were performed in each experiment.

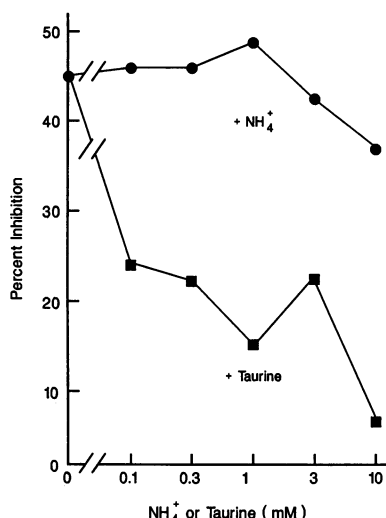


Figure 2. Effect of NH_4^+ and taurine on neutrophil inhibition of CEM cell glutamine uptake. Neutrophils ($4 \times 10^6/\text{ml}$), CEM cells ($2 \times 10^6/\text{ml}$), and PMA were incubated 1 h as a suspension with NH_4^+ (●) or taurine (■).

was much less effective when cells were incubated in suspension.

Comparison of cytotoxic and cytolytic activities. CEM cells were labeled with [^{51}Cr]chromate and incubated with neutrophils under the same conditions that resulted in inhibition of glutamine uptake. Whereas inhibition of uptake was rapid and was observed when cells were incubated as a suspension or a pellet, ^{51}Cr release was observed only in prolonged incubations and only when cells were incubated as a pellet. Moreover, the amount of ^{51}Cr release was small. Therefore, loss of the ability to take up glutamine was not the result of CEM cell lysis.

Fig. 3 A shows that only 33% ^{51}Cr release was obtained during incubations of up to 2 h with cells in a pellet. The highest release was obtained at E/T ratios of 2:1 to 4:1. In parallel experiments, < 20% ^{51}Cr release was obtained when cells were incubated in suspension. Fig. 3 A also shows that negative values were obtained at high E/T ratios. That is, ^{51}Cr release was lower with PMA than without PMA, and both values were lower than the spontaneous release observed in the absence of neutrophils. These results suggest that neutrophils absorbed, precipitated, or phagocytized labeled cell components.

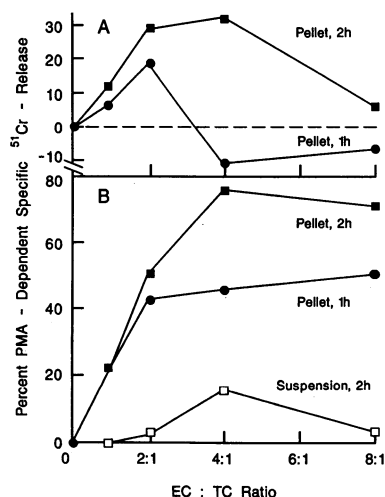


Figure 3. Effect of cell density and E/T ratio on ^{51}Cr release. ^{51}Cr -labeled CEM cells at (A) $2 \times 10^6/\text{ml}$ or (B) $5 \times 10^5/\text{ml}$ were incubated with neutrophils and PMA for 1 h (●) or 2 h (■) as a pellet or for 2 h as a suspension (□).

Table II. Effect of MPO and Antioxidants on ^{51}Cr Release

	Percent PMA-dependent specific ^{51}Cr release
Complete (pellet)	50 ± 12 (5)*
+ MPO	70 ± 10 (5)
+ SOD	45 ± 17 (5)
+ Catalase	10 ± 10 (5)
+ Taurine	7 ± 10 (5)
+ Hypotaurine	14 ± 13 (4)
+ Thiodipropionate	11 ± 10 (4)

The complete system consisted of neutrophils ($1 \times 10^6/\text{ml}$) and ^{51}Cr -labeled CEM cells ($5 \times 10^5/\text{ml}$) with PMA incubated 2 h at 37°C as a pellet. When added, MPO was 50 nM, SOD was $50 \mu\text{g}/\text{ml}$, catalase was $30 \mu\text{g}/\text{ml}$, and taurine, hypotaurine, or thiodipropionate was 10 mM.

* Mean \pm SD from the number of experiments in parentheses. Duplicate determinations were performed in each experiment.

High levels of ^{51}Cr release similar to those reported in other studies (7) were obtained by adopting conditions used in those studies. This change in conditions consisted of lowering the number of labeled CEM cells fourfold to $5 \times 10^5/\text{ml}$ and lowering the numbers of neutrophils to maintain the same E/T ratios. Fig. 3 B shows that ^{51}Cr release of 50% or higher was obtained when cells were incubated as a pellet for 1 h with an E/C ratio of 8:1, or for 2 h with ratios of 4:1 or higher. Only a low level of ^{51}Cr release was obtained in 2 h when cells were incubated in suspension. These results confirm reports that prolonged incubations, high E/T ratios, and incubation as a pellet are required for extensive cytotoxicity.

As described above, a nonoxidative cytotoxic mechanism was observed when cells were incubated as a pellet but not as a suspension. Because ^{51}Cr release was much higher when cells were incubated as a pellet, it might be expected that ^{51}Cr release was due to the nonoxidative mechanism. However, results in Table II confirm reports that ^{51}Cr release in the pellet system requires production of oxidants by the MPO system. Cytotoxicity was increased by adding MPO and blocked by catalase or compounds that detoxify oxidants produced by the MPO system. SOD did not have a significant effect.

Fig. 4 shows results of adding NH_2Cl to CEM cells in the absence of neutrophils. Small amounts of NH_2Cl inhibited glutamine uptake, and larger amounts caused lysis. Inhibition of uptake was nearly complete at concentrations that did not

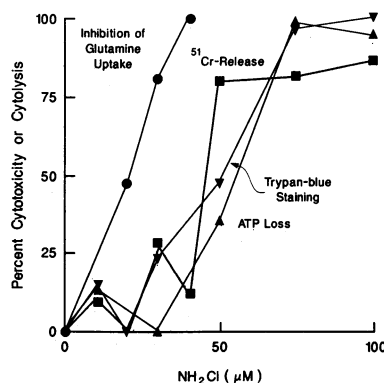


Figure 4. Effect of NH_2Cl on CEM cells. NH_2Cl was added to CEM cells ($1 \times 10^6/\text{ml}$) at the start of a 1-h incubation as a suspension, and then [^{14}C]glutamine uptake (●), ^{51}Cr release (■), trypan blue staining (▼), and loss of ATP (▲) were measured.

cause lysis. Similar results were obtained by adding HOCl rather than NH_2Cl (not shown). Differences in the activities of these two oxidants were smaller than those observed with other cells as targets (46). The NH_4^+ released by CEM cells may have converted most of the HOCl to NH_2Cl , so that the cells were exposed to NH_2Cl regardless of whether HOCl or NH_2Cl was added.

Fig. 4 also shows that similar results were obtained by measuring ^{51}Cr release, staining with trypan blue, or loss of cell ATP as indicators of cell damage. Therefore, ^{51}Cr release did not underestimate the number of dead or lysed cells. Moreover, inhibition of ATP synthesis or depletion of ATP did not account for inhibition of glutamine uptake. The results indicate that oxidants produced by the MPO system had both cytotoxic and cytolytic activities toward CEM cells and that inhibition of glutamine uptake was more sensitive than other indicators of cell injury.

Glutamine uptake. CEM cell glutamine uptake was characterized to determine the probable basis for inhibition. Uptake of label from 0.5 mM [^{14}C]glutamine was rapid and linear with time for at least 8 min (Fig. 5 A). About 5% of the label was taken up in 15 min and 8% was taken up in 1 h. Total uptake consisted of a saturable, temperature-dependent transport process with an apparent K_m of 0.33 mM and V_{\max} of 2.7 $\mu\text{M}/\text{min}$ plus a nonsaturable, temperature-independent binding or diffusion process. In all experiments, values for uptake at 37°C were corrected by subtracting the nonsaturable process measured at 4°C, as shown in Fig. 5 B. Stimulated neutrophils had no effect on the nonsaturable process. Incubation with 0.5 mM [^{14}C]glutamine for 15 min at 37°C was chosen as the standard conditions for uptake assays. Under these conditions, the rate of uptake was about equal to the initial rate, the nonsaturable portion was low relative to total uptake, and the amount taken up did not significantly change the extracellular concentration.

All of the label taken up was acid soluble, indicating that glutamine was not used in protein synthesis. Instead, glutamine was rapidly metabolized, as indicated by release of NH_4^+ into the medium (Fig. 6). Glutamine-dependent NH_4^+ release

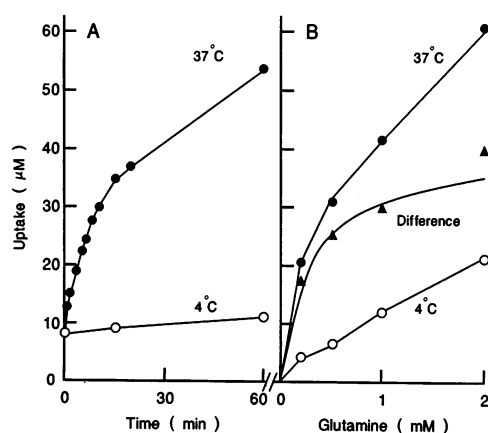


Figure 5. Effect of time and glutamine concentration on CEM cell glutamine uptake. (A) CEM cells ($2 \times 10^6/\text{ml}$) were incubated with 0.5 mM [^{14}C]glutamine and 5 mM glucose at 37°C (●) or 4°C (○). (B) CEM cells ($2 \times 10^6/\text{ml}$) were incubated with [^{14}C]glutamine for 15 min at 37°C (●) or 4°C (○), and the difference between values at 37°C and 4°C was plotted (▲). The curve drawn through the points was calculated assuming a K_m of 0.33 mM and V_{\max} of 2.7 $\mu\text{M}/\text{min}$.

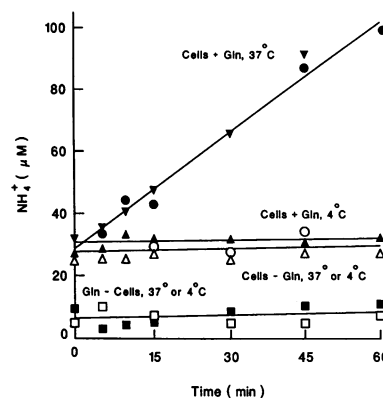


Figure 6. NH_4^+ release. CEM cells were incubated at 37°C with glutamine (Gln) and glucose (●), glutamine alone (▼), or glucose alone (▲). Cells were also held at 4°C with (○) or without glutamine (△). Glutamine was also incubated at 37°C (■) or held at 4°C (□) without cells. When added, cells were $2 \times 10^6/\text{ml}$, glutamine was 0.5 mM, and glucose was 5 mM.

required incubation at 37°C, was linear with time, and had a concentration dependence similar to that of glutamine uptake ($K_m = 0.5 \text{ mM}$, $V_{\max} = 1.6 \mu\text{M}/\text{min}$). In the first 15 min, about one NH_4^+ was released per glutamine taken up. Therefore, glutamine was rapidly deaminated to glutamate or a mixture of glutamate and α -ketoglutarate, and 50% or less of the label in the cells was in the form of [^{14}C]glutamine.

Although glutamine was rapidly metabolized, the results indicate that the amount of label in the cells was about equal to the amount taken up. If a large part of the [^{14}C]glutamine taken up was converted to labeled products that were released into the medium, then NH_4^+ release would be much greater than the amount of labeled products remaining in the cell. Other experiments confirmed this observation in that conversion of [^{14}C]glutamine to volatile products, including $^{14}\text{CO}_2$, was undetectable at 15 min and only 3% at 1 h.

Fig. 6 also shows that CEM cells released substantial amounts of NH_4^+ into the medium before the incubations. NH_4^+ release was complete during the 15–30-min period in which the cells were at 4°C before the start of the incubations. The cells were thoroughly washed, and NH_4^+ in the incubation mixtures was not due to contamination by NH_4^+ from the growth medium. Fig. 6 also shows that the amount of NH_4^+ added as a contaminant in the glutamine was < 1% of the amount of glutamine. Total glutamine decomposition measured as NH_4^+ release in the absence of CEM cells was < 2% at the end of 1 h.

The presence or absence of glucose had no effect on glutamine uptake or glutamine-dependent NH_4^+ release. Therefore, glucose was not required as an energy source for glutamine uptake, and glucose did not inhibit glutamine uptake or metabolism by competing with glutamine as an energy source.

Preincubating the cells for 1 h at 37°C with or without glucose had no effect on glutamine uptake during a subsequent incubation with 0.5 mM [^{14}C]glutamine. Similarly, preincubating for 1 h with unlabeled glutamine at concentrations up to 20 mM, and subsequent washing had no effect on [^{14}C]glutamine uptake. Therefore, the rate of uptake was a stable parameter and did not respond rapidly to the presence or absence of energy sources. Nevertheless, glutamine uptake appeared energy dependent. Preincubating for 1 h at 37°C with glucose and 10 mM cyanide lowered uptake to 9% of control. Other inhibitors were less effective. Preincubating for 1 h at 37°C with glucose and 10 mM fluoride, arsenate, or azide, or

with 2-deoxyglucose in the absence of glucose, lowered uptake to 91, 68, 57, or 69% of control. CEM cells were quite resistant to all the inhibitors. Preincubations of 1 h or more with inhibitor concentrations > 1 mM were required for inhibition of glutamine uptake. There was no inhibition of uptake when metabolic inhibitors were added with [14 C]glutamine at the start of a 15-min incubation.

Competition between glutamine and other amino acids for uptake was examined to define the transport system(s) responsible for uptake. Glutamate had little effect, indicating that extracellular deamination of glutamine and transport of glutamate did not account for uptake. Neutral amino acids such as glutamine are transported into mammalian cells by systems with broad, overlapping specificities and differing ionic requirements. The *A* and *ASC* systems are found in most tissue cells and differ most clearly in their ability to transport *N*-methyl derivatives of amino acids. *N*-Methyl α -aminoisobutyric acid is a model substrate for the *A* transport system, whereas α -aminoisobutyric acid is taken up by both the *A* and *ASC* systems (47, 48). Neither is taken up by the *L* system (49), which is also widely distributed. MeAIB and AIB inhibited glutamine uptake by 26 and 64%, indicating that ~ 26% of uptake was by the *A* system and 38% by the *ASC* system (Table III). The observation that ~ 64% of uptake was due to the Na^+ -dependent *A* and *ASC* systems was confirmed by replacing Na^+ with Li^+ or the choline cation. The Na^+ -independent *L* system accounted for the remaining uptake. Leucine and serine were about equally effective as inhibitors. Leucine competes more effectively than serine for uptake by the *L* system, whereas serine competes more effectively than leucine for uptake by the *A* and *ASC* systems.

Histidine competes with glutamine for uptake by the Na^+ -dependent *N* system, which is the major transport system for glutamine in hepatocytes (50, 51). However, the results did not indicate a contribution by the *N* system to CEM cell glutamine uptake. The cation dependence of the *N* system differs from that of *A* and *ASC* in that Li^+ substitutes for Na^+ as a cosubstrate for the *N* system. Uptake in Li^+ medium was no higher than in the choline medium, indicating that there was no Li^+ -dependent uptake by the *N* system. Histidine inhibited uptake, but most of this inhibition was due to competition for uptake by the *L* system. The results indicate that the *A*, *ASC*, and *L* systems made major contributions to glutamine uptake and that about two-thirds of the uptake was Na^+ dependent.

Table III. Effect of Amino Acid Competition and Cation Substitution on CEM Cell Glutamine Uptake

Inhibitor	Percent of uptake in Na^+ medium		
	Na^+ medium	Li^+ medium	Choline medium
None	(100)	36	39
MeAIB	74	ND	ND
AIB	36	ND	ND
Leucine	22	4	0
Serine	19	9	7
Histidine	46	4	0

CEM cells ($2 \times 10^6/\text{ml}$) were incubated for 15 min at 37°C with 0.5 mM [14 C]glutamine and 5 mM glucose in the presence and absence of 20 mM unlabeled amino acids. Determinations were in triplicate.

Although it is likely that attack on membrane components was responsible for inhibition, inactivation of intracellular enzymes could also result in inhibition of glutamine uptake. Transport systems catalyze the movement of substrates into and out of the cell, but intracellular [14 C]glutamine was rapidly deaminated to NH_4^+ and labeled products that were not transported out of the cell. The labeled products were also not rapidly metabolized to other products such as $^{14}\text{CO}_2$ that could diffuse out of the cell. Therefore, deamination trapped the label in the cell and may have permitted a higher level of intracellular accumulation of label. If the deaminating enzymes were inactivated, intracellular accumulation of label might be inhibited even if membrane transport systems were fully active. To determine whether deaminating enzymes were inhibited, CEM cells were incubated 10 min at 37°C in Cl^- medium, in Cl^- medium with 100 μM HOCl to lyse the cells, or in 15 mM phosphate buffer to osmotically lyse the cells. DTT (0.1 mM) was added to reduce any oxidants derived from HOCl, the incubation was continued for 1 h at 37°C with and without 10 mM glutamine, and NH_4^+ production was measured. Glutamine-dependent NH_4^+ production by the HOCl lysate was only 14% less than that of intact cells, and osmotic lysis caused a similar small inhibition. Therefore, inhibition of deamination did not account for inhibition of uptake.

Discussion

Inhibition of CEM cell glutamine uptake was a sensitive indicator of the cytotoxic activity of stimulated neutrophils. Inhibition was observed at low E/T ratios, in short incubation times, and regardless of whether cells were incubated as a suspension or a pellet. The results indicate that stimulated neutrophils are potent antitumor effector cells when cytotoxicity rather than cytotoxicity is the measure of antitumor activity.

Cytotoxicity as measured by ^{51}Cr release was observed under a more limited set of conditions. Higher E/T ratios, longer incubations, and incubation of the cells as a pellet were required. Results were also influenced by the total number of cells. The best results were obtained with low numbers of cells spread over a large area to obtain a very thin layer of cells, which may increase opportunities for cell-cell contact. Alternatively, the effect of cell number may be due to depletion of O_2 in thick cell layers (52), which would inhibit oxidative mechanisms of toxicity.

The low sensitivity of the ^{51}Cr -release assay as compared with the glutamine uptake assay indicates that more extreme conditions are required to break open the target cells than to inhibit target cell functions. Other factors may also contribute to the sensitivity of glutamine uptake to neutrophil toxins. The *N* and *ASC* transport systems are inactivated by agents that react with sulfhydryl groups (51, 53), suggesting that these systems would be susceptible to oxidative inactivation. Transport systems must also be partially exposed on the cell surface. These membrane components would be accessible to leukocyte toxins and would be subjected to higher levels of toxins than intracellular components, which are protected by the membrane barrier and by high intracellular levels of protective enzymes and reducing agents.

Although inhibition of glutamine uptake was a more sensitive indicator than ^{51}Cr release, the principal mechanism of toxicity was the same regardless of the indicator. MPO-cata-

lyzed oxidation of Cl^- to HOCl was required for toxicity. The reaction of HOCl with endogenous NH_4^+ to yield NH_2Cl and the oxidation of CEM cell components by NH_2Cl was the probable mechanism of toxicity. However, the high level of endogenous NH_4^+ ($> 50 \mu\text{M}$) made it impossible to demonstrate an effect of added NH_4^+ . Moreover, there was not a great difference in toxicity of HOCl and NH_2Cl to CEM cells, perhaps because HOCl reacted rapidly with NH_4^+ released by the cells. Therefore, it was not possible to distinguish between the toxicity of HOCl or NH_2Cl , though NH_2Cl was shown to mediate neutrophil cytotoxicity to erythrocytes (46).

The glutamine uptake assay also revealed a nonoxidative contribution to neutrophil cytotoxicity that was not detected in the ^{51}Cr -release assay. This nonoxidative mechanism was observed only when cells were incubated as a pellet, suggesting that close continuous cell-cell contact was required. Failure to detect a nonoxidative contribution to ^{51}Cr release may indicate that this mechanism was cytotoxic but not cytolytic, that longer incubations would be required for cytolysis, or that this mechanism was ineffective at the low cell numbers required for the ^{51}Cr -release assay.

The nonoxidative contribution to cytotoxicity was defined as inhibition that could not be blocked by adding catalase, dapsone, or oxidant scavengers such as DTT, taurine, hypotaurine, or thiodipropionate. Exclusion of a large molecule such as catalase from the limited area of cell-cell contact (54) might account for the failure of catalase to completely block toxicity. On the other hand, MPO and SOD are also large, but they were effective in boosting toxicity. Moreover, if the MPO system was responsible for all the toxicity, then dapsone and the small scavenger molecules would have to be physically excluded, while H_2O_2 and Cl^- were not excluded.

Further studies will be required to determine the basis for inhibition of glutamine uptake. As discussed above, transport systems are likely targets for attack by neutrophil toxins. However, inhibition of a particular transport system could not account for the results. Three transport systems made major contributions to uptake, and neutrophils almost completely inhibited uptake, indicating a general inhibitory effect. Two of the transport systems were Na^+ dependent, so that loss of the Na^+ , K^+ , and H^+ gradients across the cell membrane would be sufficient to substantially inhibit uptake. Loss of these gradients could result from increased membrane permeability to monovalent cations, inhibition of the Na^+/K^+ ATPase pump in the cell membrane, or depletion of ATP.

The results suggest that inhibition of energy metabolism and depletion of ATP were not responsible for inhibition of glutamine uptake. ATP levels remained high in cells inhibited by NH_2Cl or HOCl . In addition, preincubating CEM cells without glucose did not inhibit glutamine uptake, so that neutrophil inhibition of CEM cell glucose uptake or metabolism would not be sufficient to inhibit glutamine uptake. Prolonged incubations with inhibitors of energy metabolism were required to inhibit uptake. Inhibition of uptake by neutrophils was much faster, suggesting that neutrophil toxins had a direct effect on amino acid transport, the Na^+/K^+ ATPase, or cation permeability.

Regardless of the basis for inhibition, decreased glutamine transport and metabolism would inhibit growth of cells that require glutamine and might result in cell death if this inhibition were not reversed or overcome. Inhibition of glutamine uptake is probably not specific to tumor cells, but would be

more significant for cells that require glutamine, which may include many tumor cells. Therefore, inhibition of glutamine uptake may be not only useful as an indicator of neutrophil cytotoxicity, but may represent a significant tumoristatic or tumoricidal effect.

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