

872-96

Tumor Growth and Disulfide Reduction: Possible Dependence on Protein-Disulfide Reductase^{1,2,3}

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SUMMARY—Tumor cells, washed and resuspended in balanced salt solution with 2–5 mM disodium ethylenediaminetetraacetate (EDTA), lost transplantability after incubation with 1 mM iodoacetate or *N*-ethylmaleimide. All 7 ascites and 2 solid murine tumors investigated were inactivated by the sulfhydryl (SH)-blocking procedure though their cells were still metabolizing and excluding supravital stains. Protection was conferred by the SH compounds dithiothreitol (DTT), L-cysteine, and reduced glutathione (1 mM). Inactivated cells could be reactivated by further incubation with an SH compound or with native cell-free ascitic fluid. Therefore, loss of transplantability was not attributable to a lethal effect of the blocking agents. Protection with DTT or incubation with it alone resulted in more malignant tumors. Both ascitic fluid and intercellular material of solid tumors protected against inactivation. This protection was overcome when a) the disulfides were converted into reactive SH's before SH blocking, or b) the nucleotide (NADPH)-dependent protein-disulfide reductase (PDR) of the fluid was inhibited by heating 30 minutes at 70° C or by 0.1 mM arsenite. Activity of the NADPH-dependent PDR was much higher in tumor cells than in normal cells. We found no NADPH-dependent PDR activity in normal serum, but detectable activities in cell-free ascitic fluid and in serum of tumor-bearing mice. Pretreatment of the fluid with DTT, EDTA, arsenite, or heat increased the immunogenicity of SH-blocked ascites tumor. Thus, proliferation and immunogenicity of (murine) tumors appeared to depend on reactive SH's, protein-disulfide reduction, and implicated enzyme(s).—*J Natl Cancer Inst* 51: 575–583, 1973.

IN PREVIOUS STUDIES (1, 2), certain ascites tumors, alkylated in vitro with sulfhydryl(SH)-blocking agents, lost transplantability though their cells still metabolized and excluded supravital stains. However, results were not always reproducible. With improved methods (3), all tumors investigated have been inactivated, reproducibly, with the same low concentrations of SH-blocking agents and despite apparent cell survival. This phenomenon, which can now be analyzed, tends to confirm the relevance of disulfide-SH interchange to tumor growth (4). Tietze's work on the disulfide reductases which control the interchange (5, 6) has encouraged our investigation of disulfide reduction as it relates to growth and immunogenicity of tumors, and of disulfide reductase activities in tumor cells, exudates, and fluids.

Three disulfide reductases are found in high-speed supernatant of rat liver homogenate (5, 6): nicotinamide-adenine dinucleotide phosphate

(reduced)—synonymous with triphosphopyridine nucleotide—(NADPH)-glutathione reductase, reduced glutathione (GSH)-disulfide transhydrogenase (oxidoreductase), and NADPH, i.e., nucleotide-dependent protein-disulfide reductase (PDR). It is still unsettled whether the last enzyme is related to the thioredoxin system in yeast, *Escherichia coli*, and Novikoff hepatoma (7–9). This system consists of thioredoxin, a small protein with a single disulfide bond, and thioredoxin reductase, a flavoprotein that catalyzes the reduction of thioredoxin by NADPH (9). NADPH-dependent PDR, viz. the thioredoxin system, are part of electron-carrying

¹ Received February 5, 1973; accepted April 25, 1973.

² Supported by the American Cancer Society (Massachusetts Division) #1408-C.

³ Publication #306, Pondville Hospital.

⁴ We thank Prof. M. Pitzurra and Dr. D. G. Therriault for their cooperation, Miss J. Donohue for technical assistance, and Mrs. D.E. Noonan for clerical help.

chains implicated in the reduction of ribonucleotides, i.e., in the biosynthesis of deoxyribonucleotide (9).

In this report, evidence is presented confirming that proliferation and immunogenicity of murine tumor cells are controlled by the disulfide-sulfhydryl system. We also discuss preliminary data on the activity of the 3 disulfide reductases in normal and tumor-derived murine cells and fluids.

MATERIALS AND METHODS

Animals and tumors.—Strains from The Jackson Laboratory, Bar Harbor, Maine, were maintained by brother \times sister mating in our colony. Ascites tumors were carried by serial transplantation twice weekly of 2×10^5 to 10^6 cells. Inocula stemmed from tumors grown for 10–12 days in donor hosts. Tumors were Ehrlich ascites carcinoma and Krebs-2 carcinoma in Swiss, adenocarcinoma 755 and leukemia C1498 in C57BL/6J, methylcholanthrene-induced sarcoma (MCT) in C57BL/P (10), Sarcoma 1 in strain A/J, leukemia L1210 in DBA/2J, and breast adenocarcinoma (BAC/P) in C3H/HeJ. The last tumor originated from a spontaneous breast adenocarcinoma at Pondville Hospital, was carried in the original strain since 1967, and was converted to the ascites form in 1969. We have also used the solid, original form of MCT and BAC/P, transplanted subcutaneously (sc) into the scapular region with trocars carrying 2×4 mm blood- and necrosis-free pieces of tumors, 9–15 days old.

Homogenates of normal and tumorous cells.—Normal spleens and livers or solid tumors of C57BL/P and Swiss mice were minced in a solution of Ca^{++} - and Mg^{++} -free balanced salt solution (BSS) + 5 mM disodium-ethylenediaminetetraacetate (EDTA), pH 7.4 (medium A). The cells were mechanically dispersed in a VirTis-45 homogenizer at lowest speed for 60–90 seconds; the switch was opened and closed to maintain low speed. The dispersed material was passed through 2, then 4, layers of gauze. Cells were separated by centrifugation at $250 \times g$ for 6 minutes, washed twice in the described medium, and resuspended in 8 volumes isotonic saline. Micro-glass beads were added ($\frac{1}{2}$ vol), and the suspensions were homogenized in the VirTis-45 at about 20,000 rpm for 10 minutes while refrigerated. Homogenates were finally spun at $100,000 \times g$ for 1 hour. Pellets were discarded, and the supernatants dialyzed against a 0.1M Tris-0.01M EDTA buffer, pH 8.

Ascites tumor was mixed with about one-third vol of medium A. Cells were separated from fluid at $250 \times g$ for 8 minutes, then washed twice in medium A. The washed cells were resuspended in 8 volumes 0.9% NaCl, homogenized, and processed as were normal cells.

Measurements of reductase activities.—The procedure was that of Tietze (5). Enzymatic reduction of disulfide bonds was followed either directly, with 5,5'-dithiobis-(2-nitrobenzoic acid) (DNTB) as the substrate and spectrophotometrical

observation of the formation of SH groups [i.e., the reduction of DNTB (11)], or by measurement of the rate of oxidation of NADPH.

a) Nucleotide-dependent disulfide reductase activity.—To a 0.1M Tris-0.01M EDTA buffer, pH 8.0, containing 2–6 mg protein was added $1.33 \mu\text{M}$ DNTB and $3.28 \mu\text{M}$ NADPH. The course of the reaction was followed by the change in absorbance at 412 nm over 30 minutes; a Gilford Model 2400-5 recording spectrophotometer was used. Temperature was controlled at $35 \pm 0.5^\circ \text{C}$ in the cell compartment. All reacting solutions were increased to a final volume of 3.5 ml.

b) Glutathione reductase activity.—To a 0.1M phosphate-EDTA buffer, pH 7.2, containing 2–6 mg protein were added $1.65 \mu\text{M}$ NADPH and $1.50 \mu\text{M}$ oxidized glutathione (GSSG). As the enzymatic reaction was progressing, we measured the rate of oxidation of NADPH by the decrease in absorbance at 340nm.

c) Reduced glutathione (GSH)-disulfide transhydrogenase activity.—To a 0.1M Tris-EDTA buffer, pH 7.5, containing 2–6 mg protein and $50 \mu\text{g}$ glutathione reductase were added $1.65 \mu\text{M}$ NADPH, $1.4 \mu\text{M}$ GSH, and a disulfide substrate such as 0.5 ml of a saturated L-cystine solution. Rate of reaction (i.e., oxidation of NADPH) was followed at 340 nm.

SH blocking.—SH-alkylating agents sodium iodoacetate (IA) and *N*-ethylmaleimide (NEM) were used at 0.1 and 1 mM concentrations. Alkylation was done by incubation at pH 7.4 in a thermo regulated water bath at 37°C for 1 hour.

In 1 group of experiments, ascites tumor cells or cells of solid tumors were alkylated with their fluid or intercellular material. They were either treated directly or pretreated by incubation for 1 hour at 25°C , pH 7.4, with 1 mM dithiothreitol (DTT) or 0.1 mM arsenite or 5 mM EDTA (viz. 2 mM calcium disodium EDTA). Dispersed cells of solid tumors or of normal spleens were adjusted to a volume ratio of 1:4 packed cells to medium before alkylation.

In a second group of experiments, the tumor cells were separated from their fluid or intercellular material and alkylated in BSS. Ascites tumor was spun at $250 \times g$, 5–10 minutes. The cells were washed twice in medium A; then they were resuspended and alkylated as described. Solid tumors or normal spleens were minced, dispersed, and stirred for 30 minutes in medium A. The product was passed through 4 layers of gauze, and the cells were washed twice, resuspended, and alkylated in medium A.

In a third set of experiments, we alkylated the tumor cells, prepared as for group 2, in pretreated ascitic fluid or intercellular material. Cell-free ascitic fluid (or material released from dispersed cells of solid tumors) was reduced with 1 mM DTT or incubated with 0.1 mM arsenite (viz. 5 mM EDTA) or heated at 70°C for 30 minutes. The washed tumor cells were resuspended and alkylated in the pretreated media. After incubation with DTT, alkylation was performed with 3 mM IA or NEM, which can alkylate the 2 SH groups contributed by each molecule of DTT and leaves the same concentration of IA or NEM as in previous procedures.

Sulfhydryl compounds were tested for their capacity to protect against inactivation. Washed cells of Krebs-2 ascites tumor were resuspended in washing medium containing 1 mM NEM and 1 mM of DTT or L-cysteine (free base) or GSH and incubated 1 hour at 37° C. Control cells were incubated with NEM only.

To see if inactivated tumor cells could be reactivated by the same SH compounds, 1.5 mM DTT or L-cysteine or GSH was added to suspensions of Krebs-2 ascites cells already treated with NEM. After a second hour of incubation, transplantability was tested; controls were inoculated with cells treated with NEM only.

Testing the viability of tumor cells.—As noted by Grinell and Srere (12), tumor cells treated with SH-blocking agents at these concentrations no longer attach to glass substrate in tissue culture. Other criteria were used.

Supravital staining: Cell death was determined by the trypan-blue and eosin-Y techniques (13).

Transplantability: Ascites tumor cells (2×10^6) and 6×10^8 cells of solid tumors, treated as described, were injected intraperitoneally (ip) or sc, respectively, into 10 mice per group; the animals were observed for 30 days. The higher number of cells from solid tumors is on account of their lower apparent survival rate by supravital stain exclusion. Control groups received equivalent amounts of untreated cells. In view of Tennant's (14) critical evaluation of the trypan-blue exclusion technique, we determined whether an increased dosage of alkylated cells would result in tumor growth. To that effect, the following doses of SH-alkylated cells of the 755 ascites carcinoma were injected ip into groups of 10 C57BL/6J mice: 4×10^6 , 8×10^6 , 1.6×10^7 , and 3.2×10^7 .

Respirometry: Oxygen uptake of ascites tumor cells was measured in a Warburg constant-volume respirometer (20-unit refrigerated Cenco). Three flasks were loaded with 10^8 treated cells, 3 with 10^8 untreated cells, and 3 with 10^8 heat-killed cells (56° C, 1 hr). Liberated CO₂ was trapped by folded KOH papers, and oxygen uptake was determined at intervals of 15 minutes for 2½ hours. Each sample was checked for transplantability by ip inoculation of 2×10^6 cells into 10 susceptible mice.

Immunogenicity: Criterion was the tumor immunity induced by vaccination with the altered tumor cells. Since the protective effect of ascitic fluid was abolished by opening disulfides with DTT, by treating with arsenite, EDTA, or heat, the influence of these agents on immunogenicity of 755 ascites tumor in C57BL/6J was explored. The various suspensions (one-tenth ml doses), containing 2×10^6 cells, were injected ip into groups of 10 C57BL/6J male mice. Booster injections were given twice at 14-day intervals. A group of 10 was sensitized with the same number of normal spleen cells of C57BL/6J, resuspended and alkylated with 1 mM IA in Krebs-Ringer buffer + 5% isogenic serum. The animals of these groups plus 10 unprepared controls were challenged 7 days after the last injection with 2×10^6 fresh, untreated cells from the same tumor; each was observed for tumor growth for 90 days.

RESULTS

Activity of Disulfide Reductases

Measurements of nucleotide-dependent PDR are given in table 1. Activities are expressed in Δ optical density (Δ OD) at 412 m μ /mg protein in 4 minutes.

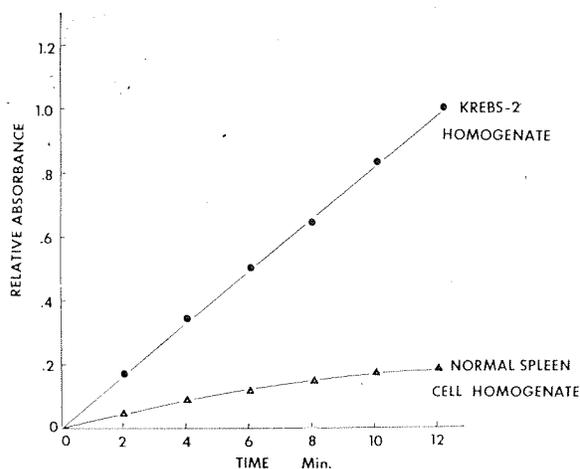
Activity was nil in normal serum but detectable in cell-free ascitic fluid and in sera of tumor-bearing mice. Low activity levels were found in normal spleen cells of C57BL/P and Swiss mice. Values were high in tumor cells (i.e., from 5 to 20 times above levels in normal spleen cells). This is illustrated by text-figure 1. Interestingly, the value for liver was quite below that of tumor cells. Activity in mouse embryo homogenate was between that in liver and that in tumor cells, though still clearly below the latter.

Differences in activity of the GSSG reductase between normal and tumorous cells were less conspicuous. Practically none were found with GSH-disulfide transhydrogenase (table 2).

TABLE 1.—NADPH-dependent protein disulfide reductase

Sample	Activity	
	Δ OD: 412 m μ /4 min/mg protein	SD \pm
Fluid:		
Krebs-2	0.057	0.004
755	.143	.016
MCT	.013	.004
BAC/P	.060	.015
C1498	.100	.013
L1210	.025	.016
Cell homogenate:		
Krebs-2	.552	.013
755	.947	.103
MCT	.339	.005
BAC/P	1.255	.022
C1498	1.180	.016
L1210	1.027	.004
Serum of tumor-bearing mice:		
C57BL/P with MCT solid	.003	.001
Normal mouse serum:		
C57BL/P	None*	
Swiss	None	
Pooled	None	
Normal spleen cell homogenate:		
C57BL/P	.032	.013
C57BL/P	.097	.005
Swiss	.087	.014
Normal liver cell homogenate:		
Swiss	.130	.014
Embryo homogenate: Swiss	.232	.013

*No observable increase in optical density during 30-minute reaction time.



TEXT-FIGURE 1.—NADPH-dependent protein-disulfide reductase activity of Krebs-2 ascites and of normal spleen cells (Swiss).

TABLE 2.—GSSG reductase

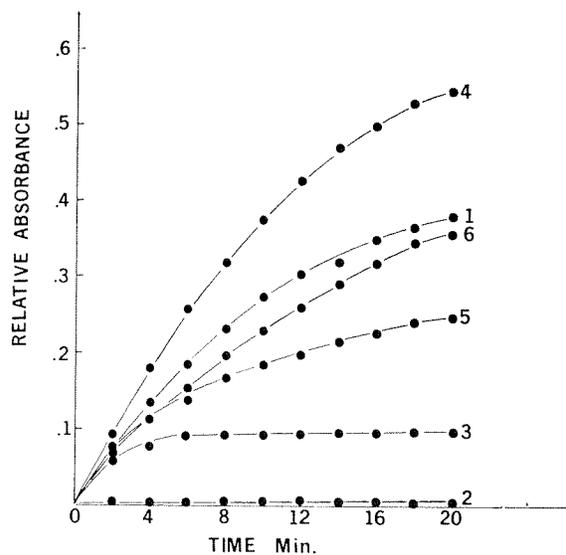
Sample	Activity	
	$\Delta OD: 412$ $m\mu/4 \text{ min}/$ mg protein	$\pm SD$
Fluid:		
Krebs-2	0.134	0.010
755	.197	.011
MCT	.151	.013
BAC/P	.130	.007
C1498	.232	.040
L1210	.062	.018
Cell homogenate:		
Krebs-2	.529	.021
755	.789	.053
MCT	.468	.004
BAC/P	.488	.042
C1498	.688	.006
L1210	.100	.008
Normal spleen cell homogenate:		
Swiss	.386	.007
C57BL/P	.172	.013
C57BL/6J	.160	.003
GSH-disulfide transhydrogenase:		
Krebs-2 fluid	None*	
MCT	.044	.016
Krebs-2 cell homogenate	None	
MCT	.009	.004
Normal mouse serum (C57-BL/P)	None	
Serum of tumor-bearing mice:		
57BL/P with MCT solid	None	
Normal spleen cell homogenate:		
C57BL/P	None	
C57BL/6J	None	

*No observable increase in optical density during 30-minute reaction time.

Heating at 70° C for 30 minutes, or incubating with 0.1 mM arsenite, inhibited the NADPH-dependent PDR of the ascitic fluid. Activity was increased after treatment with 1 mM DTT, incompletely inhibited by 1 mM NEM, and inhibited somewhat more by 3 mM NEM after 1 mM DTT (text-fig. 2). In homogenate of washed Krebs-2 ascites cells, activity was abolished by heating at 70° C for 30 minutes, as it was by 1 mM NEM but not by 0.1 mM arsenite.

Disulfide Reduction, SH Blocking, and Tumor Growth

The allogeneic Krebs-2 ascites carcinoma and the syngeneic MCT ascites sarcoma could still induce tumor growth after treatment with 1 mM IA or NEM. They did, however, lose transplantability, in a reproducible way, when the cells were separated from their fluid, washed, resuspended, and treated with the SH-blocking agents in the washing medium at pH 7.4. Supravital stain exclusion was 40–70%, and the treated cells were still metabolizing in a Warburg respirometer (text-fig. 3). Whereas IA-treated cells respire at



TEXT-FIGURE 2.—NADPH-dependent protein-disulfide reductase activity of Krebs-2 ascites supernatant: 1) untreated; 2) heat-treated at 70° C for 30 minutes; 3) treated with 0.1 mM arsenite; 4) treated with 1 mM DTT; 5) treated with 1 mM DTT followed by 3 mM NEM; 6) treated with 1 mM NEM.

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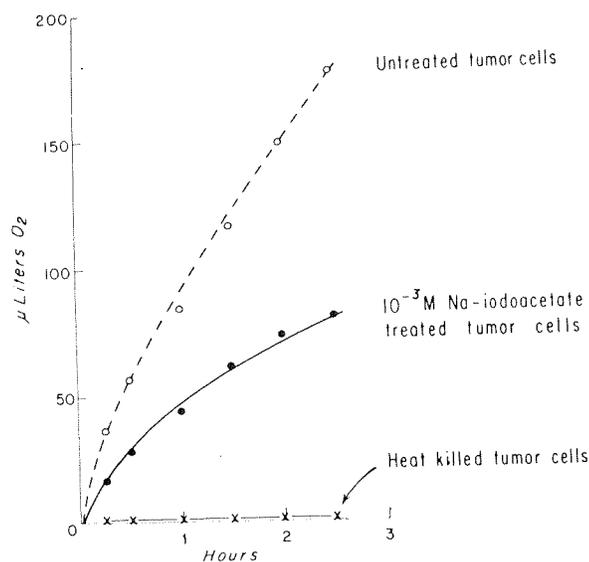
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*pH 7
†10/10
‡DTT
§Ca⁺⁺
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ating PH- was some- text- cites)° C at by mor and still mM ant- vere led, the tain vere eter at about half the normal rate, NEM-treated cells behave as control cells.⁵ Loss of transplantability was independent of dosage. Increasing the number of treated cells up to 16-fold did not result in tumor growth. Inactivation also occurred regularly when the washed tumor cells were resuspended and alkylated in ascitic fluid in which the NADPH-dependent PDR had been inhibited by heating at 70° C for 30 minutes or by 0.1 mM arsenite before SH blocking (table 3). Total inactivation was possible also when whole ascites tumor or only the fluid had been incubated 1 hour with 5 mM EDTA (viz. 2 mM calcium-disodium EDTA) before SH blocking with 1 mM NEM, or by the opening of the disulfides with 1 mM DTT before alkylation with 3 mM NEM. As much as 50-70% of the cells still seemed alive when tested by supravital stain exclusion. Treatment of the fluid with heat or arsenite or EDTA without exposing the cells to SH-blocking agents did not affect transplantability.

These results were confirmed with the 5 other ascites and 2 solid tumors investigated. Solid MCT and BAC/P were not inactivated by dispersion and alkylation in Krebs-Ringer buffer. However, both lost transplantability after their washed cells were alkylated with the SH-blocking agents in medium A with EDTA or in the released intercellular material pretreated with DTT. Apparent survival in this case was 15-30%. Reduction of

viable cells by 70-85% alone did not explain the loss of transplantability; tumor cell suspensions



TEXT-FIGURE 3.—Direct respirometry (oxygen uptake) of Krebs-2 ascites carcinoma cells in a Warburg constant volume respirometer (20-unit, refrigerated Cenco). Three flasks for each modality were loaded with 10⁸ tumor cells in a 2 ml Krebs-Ringer buffer, pH 7.4. Coefficient of variance <2%.

⁵ Respirometry on NEM-treated ascites tumor cells was performed by Prof. M. Pizzurra, Institute of Microbiology, University of Perugia, Italy.

TABLE 3.—Transplantability of murine ascites tumor cells after alkylation with 1 mM IA or NEM*

Ascites tumor	Host	Medium	Pretreatment†‡	Growing tumors/10
Krebs-2	Swiss	Fluid	—	10†
Krebs-2	Swiss	Fluid	DTT	0
Krebs-2	Swiss	Fluid	Heat	0
Krebs-2	Swiss	Fluid	Arsenite	0
Krebs-2	Swiss	Fluid	EDTA	0
Krebs-2	Swiss	BSS§	—	0
Krebs-2	Swiss	BSS + 1mM DTT	—	10
Krebs-2	Swiss	BSS + 1mM L-cysteine	—	10¶
Krebs-2	Swiss	BSS + 1mM GSH	—	10**
MCT	C57BL/P	Fluid	—	8
MCT	C57BL/P	Fluid	DTT	0
MCT	C57BL/P	Fluid	Heat	0
MCT	C57BL/P	Fluid	Arsenite	0
MCT	C57BL/P	BSS§	—	0

*pH 7.4, 37° C, 1 hour.

†10/10 survivors on day 10.

‡DTT=1 mM, pH 7.4, 25° C, 1 hour, followed by 3 mM IA, pH 7.4, 37° C, 1 hour; Heat=of fluid alone: 76° C, 1 hour; Arsenite=0.1 mM Na-arsenite, pH 7.4, 37° C, 1 hour; EDTA=5 mM disodium EDTA, pH 7.4, 37° C, 1 hour.

§Ca⁺⁺- & Mg⁺⁺-free BSS + 5 mM EDTA, pH 7.4.

||More lethal, 0/10 survivors on day 10.

¶More lethal, 4/10 survivors on day 10.

**8/10 survivors on day 10.

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with as little as 1-3% unstained cells gave rise to tumor growth.

There was total SH protection against inactivation if washed tumor cells were incubated in BSS containing 1 mM of both NEM and an SH compound, e.g., DTT, L-cysteine (free base), or GSH (table 3). When the cells were incubated in 1 mM DTT + 1 mM NEM, resulting tumors were of increased malignancy, killing all 10 animals within 10 days. When tumor cells were incubated in a fluid reduced with 1 mM DTT but not alkylated, they also induced tumors of greater malignancy; all 10 inoculated animals were dead by day 8. Controls, inoculated with the same number of untreated cells, survived an average of 20 days. When tumor cells were protected with L-cysteine, resulting tumors were more malignant than in controls but less so than after DTT protection. Protection with GSH resulted in tumors of usual malignancy.⁶ All 10 controls, inoculated with the same tumor cells and treated with NEM only, remained free of tumor.

Washed tumor cells, inactivated by 1 mM NEM, could be reactivated by the same SH compounds and by native cell-free ascitic fluid. However, ascites was only noticeable 10 days after inoculation and appeared first after reactivation with DTT. No tumors developed in 10 controls when no SH compound or native fluid was added to the NEM-treated tumor cells.

SH Blocking, PDR, and Immunogenicity

Pretreatment of the ascitic fluid with DTT, EDTA, arsenite, or heat had a marked effect on the immunogenicity of ascites tumor. When washed tumor cells were alkylated with IA in BSS, it did not matter whether they had been previously reduced with DTT or treated with arsenite. However, protection was more extensive when the suspending medium was the ascitic fluid proper, where the disulfides had been opened by DTT before alkylation with an excess of IA. Results were best if the SH's of the fluid had been stabilized (15) (viz. made more accessible) by EDTA. Immunogenicity was also increased if the NADPH-dependent protein disulfide reductase of the fluid had been inactivated by either heat or arsenite (table 4). Under these conditions, the whole ascites seems more immunogenic than the cells alone; the treated fluid adds to the protection conferred. Isogenic controls, sensitized with SH-blocked normal cells (spleen) of their own host strain, did not exhibit any tumor immunity; thus tumor-specific antigens were involved when SH-blocked tumor cells were used. Apparently, the SH-blocking agents did not act by a simple effect of adjuvancy.

⁶ The GSH used was subsequently found partially oxidized.

TABLE 4.—Immunization of C57BL/6J against 755 ascites carcinoma with the IA-treated tumor* depending on disulfide reduction†

Medium	Pretreatment	Number of tumors/10 after challenge	Survival with tumor (days)
Fluid	1 mM DTT‡	3	60.0 ± 1.13
Fluid	0.1 mM arsenite§	2	65.0 ± 0.9
Fluid	Heat	4	52.0 ± 1.8
Fluid	5 mM EDTA§	1	32.0
Fluid	—	7	28.1 ± 0.85
BSS	—	8	30.2 ± 0.66
BSS + 1 mM DTT	—	8	28.4 ± 2.1
BSS + 0.1 mM arsenite	—	10	27.8 ± 0.8
Challenge controls¶	—	—	—

*1 mM, pH 7.4, 37° C, 1 hour; 3 mM after 1 mM DTT.

†Reduction of disulfides with DTT; stabilization of SH's with EDTA; inhibition of NADPH-dependent PDR with arsenite or heat.

‡pH 7.4, 25° C, 1 hour.

§pH 7.4, 37° C, 1 hour.

||Of fluid alone; 70° C, 30 minutes.

¶Controls and sensitized mice challenged with 2×10⁶ fresh, untreated cells of 755 ascites tumor. Sensitization ip with 2×10⁶ IA-treated tumor cells +2 boosts at 14-day intervals.

DISCUSSION

SH blocking yields loss of transplantability, but not necessarily cell death. Cell survival is established by supravital stain exclusion, by respirometry, and, even more convincingly, by reactivation with SH compounds or native cell-free fluid.

Protection of tumor cells by SH compounds adds evidence that their proliferation depends on reactive SH's. The shortened survival after DTT protection and the effects of DTT alone suggest that disulfide reduction and reactive SH's affect the degree of malignancy.

Ascitic fluid and intercellular material of solid tumors also protect the tumor cells against inactivation by SH blocking. This protection can be overcome by 1) opening disulfides with DTT before blocking with an excess of alkylating agent; or 2) washing the cells with BSS containing EDTA and incubating them in ascitic fluid with 2–5 mM EDTA [1 mM EDTA completely prevents oxidation of GSH (15)]. It appears that when active PDR is present in the fluid, sulfhydryls have to be stabilized for the SH alkylation to inactivate the tumor cells. However, this is no longer prerequisite if the PDR of the fluid is inhibited. The protection by extracellular material is consistent with the failure of 1 mM NEM to inactivate the NADPH-dependent PDR of the fluid (text-fig. 2). It can be suppressed with 0.1 mM arsenite or by heat (70° C for 30 min) before alkylation. Such measures have no influence on GSSG reductase, and arsenite does not inactivate GSH transhydrogenase. Both, however, inhibit the PDR of the fluid. Ascites tumor can be inactivated by the SH blocking if its fluid does not contain disulfides that can be reduced by PDR or active reductase capable of converting available disulfides.

It may seem paradoxical that SH blocking should inactivate after reduction with DTT, which mimics the reductase. However, 3 molecules of the blocking agent are used for each molecule of DTT, which accounts for the 2 SH's of DTT and leaves a sufficient excess of alkylating agent. It is less obvious why separated washed tumor cells can be inactivated though they contain disulfides and NADPH-dependent PDR. However, these cells have been exposed to EDTA, the effects of which are comparable to those of DTT, it stabilizes SH's and helps to convert disulfides to reactive SH's (15).

Inactivation by IA or NEM after pretreatment with arsenite is not due to residual arsenite at a number of intracellular loci, since arsenite alone did not inactivate. Failure to suppress NADPH-dependent PDR activity in homogenate of Krebs-2 ascites tumor cells by 0.1 mM arsenite contrasted with extensive inhibition in the fluid and opposed the findings of Tietze (5) with normal rat liver. The enzyme may be protected by an increased number of SH's in tumorous versus normal cells.

We found that tumor growth seemed dependent on the free, reversible reduction of cellular and extracellular disulfides. This reduction is normally performed by GSSG reductase, GSH disulfide transhydrogenase, and the nucleotide-dependent PDR. Activities of the third of these were conspicuously higher in tumor cells—even higher than in normal liver and in embryo. While Tietze (16) never found activity outside the boundaries of normal cells, we noted activity in tumor exudate and in serum of tumor-bearing mice. Values for GSSG reductase and GSH transhydrogenase were close to normal. NADPH-dependent PDR could, therefore, play the greater role in malignant proliferation. In preliminary assays of human samples, values for the NADPH-dependent PDR in metastases of a carcinoma of the lung were considerably higher than in nontumorous liver; thus our findings may have validity for human tumors.

In contradistinction to our data on the NADPH-dependent PDR, the thioredoxin-thioreductase complex is present at comparable levels in both tumor extracts and normal adult liver (17). While this suggests that the thioredoxin system may be unrelated, the discrepancy could actually be due to differences in procedure. Criterion for thioredoxin system activity was the amount of deoxyribonucleotide formed, and EDTA was absent from the reaction.

Immunogenicity of ascites tumors may well vary with their disulfide-SH balance. A loss of antigenic activity has been induced repeatedly by reduction of the disulfides of isolated proteins. Peters and Goetzl saw this with bovine serum albumin (18–20), Freedman and Sela (21) with IgG. Caputo et al. (22) found that a glycoprotein from normal human serum and one from Yoshida ascites sarcoma lost the capacity of precipitating with specific antibody when >1 of their 4 disulfides was opened

by reduction. Antigenic activity was entirely recovered with reoxidation (18-21). When native BSA is completely reduced and then SH-blocked with IA, it becomes a new potent immunogen which still lacks precipitating and complement-fixing activity but induces high titers of non-IgG antibodies and brings about passive hemagglutination and cutaneous anaphylaxis (23). Higher SH levels in proliferating cells (24) and in fetal and malignant tissues (25) are paralleled by immunologic sluggishness (26-31). Consistent with these premises, significant immunity to transplantable tumors was brought about by Apffel et al. (1, 2) with SH-blocked tumor cells. The increased immunogenicity of SH-blocked tumor cells was confirmed by Jasmin et al. (32) with a Rauscher virus-induced leukemia and, more recently, by Prager et al. (33, 34) with an ascites lymphosarcoma. Grassetti (35) found that the dissemination of tumors from inocula of cells treated with the SH-oxidizing agent di-thiodinitric acid was reduced. With our latest technique of SH alkylation, we now achieve more reproducible and more extensive tumor immunities.

Our data and the preceding considerations suggest that reactive SH's, convertible disulfides,⁷ the nucleotide-dependent PDR, and hydrogen-providing NADPH are prerequisites of tumor growth in mice and affect the immunogenicity of the tumor cells.

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⁷ After this study was completed, a corroborating communication came to our attention; it stated that mouse leukemia cells in culture are highly dependent on thiols and disulfides (J.D. Broome and M.W. Jeng, *J Natl Cancer Inst* 49:579-581, 1972).

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