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Increased cystine uptake capability associated with malignant progression of Nb2 lymphoma cells

PW Gout[1], YJ Kang[2], DJ Buckley[3], N Bruchovsky[1] and AR Buckley[3]

[1]*Department of Cancer Endocrinology, British Columbia Cancer Agency, Vancouver, Canada;* [2]*Department of Medicine, University of Louisville, KY, USA; and* [3]*Department of Pharmacology and Toxicology, University of North Dakota School of Medicine, Grand Forks, ND, USA*

Analysis of rat, pre-T cell 'Nb2 lymphoma' sublines, manifesting different degrees of malignant progression, can indicate phenotypic changes potentially useful as therapeutic targets. In this study, the prolactin (cytokine)-dependent Nb2-11 and autonomous Nb2-SFJCD1 sublines were compared for *in vitro* thiol growth requirements. Whereas Nb2-11 culture growth depended on 2-mercaptoethanol (2-ME; 33–100 μM), Nb2-SFJCD1 cells were 2-ME-independent. This difference stemmed from differential uptake of exogenous L-cystine, critically required for proliferation. Uptake of ^{35}S-L-cystine (10 μCi/ml; 40 μM) showed Nb2-11 cells had low cystine uptake capability; 2-ME enhanced cystine uptake to growth-sustaining levels. Nb2-SFJCD1 cells did not require 2-ME due to intrinsic, 11-fold higher cystine uptake via the x_c^- cystine/glutamate transport system. In absence of 2-ME, monosodium glutamate abrogated Nb2-SFJCD1 proliferation by specifically inhibiting cystine uptake (85% at 10 mM). Elevated glutathione (GSH) levels were not essential for growth of either line as shown with L-buthionine-(S,R)-sulfoximine (0.1–4 mM) treatment. The cyst(e)ine requirement therefore did not primarily involve maintenance of normal GSH levels, reported critical for T lymphocyte replication. These and other results suggest increased cystine uptake capability constitutes another potential step in progression of T cell cancers which is not coupled to cytokine autonomy or metastatic ability development. The x_c^- transport system apparently provides a novel target for T cell cancer therapy. Its inhibition would suppress cystine uptake by certain progressed cells, and also interfere with cystine uptake, and subsequent cysteine release, by eg macrophages, thought to have a role in cysteine delivery to lymphoid cells.

Keywords: T cell lymphoma; tumor progression; 2-mercaptoethanol; x_c^- cystine-glutamate transport

Introduction

Cancers typically become more aggressive with time and following ineffective therapy. This phenomenon, termed tumor or malignant progression, is manifested by phenotypic property changes of the cancers, including an increase in growth rate; loss of a growth requirement for hormones and growth factors; development of tissue invasiveness, metastatic ability and drug resistance.[1] At the cellular level, tumor progression is typified by chromosomal alterations and gene mutations leading to tumor cell heterogeneity and outgrowth of more virulent, autonomous and therapy-resistant sublines (clonal evolution).[2,3] Tumor progression poses a very serious problem to the clinical management of cancer.[1–3] Analysis of phenotypic and genotypic alterations involved in tumor progression may lead to identification of novel therapeutic targets which are essential for more effective therapy.[4]

In previous studies, we have developed a system of cultured cell lines[5,6] which can be used for investigating biochemical, chromosomal and molecular genetic mechanisms underlying the malignant progression of certain T cell cancers, including loss of a dependency on growth factors (cytokines)[7–9] and development of tissue invasiveness and metastatic ability.[10] The system consists of a parental line of cultured rat pre-T lymphoma cells, designated 'Nb2 lymphoma', and a number of its genetically related sublines which developed via clonal evolution as indicated by karyotypic analysis.[7] The viability and growth of the parent Nb2 lymphoma cell line are critically dependent on the polypeptide hormone, prolactin (PRL), acting as a growth factor (cytokine).[10] In contrast, the growth of Nb2 sublines, obtained by culturing the parent cells in growth factor-deficient medium and cloning of surviving cells, is completely independent of growth factors.[6,9,10] Whereas subcutaneous tumors generated by parent line cells in Noble rats are nonmetastatic, tumors from certain Nb2 subline cells are highly tissue invasive and metastatic.[10] By comparing the various Nb2 cell lines, phenotypic and genotypic changes can be identified which are associated with, and may underlie, their progression towards increased malignancy.[7–10] In view of its pre-T cell origin, the system may serve as a model for T cell malignancies such as childhood acute T lymphoblastic leukemia.[10]

The use of exogenous thiols is obligatory for the culturing of a wide variety of lymphoid cells.[11] 2-Mercaptoethanol (2-ME), for example, was an absolute requirement for the establishment of the first Nb2 lymphoma cell cultures.[5] As reported by various groups, 2-ME can enhance the uptake of L-cystine by lymphoid cells.[12–14] L-Cystine or its reduced form, L-cysteine, is an essential amino acid for mammalian lymphocytes, and a number of malignant human and animal cell lines of lymphoid origin, which lack the ability to synthesize the amino acid.[15,16] Consequently, the growth of such cells is critically dependent on the levels of the amino acid in their microenvironment. While lymphoid cells can readily take up extracellular cysteine, they have, in general, a very low uptake capability for cystine,[16,17] which is the dominant form of the amino acid in tissue culture medium or in the circulation, since cysteine is rapidly oxidized to cystine.[11,18] *In vitro*, the cyst(e)ine requirement of lymphoid cells can be accommodated by the presence in the medium of cystine at elevated concentrations or, alternatively, by the presence of a thiol such as 2-ME.[11,12,16] *In vivo*, lymphoid cell proliferation apparently depends significantly on the supply of cysteine by neighboring body cells, eg macrophages and fibroblasts. These cells can take up cystine from the circulation via a cystine/glutamate antiporter plasma membrane transport system, designated x_c^-, and release cysteine into the extracellular space.[19–21]

Intracellular cysteine levels are critically important for the synthesis of glutathione (GSH), a tripeptide thiol produced from glutamate, cysteine and glycine.[22] GSH, the most abundant nonprotein thiol present in animal cells, has important

Correspondence: PW Gout, Department of Cancer Endocrinology, BC Cancer Agency, 600 West 10th Avenue, Vancouver, BC V5Z 4E6, Canada or YJ Kang, Department of Medicine, University of Louisville, 530 S. Jackson St, 3rd Floor ACB, Louisville, KY 40292, USA
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roles in a broad spectrum of cellular processes, including a protective role as an antioxidant, maintenance of the cellular redox equilibrium, as well as regulation of enzyme activity and mitogenic responses, eg of lectin-stimulated T lymphocytes.[22,23]

In the present study , we have investigated the 2-ME growth requirements of various Nb2 lymphoma cell lines as a function of malignant progression, with special attention to the cyst(e)ine and GSH requirements of the cells. We have compared two Nb2 sublines in particular, ie the Nb2-11 line, which is PRL- or interleukin-2 (IL-2)-dependent for growth, and the more progressed Nb2-SFJCD1 line, which is growth factor-independent and possesses additional chromosomal alterations.[6,7,10] The results indicate that malignant progression of Nb2 lymphoma cells can be associated with an increased ability to take up extracellular cystine, found to be an essential amino acid for Nb2 cells. The x_c^- cystine/glutamate plasma membrane transport system, mediating the increased cystine uptake, may provide a novel target for the therapy of progressing T cell cancers.

Materials and methods

Materials

Bathophenanthroline disulfonic acid, L-buthionine-(S,R)-sulfoximine (BSO), L-cystine, L-cysteine, 1-fluoro-2,4-dinitrobenzene, GSH, insulin (bovine pancreatic), monosodium L-glutamate (MSG), prolactin (ovine), *N*-ethylmaleimide and transferrin (iron-saturated; human) were acquired from Sigma (St Louis, MO, USA). M-cresol purple, 2-hydroxyethyl disulfide, iodoacetic acid and perchloric acid were obtained from Aldrich (Milwaukee, WI, USA). ^{35}S-L-cystine (specific activities 154 and 245 μCi/μmol) was obtained from Amersham Canada (Oakville, Ontario, Canada). Solvents used for high performance liquid chromatography (HPLC) were obtained from Fisher Scientific (Chicago, IL, USA). A 3-amino-propyl-spherisorb column (20 cm × 4.6 mm, 5-μM particles) was obtained from Custom LC (Houston, TX, USA). All other chemicals were acquired from Sigma or Aldrich. Regular and cyst(e)ine-free Fischer's media were obtained from Stem Cell Technologies (Vancouver, BC, Canada).

Cell cultures

The PRL/IL-2-dependent cell line, Nb2-11 (cloned in Dr HG Friesen's laboratory, Winnipeg, Manitoba, Canada), was propagated at 37°C in an atmosphere of 5% CO_2/95% air as suspension cultures in 80 cm^2 tissue culture flasks in Fischer's medium containing 10% fetal bovine serum as a source of lactogen (PRL) (Gibco BRL, Burlington, Ontario, Canada), 10% nonmitogenic horse serum (ICN Flow, Costa Mesa, CA, USA), penicillin (50 U/ml), streptomycin (50 μg/ml) and 2-ME (50 μM), as previously described.[6] The cloned, growth factor-independent Nb2-SFJCD1, D5, F1 and F5 Nb2 lymphoma cell lines were similarly propagated in the above, 2-ME-containing medium from which fetal bovine serum had been omitted.[6] Cultures were subcultured twice a week.

For experiments, cells were used from exponentially growing cultures, within 2–6 weeks of their resurrection from storage in liquid nitrogen. Cell suspensions (2.0 ml aliquots) were cultured in triplicate in 12-well Linbro culture plates (Flow Laboratories, Mississauga, Ontario, Canada). When comparing cell lines with regard to drug–cell interactions, the serum supplement of the culture medium for the growth factor-independent Nb2 cells was increased to 20% horse serum to match the serum content of the medium used for the Nb2-11 cells and thus ensure a more valid comparison. *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (10 mM) was in many experiments used for extra buffering action. To remove extracellular 2-ME, cystine or serum proteins from cultures, cells were centrifuged (3.5 min at 350 *g*) and the cell pellets resuspended in medium lacking the compounds/serum; this procedure was repeated twice. Chemically defined, cyst(e)ine-free culture medium consisted of Fischer's medium, from which cyst(e)ine had been omitted, supplemented with insulin (bovine pancreatic; 15 μg/ml), iron-saturated transferrin (human; 15 μg/ml), PRL (ovine; 100 ng/ml), HEPES (10 mM; pH 7.2) and antibiotics. Cell populations were determined using an electronic cell counter (Coulter Electronics, Hialeah, FL, USA).

Cellular uptake of ^{35}S-L-cystine

The uptake of cystine by cells was measured using the method of Novogrodsky *et al*,[24] as modified by Hishinuma *et al*.[25] Toward this end, cells were washed free of extracellular cystine, serum proteins or 2-ME (as described above) and resuspended in cyst(e)ine- and protein-free Fischer's medium, containing 10 mM HEPES (pH 7.2), at a concentration of (12–16) × 10^6 cells/ml. Aliquots of the cell suspensions (300, 350 or 400 μl) were distributed into 14 ml round bottom polypropylene tubes (Falcon; Becton Dickinson Canada, Mississauga, Ontario, Canada) and incubated in a shaking water bath (37°C). Chemicals to be tested for effects on cystine uptake (eg 2-ME, MSG, BSO) or medium were then added (50 μl) in various combinations. Following a short incubation, ^{35}S-L-cystine (50 μl) was added to a final volume of 500 μl (final concentration approximately 10 μCi/ml, 40 μM). At the desired times, 100 μl aliquots of labeled cell suspensions were removed (triplicates) and layered on 200 μl amounts of a 15:85 (vol/vol) mixture of mineral oil and di-*n*-butyl phtalate prepipetted into 1.5 ml plastic Eppendorf centrifuge tubes. After centrifugation in a microfuge (full speed, 30 s) and removal of the supernatants, the tips of the tubes containing the pellets were cut off and the cell pellets solubilized in 500 μl 1 N KOH in scintillation vials (overnight; 37°C). Glacial acetic acid (500 μl) was then added plus 10 ml of liquid scintillation fluid (ScintiVerse; Fisher Scientific, Nepean, Ontario, Canada) and the amount of cell-associated radioactivity determined in a scintillation counter. Data were expressed as counts/min/10^6 cells.

In some experiments, cellular uptake of cystine was measured using medium in which Na^+ had been replaced by choline. Instead of Fischer's medium, potassium phosphate-buffered salt solution (3 mM KCl, 0.01% $CaCl_2$, 0.012% $MgSO_4 \times 7H_2O$, 0.1% glucose and 10 mM potassium phosphate, pH 7.2) was used containing either 137 mM NaCl or 137 mM choline chloride.

Determination of cellular GSH concentration

Total cellular GSH levels were determined by the HPLC assay described by Fariss and Reed[26] with some modifications previously reported.[27] Briefly, cells were washed free of culture medium by centrifugation and resuspension in phosphate-

buffered saline. Following another centrifugation, cell pellets were snap-frozen and stored at –70°C prior to assay. For determination of GSH, cell pellets were mixed with 10% perchloric acid containing 1.0 mM bathophenanthroline disulfonic acid. Following centrifugation and separation of pellets and supernatants, 100 mM iodoacetic acid containing 0.2 mM m-cresol purple was added to the supernatants. This was followed by addition of excess $KHCO_3$ in small increments until CO_2 no longer developed. After 15 min incubation of the reaction mixtures at room temperature in the dark, equal volumes of 3% 1-fluoro-2,4-dinitrobenzene in absolute ethanol were added. Following vortexing, the mixtures were placed for 24 h at 4°C in the dark for subsequent analysis using a Beckman model 338 gradient HPLC system equipped with a 507 autosampler and GOLD software (Beckman, Fullerton, CA, USA). The GSH derivatives were separated with a 3-aminopropyl column and quantitated at 365 nm with a UV-vis variable detector. Both GSH and GSH disulfide were used as standards; gamma-glutamylglutamate was used as an internal control.

Statistical analysis

Experimental results from triplicate samples were analyzed using model-I ANOVA to obtain P values. Differences are considered to be significant when $P < 0.05$.

Results

Growth requirement for exogenous 2-ME

Figure 1 shows the growth of Nb2-11 and Nb2-SFJCD1 cell cultures over a 3-day period as a function of exogenous 2-ME concentration. The results demonstrate that the proliferation of the Nb2-11 cells was critically dependent on the presence in the culture medium of exogenous 2-ME in a narrow concentration range (approximately 33–100 μM), as previously reported for the parent Nb2 lymphoma cells.[5] In contrast, the proliferation of Nb2-SFJCD1 cells was completely independent of exogenous 2-ME. Following transfer to 2-ME-deficient medium these cells did not show a lag period and proliferated at about the same rate as in the presence of 2-ME (1–100 μM). Moreover, Nb2-SFJCD1 cells could be propagated for indefinite periods in their standard, 10% horse serum-containing culture medium from which 2-ME had been omitted (data not shown). 2-ME abolished growth of both cell lines at concentrations $\geqslant$333 μM (Figure 1).

In separate experiments it was established that Nb2-11 cells grew readily when 2-ME was replaced by 2-hydroxyethyl disulfide, the oxidation product of 2-ME, but not when 2-ME was replaced by 1–1000 μM GSH (data not shown). These results closely resemble observations by Broome and Jeng using L1210 lymphoma cell cultures.[11]

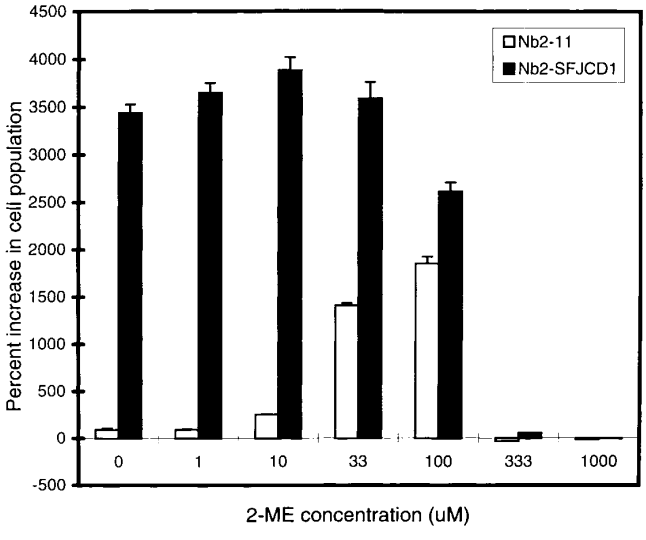

Figure 1 Growth of Nb2-11 and Nb2-SFJCD1 cell cultures as a function of exogenous 2-ME concentration. Cells were washed free of extracellular 2-ME for a 72-h incubation in culture media containing 20% serum and various concentrations of 2-ME, as indicated. Initial cell concentrations: 25×10^3 cells/ml. Population growth is expressed as percent increase relative to the initial cell populations. Results are presented as means ± s.d. and represent data from three experiments.

Growth requirements for L-cystine

Since growth requirements of cultured lymphoid cells for 2-ME had previously been linked to a cystine requirement,[11,12–14] Nb2-11 and Nb2-SFJCD1 cells were compared with regard to their growth requirements for this amino acid. Figure 2 shows that Nb2-SFJCD1 cells grew readily – in the absence of 2-ME and cysteine – in chemically defined medium containing L-cystine at 9, 27 and 81 μM, showing increases in cell population of approximately 400, 600 and 800%, respectively. In contrast, the Nb2-11 cells showed, under the same conditions, massive lysis at 9 μM cystine and increases in cell population of only 20 and 200% at 27 and 81 μM cystine, respectively. However, the Nb2-11 cells proliferated readily at 27 μM cystine when 50 μM 2-ME was

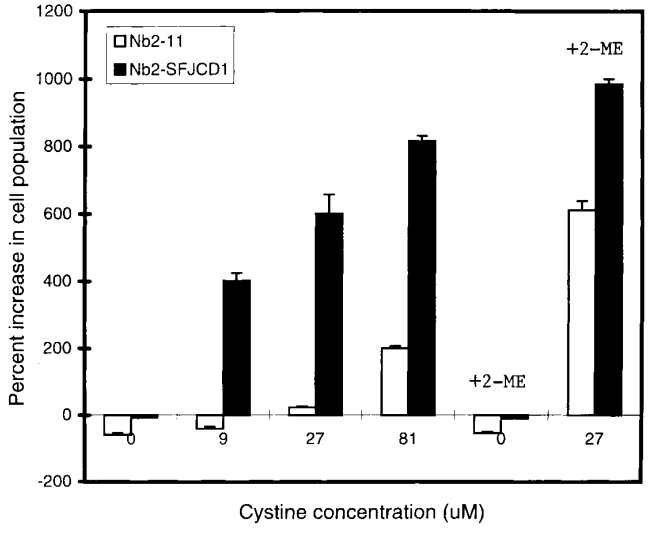

Figure 2 Effect of exogenous cystine on growth of Nb2-11 and Nb2-SFJCD1 cell cultures in the absence and presence of 2-ME. Cells were washed free of extracellular cystine, serum proteins and 2-ME and resuspended in chemically defined, cysteine-free culture medium for a 65-h incubation with various concentrations of L-cystine in the absence and presence of 2-ME (50 μM). Initial cell concentrations: Nb2-11, 80×10^3 cells/ml; Nb2-SFJCD1, 51×10^3 cells/ml. Population growth is expressed as percent increase relative to the initial cell populations. Results are presented as means ± s.d. and represent data from three experiments.

present in the medium, showing a population increase of approximately 600%. 2-ME also stimulated the population growth of the Nb2-SFJCD1 cells at 27 μM cystine. In the absence of exogenous cystine, neither Nb2-SFJCD1 cells nor Nb2-11 cells were able to proliferate and showed cell lysis, irrespective of whether or not 2-ME was present. The results demonstrate that exogenous L-cystine is essential for the viability and proliferation of both Nb2-11 and Nb2-SFJCD1 cells and that the Nb2-SFJCD1 cells utilize the amino acid much more readily than the Nb2-11 cells. The growth-promoting effect of 2-ME, which was obtained only when cystine was present in the culture medium, is consistent with its enhancement of cystine utilization.[12]

An attempt was made to propagate Nb2-11 cells in cystine-, 2-ME- and albumin-free, chemically defined culture medium by adding L-cysteine at a range of concentrations. Cysteine did support some growth when used at 100 μM, giving a 100% increase in cell population; no improvement was obtained using repeated additions of 50 or 100 μM cysteine (every 12 h) and one-time additions at higher concentrations ($\geqslant$250 μM) were toxic (data not shown). *In vitro*, cysteine has a half-life of only a few h[11,17,18] and toxic effects of cysteine at elevated concentrations (eg 300 μM) have been reported for lymphoid cells.[16] It appears likely that cysteine-mediated proliferation of Nb2-11 cells is critically dependent on a steady supply of extracellular cysteine at a narrow concentration range, as reported for L1210 cells: above this range toxicity results, below this range no growth-promoting activity is observed.[16] Apparently, 2-ME facilitates delivery of cysteine to lymphoid cells at a constant rate.[12]

Cellular uptake of cystine: effect of 2-ME

To explain the difference in cystine utilization found between Nb2-SFJCD1 and Nb2-11 cells, the two cell lines were compared with regard to cellular uptake of cystine, using methodology developed by other workers.[24,25] Figure 3 shows the time course over a 17 min period of the association of ^{35}S-L-cystine (40 μM) with Nb2-SFJCD1 and Nb2-11 cells. In the absence of 2-ME, Nb2-SFJCD1 cells concentrated cystine much more rapidly than the Nb2-11 cells, showing 11-, 11- and seven-fold higher uptake of cystine at 2, 5 and 17 min, respectively. Addition of 2-ME (50 μM) to Nb2-11 cells markedly increased their cystine uptake, leading to cell-associated cystine levels which, at 17 min, were eight-fold higher than those observed in the absence of 2-ME and exceeded the levels found in Nb2-SFJCD1 cells cultured without 2-ME. The results demonstrate that, relative to Nb2-11 cells, the Nb2-SFJCD1 cells have a substantially increased cystine uptake capability ($P < 10^{-6}$). Furthermore, 2-ME (50 μM) enhances the cellular uptake of cystine dramatically, as shown with the Nb2-11 cells ($P < 10^{-6}$). In view of the finding that cystine is essential for the growth of both cell lines (Figure 2), the results indicate that the growth-promoting activity of 2-ME in the Nb2-11 cell cultures in standard Fischer's medium (Figure 1) is based on its ability to enhance the cellular uptake of cystine to growth-sustaining levels which the Nb2-11 cells, in contrast to Nb2-SFJCD1 cells, cannot obtain on their own in this particular medium. It is expected that the 2-ME requirement of the Nb2-11 cells can be overcome by using culture medium which contains substantially higher cystine levels than Fischer's medium. It may be noted that the concentration of cystine used in the above uptake studies, ie 40 μM, is in the physiological range.[28]

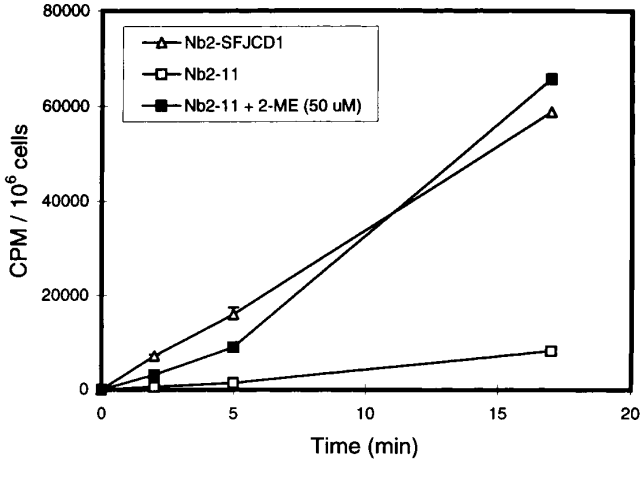

Figure 3 Uptake of ^{35}S-L-cystine by Nb2-11 and Nb2-SFJCD1 cells as a function of time: effect of 2-ME addition. Nb2-SFJCD1 cells, precultured for 3 days in 2-ME-free medium, and Nb2-11 cells were washed free of extracellular cystine, serum proteins and 2-ME and resuspended in cyst(e)ine-free, protein-free Fischer's medium for incubation at 37°C. ^{35}S-L-cystine (10 μCi/ml; 40 μM) was then added to replicate cultures, including Nb2-11 cell cultures to which 2-ME (50 μM) had been added (0 h). Cell-associated radioactivity was determined at the times indicated. Results are expressed as c.p.m./10^6 cells (mean $\pm$ s.d) and are representative of three experiments. In most cases the s.d. signs are obscured by the symbols.

Characterization of cystine uptake by Nb2-SFJCD1 cells

Exogenous cystine can be taken up by a variety of mammalian cells via the x_c^- cystine/glutamate transport system, as established for normal macrophages and fibroblasts,[19,21] HTC hepatoma cells[29] and also for L1210 mouse lymphoma cells following their conversion to 2-ME independence in 2-ME-free medium.[25] The uptake of cystine via the x_c^- transport system is characterized both by a very marked inhibition by glutamate, acting at mM concentrations in a competitive manner, and an independence from Na^+ ions.[19,25,29,30] To investigate whether cystine uptake by Nb2-SFJCD1 cells was mediated by the x_c^- transport system, the uptake of ^{35}S-L-cystine by these cells was evaluated using these two criteria. To ensure that data were obtained on the uptake of the amino acid *per se*, as distinct from cellular uptake coupled to metabolism, use was made of *N*-ethylmaleimide, an inhibitor of cystine metabolism, and a short measuring period (2 min) during which the uptake was linear.[25,30] During this period 0.1 mM *N*-ethylmaleimide inhibited the uptake of cystine only marginally ($\leqslant$10%). The results in Table 1 show that monosodium glutamate (MSG) severely inhibited the uptake of the amino acid by Nb2-SFJCD1 cells in the absence of 2-ME, ie by 82 and 85% at concentrations of 2.5 and 10 mM, respectively. Furthermore, the Nb2-SFJCD1 cells took up cystine readily in the complete absence of extracellular Na^+ ions, although at a somewhat lower rate than when Na^+ ions were present. Taken together, the results demonstrate that the uptake of cystine by Nb2-SFJCD1 cells is mediated mainly by the x_c^- cystine/glutamate transport system.

Growth-inhibitory effects of MSG: influence of 2-ME

The effect of MSG on the growth of Nb2 cell cultures is shown in Figure 4. Even at 10 mM, MSG only marginally inhibited

Table 1 Effect of MSG, Na^+-free medium and BSO on ^{35}S-L-cystine uptake by Nb2-SFJCD1 cells

Treatment	*Inhibition of cystine uptake relative to controls (%) (mean ± s.d.)*
MSG (2.5 mM)	82 ± 0.7
MSG (10 mM)	85 ± 1.5
Na^+-free medium	31 ± 0.8
BSO (4 mM)	43 ± 0.7
BSO (4 mM) + 2-ME (100 μM)	–21 ± 2.6

Nb2-SFJCD1 cells, cultured for 2 days in 2-ME-free medium, were transferred to cyst(e)ine-free, protein-free medium for determination of uptake of ^{35}S-L-cystine (approximately 10 μCi/ml, 40 μM) during a 2-min incubation at 37°C in the presence and absence of drugs or Na^+ ions. Cells treated with MSG and BSO (±2-ME) received preincubations with these drugs of 2 and 10 min, respectively. The effect of MSG was measured in the presence of 0.1 mM *N*-ethylmaleimide to inhibit cystine metabolism (see text). In the Na^+-free medium, choline chloride replaced the NaCl component of the uptake medium. MSG and BSO results represent data from three experiments.

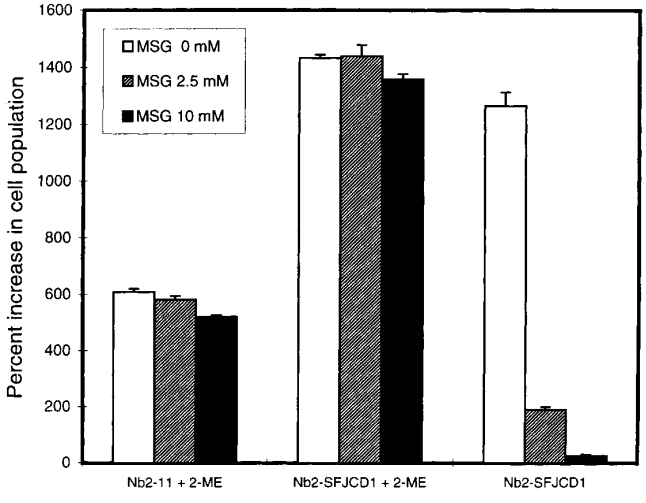

Figure 4 Effect of MSG on the proliferation of Nb2-11 cells (65 h) and Nb2-SFJCD1 cells (72 h) in chemically defined culture medium, supplemented with L-cystine (27 μM), in the presence and absence of 2-ME (50 μM), as indicated. Initial cell concentrations: Nb2-11, 80×10^3 cells/ml; Nb2-SFJCD1, 70×10^3 cells/ml. Population growth is expressed as percent increase relative to the initial cell populations. Results are presented as means ± s.d. and are representative of three experiments.

the proliferation of Nb2-11 or Nb2-SFJCD1 cells cultured in the presence of 2-ME (50 μM). In contrast, MSG profoundly inhibited the proliferation of the 2-ME-independent Nb2-SFJCD1 cells in the absence of 2-ME, showing an 85 and 98% inhibition of growth at 2.5 and 10 mM, respectively. This growth inhibition is consistent with the ability of MSG to severely inhibit uptake by the cells of essential cystine (via the x_c^- transport system) in the absence of 2-ME (Table 1). When 2-ME is present in the culture medium, it can facilitate uptake of exogenous cystine (Figure 3), very likely via a route which is not sensitive to MSG. As reported for L1210 lymphoma cells, 2-ME-mediated uptake of cystine involved the formation of a mixed disulfide, Cys-2-ME, which was taken up via the L transport system, a process which was not inhibited by glutamate.[12] Taken together, the results indicate that the severe growth arrest of 2-ME-free Nb2-SFJCD1 cell cultures by MSG (at 2.5 and 10 mM) is not based on nonspecific cytotoxicity, but most likely represents specific action of MSG, ie inhibition of cystine uptake via the x_c^- cystine/glutamate transport system.

2-ME growth independence of other Nb2 cell lines

Additional Nb2 cell lines were tested for 2-ME growth requirements and growth inhibition by MSG. They include sublines D5, F1 and F5, which are growth factor-independent as shown by propagation in growth factor-deficient, chemically defined culture medium.[6,9] Figure 5 shows that, in contrast to the Nb2-SFJCD1 cells, none of the three sublines grew as well in the absence of 2-ME as in its presence. The growth of subline D5 was particularly poor in the absence of 2-ME. Sublines F1 and F5, however, were capable of growing in the absence of the thiol, showing up to 20% of the growth rate obtained with 100 μM 2-ME. In the absence of 2-ME, the growth of all sublines was completely arrested by 10 mM MSG. In view of the specific, cystine uptake-inhibiting action of MSG established above, this finding indicates that the growth of the F1 and F5 cultures in the absence of 2-ME was based on an ability of the cells to take up growth-sustaining amounts of cystine via the x_c^- transport system, as demonstrated for the Nb2-SFJCD1 subline (Figure 3, Table 1). Since the growth rates of sublines F1 and F5 were much lower in the absence of 2-ME than in its presence, their cystine uptake capabilities are apparently only marginally adequate to meet their cystine growth requirements and substantially lower than the cystine uptake capability of the Nb2-SFJCD1 cells.

Sublines D5, F1 and F5 were generated, similar to the Nb2-SFJCD1 subline, by culturing the original Nb2 parent cells in growth factor-deficient medium – in the continuous presence of 2-ME – followed by cloning of surviving cells.[6] In view of this, the 2-ME independencies of these sublines are not a result of lengthy adaptations in 2-ME-free medium, as reported

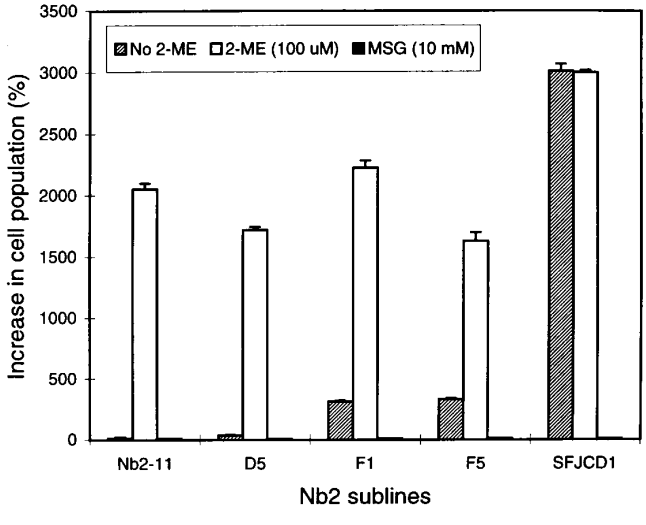

Figure 5 Growth of Nb2 sublines in the absence and presence of 2-ME: effect of MSG. Cells of sublines Nb2-11, D5, F1, F5 and Nb2-SFJCD1 were washed free of 2-ME and resuspended in culture medium containing 20% serum. Cultures were incubated (in triplicate) for 3 days (1) in the absence of 2-ME; (2) with 2-ME (100 μM) and (3) with 10 mM MSG in the absence of 2-ME, as indicated. Population growth is expressed as percent increase relative to the initial cell populations. Results are presented as means ± s.d.

for 2-ME-independent L1210 cells,[25] but apparently are intrinsic properties. As indicated by chromosomal analysis, the Nb2-11 subline has the same karyotype as the parent lymphoma line, containing the same chromosomal alterations, whereas the Nb2-SFJCD1 subline obtained six additional chromosomal alterations during its clonal evolution.[7,10] Subsequent screening of the D5, F1 and F5 sublines has indicated that these sublines have no more than two additional chromosomal changes relative to the parent or Nb2-11 lines. It may also be noted that both the Nb2-11 and Nb2-SFJCD1 sublines are highly metastatic in Noble rats.[10,31] In view of the above, the results of Figure 5 indicate that development of 2-ME growth independence in Nb2 cells, ie increased cystine uptake capability, can be associated with their malignant progression but is not coupled to development of growth factor autonomy or metastatic ability.

Intracellular GSH levels as a function of exogenous 2-ME concentration

GSH synthesis in lymphoid cells is dependent on intracellular cysteine levels which, in turn, are subject to the cellular uptake of cyst(e)ine.[20,22,23] Consequently, the cellular levels of GSH can be substantially affected by changes in the concentration of exogenous 2-ME if this thiol plays a major role in mediating the cellular uptake of the amino acid. With this in mind, GSH contents of Nb2-11 and Nb2-SFJCD1 cells have been determined as a function of the concentration of 2-ME in the culture medium. Table 2 shows that cells of the two lines contained about the same amounts of GSH (approximately 3 nmol/10^6 cells) when grown in the presence of 100 μM 2-ME. Culturing the cells at lower 2-ME concentrations led to a decrease in each of their GSH levels, especially in the case of the Nb2-11 cells. Thus at 33 μM 2-ME, the GSH levels had decreased by about 90 and 60% for actively growing Nb2-11 and Nb2-SFJCD1 cells, respectively. The effect of much lower 2-ME concentrations on GSH levels in Nb2-11 cells could not be examined since these cells do not grow well at 2-ME concentrations <33 μM (see Figure 1). However, when the 2-ME-independent Nb2-SFJCD1 cells were cultured in the absence of exogenous 2-ME it was found that these cells still contained GSH at levels of about 0.3 nmol/10^6 cells. The decline in the GSH contents of the Nb2 cells found with decreasing concentrations of exogenous 2-ME is in agreement with a reduction in the intracellular levels of cysteine expected to occur when the concentrations of 2-ME, mediating the uptake of the amino acid, are lowered. The finding that Nb2-SFJCD1 cells retained more GSH than Nb2-11 cells as the 2-ME concentrations were lowered, is consistent with the greater, 2-ME-independent cystine uptake capability of the Nb2-SFJCD1 cells (Figure 3).

Effects of L-buthionine-(S,R)-sulfoximine (BSO) on intracellular GSH levels and population growth

BSO, a selective inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in *de novo* GSH biosynthesis, is commonly employed to obtain a decrease in intracellular GSH levels.[14,22,23] In the present study, BSO has been used to determine whether Nb2-11 and Nb2-SFJCD1 cell lines had a critical growth requirement for intracellular GSH. As noted in Table 2, incubation of Nb2-SFJCD1 cells for 21 or 36 h with BSO (0.1 and 4 mM) led, both in the absence and presence of 2-ME (100 μM), to a decrease in GSH concentrations to undetectable levels, ie <0.2 nmol GSH/10^6 cells. To determine the effect of GSH depletion on population growth, Nb2-11 and Nb2-SFJCD1 cultures were incubated with 4 mM BSO for extended periods. Figure 6 shows that the BSO treatment

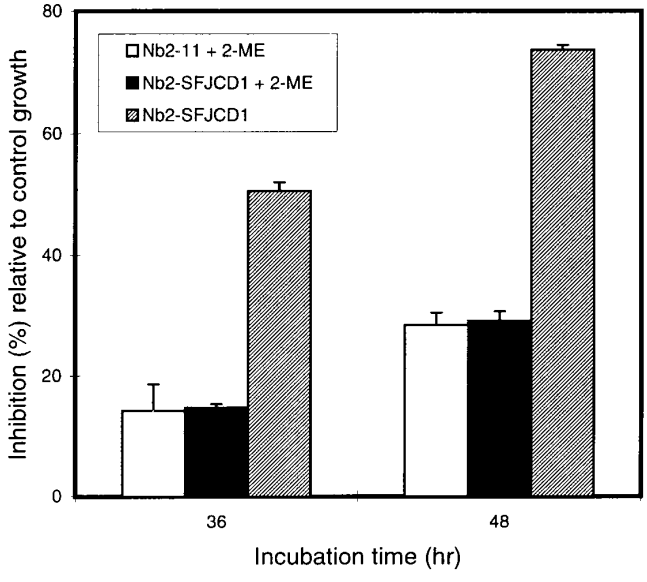

Figure 6 BSO-induced growth retardation of Nb2-11 and Nb2-SFJCD1 cell cultures. Cells were grown for 36 and 48 h with 4 mM BSO or without BSO (control) in 20% serum-containing culture medium in the presence or absence of 2-ME (100 μM), as indicated. Initial cell concentrations: Nb2-11, 144×10^3 cells/ml; Nb2-SFJCD1, 54×10^3 cells/ml. Growth inhibitions were calculated from the differences in population increase found between BSO-containing and control cultures. Results are presented as means ± s.d. and represent data from three experiments.

Table 2 GSH levels in Nb2 cells as a function of 2-ME concentration and after treatment with BSO[a]

Treatment	*Nb2-11 2-ME concentration*		*Nb2-SFJCD1*		
	100 μM	*33 μM*	*100 μM*	*33 μM*	*0 μM*
None (at 72 h)	2.87 ± 0.201	0.28 ± 0.008	2.66 ± 0.076	1.11 ± 0.134	0.29 ± 0.031
BSO 0.1 and 4 mM (after 21 and 36 h)			ND		ND

Cultures of Nb2-11 and Nb2-SFJCD1 cells in medium containing 20% serum were incubated with various concentrations of 2-ME, without or with BSO, for determination of intracellular GSH levels. Results are presented as means (± s.d.) and are representative of two experiments.
[a]GSH nmol/10^6 cells.
ND, not detectable (<0.2 nmol GSH/10^6 cells).

induced a moderate retardation of growth which was very similar for both cell lines when 2-ME (100 μM) was present in the culture medium (differences were not significant, $P = 0.8$). At 48 h, for example, growth inhibition for both cell lines was approximately 29%. Although at 21 h the levels of GSH had been largely depleted (Table 2), population growth during the next 27 h was affected to only a minor extent.

The growth retardation of Nb2-SFJCD1 cultures induced by 4 mM BSO was much greater in the absence of 2-ME than in the presence of the thiol, showing an inhibition of 74% as distinct from 29% (Figure 6). As shown in Figure 7, a substantial amount of the growth-inhibitory effect of BSO in 2-ME-free Nb2-SFJCD1 cultures could be prevented by addition of 2-ME to 100 μM. For example, 100 μM 2-ME overcame the growth inhibition obtained with 0.1 mM BSO almost completely (by about 90%; $P < 0.0004$) without raising the diminished GSH levels to detectable values (Table 2). 2-ME was less effective in reducing the growth inhibition obtained with higher BSO concentrations. Taken together, the results in Figures 6 and 7 indicate that elevated GSH levels are not essential for the proliferation of Nb2 cells. The 2-ME reversal observations are similar to previously reported findings that 2-ME was able to partially prevent BSO-induced growth inhibition of concanavalin A-stimulated human peripheral blood mononuclear cells without raising their decreased intracellular GSH levels.[14]

BSO is not completely specific in its inhibition of GSH biosynthesis and has been found to inhibit the uptake of exogenous cystine by, for example, human lung A549 carcinoma cells.[32–34] This raised the possibility that the growth-inhibitory effect of BSO found in the Nb2-SFJCD1 cultures resulted, in part, from an inhibition of cystine uptake which could be overcome by 2-ME. As shown in Table 1, a 10-min incubation of Nb2-SFJCD1 cells with BSO (4 mM) in the absence of 2-ME led to a 43% inhibition of their uptake of ^{35}S-L-cystine. This inhibition could be totally overcome by the presence of 2-ME (100 μM). These results suggest that the BSO growth-inhibitory effect in the 2-ME-free Nb2-SFJCD1 cultures was indeed due, in part, to inhibition of cystine uptake. Whereas the BSO inhibited cystine uptake mediated by the x_c^- system, it did not interfere with the 2-ME-mediated uptake of the amino acid.

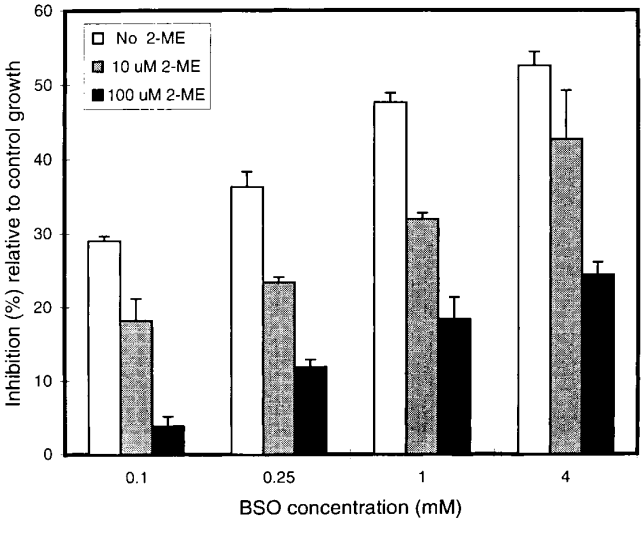

Figure 7 Partial reversal by 2-ME of the growth-inhibitory effect of BSO in Nb2-SFJCD1 cell cultures. Cells were grown for 34 h in 20% serum-containing culture medium with BSO (0, 0.1, 0.25, 1 and 4 mM) in the presence of 2-ME at three different concentrations (0, 10 and 100 μM). Initial cell concentration: 122×10^3 cells/ml. Growth inhibitions were calculated from the differences in population increase found between BSO-containing cultures and control cultures (no BSO). Results are presented as means ± s.d. and represent data from three experiments.

Discussion

Comparative analysis of cultured rat Nb2 lymphoma cell lines, showing various degrees of malignant progression, can lead to identification of differences in phenotypic and genotypic properties which could prove useful in the search for novel therapeutic targets in T cell malignancies.[7–10] In the present study, the difference observed between the 2-ME growth requirements of Nb2-11 and Nb2-SFJCD1 cells *in vitro* (Figure 1), was found to be based upon a difference in the uptake by the cells of L-cystine, which was critically needed for the growth and viability of either line (Figures 2 and 3). Whereas the Nb2-11 cells had a low intrinsic cystine uptake capability and required 2-ME to increase their uptake of cystine to growth-sustaining levels, the Nb2-SFJCD1 cells were, in contrast, able to grow in the complete absence of exogenous 2-ME – a result of an acquired, increased cystine uptake capability (Figure 3). The latter was mediated by the x_c^- cystine/glutamate plasma membrane transport system, as indicated by MSG inhibition and independence from Na^+ ions (Table 1). Lymphoid cells have, in general, a low uptake capability for cystine, but can take up cysteine readily.[16,17,20] However, cysteine is available in only limited amounts in the extracellular compartment since it is rapidly oxidized to cystine.[11,18] Studies by Dröge *et al*[19,20] have suggested that, *in vivo*, growth of lymphoid cells significantly depends on the secretion of cysteine in their microenvironment by macrophages and macrophage-like cells. In view of this, increased cystine uptake capability, as found in the present study, could render malignant lymphoid cells independent of cell-mediated cysteine supply *in vivo* and, as such, this property may be considered another feature in the progression of malignant lymphoid cells to greater autonomy. As indicated by the present study, increased cystine uptake capability can be associated with malignant progression of Nb2 lymphoma cells but is apparently not coupled to development of growth factor autonomy or metastatic ability (Figure 5).

The roles of cysteine and GSH in the function of cells of the immune system are not clearly understood. It has recently been reported that variations in cysteine and GSH levels in a human T cell line can modulate activity of the transcription factor, NF kappa B, which controls expression of a number of genes important in the proliferation and function of T cells.[35,36] The activity of NF kappa B apparently depends on optimal levels of cysteine and GSH, and is negated when their levels are lowered or increased.[35] In this regard it is of interest that the proliferation of Nb2-11 lymphoma cells is critically dependent on optimal concentrations of exogenous 2-ME: both lowering and increasing 2-ME concentrations led to growth inhibition (Figure 1). Since 2-ME mediates the cellular uptake of cyst(e)ine (Figure 3), the critical growth requirement for optimal concentrations of this thiol indicates that cysteine has a key regulatory role in the proliferation of Nb2 cells, as reported for other lymphoid cell systems.[35] In contrast, GSH, which is essential for the replication of for example normal T lymphocytes,[23] is not critically required for the proliferation of Nb2 cells. This is indicated by the lack of a major effect of

GSH depletion on the growth of 2-ME-containing Nb2 cell cultures (Table 2, Figures 6 and 7). Taken together, the observations indicate that the growth-stimulatory effect of cysteine in the Nb2 cell cultures is not primarily based on its maintenance of normal GSH levels (as a precursor in GSH *de novo* biosynthesis[22]), but on other, presently undefined mechanisms. Further research into the regulatory role of cysteine in Nb2 cell replication, including its relationship to NF kappa B, is indicated.

The finding that cyst(e)ine is an essential amino acid for Nb2 lymphoma cells was not unexpected since it has long been recognized as an essential amino acid for many normal and malignant human and animal cells of lymphoid origin,[15,16] including human acute T lymphoblastic leukemia cells.[37] The inability to synthesize cyst(e)ine makes the proliferation of such cells critically dependent on extracellular supply of the amino acid. In view of this, cyst(e)ine starvation could be useful in the therapy of for example leukemias, similar to the well-established clinical treatment of acute lymphocytic leukemia and lymphoma with L-asparaginase, aimed at depletion of the essential amino acid, L-asparagine.[38] In fact, attempts have previously been made to devise therapeutic regimens aimed at depleting circulating L-cyst(e)ine using an L-cyst(e)ine degrading enzyme in combination with an inhibitor of L-cysteine biosynthesis. However, clinical trials were precluded by the rapid plasma clearance of the enzyme.[39]

At present, not much information is available on the x_c^- cystine transport system at the molecular level. The x_c^- cystine transporter expressed in mouse macrophages is apparently encoded by 1.5–2.9 kb mRNA.[40] Mediation by the x_c^- transport system of acquired, increased cystine uptake capability, as found for Nb2-SFJCD1 cells (Figure 3, Table 1), has previously been reported for L1210 mouse lymphoma cells which had become 2-ME-independent following adaptation in 2-ME-free medium.[25] The x_c^- cystine transport system is also used by murine macrophages and human monocytes and fibroblasts which, following uptake of cystine, can release it as cysteine and, in doing so, supply cysteine to neighboring cells.[19–21] It is of great interest that the x_c^- cystine transport in these different cell types can be severely inhibited by MSG leading, for example, to inhibition of cysteine secretion by macrophages[19] and to complete growth arrest of cyst(e)ine-dependent Nb2 lymphoma cells (Figures 4 and 5). In view of this, we propose that the x_c^- cystine plasma membrane transport system constitutes a novel, potential therapeutic target for the control of progressing T cell cancers. Its inhibition would not only block the supply of cysteine by normal cells (eg macrophages) to cysteine-dependent lymphoma or leukemia cells, but also inhibit the uptake of cystine by malignant lymphoid cells which have developed an active x_c^- cystine transport system. The feasibility of this approach, including side-effects on normal tissues, could be investigated using Noble rats bearing subcutaneous tumors of the various Nb2 lymphoma cell lines.[10]

In a literature search for drugs, other than MSG, which could potently inhibit the x_c^- cystine transport system, it was found that nonsteroidal anti-inflammatory drugs have been reported to severely inhibit the x_c^- cystine transport system in human fibroblasts in a noncompetitive manner.[41] Furthermore, one of the anti-inflammatory drugs, indomethacin, has recently proven useful in developmental cancer therapy, arresting the growth and metastatic spread of certain non-lymphoid cancers in animals[42] and humans.[43] The use of such drugs for arresting the growth of Nb2 lymphoma cell lines will be the subject of further studies.

In conclusion, the present study suggests that increased cystine uptake capability constitutes another potential step in the malignant progression of T cell cancers. Furthermore, the x_c^- plasma membrane transport system, involved in the cystine uptake, appears to provide a novel therapeutic target which could be potentially useful in, for example, adjuvant therapy of T cell cancers such as acute T lymphoblastic leukemia.[44]

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