[CANCER RESEARCH 42, 2980–2984, August 1982]

Zinc-induced Resistance to Alkylating Agent Toxicity[1]

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ABSTRACT

Suspension cultures of Chinese hamster ovary cells and three derived cadmium-resistant variants were exposed to 100 μM $ZnCl_2$ prior to treatment with the alkylating agent, melphalan, and cytotoxicity was then determined by measuring colony-forming ability. A 10-fold or greater enhancement in survival of all zinc-pretreated cultures subsequently exposed to melphalan was observed which was unrelated to metallothionein induction capacity. Although the maximum achievable protection afforded by zinc occurred in cultures receiving 100 μM $ZnCl_2$, concentrations of zinc only slightly in excess of levels found in human serum were shown to provide a 4.5-fold enhancement of protection, indicating that the phenomenon can also be induced at physiologically reasonable levels. These results suggest the existence of a novel zinc-inducible mechanism which protects cells against the toxic effects of alkylating agents.

INTRODUCTION

Alkylating agents comprise an extremely useful class of anticancer drugs effective against a wide variety of tumors. One of the major limitations to their use, however, is their toxicity for normal cells, with cells of the hematopoietic system being particularly sensitive, resulting in cumulative myelosuppression (5, 11). If a method could be found to enhance selectively the resistance of normal cells to alkylating agent-induced toxicity, higher, more effective dose levels could be utilized without an accompanying increase in normal cell toxicity.

Relative to those considerations, it is known that a variety of chemical and physical agents (*e.g.*, alkylating agents, transition series metals, chelating drugs, amino acid analogs, heat shock, etc.) elicit the induction of a series of protein species (6, 9, 12, 14, 15, 17), some of which react with the agents and render them nontoxic. The most thoroughly studied type of inducible, protective protein is MT,[3] a class of low-molecular-weight, cysteine-rich proteins which are synthesized following exposure of animals or cultured cells to a variety of heavy metals, including cadmium (9, 12, 16), zinc (1, 28, 29), and mercury (20, 29).

Of particular interest in this regard is the observation by Kotsonis and Klaassen (14) of an increase in MT content in hepatic tissue from rats which received alkylating agents. In view of the thiol-rich nature of MT [approximately one-third of the amino acids comprising MT are cysteine (13)] and the propensity for alkylating agents to intereact with sulfhydryl moieties, it appeared that the induction of MT following exposure to alkylating agents observed in the animal studies of Kotsonis and Klaassen (14) might represent a potential cellular detoxification response; this putative response would involve induction of MT which would bind the alkylating agents, thereby preventing them from interacting with essential cellular targets.

If this notion is correct, cells which differ in their capacities to induce synthesis of MT should exhibit a corresponding variability in sensitivity to alkylating agent-induced cytotoxicity (*i.e.*, cells possessing high levels of MT should be much more resistant than MT-poor cells). We were able to test this hypothesis directly due to the isolation of a series of cultured cell variants of CHO cells which differed in their capacity to synthesize MT when exposed to $ZnCl_2$.

In this report, we have monitored the relationship between MT inducibility and alkylating agent-induced toxicity to provide information on the role of MT in detoxification of alkylating agents. Our studies demonstrate the existence of a novel zinc-inducible protective response which results in an approximately 10-fold enhancement of survival of cells exposed to zinc prior to treatment with L-phenylalanine mustard. Contrary to our expectations, this dramatic degree of protection is not attributable to the synthesis of MT.

MATERIALS AND METHODS

The cells utilized in this report were derived from CHO cells, established originally by Tjio and Puck (25). The selection procedure utilized to isolate variants differing in their capacity to survive treatment with cadmium is similar to the method used by Rugstad and Norseth (21). Briefly, cells in monolayers were exposed to gradually increasing concentrations of $CdCl_2$ ranging from 0.2 to 30 μM (10), and cultures exhibiting the growth rate of the untreated CHO cell when maintained in medium containing 2, 20, or 30 μM $CdCl_2$ were cloned, and the derived variants were designated Cd^{r}2C10, Cd^{r}20F4, and Cd^{r}30F9, respectively. The cloned variants retained their resistance to cadmium during a 6-month period of continuous maintenance in cadmium-free medium (10). Detailed biological and biochemical characterization of the variants and line CHO is presented elsewhere (3, 4, 9, 10). Cells utilized in experiments were grown in suspension culture in F-10 medium supplemented with 15% newborn calf serum, penicillin, and streptomycin. The medium utilized for studies with the 20F4 cell was additionally supplemented with 10 μM $ZnCl_2$ to maintain a stable growth rate; this concentration of zinc did not cause induction of MT synthesis.

Stock solutions of zinc chloride (0.1 M) were prepared in 0.01 N HCl, sterilized by filtration through a 0.22-μm diameter Swinnex GS filter unit (Millipore Corporation, Bedford, Mass.), dispensed as small aliquots into polyethylene tubes, and stored frozen at −20°. Aliquots were thawed and diluted in sterile distilled water (in polyethylene tubes) immediately prior to addition to cultures. Except where stated to the contrary, $ZnCl_2$ was utilized at a concentration of 100 μM, with cells incubated with zinc for 9 hr prior to addition of the alkylating agent.

Melphalan (L-phenylalanine mustard, sarcolysin), NSC 8806, was a gift from Dr. David Abraham, Investigational Drug Branch, National Cancer Institute, Bethesda, Md. Solutions of melphalan were prepared immediately prior to utilization by dissolving the drug in acidic ethanol [0.05 N HCl-70% (by volume) ethanol]. Suspension culture cells were utilized in all drug exposure studies. The concentration of melphalan

[1] This work was sponsored by the United States Department of Energy.

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[3] The abbreviations used are: MT, metallothionein; CHO, Chinese hamster ovary.

Received November 23, 1981; accepted May 11, 1982.

utilized in these studies (8 μM) was selected on the basis of preliminary experiments as a level which would induce a 2- to 3-log decrease in survival.

In view of the extreme sensitivity of line CHO and its derivatives to serum-free medium, the mephalan was added directly to cultures in normal (*i.e.*, serum-containing) medium. A linear decrease in survival was obtained during the exposure periods used in the studies described in this report, and the degree of experiment-to-experiment variability was minimal, indicating that the presence of serum was not adversely affecting melphalan activity. Our results agree with the findings of O'Neill *et al.* (18), who found that the cytotoxicity and mutagenicity stemming from treatment of CHO cells with a variety of alkylating agents was the same whether or not the medium was supplemented with serum.

For survival measurements, aliquots of cells were removed from suspension culture, washed, counted, diluted in drug-free medium, and plated (from 200 to 10^4 cells/dish) directly into 60-mm plastic tissue culture dishes. Following a 7-day incubation period, the plates were washed, fixed, and stained with crystal violet prior to determining survivors (*i.e.*, colonies of 50 or more cells). Plating efficiencies ranged from 88 to 96%, with no significant differences in plating efficiencies observed between zinc-pretreated and control cultures. Results were calculated as a percentage of the appropriate sham-treated control. This technique permits detailed cell accountability and, since it does not involve a trypsinization step, it also eliminates possible trypsin-specific effects on survival which would complicate interpretation of data.

The method utilized to determine the MT synthesis rate (7) is a variation on a technique developed by Steinberg *et al.* (23). In this procedure, Sephadex G-75 column chromatography of cytoplasmic fractions pulse-labeled with [^{35}S]cysteine was utilized to separate the [^{35}S]MT from larger, ^{35}S-containing cytoplasmic components and from unincorporated amino acid. The relative rate of MT synthesis was calculated as 100 times the fraction of ^{35}S cpm in the MT peak relative to the ^{35}S cpm in non-MT proteins which eluted prior to MT.

RESULTS

Previous studies from this laboratory (9) indicated a differential effect of zinc on induction of MT in the 2C10 and CHO cells. When comparable studies were performed with the entire set of cells utilized in this report, the results presented in Chart 1 were obtained. All 3 cadmium-resistant variants responded to treatment with zinc by elaborating synthesis of MT. In contrast, zinc was without effect on MT synthesis in the CHO cell.

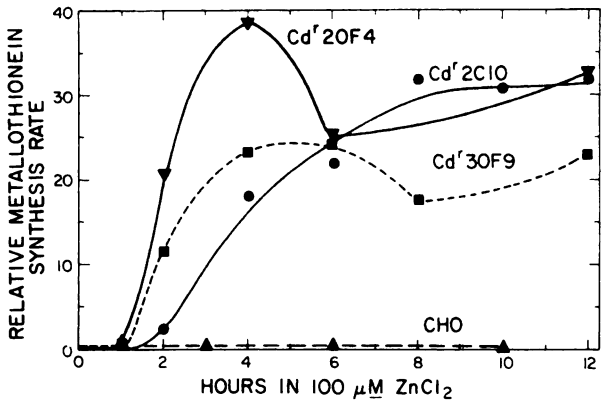

Chart 1. MT induction kinetics in CHO cells and in a series of CHO-derived, cadmium-resistant variant cells as a result of exposure to zinc. Zinc chloride was added to each culture (100 μM) and maintained throughout the entire course of the experiment. Relative rates of MT synthesis were determined in the manner described previously (7). ▲, CHO; ●, 2C10; ▼, 20F4; ■, 30F9.

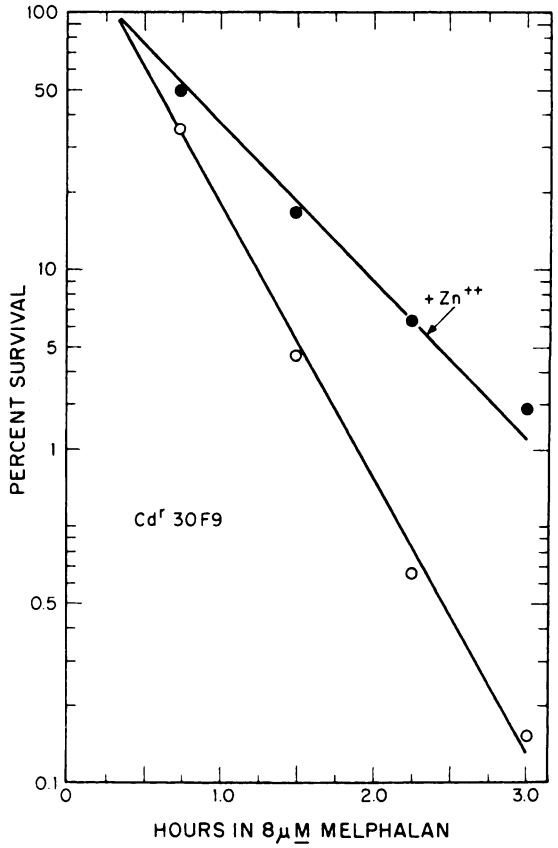

Chart 2. Survival of melphalan-treated 30F9 cells in the presence or absence of zinc pretreatment. $ZnCl_2$ was added to a final concentration of 100 μM in the zinc-pretreated culture. After a 9-hr incubation period, melphalan (8 μM) was added to both cultures, and aliquots were removed from each at varying times thereafter and plated in melphalan-free normal medium. Following a 7-day incubation period, colonies containing 50 cells or more were scored as survivors. ●, zinc-pretreated culture; ○, results obtained with the non-zinc-pretreated control culture.

If MT plays a major role in reducing alkylating agent-induced cytotoxicity as postulated, then the zinc-pretreated 2C10, 20F4, and 30F9 cells, which possess high rates of MT synthesis when first exposed to melphalan, should exhibit enhanced resistance relative to their non-zinc-treated counterparts. Similarly, CHO cells should not benefit from pretreatment with zinc since $ZnCl_2$ failed to enhance the synthesis of MT in that cell. Any variation from that pattern (such as a lack of enhancement of survival in zinc-pretreated 2C10, 20F4, or 30F9 cells or an increase in resistance of zinc-treated CHO cells) would indicate that MT does not play a major role in the detoxification of alkylating agents.

In Charts 2 to 5 are presented the results obtained when each of the 4 cell types was exposed to 8 μM melphalan ± a 9-hr pretreatment period with 100 μM $ZnCl_2$. After 3 hr in melphalan, the 30F9 (Chart 2), 20F4 (Chart 3), and 2C10 (Chart 4) cultures which were pretreated with zinc all exhibited an approximately 10-fold enhancement in survival (measured at the end of the 3-hr experiment) relative to the noninduced control cultures, a finding that appears to be consistent with the notion that MT synthesis provides an important mechanism for detoxification of alkylating agents. The data in Chart 5, however, clearly disprove the validity of this hypothesis, since CHO cells pretreated with zinc exhibit an increase in survival which is nearly identical to that observed in the variant cells,

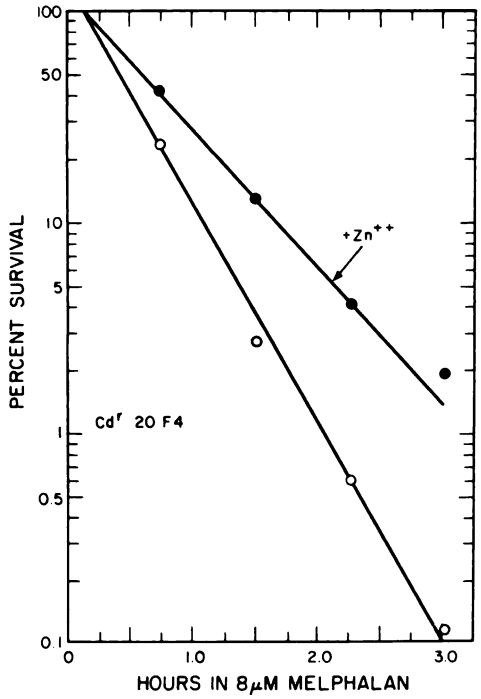

Chart 3. Survival of melphalan-treated 20F4 cells in the presence or absence of zinc pretreatment. Experimental details and symbols utilized are those described in Chart 2.

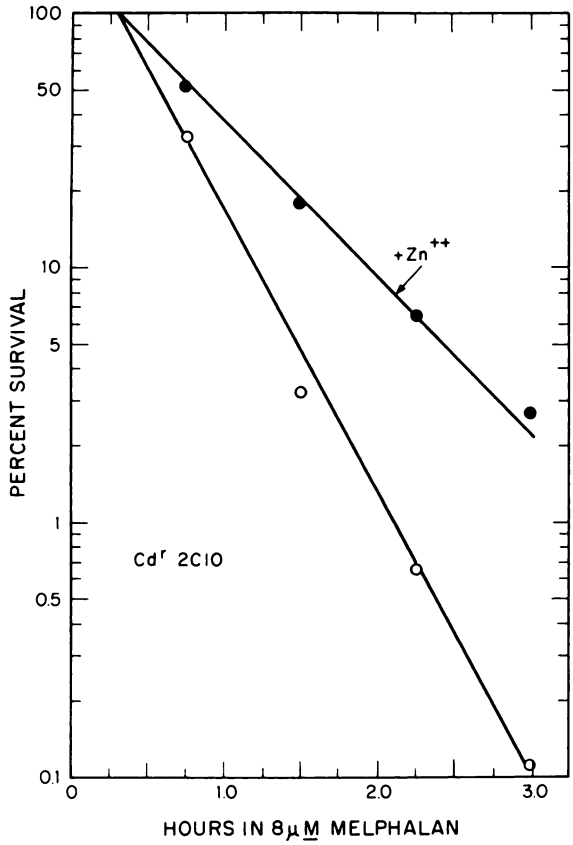

Chart 4. Survival of melphalan-treated 2C10 cells in the presence or absence of zinc pretreatment. Experimental details and symbols utilized are those described in Chart 2.

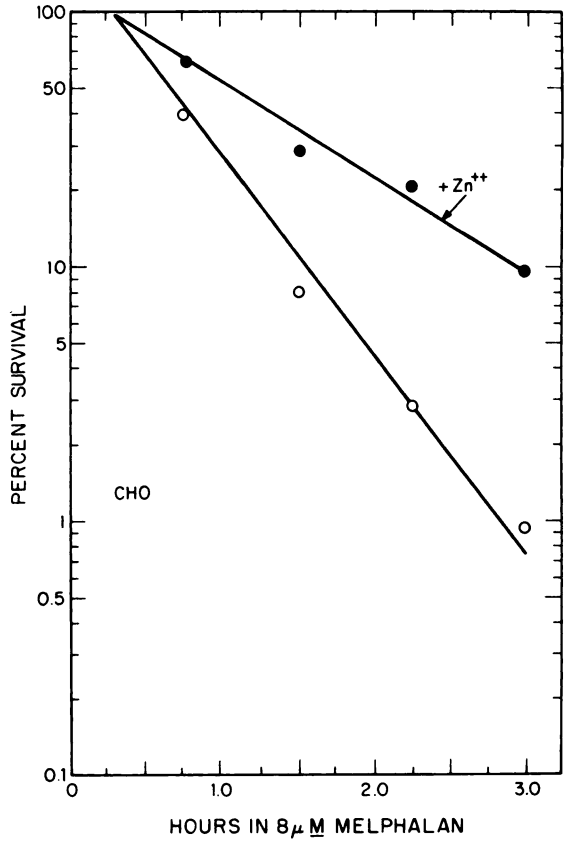

Chart 5. Survival of melphalan-treated CHO cells in the presence or absence of zinc pretreatment. Experimental details and symbols utilized are those described in Chart 2.

even though the rate of MT synthesis remained unchanged as a result of treatment with $ZnCl_2$ (Chart 1).

A further indication that the zinc-induced response is not attributable to MT synthesis is provided by a companion set of studies with this same series of cells exposed to a different alkylating agent, iodoacetate (26). Not only did zinc-pretreated CHO cells exhibit a greatly enhanced resistance to the toxic effects of iodoacetate in the absence of an elevation in MT synthesis rate, but also zinc-pretreated 2C10 cells, under conditions which greatly enhanced MT synthesis rates, failed to exhibit an increase in survival, providing further proof that resistance and MT synthesis capability are not related in any simple fashion. Additional support for this conclusion arises from measurements of intracellular localization of radiolabeled iodoacetate in zinc-pretreated CHO and 30F9 cells in which there is little binding of alkylating agent to MT species in either cell (27). Taken together, these data indicate the existence of a zinc-inducible protective response which does not involve synthesis of MT.

Because the experiments described in this manuscript were designed to determine whether MT could protect cells against the toxic effects of alkylating agents, the conditions selected for zinc pretreatment were those which maximized synthesis-stability of MT (8). That is, there is no reason to assume *a priori* that the conditions which maximize MT synthesis are necessarily ideal for induction of the protective response. Accordingly, 2 experiments were carried out in which either the length of the zinc preinduction period or the concentration of $ZnCl_2$ were varied, with survival the endpoint.

Table 1

Effect of varying the time of $ZnCl_2$ preincubation period on enhancement of resistance of CHO cells to melphalan-induced cytotoxicity

The cells were treated with 8 μM melphalan for 3 hr prior to plating for survival.

Duration (hr) of pretreatment period with 100 μM $ZnCl_2$	% of survival	Relative enhancement of survival (−fold)
0	0.50	
3	0.66	1.3
6	5.8	11.6
9	10.4	20.8
12	8.4	16.8

Table 2

Effect of varying the concentration of $ZnCl_2$ during the preincubation period on enhancement of resistance of CHO cells to melphalan-induced cytotoxicity

The cells were treated with 8 μM melphalan for 3 hr prior to plating for survival.

Concentration (μM) of $ZnCl_2$ during pretreatment period	% of survival	Relative enhancement of survival (−fold)
0	0.58	
20	2.6	4.5
40	2.4	4.1
60	1.6	2.8
80	5.1	8.8
100	9.5	16.4

CHO cells were utilized for these studies. In Table 1 are results obtained when the $ZnCl_2$ pretreatment period was varied over the range of 3 to 12 hr. The maximum enhancement of survival was obtained in the culture pretreated for 9 hr, although a 12-hr incubation period appeared to yield nearly equivalent results. Thus, it appears that the kinetics of induction of the protective response closely mimics those established empirically for induction of MT.

The effect of varying the concentration of $ZnCl_2$ utilized during the pretreatment period (prior to addition of melphalan) is shown in Table 2. It is readily apparent that the 100 μM concentration of $ZnCl_2$ yields the greatest enhancement. This is the highest nontoxic concentration of $ZnCl_2$ that can be utilized in line CHO and its derivatives; a concentration of 125 μM $ZnCl_2$ produces extensive cell killing (9), indicating an extremely narrow concentration range between nontoxic and toxic levels of this essential trace element. Note, however, that even at the 20 μM level there is a significant enhancement in survival; this level of zinc is approximately 2 times the lowest concentration of this element present in adult human serum (24). Thus, this novel protective response can be elicited under nearly physiological conditions.

DISCUSSION

The results presented in this report indicate the existence in cultured Chinese hamster cells of a novel zinc-inducible protective response which grossly decreases alkylating agent cytotoxicity. Preliminary experiments presented elsewhere (27) indicate that the protection is not attributable to decreased extracellular levels of available alkylating agent in media containing high concentrations of zinc, nor is it due to a zinc-induced alteration in permeability to the alkylating agent. Furthermore, based upon the differences in inducibility of MT achieved among the various types of cells used (Charts 1 to 5), it is clear that MT is not involved to any major extent in the detoxification of alkylating agents.

Cagen and Klaassen (2), utilizing an entirely different experimental approach and intact animals rather than cultured cells, arrived at a similar conclusion regarding the noninvolvement of MT as a major source for conjugation of alkylating agents. Those authors examined the binding of radiolabeled iodoacetate to MT isolated from hepatic homogenates of rats which had been pretreated with zinc to boost the level of MT. Their results suggested that, while the sulfhydryl groups on MT can act as binding sites for alkylating agents, the greater affinity of alkylating agents for glutathione (in preference to MT) implied that MT did not represent a major pathway for conjugation of alkylating agents. Thus, our *in vitro* findings utilizing cytotoxicity as the end point are in excellent agreement with the *in vivo* results obtained by Cagen and Klaassen (2), indicating that comparable mechanisms (in regard to zinc-induced MT synthesis and interaction of MT with alkylating agents) are operative in these 2 very different biological systems.

Not only are our results biochemically similar to those occurring in intact animals, but at least in this instance they appear to be physiologically relevant as well. The lowest level of zinc which induces the protective response in our Chinese hamster cells (Table 2) is not grossly above the normal levels of zinc found in human serum (24), implying that the response is inducible within a reasonable physiological range, an important consideration if this phenomenon one day is exploited for use in the clinic.

We have not yet identified the mechanism responsible for this interesting zinc-induced protective response, but several speculative explanations may be proposed for consideration. One possibility is that zinc induces an increase in the intracellular level of glutathione or one of its metabolites. The known conjugation of electrophiles with the cysteine moiety of glutathione plus the demonstrated preferential binding of alkylating agents in rat liver to glutathione as opposed to MT (7) suggest such an explanation. Among other possibilities as zinc-induced products are non-MT proteins which might be capable of binding and inactivating the alkylating agents or enhancing their catabolism. Regarding the first point, while there is no direct evidence which bears on this notion, stressful conditions which cause the induction of MT also induce the synthesis of a series of additional proteins, the function of which has not yet been determined (6, 9, 12, 15). The catabolism of alkylating agents would result in a reduction in the effective intracellular concentration of the active forms of the alkylating agents.

The work of Samson and her colleagues with bacteria and mammalian cells perhaps suggests a zinc-induced enhancement in DNA repair capacity. With regard to this possibility, Samson and Schwartz (22) have demonstrated an enhancement of resistance to high levels of the alkylating agent, *N*-methyl-*N'*-nitronitrosoguanidine in CHO and cultured human skin fibroblasts which were chronically exposed to sub-toxic levels of *N*-methyl-*N'*-nitronitrosoguanidine prior to challenge with normally toxic levels of the drug. The degree of protection observed in these cells was roughly equivalent to that observed in the present zinc enhancement studies. The protection did not appear to be attributable to changes in cell cycle distribution or altered drug permeability or detoxification, leading Samson and Schwartz to conclude that the low levels of *N*-methyl-*N'*-nitronitrosoguanidine induced an adaptive DNA repair system in the resistant cells. While the precise nature of the mechanism responsible for the observed resistance might be open to question, it is important to note that both the studies

of Samson and Schwartz and the present report describe an inducible (adaptive) response which greatly increases the resistance to alkylating agent-induced toxicity, perhaps suggesting a similar or identical domain of responses induced by zinc and low levels of alkylating agents.

Although it is not yet possible to determine the relevance of these cultured cell studies to the clinic, at least one line of evidence suggests that zinc triggers the induction of protective proteins in human tissue and, under appropriate conditions, elicits a differential response in normal and tumor tissue. Phillips (19) demonstrated that incubation of freshly collected normal human lymphocytes with zinc transferrin resulted in synthesis of an inducible protective protein species at a level which was 3.6 to 21 times greater than the level of that protein induced in lymphocytes from leukemic patients. While the protective protein induced in Phillips' study (MT) is clearly not responsible for the phenomenon observed in this report, Phillips' findings suggest that conditions can be found in which normal cells mount a much greater inducible protective response than do tumor cells. If the zinc-inducible substance responsible for protecting cells against alkylating agent toxicity behaves in similar fashion, pretreatment of a patient with an appropriate level of zinc prior to initiation of alkylating agent therapy could result in a selective sparing of normal tissue. A detailed description of possible ramifications of our findings to cancer chemotherapy is presented elsewhere (27).

Studies designed to elucidate the mechanism responsible for this novel protective response are continuing.

ACKNOWLEDGMENTS

We gratefully acknowledge the excellent technical assistance of Joseph G. Valdez, John L. Hanners, and Brenda V. Aguirre.

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