Inhibitory Effect of Trichothecene Mycotoxins on Bovine Platelets Stimulated by Platelet Activating Factor

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ABSTRACT

Several species of fungi, which infect cereals and grains, can produce a class of compounds, known as trichothecene mycotoxins, which is characterized by a substituted epoxytrichothecene ring structure. Cattle are susceptible to intoxication from feeds contaminated with T-2 toxin, one of the more potent trichothecene mycotoxins, while swine refuse to ingest feed contaminated with T-2 toxin. The bovine platelet has been used as a model cell system to evaluate the effects of T-2 toxin and its natural metabolites, HT-2 toxin and T-2 tetraol, on cell function *in vitro*. Due to the lipophilic nature of these mycotoxins, a biologically active phospholipid was used to stimulate the platelets in the presence and absence of the toxins.

The mycotoxin T-2 toxin and its major metabolite HT-2 toxin inhibited platelet activating factor-stimulated bovine platelets, suspended in homologous plasma, in a concentration but not time dependent manner. Significant inhibition of platelet function ($p<0.01$) occurred with 135 ng T-2 toxin per 10^6 platelets and with 77 ng HT-2 toxin per 10^6 platelets. These mycotoxins exerted an additive inhibitory effect on the platelet aggregation response. In contrast, the minor metabolite T-2 tetraol had no inhibitory effect on platelet function and had no influence on the responses of T-2 toxin or HT-2 toxin when the mycotoxins were present together in the platelet suspensions. The results suggest that the apparent potency of T-2 toxin may be due, at least in part, to the fact that, as it is degraded in the animal tissue, major metabolites, such as HT-2 toxin, inhibit cellular function as effectively as the parent compound.

Key words: Trichothecene mycotoxins, platelet activating factor, bovine platelets.

RÉSUMÉ

Plusieurs espèces de champignons qui infectent les céréales et les grains peuvent produire une classe de composés qu'on appelle mycotoxines trichothécènes et qui se caractérise par la substitution d'un anneau époxytrichothécène. Les bovins peuvent s'intoxiquer en mangeant des aliments contaminés par la toxine T-2, une des plus puissantes mycotoxines trichothécènes, tandis que les porcs refusent de manger de tels aliments. Les plaquettes bovines ont servi de modèle de système cellulaire pour évaluer les effets de la toxine précitée et de ses métabolites naturels: la toxine HT-2 et le tétraol T-2, sur la fonction cellulaire *in vitro*. À cause de la nature lipophile de ces mycotoxines, les auteurs utilisèrent un phospholipide à activité biologique, pour stimuler les plaquettes, en présence et en l'absence des dites toxines.

La mycotoxine T-2 et son principal métabolite, la toxine HT-2, inhibèrent les plaquettes bovines stimulées par leur facteur activant, en suspension dans du plasma homologue, d'une manière dépendante de la concentration, mais non du temps. Une inhibition significative de la fonction plaquettaire ($p<0,01$) survint dans un mélange de 135 ng de toxine T-2, ou de 77 ng de toxine HT-2, et de 10^6 plaquettes. Ces mycotoxines exercèrent un effet inhibiteur additionnel sur la réaction de l'agrégation thrombocytaire. Par contre, le métabolite mineur tétraol T-2 n'exerça aucune influence inhibitrice sur la fonction des plaquettes et n'affecta pas l'effet des mycotoxines T-2 ou HT-2, quand elles se retrouvaient toutes deux dans les suspensions de plaquettes. D'après ces résultats, la puissance apparente de la toxine T-2 résulterait, au moins partiellement, du fait qu'à mesure qu'elle se dégrade dans les tissus animaux, ses métabolites majeurs tels que la toxine HT-2, inhibent la fonction cellulaire aussi bien qu'elle-même.

Mots clés: mycotoxines trichothécènes, facteur activant des plaquettes, plaquettes bovines.

INTRODUCTION

Tricothecene mycotoxins are a group of substituted 12,13-epoxytrichothecene compounds which can be produced by several species of *Fusarium* fungi (1,2). Animals ingesting moldy feeds contaminated with trichothecene mycotoxins exhibit a number of disease symptoms including lack of weight gain, anorexia, gastrointestinal disorders and immunosuppression (3-6).

In part because of its potency, the mycotoxin, T-2 toxin, has been the subject of extensive investigation in recent years, however the biochemical mechanism of action of this and related mycotoxins has still not been fully elucidated. Following chronic

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exposure to T-2 toxin, some of the clinical signs observed include leukopenia, agranulocytosis and impaired central nervous system function (7-9). The inhibitory action of T-2 toxin on DNA, RNA and hence protein synthesis is thought to be responsible for some of these toxic effects (10-14). However, the inhibition of protein synthesis cannot account for some of the acute effects of T-2 toxin. For example, a single intravascular dose of T-2 toxin administered to swine produced a shock syndrome characterized by reductions in cardiac output and blood pressure (15). Furthermore, both *in vitro* and *in vivo* studies in a number of species have shown that T-2 toxin can impair blood platelet function (16-19) but the platelet has only limited protein synthesizing ability. Consequently it has been suggested that T-2 toxin can affect other cellular parameters such as membrane permeability (16,20,21). In this study the effects of T-2 toxin, HT-2 toxin and T-2 tetraol, alone and in combination, on bovine platelet function *in vitro* have been examined. In a number of studies, involving various species of animals and birds, it has been shown that HT-2 toxin is a major metabolite and T-2 tetraol a minor metabolite of T-2 toxin (22-28). Consequently, animals which are exposed naturally or in experimental studies to T-2 toxin, will also have HT-2 toxin and T-2 tetraol present, at least transiently, in circulation.

Platelet activating factor (acetylglycéryl ether phosphocholine, AGPAF), in addition to being a potent platelet stimulator, is known to be a mediator of cardiac anaphylaxis and inflammation (29). This lipid mediator can be released from mast cells, basophils and monocytes and may be one of the links between the immunological and hemostatic defense mechanisms *in vivo* (29). The effects of trichothecene mycotoxins on reactions initiated by this compound have not been previously reported. The bovine platelet has been selected as the model cell system for this investigation since not only is the bovine platelet extremely sensitive to stimulation by AGPAF *in vitro* (30), but also the bovine platelet is as sensitive as human and ovine platelets to the inhibitory effects of T-2 toxin (16,17,31).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Blood was collected from the external jugular vein of healthy, mature Holstein cows from the dairy herd maintained at the Ontario Veterinary College.

PREPARATION OF PLATELET RICH PLASMA

Blood was collected with 18-gauge needles and plastic syringes and immediately mixed in siliconized glass tubes with one tenth of its volume of 0.13 M trisodium citrate. Platelet rich plasma (PRP) was prepared by centrifuging the citrated blood at 200 *g* for 15 min at 21°C. The PRP was harvested using siliconized pipettes and the remaining blood was further centrifuged at 2500 *g* for 20 min at 4°C to obtain platelet poor plasma (PPP). The platelet counts for both PRP and PPP were determined with Unopettes (Becton-Dickinson Co., Rutherford, New Jersey). Platelet rich plasma was adjusted with autologous PPP to give a platelet count of $2 \times 10^5/\mu L$. The platelet suspensions were maintained at 21°C for not more than 1 h before the platelet-aggregation response was studied.

PREPARATION OF REAGENTS

The aggregating agent, acetylglyceryl ether phosphorylcholine (platelet activating factor, AGPAF, Calbiochem, San Diego, California) was dissolved in 100% ethanol to give a stock solution of 5 mg per mL which was subsequently diluted with Tyrodes-BSA buffer (pH 7.35) containing 1 mM $CaCl_2$ (30). The adenosine-di-phosphate (ADP, Sigma Chemical Co., St. Louis, Missouri) was dissolved in 0.05 M-Tris buffered saline (TBS, pH 7.4) to give a 1×10^{-2} M stock solution. For platelet aggregation further dilutions were made with TBS.

The mycotoxins, T-2 toxin (Myco-Lab Co., Chesterfield, Missouri), HT-2 toxin and T-2 tetraol (Sigma Chemical Co., St. Louis, Missouri) were dissolved in methanol to yield stock solutions containing 22.3 mg/mL, 21.2 mg/mL and 149 mg/mL respectively. These preparations were further diluted before use so that the solvent solution added to the PRP would not exceed 1:3 (v/v) methanol and 0.18 M sodium chloride.

MEASUREMENT OF PLATELET AGGREGATION

Platelet aggregation was measured in a Payton dual channel model instrument (Payton Associates Ltd., Scarborough, Ontario). Before the addition of 25 μL of the aggregating agent, 215 μL PRP was incubated at 37°C with 10 μL of toxin solution or solvent as control. Maximum or 100% aggregation for each experiment was established as the difference between the light transmission in the PRP sample and the PPP sample. All assays were run in duplicate on a minimum of four samples obtained from different animals. Statistical analyses using the Stüdent's t-test and a Monroe programmable calculator (Monroe Calculator Corp., Kitchener, Ontario) were used to compare the control and toxin treated samples.

RESULTS

Both T-2 toxin and its metabolite, HT-2 toxin, inhibited bovine platelets stimulated *in vitro* with AGPAF in a concentration dependent manner (Fig. 1). In this system HT-2 toxin was a more effective inhibitor of platelet function than the parent compound, T-2 toxin, since the relative concentrations of toxins required to produce a significant ($p<0.01$) reduction in the platelet aggregation response were 0.071 μg and 0.135 μg per 10^6 platelets respectively. The other metabolite tested, T-2 tetraol, appeared to be ineffective as an inhibitor of platelet aggregation even when concentrations as high as 1.73 μg per 10^6 platelets were added to the platelet suspensions (Table I). The inhibitory effects of T-2 toxin and HT-2 toxin were not dependent on the time of exposure of the platelets to the toxins. The relative aggregation responses for the control and toxin treated samples were similar irrespective of whether the platelets were incubated for 0.5, 1.0 or 5.0 min before the addition of the aggregating agent (Table I). For subsequent experiments a 2.0 min preincubation period was selected.

The addition of T-2 toxin and HT-2 toxins together to platelet rich plasma suspensions produced an additive inhibitory response (Table II). The inhibitory response induced by the combination of T-2 toxin and HT-2 toxin

TABLE I. The Effect of Different Incubation Periods on the Platelet Aggregation Response in the Presence of T-2 Toxin, HT-2 Toxin and T-2 Tetraol (Mean ± SD)

Time of Incubation (min)	Percent Maximum Aggregation[a]					
	Control	T-2 Toxin 0.68 μg/10⁶ Cells	Control	HT-2 Toxin 0.31 μg/10⁶ Cells	Control	T-2 Tetraol 1.73 μg/10⁶ Cells
0.5	60.6±4.4	27.8±4.3	57.4±4.0	24.0±7.2	60.4±1.8	65.7±1.9
1.0	57.0±4.0	25.5±4.2	57.0±7.2	25.6±2.7	64.6±3.4	60.9±4.6
5.0	56.4±5.1	29.9±2.6	57.6±8.9	27.1±7.3	54.8±3.0	51.8±4.2

[a]Platelet aggregation initiated with 4.5×10^{-9} M AGPAF

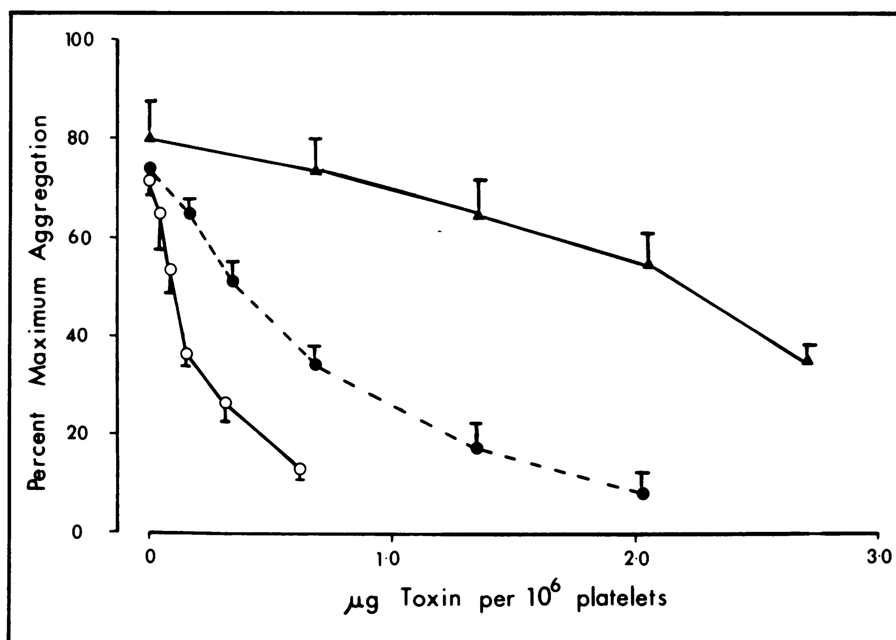

Fig. 1. The platelet aggregation response in platelet rich plasma suspensions stimulated with 9.0×10^{-9} M AGPAF in the presence of T-2 toxin (●---●), HT-2 toxin (o — o) and the response of platelets stimulated with 1.0×10^{-5} M ADP in the presence of T-2 toxin (▲ — ▲) expressed as mean ± SD, n=8.

TABLE II. The Effects of T-2 Toxin, HT-2 Toxin and T-2 Tetraol Alone and in Combination on the Platelet Aggregation Response (Mean ± SD, n=8)

Treatment	Percent Maximum[a] Aggregation	Percent Inhibition[b]
Control	78.0±4.5	0
T-2 toxin (0.34 μg/10⁶ cells)	52.2±6.1	34.2±4.5
HT-2 toxin (0.08 μg/10⁶ cells)	59.9±7.2	24.3±5.0
T-2 toxin + HT-2 toxin	42.2±5.1	46.7±4.6
T-2 tetraol (1.73 μg/10⁶ cells)	76.4±4.5	1.9±2.5
T-2 toxin + HT-2 toxin + T-2 tetraol	43.6±4.1	44.3±3.5

[a]Platelet aggregation initiated with 9.0×10^{-9} M AGPAF
[b]Compared to control value

was significantly greater ($p<0.01$) than the inhibitory response induced by the toxins separately. When the concentration of each toxin was increased twofold the relative percent inhibitory responses were also increased from 34.2 ± 4.5 to 50.9 ± 3.3 for T-2 toxin, from 24.3 ± 5.0 to 53.6 ± 10.0 for HT-2 toxin and from 46.7 ± 4.6 to 82.8 ± 2.1 for the combination of toxins. When T-2 tetraol was included together with T-2 toxin and HT-2 toxin in the platelet rich plasma it neither enhanced nor inhibited the aggregation response. The mean percent aggregation response was 43.6 ± 4.1 in the sample treated with all three toxins which is similar to the response when only T-2 toxin and HT-2 toxin were present (Table II).

Both T-2 toxin and HT-2 toxin appeared to be more potent inhibitors of platelets stimulated to aggregate with AGPAF than with ADP. The concentration of T-2 toxin required to inhibit platelet aggregation by 50% was 0.67 μg per 10⁶ cells for AGPAF stimulated platelets and 2.5 μg per 10⁶ cells for ADP stimulated platelets (Fig. 1). Similarly the addition of HT-2 toxin at 1.24 μg per 10⁶ cells reduced the platelet aggregation response in AGPAF stimulated platelets from 71.2 ±7.7% in the control to 8.9 ±4.5% while this concentration of HT-2 toxin had no effect on ADP induced aggregation.

In inducing platelet aggregation AGPAF first binds to receptors on the platelet surface. Platelets then undergo a change in shape and throw out pseudopods which enhance platelet to platelet adhesion (32). On the aggregometer tracing this initial shape change is indicated by the rise in the curve immediately following the addition of the aggregating agent. Neither T-2 toxin or HT-2 toxin, alone or in combination, interfered with the initial shape change response induced by AGPAF. The toxins only inhibited the extent to which aggregates form. Typical aggregometer tracings which illustrate these effects are shown in Fig. 2.

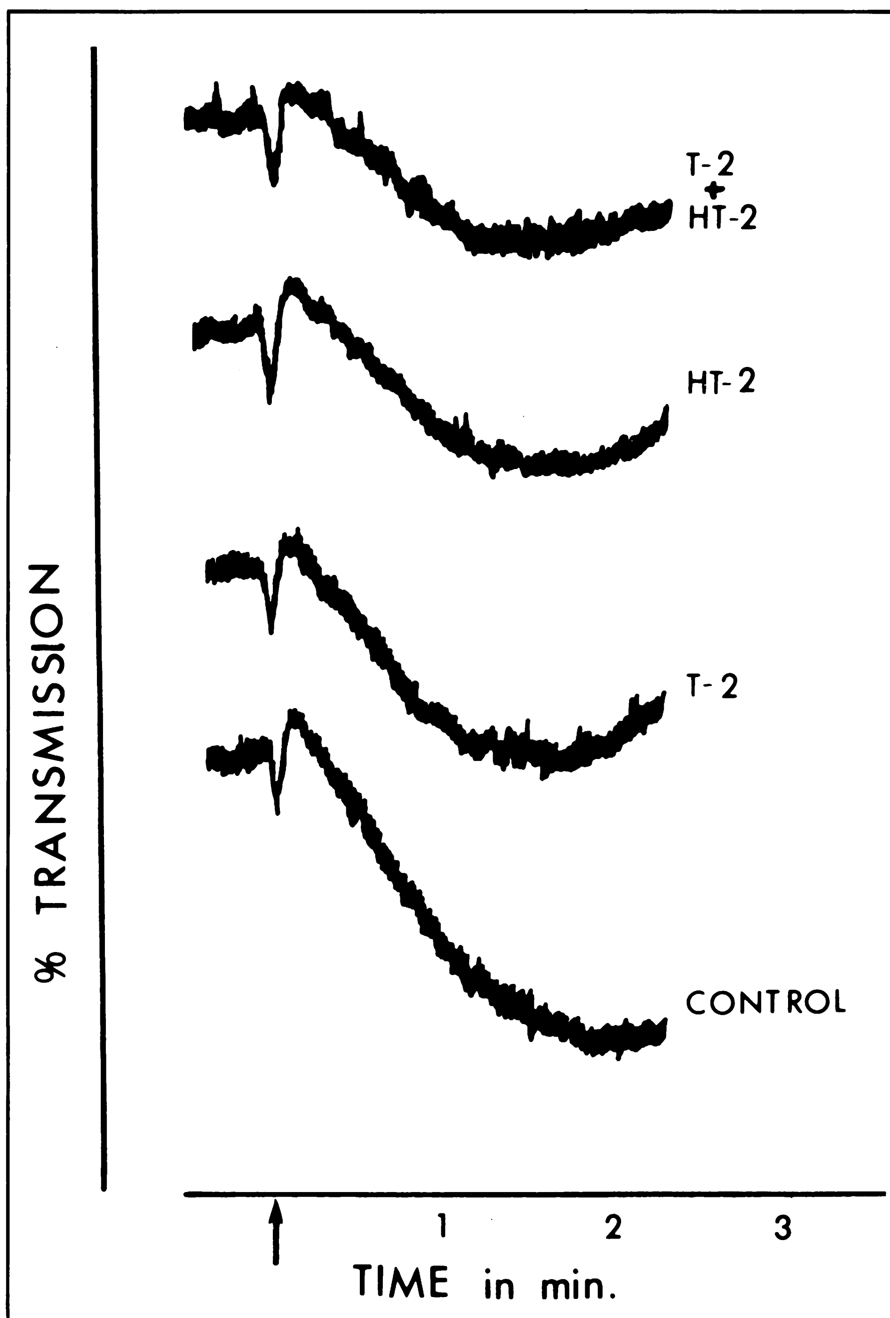

Fig. 2. Representative platelet aggregation response curves in platelets stimulated with 4.5×10^{-9} M AGPAF in the presence of T-2 toxin, HT-2 toxin alone and in combination. The arrow represents the time of addition of AGPAF to the platelet suspension.

DISCUSSION

Both toxin and HT-2 toxin appear to be effective inhibitors of AGPAF induced bovine platelet aggregation *in vitro* while T-2 tetraol is ineffective in this system. This observation is in agreement with previous studies which have shown that the toxic potency of trichothecenes vary depending on the modifications on the side chains of the molecules and that T-2 tetraol is significantly less toxic than either T-2 toxin or HT-2 toxin (22). Not only does T-2 tetraol fail to inhibit platelet aggregation, it does not interfere with the inhibitory effects of T-2 toxin or HT-2 toxin when it is present in the platelet suspension along with these compounds. It has been suggested that the first line of defense of ruminants against toxic compounds is their metabolism by rumen microbes, for example the conversion of T-2 toxin to HT-2 toxin (33). The fact that, in the bovine-AGPAF induced aggregation system, not only is HT-2 toxin a more potent inhibitor of platelet function than T-2 toxin but also that T-2 toxin and HT-2 toxin exert an additive effect indicates that for some cellular systems the initial steps of T-2 toxin metabolism may not reduce but may in fact enhance the toxic potential of T-2 toxin.

Although it has not been a consistent observation, hemorrhagic episodes have been observed in experimental studies following the administration of T-2 toxin (4,34-37). While the inhibitory effects of T-2 toxin and HT-2 toxin on platelet function may contribute to the observed bleeding problems, the failure of T-2 tetraol to inhibit platelet function may also be a factor in the absence of hemorrhage on other occasions. The development of hemorrhage in animals may be related to the rate at which T-2 toxin is metabolized and the specific metabolites produced. Since it has been suggested that T-2 toxin may have a relatively short half-life, of the order of 14 to 18 minutes (37), the relative quantities of specific metabolites produced may be the more important parameter. This conclusion is supported by the evidence that the time of exposure of platelets to T-2 toxin and HT-2 toxin had no effect on the inhibitory response. The trichothecene mycotoxins resemble other known platelet inhibitors, such as acetylsalicylic acid and indomethacin, in their ability to depress platelet function without inducing clinical hemorrhagic conditions (38).

The mechanism by which AGPAF induces platelet aggregation is distinct from the mechanism of action of other aggregating agents such as ADP and collagen (40,41). It has been shown that AGPAF binds to receptors on the platelet membrane and initiates aggregation by a pathway independent of the generation of thromboxane A_2 from endogenous arachidonic acid (29,40). Although both T-2 toxin and HT-2 toxin suppress thromboxane release from collagen stimulated platelets (19), this may not be the primary route by which T-2 toxin inhibits platelet aggregation (42). This conclusion is compatible with the observation that AGPAF induced platelet aggregation is more sensitive to inhibition by

both T-2 toxin and HT-2 toxin than ADP induced aggregation (Fig. 1).

Since neither T-2 toxin nor HT-2 toxin affect the initial shape change induced in platelet rich plasma by the addition of AGPAF, it would appear that these mycotoxins do not interfere with AGPAF binding to platelet receptors (Fig. 2). However, this observation does not preclude the possibility that these mycotoxins can bind to other receptor sites on the platelet membrane in a manner analogous to the receptor binding demonstrated between T-2 toxin and murine lymphocytes (43).

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