0022-3565/89/2492-0552$02.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 249, No. 2
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Printed in U.S.A.

Prophylaxis of Cyanobacterial and Mushroom Cyclic Peptide Toxins[1]

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Accepted for publication February 6, 1989

ABSTRACT

The pathogenicity of virulent cyclic peptide toxins of the cyanobacterium, *Microcystis aeruginosa*, and the mushroom, *Amanita phalloides*, was prevented in mice by pretreatment with a variety of chemically unrelated agents including hydrocortisone, shellac, certain diazo and triazine dyes and cyclosporine A. Despite the diverse nature of the protective agents, a feature commonly associated with protection was the ability to impair hepatic uptake of ^{51}Cr-labeled sheep erythrocytes, a function of hepatic macrophages (Kupffer cells). In addition, several of the protective agents are known to affect other aspects of reticuloendothelial cell function. Therefore it seems likely that the hepatic macrophage is involved in the observed protection, although by what mechanism(s) is unknown. The most remarkable prophylaxis was seen with a single injection of Trypan red, which provided nonimmunologic protection against a lethal dose of a cyanobacterial toxin, cyanoginosin-LR, for periods up to 3 months.

Ingestion of *Amanita phalloides* can be lethal to humans and several hundred deaths occur annually worldwide due to this and related mushrooms (Wieland and Wieland, 1959). Lethality is attributable to cyclic peptides, primarily the amanitins. Although only indirect evidence links the cyanobacterium, *Microcystis aeruginosa*, to human illness, ingestion of these bacteria (formerly termed blue-green algae) can rapidly kill other mammals and birds (Falconer *et al.*, 1983; Konst *et al.*, 1965). Virulence is attributable, at least in part, to the cyanoginosins, a group of cyclic heptapeptides which contain five homologous amino acids and two variable L-amino acids (Elleman *et al.*, 1978; Botes *et al.*, 1984). As part of our continuing study of cyanoginosin pathophysiology, inhibitors were sought which might provide insight into the mechanism of action of these toxins. As a result, we have identified several chemically unrelated agents that prevent the lethality of not only some cyanoginosins, but the mushroom toxins, α-amanitin and phalloidin, as well. The cyanoginosin containing leucine and arginine as the variable amino acids (cyanoginosin-LR) was selected for detailed study because its dose-response curve has been well established with the Brookhaven National Laboratory Hale-Stoner outbred strain of Swiss albino mice and the pathogenesis of its toxicity in mice has been partially characterized (Slatkin *et al.*, 1983; Adams *et al.*, 1985, 1988). However, our findings also extend to cyanoginosin-RR and, in part, to cyanoginosins -LY and -LA (R. D. Stoner, unpublished observations).

Received for publication October 7, 1988.
[1] This work was supported in part by Grant ES03302-01A1 from The National Institutes of Health, Project Order 85PP5825/DCRN from the U.S. Army Medical Research and Development Command and Contract DE-AC02-76CH00016 with the U.S. Department of Energy. Accordingly, the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or allow others to do so, for U.S. Government purposes.

Materials and Methods

Animals. Female Swiss mice of the Brookhaven National Laboratory Hale-Stoner strain aged 8 to 12 weeks and weighing 25 to 30 g were used in most experiments, the exception being the use of 4-month-old male C57 mice for the initial screening of some dyes for their ability to block the toxins.

Toxins. The three toxins studied were cyanoginosin-LR, phalloidin and α-amanitin. Cyanoginosin-LR was isolated from *M. aeruginosa* strain 006 furnished by Professor G. H. J. Krueger, Department of Botany, University of the Orange Free State, Bloemfontein, South Africa. Purification of the toxin was by the procedure described previously (Slatkin *et al.*, 1983; Siegelman *et al.*, 1984). The median lethal dose of cyanoginosin-LR is 0.06 mg/kg b.wt. (mg kg^{-1}) (Slatkin *et al.*, 1983). A dose of 0.1 mg kg^{-1}, which is 100% lethal by i.v. or i.p. injection, was chosen for the present study. Except where otherwise stated, the doses of phalloidin and α-amanitin (Sigma Chemical Co., St. Louis, MO) were 2.0 and 0.4 mg kg^{-1}, respectively.

Chemicals. The agents tested for their ability to induce protection and to alter hepatic macrophage phagocytic function included hydrocortisone (Solu-Cortef, The Upjohn Co., Kalamazoo, MI), Higgins India Ink (Waterproof Drawing Ink, No. 4415, Faber-Castell, Newark, NJ), solubilized shellac (as a 23% solution of the ammonium salt, kindly provided by Faber-Castell), Trypan blue (Mallinckrodt Inc., St. Louis, MO), Trypan red (Gurr, Santa Monica, CA), Reactive Red 120 and Reactive Blue 4 (Sigma, St. Louis, MO), injectable cyclosporine A (Sandimmune, Sandoz Pharmaceuticals, East Hanover, NJ) and carbon tetrachloride. Other agents tested only for their ability to block the lethality of the cyclic peptide toxins included 10% suspensions of carbon black particles (0.0236 and 0.1796 mm, Duke Standards, Palo Alto, CA), the carbon sediment obtained after centrifuging Higgins India Ink at 5 × 10^4 *g* for 4 hr, the diazo dyes Evan's blue, Congo red, Biebrich scarlet and benzopurpurin 4B (Sigma, St. Louis, MO) and suramin, a colorless derivative of the diazo compounds (Mobay Chemical Corp., New York, NY). In addition, Faber-Castell provided two

other components of India ink: Carbon Black Dispersion No. 8, a 10% dispersion of carbon black in a 4% solution of alkali hydrolized gelatin, and a 39% solution of ammonium hydroxide-hydrolyzed gelatin.

Procedures. Screening of an agent for toxin prophylaxis was performed using 1) three groups of mice pretreated with the agent and 2) a group of control mice; 5 to 10 mice were placed in each group. Toxin challenge was carried out 1 hr, 1 day and 1 week after administration of the agent being screened. In every experiment involving toxin challenge, all administered toxins were lethal to 100% of unprotected control mice at the doses stated, and the i.v. and i.p. routes of administration were equally effective. Each dye was tested initially as a 1% solution (w/v) in distilled water or phosphate-buffered saline, the i.p. dose being 100 to 200 mg kg^{-1}.

To determine if hydrocortisone-induced protection was nongenomic (Duva *et al.*, 1983), cycloheximide, 3 mg kg^{-1}, was injected s.c. with, and 1.5 hr after, the i.p. injection of hydrocortisone, 500 mg kg^{-1}. Another group of mice received progesterone, 200 mg kg^{-1} s.c. 1 hr before hydrocortisone (Tsurufuji *et al.*, 1979). Three hours after the initial injections the two groups of mice were challenged i.p. with 0.1 mg kg^{-1} of cyanoginosin-LR.

For *in vivo* study of hepatic macrophage (Kupffer cell) phagocytic function ^{51}Cr-labeled sheep erythrocytes were prepared by the method of Souhami (1972), and 1×10^7 cells were injected into mice *via* a tail vein. Two hours later the mice were sacrificed under ether anesthesia. The spleens and livers were removed and counted separately in a gamma scintillation counter, and the ratio of splenic to hepatic radioactivity was calculated. An increase in the spleen-to-liver ratio of the label is primarily an index of impaired hepatic macrophage uptake of the labeled cells (Souhami, 1972). Although more sensitive assays of reticuloendothelial cell particle clearance function are available, this technique was chosen because it required only 2 hr for completion.

To determine if selected diazo dyes caused hepatocellular damage alanine aminotransferase activity was determined on an autoanalyzer (Technicon, Tarrytown, NY) on serum collected at various times after administration of a protective dose of the dyes. Mice pretreated with the hepatotoxin, CCl_4, were used as controls.

Statistical analysis. A comparison of spleen/liver radioactivity between test and control mice were performed using Student's *t* test. Spearman's rank correlation and χ^2 analysis were used to identify an association between protection, defined as percentage of survivors after challenge with a lethal dose of cyanoginosin-LR, and hepatic macrophage blockade, defined as an elevation of spleen/liver radioactivity significantly greater, by *t* test analysis, than control. The only level of statistical significance tested was $P < .05$.

Results

Eight compounds were found to be protective. Effective doses and the approximate duration of protection are listed in table 1. In many experiments not reported herein the effectiveness of all the protective agents was confirmed and found to span a wide range of doses, with the duration of protection being dose-dependent. However, hydrocortisone was effective only in very large doses, incomplete protection occurring below 400 mg kg^{-1}. One agent, carbon tetrachloride, had been noted previously to block cyclic peptide toxicity (Adams *et al.*, 1985).

Because there may be significant variation in chemical composition and purity of individual diazo dyes, nominal Trypan blue dyes from several suppliers were tested. A sample of one batch was found to be virtually ineffective in prophylaxis. Thin layer chromatography revealed an uncharacteristic band pattern, *i.e.*, the substance was not Trypan blue, a conclusion confirmed subsequently by the supplier. Mallinckrodt product number E124 was the most effective in inducing protection and was selected as the standard Trypan blue test dye.

Pretreatment of mice with either cycloheximide or progesterone did not abrogate the protection provided by hydrocortisone.

TABLE 1

Agents found to protect mice against lethal doses of the specified cyclic peptide toxins; duration of protection

Protective Agent	Toxin		
	Cyanoginosin-LR	Phalloidin	α-Amanitin
mg kg^{-1}, route			
Hydrocortisone (500, i.p.)	[a]	[a]	[a]
Shellac (92, i.v.)	[b]	[b]	[b]
Diazo dyes			
Trypan blue[c] (100, i.v.)	[b]	[b]	[b]
Trypan red (100, i.v.)	[d]	[d]	no protection
Evan's blue (100, i.p.)	[b]	not tested	not tested
Triazine dyes			
Reactive Red 120 (200, i.p.)	[b]	[b]	[b]
Cyclosporine A (24, i.v.)	[a]	[a]	no protection
CCl_4[c] (50 μl/mouse, s.c.)	[b]	[b]	not tested

[a] Protective for at least 1 hr; [b] protective for at least 1 day; [c] protection developed 24–48 hr after injection of the protective agent; [d] protective for at least 1 wk.

All but one of seven protective agents tested altered the spleen-to-liver distribution of ^{51}Cr-labeled sheep erythrocytes. In several instances the decrease in hepatic clearance of the labeled cells (and thereby an increase in the ratio of splenic to hepatic uptake) paralleled the appearance of protection against cyanoginosin-LR, as determined in groups of mice concurrently given a lethal dose of that toxin (fig. 1). An analysis of data obtained 24 hr after injection of the protective agents, the only time point common to all experiments, revealed a significant correlation between protection and the ability of an agent to elevate spleen/liver radioactivity significantly (Spearman's rank correlation: rho = 0.85, $n = 8$, $P < .05$). In addition, concurrence of protection and hepatic macrophage blockade was present at 24 of the 32 time points evaluated in figure 1a through 1f, which, by χ^2 analysis, is also significant ($\chi^2 = 8$, dF = 1, $P < .05$). However, an agent known to be effective at inducing reticuloendothelial cell blockade, dextran sulfate-500, was used as a positive control for these experiments, and it did not protect against the toxin (fig. 1h). In contrast the agent inducing the most prolonged protection against the toxin, Trypan red, was atypical, for it did not alter the uptake of the labeled cells at any point (fig. 1g). Therefore it was not included in the statistical analysis given above. When Trypan red is included both analyses lose statistical significance, in part because Trypan red was tested at so many (nine) time points.

Preliminary investigations have shown the hepatotoxin, carbon tetrachloride, to be capable of blocking cyanoginosin-LR toxicity (Adams *et al.*, 1985). To determine if the diazo dye-induced protection was a consequence of hepatocellular damage, serum activity of alanine aminotransferase was determined in groups of mice given protective doses of either Trypan blue, Trypan red or CCl_4. Carbon tetrachloride and Trypan blue, but not Trypan red, increased serum enzyme activity (table 2).

To confirm the effectiveness of the protection against phalloidin, several of the agents were tested concurrently against a separate sample of the toxin kindly provided by Professor Theodor Wieland of the Max-Planck-Institut für Medizinische Forschung (table 3).

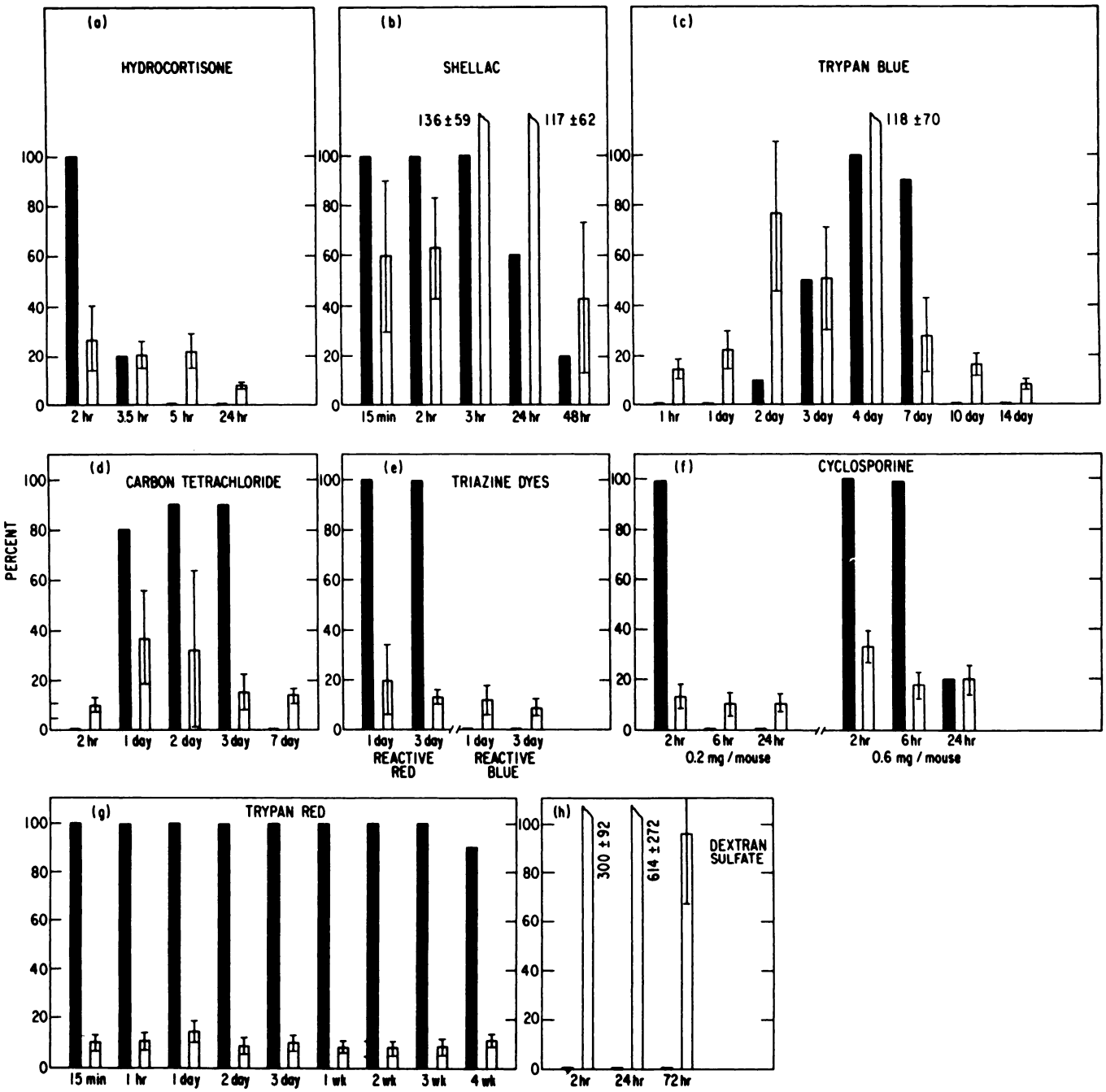

Fig. 1. Alterations of splenic and hepatic reticuloendothelial cell activity in mice after administration of agents found to prevent lethality of cyclic peptide toxins. The normal ratio (± S.D.) of the spleen/liver uptake of i.v. injected ^{51}Cr-labeled sheep erythrocytes was 0.08 ± 0.03 (n = 55). The ordinate scale represents 1) the percentage of survival of mice given the specified agent and then injected with a lethal dose of cyanoginosin-LR (solid bars), and 2) the degree of hepatic reticuloendothelial cell blockade induced by the same agent expressed as percentage × 10^{-1} of concurrently determined control value (open bars). Brackets indicate S.D. The interval between the administration of the protective agents and injection of the toxin is indicated on the abscissa. Doses and injection routes: hydrocortisone, 500 mg kg^{-1}, i.p.; shellac, 92 mg kg^{-1}, i.v.; Trypan blue, 100 mg kg^{-1}, i.p.; CCl_4, 50 μl/mouse, s.c.; triazine dyes, 100 mg kg^{-1}, i.p.; Trypan red, 200 mg kg^{-1}, i.p.; dextran sulfate-500, 15 mg kg^{-1}, i.v. Cyclosporine A, 0.2 or 0.6 mg/mouse (8 and 24 mg kg^{-1}, respectively), was given i.v. Control mice for the cyclosporine experiments received either normal saline or the vehicle for the i.v. formulation of the drug, kindly supplied by the manufacturer (Sandoz Pharmaceuticals, East Hanover, NJ). Because there were no significant differences between the two control groups, their data were pooled for analysis. The individual bars represent the results of two or more experiments. The number of mice used for ^{51}Cr-labeled sheep erythrocyte distribution ranged from 4 to 10 per experiment. The number of mice for toxicity testing, injected concurrently, ranged from 5 to 10 per experiment. For statistical analysis, see under "Results."

Discussion

Hydrocortisone was the first compound found to inhibit the lethality of cyanoginosin-LR, phalloidin and α-amanitin. This activity is clearly nongenomic, for hydrocortisone-induced protection is effective within minutes of injection, requires pharmacologic doses and is not blocked by pretreatment with cycloheximide or progesterone. One nongenomic effect of glucocorticoids such as hydrocortisone is reticuloendothelial cell blockade. Therefore other agents known to affect macrophage function were tested.

India ink, a stabilized suspension of lampblack, induces

TABLE 2

Serum alanine aminotransferase activity (IU/ml, mean ± S.D.) in mice after injection of three protective agents[a]

Time after Injection	Trypan blue	Trypan red	CCl_4 Controls
	100 mg kg^{-1} i.v.	*200 mg kg^{-1} i.v.*	*50 µl/mouse s.c.*
6 hr	44.6 ± 8.2	38.6 ± 10.8	38.6 ± 9.7
2 days		54.6 ± 5.6	6827 ± 1018.5
4 days	>3000		
6 days	396.8 ± 229.2	41.0 ± 11.1	
14 days	51.0 ± 18.8	36.3 ± 7.7	
21 days		39.6 ± 9.5	
28 days		41.3 ± 19.5	

[a] Each group included five or more mice. Addition of the dyes to mouse serum having either normal or elevated alanine aminotransferase activity did not affect enzyme activity readings.

TABLE 3

Fraction of variously pretreated mice dying after i.p. administration of a lethal dose (3.0 mg kg^{-1}) of phalloidin

Protective Agent	Dose and Route	Time from Pretreatment to Phalloidin Injection		
	mg kg^{-1}	*1 hr*	*1 day*	*1 wk*
Shellac	92, i.v.	0/10	0/10	8/10
Trypan red	100, i.v.	0/10	0/10	0/10
Hydrocortisone	500, i.p.	0/10	7/10	
Reactive Red 120	200, i.p.	0/10	0/10	10/10
Cyclosporine A	8, i.v.	0/10	10/10	

reticuloendothelial cell blockade (Markham and Florey, 1951). Intravenous injection of 50 µl of Higgins India Ink prevented the lethality of subsequently administered cyanoginosin-LR, phalloidin or α-amanitin. Protection developed immediately and persisted for 24 hr. It is notable that α-amanitin toxicity, which usually takes several days to evolve, did not develop in India ink-treated mice observed for several weeks after injection of the toxin if it was given during that 24-hr interval. Activated charcoal is used as an antidote after ingestion of a variety of toxic substances because it adsorbs the agents and thus limits gastrointestinal absorption. However, inactivation by adsorption did not occur with cyanoginosin-LR, for India ink is ineffective in prophylaxis unless given i.v., and a nonincubated mixture of a protective dose of ink and a lethal dose of cyanoginosin-LR, when given i.p., caused severe thrombocytopenia, massive intrahepatic hemorrhage and death within 1 to 2 hr, characteristic features of cyanoginosin toxicity. Unexpectedly, India ink protection was found to reside in the shellac component of the ink fluid rather than in the suspended carbon. However, shellac, like colloidal carbon, is known to affect reticuloendothelial cell function, as indicated by impaired clearance of carbon particles (Halpern *et al.*, 1953). Like India ink, the ammonium salt of shellac was protective immediately on injection.

Trypan blue (Color Index: 23850), a supravital diazo dye used to identify macrophages *in vivo*, is an inhibitor of certain lysosomal enzymes and may interfere with pinocytosis by macrophages (Padawer, 1973; Shau and Dawson, 1984; Williams *et al.*, 1976). Both Trypan blue and its close structural relative, Evan's blue, were found to be protective against cyanoginosin-LR, although the latter was more toxic to mice. Because of the effectiveness of Trypan blue in toxin prophylaxis, other diazo dyes were tested. Strikingly, a single i.p. injection of Trypan red (Color Index: 22850), 200 mg kg^{-1}, provided protection against cyanoginosin-LR for up to 3 months, approximately 1/6 of the mouse median life span. This period also approximates the life span of hepatic macrophages in rats (Volkman, 1977). Suramin, a colorless diazo derivative developed as a consequence of Ehrlich's discovery of the antiinfective nature of Trypan red and most recently used in the therapy of the acquired immunodeficiency syndrome, delayed cyanoginosin-LR lethality only when given in highly toxic doses. Pretreatment of mice with carbon tetrachloride blocks the toxins (Adams *et al.*, 1985) and impairs hepatic macrophage function (fig. 1d), but it also damages hepatocytes. Because normal hepatocyte function might be required for toxin activation, evidence for hepatocellular damage was sought in mice given protective doses of diazo dyes. Serum alanine aminotransferase activity increased after administration of Trypan blue, confirming the hepatotoxicity of that dye (Gillman and Gillman, 1951), but not after Trypan red (table 2), and the liver appeared histologically normal for 1 month after Trypan red injection. Hepatocyte damage does not appear to be a prerequisite for protection induced by some diazo dyes against cyclic peptide toxins.

Several dyes of the triazine class were also tested as toxin inhibitors *in vivo*. Reactive Red 120 blocked lethality of the three toxins, whereas Reactive Blue 4 provided no protection. Triazine dyes belong to a class of compounds not selected for testing because of known effects on macrophage function. Instead, they were studied because of their high degree of protein binding, a characteristic of Trypan blue and other diazo dyes (Rawson, 1943).

Cyclosporine A, a potent inhibitor of cellular immunity, impairs certain nonphagocytic macrophage functions (Manca *et al.*, 1985). In addition, like the cyanoginosins, it is both strongly hydrophobic and a cyclic peptide. Cyclosporine A, when given i.v. to mice less than 2 hr before cyanoginosin-LR, blocked the lethality of cyanoginosin-LR and phalloidin but not α-amanitin.

The mechanism(s) of protection does not appear to reside in the ability of the agents to block a putative single biochemical lesion produced by the cyclic peptide intoxicants. Phalloidin kills mice in 3 to 4 hr and its acute hepatic toxicity is believed due to binding with F-actin (Wieland, 1983). Toxic extracts of *M. aeruginosa* do not alter the level of unpolymerized G-actin (Runnegar and Falconer, 1986), in contrast to phalloidin, which decreases it. α-Amanitin requires 3 to 7 days to kill mice by inhibiting DNA-dependent RNA polymerase and thereby inducing hepatic and renal failure (Wieland, 1983). Therefore, the prophylactic agents may modulate an unidentified biological process common to activation of a class of cyclic peptides.

Selection of several of the agents subsequently shown to be protective was based on their known ability to affect macrophage function. The importance of macrophages, especially the hepatic macrophage, in toxin protection is also suggested by our studies of sheep erythrocyte clearance. Impaired hepatic uptake of labeled sheep erythrocytes followed pretreatment of mice with all but one of seven protective agents so tested (fig. 1, a–g). However, the specific macrophage function associated with protection remains unidentified. The observed alteration in phagocytosis probably represents only a marker of more generally deranged reticuloendothelial cell function and not the mechanism through which protection is mediated. For example, dextran sulfate-500 provided a high level of reticuloendothelial blockade, but was ineffective in blocking toxin lethality (fig. 1h). Paradoxically, Trypan red, despite its remarkably sustained protection against cyanoginosin-LR, had no detectable effect on spleen/liver distribution of sheep erythrocytes (fig. 1g). Nevertheless, this red dye was visible in unstained bone marrow smears for at least 4 months, and examination of

smears counterstained with Prussian blue (for identifying iron) indicated the dye was present in macrophages. Our preliminary experiments indicate that cyanoginosin-LR induces extracellular release of potentially injurious lysosomal enzymes from mouse peritoneal macrophages *in vitro* (unpublished data). Conceivably some of the protective agents, in particular the diazo dyes, could inhibit a similar response by hepatic macrophages *in vivo* and thereby avert pathogenicity of the toxins.

Microsomal enzyme inducers have been found to increase the survival time of cyanoginosin-treated mice if given 48 hr before toxin challenge (Brooks and Codd, 1987). In contrast, most of the agents described herein are effective within minutes and protection is absolute for hours or days; the lethal process is not merely retarded or postponed. The protected mice develop no signs of illness from the toxins. For example, the thrombocytopenia and hemorrhagic engorgement of the liver characteristic of cyanoginosin-LR toxicity (Slatkin *et al.*, 1983) do not occur. These studies suggest a key role of the macrophage in cyclic peptide toxin pathophysiology, a role that also may be relevant to modulation of the effects of other cyclic peptides. In addition, it is conceivable that some of the protective agents or their derivatives could be clinically useful in prevention or in early therapy of selected cyclic peptide poisonings.

Acknowledgment

We thank Ms. Bettie Sylvester for technical assistance.

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