Ricin induces the production of tumour necrosis factor- α and interleukin-1 β by human peripheral-blood mononuclear cells

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Ricin induced the release of tumour necrosis factor- α (TNF- α) and interleukin-1 β by human peripheral-blood mononuclear cells in a dose- and time-dependent manner. The inhibition induced by ricin upon the phytohaemagglutinin (PHA)-driven lymphocyte proliferation was greater in cultures of mononuclear cells than that observed in monocyte-free cultures of lymphocytes,

and was decreased after addition of an anti-TNF- α antibody to PHA-activated cultures. Low levels of TNF- α were detected in plasma from rats poisoned with ricin. These results suggest that ricin induced the release of macrophage-derived cytokines which may have a role in the pathogenesis of ricin toxic effects.

INTRODUCTION

Ricin, the toxin of *Ricinus communis* seeds (castor beans), is a type 2 ribosome-inactivating protein (two-chain RIP, as opposed to type 1 single-chain RIPs; reviewed by Stirpe et al., 1992). Ricin and related type 2 toxins are N-glycosidases which cleave the N-glycosidic bond of a single adenine residue in a defined position of rRNA (A4324 of rat liver 28 S rRNA) (Endo et al., 1987). Deadened ribosomes are unable to bind the elongation factor 2, with the consequent arrest of protein synthesis and cell death.

In the rat, ricin causes severe necrotic lesions in the liver, kidney and pancreas, severe inflammation and haemorrhages in the intestine, massive ascites and pleural effusion (Derenzini et al., 1976). Lesions of hepatocytes appear to be secondary to damage to Kupffer cells (Derenzini et al., 1976; Bingen et al., 1987), which preferentially take up the toxin via the mannose receptor (Skilleter et al., 1981; Simmons et al., 1986; Magnusson et al., 1991). Furthermore, ricin induces a primary acute-phase response (Nadkarni and Deshpande, 1991), has immuno-enhancing effects (Kishima et al., 1987; Thorpe et al., 1989) and co-stimulates proliferation of lymphoid cells (Kemeny et al., 1989).

Several bacterial toxins induce the secretion of cytokines (reviewed by Flegel et al., 1991). We report now that ricin induces the release of tumour necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) by human peripheral-blood mononuclear cells. TNF- α was detected in the plasma of ricin-poisoned rats. Our results suggest that these cytokines produced by macrophages stimulated and/or damaged by ricin may contribute to the pathogenesis of the lesions and other effects observed after ricin poisoning.

MATERIALS AND METHODS

Materials

Ricin was prepared as described by Nicholson et al. (1974) and was inactivated by boiling a 100 pM solution for 20 min in a sealed ampoule. Iscove modified Dulbecco's medium was purchased from GIBCO, Bethesda, MD, U.S.A., and Lymphoprep was from Immuno, Milan, Italy. Endotoxin-free BSA (Cohn

fraction V), iron-free human transferrin, penicillin and streptomycin were from Sigma, St. Louis, MO, U.S.A. Percoll was from Pharmacia, Uppsala, Sweden. Phytohaemagglutinin-P (PHA-P) was obtained from Difco Laboratories, Detroit, MI, U.S.A. [3 H]Thymidine (sp. radioactivity 5 μ Ci/mmol) was obtained from Amersham International, Amersham, Bucks., U.K. E.l.i.s.a. kits for human cytokine determinations were from British Biotechnology, Abingdon, U.K. The e.l.i.s.a. kit for mouse TNF- α and polyclonal antiserum specific for human TNF- α were from Genzyme, Cambridge, MA, U.S.A. Experiments were performed in endotoxin-free conditions, since ricin solutions were passed twice through a Detoxi-Gel column (Pierce, Rockville, IL, U.S.A.). Endotoxin contamination was decreased from 12.5 ng/mg to < 0.006 ng/mg, i.e. the lowest limit of detection of the Limulus assay kit (Whittaker Bioproducts, Walkersville, MD, U.S.A.). Disposable sterile plastic tubes, tissue-culture medium and supplements were purchased as endotoxin-free.

Purification of mononuclear cells and lymphocytes

Human peripheral-blood mononuclear cells were separated from venous blood of healthy adult volunteers on density gradients (Bøyum, 1968), by using Lymphoprep. Cells were 95% lymphocytes and 5% monocytes, and their viability ranged from 90 to 98%. Mononuclear cells were suspended in a serum-free medium supplemented with 800 μ g/ml BSA, 2 μ g/ml iron-free human transferrin, 100 units/ml penicillin and 100 μ g/ml streptomycin.

In some experiments lymphocytes were separated from monocytes as described elsewhere (Minghetti et al., 1988), by using iso-osmotic Percoll gradients. In these cell suspensions monocyte contamination was less than 1%.

Microculture assays

Cell cultures were performed as described elsewhere (Licastro et al., 1983). Briefly, mononuclear cells or monocyte-depleted lymphocytes (2×10^5 viable cells in 0.1 ml of serum-free medium) were distributed in quadruplicate in microplate wells. Cultures were stimulated with PHA-P (20 μ g/ml) diluted in serum-free medium. Cells were cultured at 37 °C in a humidified atmosphere of air/CO $_2$ (19:1) for different incubation periods.

Cell proliferation was assessed by pulsing the cultures with [3 H]thymidine (0.5 μ Ci/well) during the last 6 h. Cells were harvested on glass-fibre filters with a multiple cell harvester (Skatron, Oslo, Norway), and [3 H]thymidine incorporation was measured by liquid-scintillation counting as previously described (Franceschi et al., 1979). Ricin toxicity *in vitro* was determined by the inhibition of [3 H]thymidine incorporation induced by the toxin added to PHA-P-stimulated cultures. The viability of cells after addition of ricin *in vitro* was also determined by the Trypan Blue exclusion assay.

Determination of cytokines

Mononuclear cells were cultured with or without ricin for 24 and 48 h, and then supernatants were collected by centrifugation and stored at -20°C . TNF- α and IL- β were determined by e.l.i.s.a.

Experiments *in vivo*

Rats were intraperitoneally injected with ricin (0.5 μ g/100 g body wt.) and blood samples were taken from the venous plexus of ocular bulbs under ether anaesthesia after 2, 4 and 8 h. The plasma levels of TNF- α were determined by an e.l.i.s.a. specific for mouse TNF- α .

RESULTS

The toxicity of ricin for proliferating human peripheral-blood mononuclear cells was determined by the inhibition of DNA synthesis induced by the toxin in PHA-stimulated cell cultures. The toxin inhibited [3 H]thymidine incorporation at concentrations between 1 and 100 pM, the latter dose causing complete suppression of lymphocyte proliferation (Figure 1). Resting mononuclear cells exposed to the toxin at the same concentrations progressively lost viability, up to 80% after 24 h with 100 pM ricin (Figure 2).

The capacity of endotoxin-free ricin to stimulate TNF- α and IL- β release was studied in supernatants from endotoxin-free cultures. Moreover, ricin was boiled to determine whether the stimulation of cytokine release was dependent on the integrity of the molecule. The toxin induced the release of TNF- α by mononuclear cells, in a concentration-dependent manner (Table 1). The maximum release was obtained with 5 pM ricin, higher concentrations being inhibitory. Ricin also induced IL- β release, which was greater at 48 h than at 24 h of culture (Table 1). Boiled ricin did not stimulate cytokine release (results not shown).

Two experiments were performed to verify whether cytokines secreted by stimulated monocytes/macrophages had a role in ricin cytotoxicity. First, the effect of ricin on monocyte-free lymphocyte cultures was investigated. The inhibition of thymidine uptake by ricin was lower in cultures of monocyte-depleted purified lymphocytes than in mononuclear-cell cultures containing monocytes (Table 2). Then the effect of ricin on thymidine incorporation by PHA-activated mononuclear cell cultures was studied in the presence of a polyclonal antibody against TNF- α . This addition abolished the inhibitory effect of 5 pM ricin (Figure 3). This effect was statistically significant ($P < 0.001$) as evaluated by a paired t test between cells treated with ricin and cells treated with ricin and the antibody. The toxicity of a higher concentration (100 pM) of the toxin was not modified by the antibody.

TNF- α was detected in plasma samples taken from two rats at 4 h after injection of ricin at 0.5 μ g/100 g body wt.: 100 and 60 pg of TNF- α per ml were found. No TNF- α was detected in the plasma of three different animals bled at 2 h and 8 h after toxin injection.

Figure 1 Effects of ricin on human peripheral-blood lymphocyte proliferation

The effect of ricin was measured on the incorporation of [3 H]thymidine by human peripheral-blood mononuclear-cell cultures stimulated with PHA-P as described in the text. Results are means of three experiments, and the S.E.M. is indicated by vertical bars. Cells were cultured for 48 h (Δ) or 72 h (●).

Figure 2 Effects of ricin on the viability of human peripheral-blood mononuclear cells

Human peripheral-blood mononuclear cells were cultured for 24 h (■), 48 h (Δ) or 72 h (●), and viability was measured by Trypan Blue exclusion.

Table 1 Effect of ricin on the release of TNF- α and of IL- β from cultures of human peripheral-blood mononuclear cells

TNF- α and IL- β were determined in the culture supernatant as described in the text.

Addition	Incubation for...	TNF- α (pg/ml)	
		24 h	48 h
None		17 $\pm$ 5	0
Ricin (5 pM)		123 $\pm$ 11	80 $\pm$ 1
Ricin (10 pM)		107 $\pm$ 6	87 $\pm$ 3
Ricin (100 pM)		0	0
	Incubation for...	IL- β (pg/ml)	
		24 h	48 h
None		0	0
Ricin (5 pM)		31 $\pm$ 12	64 $\pm$ 4
Ricin (10 pM)		12 $\pm$ 1	84 $\pm$ 7

Table 2 Inhibition of lymphocyte proliferation induced by ricin in the absence or presence of monocytes

Incorporation of [3 H]thymidine by cultures of human peripheral-blood mononuclear cells or purified human lymphocytes was measured after 48 h of culture as described in the text. Values are means $\pm$ S.E.M. of four determinations.

Additions	[3 H]Thymidine incorporated			
	Mononuclear cells		Purified lymphocytes	
	(d.p.m.)	(% of controls)	(d.p.m.)	(% of controls)
None	752 $\pm$ 35		1494 $\pm$ 210	
PHA	20473 $\pm$ 3059	(100)	7131 $\pm$ 1103	(100)
PHA, ricin (1 pM)	12786 $\pm$ 974	62	6717 $\pm$ 273	82
PHA, ricin (5 pM)	7668 $\pm$ 1310	37	5443 $\pm$ 111	76
PHA, ricin (10 pM)	4306 $\pm$ 1075	21	3534 $\pm$ 116	49

Figure 3 Effects of an antibody against TNF- α on the inhibition of human peripheral-blood lymphocyte proliferation by ricin

The incorporation of [3 H]thymidine by PHA-P-stimulated cells cultured for 48 h in the absence (Δ) or in the presence ($\bullet$) of anti-TNF- α antiserum (1:500 final dilution) was measured as in Figure 1. In the absence of ricin 11020 d.p.m. was incorporated.

DISCUSSION

Ricin was highly toxic for mitogen-activated or resting human mononuclear-cell cultures. The toxin alone induced the release of low but significant levels of TNF- α and IL-1 β in cultured human cells. The effect of the toxin on lymphocyte proliferation (i) was less severe in monocyte-depleted than in non-depleted cultures, and (ii) was decreased by an antibody against TNF- α . These results suggest that part of the inhibitory activity of ricin on lymphocyte proliferation might be mediated by TNF- α and possibly other cytotoxic factors released by monocytes. This notion is in accordance with other observations concerning the production and release of IL-1 α . In fact, it was reported that IL-1 α could be mainly released after cell injury (Hazuda et al., 1988). Precursors of IL-1 α and IL-1 β have been identified; these molecules are processed into lysosomes and transformed into the mature 17 kDa forms (Bakouche et al., 1987). However, after 18–24 h of activation by lipopolysaccharide, IL-1 β was the prevalent form released by monocyte/macrophages (Lonnemann et al., 1989), whereas the IL-1 α precursor mostly remained associated with cells. The latter IL-1 isoform could be released along with IL-1 β after cell injury (Hazuda et al., 1988). It should be recalled also that ricin B chain is mitogenic for lymphocytes

(Closs et al., 1975) and ricin co-stimulates spleen cells (Kemeny et al., 1989). Thus it is conceivable that ricin might exert a double effect on cytokine production by mononuclear cells, by inducing first a primary release of cytokines consequent to cell activation and, second, a release due to damage to activated cells. Higher doses of ricin, which exerted strong toxic effects, killed cells without inducing activation, and consequently there was no release of cytokines. A similar activation/killing notion was proposed to explain the action of *Clostridium difficile* toxin B on monocytes (Flegel et al., 1991).

Ricin might also make cells more susceptible to lysis by some cytokines. In fact, inhibition of protein synthesis was shown to enhance the toxic effects of TNF- α (Massad et al., 1991).

The release of TNF- α and IL-1 β observed in the present experiments is consistent with results previously obtained with ricin (Krakauer, 1991; B. Beutler, unpublished work, quoted by Soler-Rodriguez et al., 1992) and with the related toxin viscumin (Hajto et al., 1990; Männel et al., 1991). Our experimental conditions, however, differed from the studies quoted above in that (i) lower concentrations of ricin were used, and (ii) the stimulation of cytokine release was obtained with ricin alone, i.e. without any co-inducer.

It is commonly accepted that ricin exerts its toxic effects through the inhibition of protein synthesis consequent to the inactivation of ribosomes. However, some aspects of ricin poisoning are still obscure. Thus, the lesions observed in ricin-poisoned rats (see the Introduction) are not found in animals injected with other inhibitors of protein synthesis, such as puromycin or cycloheximide. The release of cytokines may have a role in the pathogenesis of lesions induced by ricin and possibly by other type 2 RIPs.

It is well established that ricin affects primarily tissue macrophages (see the Introduction), and it is conceivable that the toxin might stimulate these cells to produce monokines. TNF- α , IL-1 and IL-6 are known to possess pleiotropic effects and mediate many aspects of inflammation (Dinarello, 1989). The release of these cytokines might be responsible for at least some of the symptoms and lesions observed in ricin poisoning, such as fever, haemorrhagic liver necrosis, ascites and pleural effusion, intestinal inflammation with haemorrhages and necrosis, as well as the primary acute-phase response (Nadkarni and Deshpande, 1991) and the immunomodulatory effects. Consistent with this notion is the observation that the intestinal tract of ricin-poisoned rats strongly resembles that of TNF/cachectin-treated rats (Tracey et al., 1986).

The presence of TNF- α in the plasma of rats poisoned with ricin could be demonstrated only 4 h after the injection of the toxin. These results are consistent with the variability in time of the lesions observed in rats poisoned with the dose of ricin used in the experiments. It is possible that TNF- α , secreted at a certain time after injury, is rapidly consumed after its secretion. This might explain why Soler-Rodriguez et al. (1992) could not detect TNF- α in the serum of mice poisoned with ricin. Furthermore, it has been shown that TNF- α definitely contributes to the pathogenesis of liver lesions introduced by lipopolysaccharide and galactosamine in mice. However, this cytokine still could not be found in the serum of poisoned animals (Hishinuma et al., 1990).

Ricin A chain can be linked to antibodies to form 'immunotoxins', which are selectively toxic to target cells and are currently being evaluated for cancer therapy. Phase I/II clinical trials showed that immunotoxins caused side effects, among which fever, myalgias, capillary leak syndrome and oedema were most common (Hertler and Frankel, 1989). It is of practical importance to verify whether the production of cytokines might be involved in the pathogenesis of these side effects, as the present results

seem to suggest. In this case, the concomitant administration of compounds blocking or interfering with the action of these cytokines might allow the administration of higher doses of immunotoxins and improve their therapeutic efficiency.

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