Shiga toxin 1 and ricin A chain bind to human polymorphonuclear leukocytes through a common receptor

Valentina Arfilli, Domenica Carnicelli, Laura Rocchi, Francesca Ricci, Pasqualepaolo Pagliaro, Pier Luigi Tazzari, Maurizio Brigotti

Shiga toxin 1 and ricin A chain bind to human polymorphonuclear leukocytes through a common receptor

Valentina ARFILLI*, Domenica CARNICELLI*, Laura ROCCHI*, Francesca RICCI†, Pasqualepaolo PAGLIARO†, Pier Luigi TAZZARI† and Maurizio BRIGOTTI*[1]

*Dipartimento di Patologia Sperimentale, Università di Bologna, Via San Giacomo 14, 40126 Bologna, Italy, and †Servizio di Immunoematologia e Trasfusionale, Ospedale S. Orsola-Malpighi, Via Massarenti 9, 40138 Bologna, Italy

Short (page heading) title: Ribosome-inactivating proteins bind to neutrophils

Key words: neutrophils, ribosome-inactivating proteins, hemolytic uremic syndrome

The main cause of acute renal failure in children is HUS (hemolytic uremic syndrome), a consequence of intestinal infections with *Escherichia coli* strains producing Stx (Shiga toxins). Stx released in the gut by the non-invasive bacteria reach the blood stream and are targeted to cerebral and renal endothelium triggering HUS. PMN (polymorphonuclear leukocytes) seem involved in Stx delivery through an unidentified membrane receptor (K_d = 10^{-8} M; 2 x 10^5 binding sites) which does not allow internalization. Some experts in the field have defined the interaction Stx/PMN as non-specific and of little biological significance. Here, we show that the A chain of ricin, the well known plant RIP (ribosome-inactivating protein), interacts with PMN (K_d = 10^{-9} M; 2 x 10^5 binding sites) competing for the same receptor that recognizes Stx, whereas diphtheria toxin and several agonists of Toll-like receptors or the mannose-receptor were ineffective. No toxic effects of ricin A chain on PMN were observed, as assessed by measuring protein synthesis and the rate of spontaneous apoptosis of leukocytes. Moreover, two single-chain RIPs (gelonin, saporin S6) showed the same competing effect. Thus, RIPs and Stx1 share structural similarities, the same enzymatic activity and a common receptor on PMN. These observations reveal that the Stx/PMN interaction is specific confirming that PMN recognize molecular patterns common to different foreign molecules.

Abbreviations used: DABCO, 1,4 diazabizyclo[2.2.2]octane; DAPI, 4'-6-diamidino-2-phenylindole; Gb_3, globotriaosylceramide; HUS, hemolytic uremic syndrome; MCV, mean channel value of fluorescence; NEM, *N*-ethylmaleimide; PAMPS, pathogen-associated molecular patterns; PMN, polymorphonuclear leukocytes; PRR, pattern-recognition receptors; RIP, ribosome-inactivating protein; STEC, Shiga-toxin producing *E. coli*; Stx1, Shiga toxin 1; Stx2, Shiga toxin 2; Stx, Shiga toxins; Toll-like receptor, TLR.

[1] To whom correspondence should be addressed (email maurizio.brigotti@unibo.it)

INTRODUCTION

Stx (Shiga toxins) are produced by pathogenic STEC (Shiga toxin-producing *Escherichia coli*) strains which are responsible for severe human illnesses, such as hemorrhagic colitis and HUS (hemolytic uremic syndrome) [1]. HUS is the main cause of acute renal failure in children [2, 3] and is the consequence of intestinal infection by STEC.

STEC produce two main types of bipartite toxins, Stx1 (Shiga toxin 1) and Stx2 (Shiga toxin 2) that are capable of binding to glycolipid receptors, mainly Gb3 (globotriaosylceramide), present on the surface of target cells through their B subunits [4]. After endocytosis, the A subunit damages ribosomes, by removing a specific adenine from 28S rRNA [5], and DNA, by releasing multiple adenines [6, 7]. The consequences are the arrest of protein synthesis and the formation of apurinic sites in the nucleus. Target cells showed a broad spectrum of responses including production of pro-inflammatory cytokines involved in HUS pathogenesis [8, 9] and triggering of the apoptotic program [10]. There is no doubt that the major part of the histopathological lesions observed in HUS are caused by the interaction of these toxins with the endothelial lining of the brain and kidney [4]. However, as STEC are non-invasive bacteria confined to the gut, a great effort has been made to clarify the mechanism of transfer of Stx from the intestinal lumen to the endothelia of target organs. Stx have never been found in the plasma of patients [11, 12], whereas they were detected on the surface of PMN (polymorphonuclear leukocytes) from patients with HUS [13-15]. Purified Stx interact with human PMN through an unknown receptor [16-18] which allows the binding but not internalization of the toxins [16]. Stx showed lower affinity for the leukocyte receptor compared with the Gb3 receptor on target endothelial cells [16] and this might permit the transfer from circulating cells to endothelia. It has been demonstrated that Stx bound to PMN were transferred to Gb3-containing cells during co-incubation *in vitro* [16, 18] impairing protein synthesis of target cells [16]. Although direct evidence in patients or animal models are lacking, this mechanism might represent what happens in the kidney of HUS patients. However, interaction between Stx and PMN has been the object of controversy, with the presentation of conflicting results. Some authors have claimed a lack of specific binding of Stx to PMN [19, 20] or re-interpreted their own previous results as artefacts [21].

In this light, a better characterisation of the receptor responsible for the recognition of Stx by PMN would be a major goal in understanding a crucial step in the pathogenesis of HUS. The partially characterised molecule is a membrane receptor of human PMN [14, 16, 17], expressed on mature neutrophils or granulocyte-differentiated HL-60, but not on immature PMN or undifferentiated HL-60 [17], capable of binding isolated Stx in the absence of added cofactors [14, 17], but not able to internalize the toxic ligand [16]. The binding of Stx1 to PMN is highly sensitive to trypsin treatment [16].

PMN, as well as macrophages, are capable of recognizing PAMPS (pathogen-associated molecular patterns) by means of membrane or cytoplasmic PRR (pattern-recognition receptors) that belong to the innate host immune system [22-24], thus allowing a discrimination between pathogens and self. This prompted us to investigate whether the unknown Stx receptor of PMN is involved in the recognition of molecular signatures shared by other bacterial toxins (diphtheria toxin) or by toxins homologous to Stx.

The molecular mechanism of action of Stx described above is the same as that of the large family of RIPs (ribosome inactivating proteins) from plant: the removal of a specific adenine residue from 28S rRNA in ribosomes and multiple adenines from DNA, for review see [25, 26]. Most plant RIPs, such as gelonin (from *Gelonium multiflorum* seeds) and saporin S6 (from *Saponaria officinalis* seeds), consist only of a single chain (A chain) containing the catalytic site. Ricin, the well known two-chain RIP from *Ricinus communis* seeds, is highly toxic since contains, in addition to the enzymatically active A chain, a second chain (B chain)

that binds to galactose-containing surface molecules mediating endocytosis of the RIP. Stx and ricin largely differ in B chain structure and binding specificity, whereas their A chains show high degree of homology [27, 28]. In addition to the canonical route of internalization through the lectinic B chain, ricin can enter cells through a secondary route: the mannose receptor present on macrophages or Kupffer cells [29, 30]. This receptor binds to high mannose oligosaccharide chains present on both A and B chains of the toxin and this leads to the internalization of the ligand. The mannose receptor is also present on PMN as a non-canonical PRR able to bind exogenous and endogenous glycosylated molecules [31].

In this paper we provide evidence that the A chain of ricin also binds to PMN through the same receptor responsible for Stx recognition. Mannose receptor, TLR (Toll-like receptor) 1, TLR2, TLR5 and TLR6 are not involved in this unconventional role of neutrophils.

EXPERIMENTAL

Materials

Pam2CSK4 and Pam3CSK4 were purchased from InvivoGen; peptidoglycan from *Staphylococcus aureus*, lipoteichoic acid, ovalbumin and mannosylated BSA (23 mol saccharide/mol BSA) were obtained from Sigma-Aldrich and flagellin from *Salmonella typhimurium* was purchased from Alexis Corporation.

Toxin purification

The prototypes Stx1 producer *Escherichia coli* C600 (H19J) was kindly supplied by Dr Alison O'Brien (Department of Microbiology and Immunology, USU of the Health Sciences, Bethesda, U.S.A.). Stx1 was purified by receptor analogue affinity chromatography [32] on globotriose-Fractogel (IsoSep AB). Ricin was a generous gift from Prof. Andrea Bolognesi of this Department. Separation of the two ricin chains by two chromatographic runs on blue-Sepharose and blu dextran-Sepharose was performed as described in [33]. The purity of the isolated chains was verified by SDS-polyacrilamide gel electrophoresis. Isolated ricin A chain was stored at 4°C in 10 mM Tris HCl, pH 7.5, 100 mM NaCl, 0.1% mercaptoethanol. In these conditions, after 3 months, almost all of ricin A chain was in the monomer form (96-99%) as assessed by non-reducing SDS-polyacrilamide gel electrophoresis followed by densitometric analysis of the blue Comassie-labelled bands. The experiments described in the present paper (see below) have been performed with different ricin A preparations within 3 months. The IC_{50} of ricin A chain on the unfractionated rabbit reticulocyte lysate system (0.1-0.2 nM) was similar to previous reported values [25, 34]. IC_{50} was calculated by the least squares method applied to the linear regression between fractional activity and log of the inhibitor concentration. Gelonin from *Gelonium multiflorum* was from Sigma-Aldrich. Saporin S6 was a generous gift from Prof. Fiorenzo Stirpe of this Department. Diphtheria toxin was prepared by the method described in [35].

Binding of Stx1 to PMN

To obtain endotoxin-free PMN (98 % lobulated nuclei) from healthy donors, all the solutions used thorough out the method [36] were sterile and prepared with clinic water. The binding experiments were performed in Eppendorf tubes precoated with PBS containing 1% BSA to avoid non-specific loss of toxins [37]. Freshly isolated endotoxin-free PMN (0.5×10^6) were immediately incubated with native or radiolabelled Stx1 (6-60 nM) in 250 µl of PBS

containing 1% BSA in the absence or in the presence of competing molecules for 90 min at 37°C, with occasional stirring by gentle inversion of the tube. The cells were spin down at 200 ***g*** for 5 min and washed three times with 100 µl of the same buffer containing BSA at 37°C. The extent of binding of native or radiolabelled Stx1 to PMN was measured as described in the following paragraphs.

Indirect flow cytometric analysis of Stx1 bound to PMN

Stx1 bound to PMN was detected by flow cytometry as previously described [13, 14]. Briefly, PMN were incubated with appropriately diluted mouse monoclonal antibody against Stx1 in the presence of human serum to saturate Fc receptors. After incubation with FITC-goat anti-mouse IgG, flow cytometric analysis was utilized to reveal the cell-bound fluorescence. Flow cytometer (FC500 Beckman Coulter) was set to acquire and gate events both by forward scatter vs 90° side scatter, and by green fluorescence vs 90° side scatter. PMN were checked by staining with monoclonal antibodies to antigens associated to granulocytes (FITC-CD16 and FITC-CD65, Beckman Coulter). This set resulted in a prompt analysis of both morphology and fluorescence, allowing a clear evaluation of control and positive samples. The MCV (mean channel value of fluorescence) of the cells was chosen as an objective parameter to measure the extent of binding of Stx1 to cells [14]. The single values were calculated by subtracting the control MCV (range 0.4-0.6), i.e., the MCV of cells incubated with primary and secondary antibodies in the absence of the toxin. The same values (MCV = 0.4-0.6) were obtained if anti-Stx1 mouse monoclonal antibodies were omitted in the assay in the presence of toxins and secondary antibodies. The assay had been previously validated by challenging Stx-positive PMN with a negative control antibody [13] and by comparing control subjects and HUS patients in a double blind fashion [14].

Radiolabelling of Stx1 and binding to PMN

Stx1 was radiolabelled with Iodo-beads (Pierce Biotechnology) as previously described [17]. The efficiency of labelling was checked by precipitating an aliquot of [^{125}I]Stx1 with 6% TCA in the presence of 0.01% sodium deoxycholate. The biological activity of the radiolabelled toxin (specific radioactivity about 100,000 cpm/pmol) was assessed by testing the binding to immobilized Gb3 (globotriose-Fractogel), and by measuring the IC_{50} of radiolabelled and native toxins on human endothelial cell protein synthesis [6]. The binding of [^{125}I]Stx1 to PMN was quantified by counting the cell-associated radioactivity with a γ-counter after extensive washing (see above). The binding of radiolabelled Stx1 in the presence of a 50-fold excess of native toxin (nonspecific binding) was subtracted in each experiment.

Fluorescent labelling of ricin A chain

The storage buffer of ricin A chain was removed by centrifugation on Microcon YM-3 (Millipore) followed by repeated washes with PBS. Then, ricin A chain (100 µg) was conjugated with Alexa Fluor® 488 reactive fluorescent dye and purified by spin column according to the directions of the manufacturer (Molecular Probe, Invitrogen). The degree of labelling was about 1 mole of Alexa Fluor® 488 dye per mole of subunit.

Treatment of ricin A chain with *N*-ethylmaleimide

Ricin A chain was made 30 µM in PBS (pH 7.2) by centrifugation on Microcon YM-3 and repeated washes with the above buffer. Then, 30-fold molar excess of freshly prepared NEM (*N*-ethylmaleimide, Pierce Biotechnology) was added and the reaction mixture was incubated for 2 h at room temperature [38]. The reacted ricin A chain was purified by spin column as described above. The degree of free sulfhydryl groups, assessed by Ellman's reagent

according to manufacturer's instruction (Pierce Biotechnology) after 5 min incubation, was 0.86 or 0.16 mole/mole of untreated or NEM-treated ricin A chain, respectively.

Direct flow cytometric analysis of the binding of fluorescent ricin A chain to PMN

PMN (0.5 x 10^6) were treated with increasing concentrations of Alexa Fluor® 488-ricin A chain (0.5-20 nM) in 250 µl of PBS containing 1% BSA and incubated and washed as described above. The extent of binding of fluorescent ricin A chain to PMN was measured by direct flow cytometric analysis and expressed as MCV. The binding of fluorescent ricin A chain in the presence of an excess of unlabelled ricin A (non-specific binding) was subtracted in each experiment and shown in Figure 4.

Scatchard plot and non-linear regression analysis of the interaction PMN/ricin A chain

Different experiments of iodination of ricin A chain were performed with Iodo-beads (Pierce Biotechnology) with different protocols suggested by the manufacturer to limit the oxidation of the protein. In each case we observed loss of PMN binding activity after iodination. Thus, Alexa Fluor® 488-ricin A chain was used in this set of experiments. Different concentrations of fluorescent A chain (0.5-10 nM) were incubated for 90 min at 37°C in 1.5 ml PBS containing 1% BSA in Eppendorf tubes coated with BSA (see above) in the absence and in the presence of PMN (4.75 x 10^6). After centrifugation of the samples for 5 min at 200 ***g***, the fluorescence values of supernatants were measured using a Kontron spectrophotofluorimeter. Excitation and emission wavelengths were set at 494 and 519 nm. The single values of the two different series (with or without PMN) were calculated after subtracting the blank values obtained with the supernatant from untreated PMN or with the buffer alone, respectively. The amounts of fluorescent toxin bound to PMN were calculated by subtracting the values of fluorescence obtained in the presence of PMN to those obtained in the absence of cells. The K_d (dissociation constants) and the number of binding sites on PMN were determined by Scatchard plot or by nonlinear regression of the saturation binding data using the GraphPad prism computer program (GraphPad Software).

Protein synthesis of PMN

PMN (3.5 x 10^6) were incubated for 90 min at 37°C in 1.75 ml of PBS containing 1% BSA in the absence and in the presence of ricin or ricin A chain as indicated in the legend to Figure 2. At the end of the incubation, cells were washed three times with 1 ml PBS containing 1% BSA and protein synthesis was assessed immediately or after 15-h post-incubation in RPMI containing 10% FCS. PMN translation was measured as the rate of incorporation of labelled leucine during a 1 h incubation of the cells at 37°C in 1 ml RPMI containing 10% FCS and 5 µCi of [^{3}H]leucine (62 Ci/mmol, Amersham). PMN were spun down as described above and washed three times with 0.5 ml of cold PBS containing 10 mM leucine. The cellular pellets were treated with 0.5 ml of cold 10% TCA followed by 5 min incubation in ice and centrifuged 10 min at 13,000 ***g***. The procedure was repeated three times. Finally the precipitated cellular proteins were resuspended in 200 µl of KOH 0.2 N and the radioactivity was measured in a liquid scintillation counter. In each experiment blank values obtained by incubating PMN in the presence of labelled leucine (see above) for 1 h at 0 °C were subtracted.

Determination of PMN apoptosis

Apoptosis was evaluated (i) by assessing the annexin V binding to phosphatidylserine exposed on the outer leaflet of the plasma membrane of apoptotic cells and by evaluating, simultaneously, the exclusion of PI (propidium iodide) (ApoScreen Annexin V apoptosis kit-

FITC, Beckman Coulter) by means of flow cytometric analysis; and (ii) by the observation of changes in nuclear shape of PMN (from lobulated to shrunken, ovoid, and pyknotic). Nuclear shape of PMN incubated as described in the legend to Figure 2B was determined from the DAPI (4'-6-diamidino-2-phenylindole) staining. After washing in PBS and distilled water, the coverslips were air dried and mounted in a solution containing 0.2 mg/ml DAPI (Sigma-Aldrich) in water, diluted 1:500 in DABCO (1,4 diazabizyclo[2.2.2]octane) (Sigma-Aldrich) and examined with a Nikon epifluorescence microscope, equipped with a 100KV lamp and filters for FITC and DAPI.

Statistics

Student's *t* test was used to compare two groups. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of bacterial or plant toxins on the binding of Stx1 to PMN

According to the hypothesis that Stx receptors on PMN might recognize molecular signatures common to other bacterial toxins or to structurally related toxins of non-bacterial origin, we measured the binding of Stx1 to PMN by indirect flow cytometric analysis in the presence of diphtheria toxin and the plant RIP ricin. The experimental conditions were chosen to have 50% saturation of Stx receptors on PMN [14, 17]. Ricin in different forms (whole toxin, reduced toxin, isolated A or B chains) strongly interfered with the recognition of Stx1 by PMN (Table 1), whereas binding was unaffected in the presence of large molar excess of diphtheria toxin (Table 1). NEM-treated ricin A chain was equally effective indicating that the single sulfhydryl group exposed after reduction of whole ricin and neutralized by NEM [38] is not required for activity. The results obtained with the B chain of ricin, which recognizes many glycolipids and glycoproteins on cell surface, are not surprising and suggest that the Stx receptors on PMN are glycosylated. More interesting is the competing effect displayed by the isolated ricin A chain, which showed structural homology with the A chains of Stx. To characterize better the competing effects and to exclude the presence of ricin A chain/Stx interactions preventing the binding of the bacterial toxin to PMN, the latter cells were pre-incubated with ricin A chain followed by extensive washing. This treatment completely inhibited the binding of subsequently added Stx1 (Figure 1) suggesting the presence of a common binding sites for the two toxins on PMN.

Lack of cytotoxic effects elicited by ricin A chain on PMN

The internalization of isolated ricin A chain through the mannose-receptor has been described in different cells [29, 30]. To exclude the presence of cytotoxic effects on PMN explaining the results obtained above, the translation rate of PMN was measured in the presence and in the absence of ricin A chain. Although PMN are terminally differentiated non-proliferating cells, a detectable level of [^{3}H]leucine incorporation into proteins was recorded. As shown in Figure 2A, PMN protein synthesis was strongly inhibited by whole ricin, whereas ricin A chain, tested at concentrations impairing the binding of Stx1 to PMN, did not significantly affect translation even after 15-h post-incubation.

The absence of cytotoxic effects was also confirmed by comparing the rate of spontaneous apoptosis of control PMN and of PMN treated with ricin A chain. Mature PMN of the blood are highly specialized non-dividing cells with a short (1 or 2 days) life span [39] as they are constitutively engaged in the apoptotic program [40]. PMN undergoing apoptosis lose the multilobulated shape of the nucleus that became ovoid, shrunken, and pyknotic, and this is

considered one of the morphological characteristics of the apoptotic PMN [40]. Figure 2B shows that the appearance of apoptotic nuclei after 10 h or 24 h incubation of freshly isolated PMN was similar to that observed in ricin A-treated cells, whereas in the presence of ricin the whole PMN population underwent apoptosis. PMN were also analyzed for binding to annexin V and simultaneously with a PI dye exclusion test after 10 h incubation in the absence and in the presence of ricin A chain. The annexin V assay is based on the binding of FITC-conjugated annexin V to phosphatidylserine exposed on the outer leaflet of the plasma membrane lipid bilayer of apoptotic cells, followed by flow cytometric detection. Annexin V-positive cells were classified as early apoptotic cells (Figure 3, quadrants C4), and annexin V and PI double positive cells were considered late apoptotic cells (Figure 3, quadrants C2). The results depicted in the representative cytograms of Figure 3 show that the number of untreated live PMN ($0.25 \times 10^6 \pm 0.02 \times 10^6$) that are not yet initiated in the spontaneous apoptotic program (Figure 3A, quadrants C3) did not significantly change in the presence of 2 nM ($0.23 \times 10^6 \pm 0.02 \times 10^6$) or 20 nM ($0.22 \times 10^6 \pm 0.01 \times 10^6$) ricin A chain (Figures 3D and 3C, respectively). In contrast, whole ricin represented a frank apoptotic signal since nearly the totality of ricin-treated PMN were positive to annexin V (Figure 3B), while the number of live PMN ($0.01 \times 10^6 \pm 0.01 \times 10^6$, $P < 0.005$) was dramatically reduced. Necrotic cells (cell positive for PI alone; quadrant C1) or late apoptotic cells were almost absent under these conditions.

Taken together, these findings excluded the presence of cytotoxic effects induced by ricin A chain and, as previously shown for Stx1, suggest the involvement of a receptor incapable of inducing internalization in the interaction ricin A chain/PMN.

Single chain RIPs inhibit the binding of Stx1 to PMN

Table 2 shows that also the single-chain RIPs gelonin and saporin S6, as well as ricin A chain, significantly inhibited the binding of Stx1 to PMN assessed by indirect flow cytometric analysis. Since this is an antibody-based indirect method that has been questioned by some authors [21], the RIP capacity to displace Stx1 from PMN was confirmed with iodinated Stx1 obtaining similar results (Table 2). The experimental conditions were chosen to have full saturation of Stx receptors on PMN [14, 17]. The results indicate that some common structural features of the enzymatically active chains of plant RIPs and Stx are recognized by PMN. Ricin A showed a more powerful trend in displacing Stx1 from PMN either by direct (iodinated Stx1) or indirect method (cytofluorimetric assay), even though differences in the inhibitory power of the tested RIPs were not significant with both assays. However, the strong competing effect showed with ricin A chain tested at a lower concentration in respect with Stx1 (Table 2) suggests an high-affinity binding of this RIP to PMN.

Mannose receptor and other known PRR are not involved in Stx1 recognition by PMN

It should be noted that the mature form of saporin S6 is not glycosylated [41, 42] and, thus, the results obtained in Table 2 with this RIP can not be explained by engagement of the mannose receptor. Moreover, the lack of inhibition of Stx1 binding to PMN in the presence of agonists of the mannose receptor (mannosylated BSA, ovalbumin) completely rules out the involvement of this PRR in Stx recognition by PMN (Table 1). The binding of Stx1 to PMN was also unaffected by the presence of large excess of competitor molecules (Pam2CSK4, Pam3CSK4, peptidoglycan, lipotheicoic acid, flagellin) for TLR1, TLR2, TLR5, TLR6 which sense protein or peptide derivatives produced by pathogens (Table 1). TLR3, TLR7, TLR8 and TLR9 were excluded in these experiments since they are not expressed on the cell surface [22].

High-affinity binding of ricin A chain to PMN

As shown in Figure 4, the binding of ricin A chain to PMN was directly demonstrated by incubating cells with increasing amounts of fluorescent Alexa Fluor 488®-ricin A chain. The binding was saturable (Figure 4) and inhibited specifically by 500 nM unlabelled ricin A chain. The amount of fluorescence associated to PMN was identical in experiments performed at 4°C or at 37°C, indicating that ricin A chain is not actively internalized by PMN. Scatchard plot analysis fitted in a linear curve suggesting the presence of a single binding-site class. As shown in Table 3, the K_d and the number of binding sites of the interaction ricin A chain/PMN were calculated both by linear and by non linear regression employing PMN from two different healthy donors. The K_d value (10^{-9} M) showed that the affinity of ricin A chain for PMN is higher than that previously reported for Stx1 (10^{-8} M) [16] explaining the powerful inhibition of Stx1 binding obtained with lower concentrations of added ricin A chain (Table 2). The number of ricin A binding sites per cell calculated in the present study (178,000 ± 5000) was of the same order of magnitude of those previously calculated for the interaction Stx1/PMN by te Loo et al. [16] (210,000 binding sites) and in our laboratory (195,000 binding sites) [17].

Since unlabelled ricin A chain has been found to inhibit the binding of Stx1 to PMN (Figure 1) the reciprocal experiment was performed. Taking into account the 10-fold higher affinity of ricin A chain for PMN with respect to Stx1, PMN were pre-incubated with the bacterial toxin, then the unwashed leukocytes were treated with fluorescent ricin A chain at 4°C to minimize detachment of Stx1 from PMN [17]. Figure 5 shows that an excess of free Stx1 (60 nM) displaced fluorescent ricin A chain (2 nM) from PMN.

Taken together, the results obtained are in keeping with the notion that Stx1 and ricin A chain share the same receptor on PMN.

DISCUSSION

The interaction between Stx and PMN and their role in the pathogenesis of HUS have been the object of great scrutiny and stimulated intense debates. Most of the criticisms have been centred on the weak affinity of the binding ($K_d = 10^{-8}$ M) that was defined by some authors as non-specific and of little biological significance. In fact, some experts in the field failed to appreciate the relevance of binding of a toxin to a low affinity receptor, rather than to the presumably equally accessible higher affinity receptors in renal endothelium. However, to the best of our knowledge, free Stx have never been found in the plasma of patients suffering from HUS. Moreover, the low affinity displayed by PMN for Stx1 could be considered a strength in a model involving the passage from circulating cells carrying the toxin to target cells. Obviously, low affinity does not necessary mean lack of specificity.

The present paper provides strong evidence supporting the concept that PMN specifically recognize Stx1 and other closely related toxic proteins, namely RIPs. We found that the A chain of ricin and two single chain RIPs, gelonin and the non-glycosylated saporin S6, inhibit the binding of Stx1 to PMN, whereas diphtheria toxin and several peptide/protein derivatives acting as agonists of TLR or the mannose receptor are completely ineffective. This is in keeping with the notion that there are regions of sequence homology between the A chains of ricin, Stx1 and the close relatives Shiga toxin from *Shigella dysenteriae* and Stx2 variants [27, 28]. The A subunits of these toxins are structurally and functionally equivalent to single chain RIPs [25, 43]. Our results are in agreement with the convincing evidence presented by Griener et al [18] indicating that the A subunit of Stx is mainly responsible for the binding of the whole toxin to human PMN with a Gb3-independent mechanism. The implication of Gb3 in Stx1 and ricin A binding was further excluded as human granulocytes do not have the

enzyme repertoire for globo-series glycosphingolipids and do not express Gb3 and Gb4 [18, 44].

The binding of ricin A chain to human PMN was directly demonstrated by flow cytometric analysis, since iodination of the protein, even though performed in mild conditions, led to loss of PMN binding activity. It should be noted that Stx1 is fully active in binding PMN after iodination (Table 2) [17] and that its A chain possesses fewer tyrosine residues available for labelling compared to ricin A chain. Denaturation of the latter protein caused by steric hindrance as a consequence of iodination or caused by susceptibility to oxidation of some crucial protein domains might explain the result.

The evidence collected support the involvement of a common receptor for Stx1 and ricin A chain on PMN: (i) pre-incubation of PMN with ricin A chain, although non-toxic for leukocytes, prevented the binding of Stx1; (ii) pre-incubation of PMN with Stx1 displaced ricin A chain from leukocytes; (iii) co-incubation of Stx1 with single chain RIPs homologous to ricin A chain inhibited the binding of the bacterial toxin to PMN; (iv) the number of binding sites of the interactions Stx1/PMN and ricin A chain/PMN is of the same order of magnitude; (v) ricin A chain was not internalized by leukocytes in the same way as Stx1. The possibility that the data obtained may be explained by non-specific competition of Stx1 binding by the strong ionic charges of the A chains of some ribosome-inactivating proteins was ruled out since saporin S6 and gelonin which have fairly high isoelectric points (≥ 9.5) are effective as ricin A chain (Table 1 and 2) which displays a nearly neutral isoelectric point (7.3-7.6) [25, 45].

Although the importance of these findings in the mechanism of poisoning by ricin has to be established and is beyond the scope of this study, our data strongly suggest the presence of a receptor on PMN interacting with a class of enzymes which have the same mechanism of action on polynucleotides and structural homology. These observations reveal that the Stx/PMN interaction is specific confirming the important role of these circulating cells in the diagnosis and in the pathogenesis of HUS.

ACKNOWLEDGEMENTS

We thank Prof. Andrea Bolognesi and Prof. Fiorenzo Stirpe of this Department for the generous gift of ricin and saporin 6, respectively. We also thank Prof. Emanuele Papini (Dipartimento di Scienze Biomediche Sperimentali dell'Università di Padova) and Dr Christine Betts of this Department for the critical reading of the manuscript.

FUNDING

This work was supported by the University of Bologna (RFO funds, M.B).

REFERENCES

1 Trompeter, R. S., Schwartz, R., Chantler, C., Dillon, M. J., Haycock, G. B., Kay, R. and Barratt, T. M. (1983) Haemolytic-uraemic syndrome: an analysis of prognostic features. Arch. Dis. Child. **58**, 101-105

2 Tarr, P. I., Gordon, C. A. and Chandler, W. L. (2005) Shiga-toxin-producing Escherichia coli and haemolytic uraemic syndrome. Lancet **365**, 1073-1086

3 Noris, M. and Remuzzi, G. (2005) Hemolytic uremic syndrome. J. Am. Soc. Nephrol. **16**, 1035-1050

4 Paton, J. C. and Paton, A. W. (1998) Pathogenesis and diagnosis of Shiga toxin-producing Escherichia coli infections. Clin. Microbiol. Rev. **11**, 450-479

5 Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988) Site of action of a Vero toxin (VT2) from Escherichia coli O157:H7 and of Shiga toxin on eukaryotic ribosomes. RNA N-glycosidase activity of the toxins. Eur. J. Biochem. **171**, 45-50

6 Brigotti, M., Alfieri, R., Sestili, P., Bonelli, M., Petronini, P. G., Guidarelli, A., Barbieri, L., Stirpe, F. and Sperti, S. (2002) Damage to nuclear DNA induced by Shiga toxin 1 and ricin in human endothelial cells. Faseb J. **16**, 365-372

7 Barbieri, L., Valbonesi, P., Brigotti, M., Montanaro, L., Stirpe, F. and Sperti, S. (1998) Shiga-like toxin I is a polynucleotide:adenosine glycosidase. Mol. Microbiol. **29**, 661-662

8 Brigotti, M., Carnicelli, D., Ravanelli, E., Vara, A. G., Martinelli, C., Alfieri, R. R., Petronini, P. G. and Sestili, P. (2007) Molecular Damage and Induction of Proinflammatory Cytokines in Human Endothelial Cells Exposed to Shiga Toxin 1, Shiga Toxin 2, and {alpha}-Sarcin. Infect. Immun. **75**, 2201-2207

9 Matussek, A., Lauber, J., Bergau, A., Hansen, W., Rohde, M., Dittmar, K. E., Gunzer, M., Mengel, M., Gatzlaff, P., Hartmann, M., Buer, J. and Gunzer, F. (2003) Molecular and functional analysis of Shiga toxin-induced response patterns in human vascular endothelial cells. Blood **102**, 1323-1332

10 Nakao, H. and Takeda, T. (2000) Escherichia coli Shiga toxin. J. Nat. Toxins **9**, 299-313

11 Caprioli, A., Luzzi, I., Rosmini, F., Pasquini, P., Cirrincione, R., Gianviti, A., Matteucci, M. C. and Rizzoni, G. (1992) Hemolytic-uremic syndrome and Vero cytotoxin-producing Escherichia coli infection in Italy. The HUS Italian Study Group. J. Infect. Dis. **166**, 154-158

12 Karmali, M. A., Petric, M., Lim, C., Fleming, P. C., Arbus, G. S. and Lior, H. (1985) The association between idiopathic hemolytic uremic syndrome and infection by verotoxin-producing Escherichia coli. J. Infect. Dis. **151**, 775-782

13 Brigotti, M., Caprioli, A., Tozzi, A. E., Tazzari, P. L., Ricci, F., Conte, R., Carnicelli, D., Procaccino, M. A., Minelli, F., Ferretti, A. V., Paglialonga, F., Edefonti, A. and Rizzoni, G. (2006) Shiga toxins present in the gut and in the polymorphonuclear leukocytes circulating in the blood of children with hemolytic-uremic syndrome. J. Clin. Microbiol. **44**, 313-317

14 Tazzari, P. L., Ricci, F., Carnicelli, D., Caprioli, A., Tozzi, A. E., Rizzoni, G., Conte, R. and Brigotti, M. (2004) Flow cytometry detection of Shiga toxins in the blood from children with hemolytic uremic syndrome. Cytometry B Clin. Cytom. **61**, 40-44

15 Te Loo, D. M., van Hinsbergh, V. W., van den Heuvel, L. P. and Monnens, L. A. (2001) Detection of verocytotoxin bound to circulating polymorphonuclear leukocytes of patients with hemolytic uremic syndrome. J. Am. Soc. Nephrol. **12**, 800-806

16 te Loo, D. M., Monnens, L. A., van Der Velden, T. J., Vermeer, M. A., Preyers, F., Demacker, P. N., van Den Heuvel, L. P. and van Hinsbergh, V. W. (2000) Binding and transfer of verocytotoxin by polymorphonuclear leukocytes in hemolytic uremic syndrome. Blood **95**, 3396-3402

17 Brigotti, M., Carnicelli, D., Ravanelli, E., Barbieri, S., Ricci, F., Bontadini, A., Tozzi, A. E., Scavia, G., Caprioli, A. and Tazzari, P. L. (2008) Interactions between Shiga toxins and human polymorphonuclear leukocytes. J. Leukoc. Biol. **84**, 1019-1027
18 Griener, T. P., Mulvey, G. L., Marcato, P. and Armstrong, G. D. (2007) Differential binding of Shiga toxin 2 to human and murine neutrophils. J. Med. Microbiol. **56**, 1423-1430
19 Fernandez, G. C., Gomez, S. A., Rubel, C. J., Bentancor, L. V., Barrionuevo, P., Alduncin, M., Grimoldi, I., Exeni, R., Isturiz, M. A. and Palermo, M. S. (2005) Impaired neutrophils in children with the typical form of hemolytic uremic syndrome. Pediatr. Nephrol. **20**, 1306-1314
20 Flagler, M. J., Strasser, J. E., Chalk, C. L. and Weiss, A. A. (2007) Comparative analysis of the abilities of Shiga toxins 1 and 2 to bind to and influence neutrophil apoptosis. Infect. Immun. **75**, 760-765
21 Geelen, J. M., van der Velden, T. J., Te Loo, D. M., Boerman, O. C., van den Heuvel, L. P. and Monnens, L. A. (2007) Lack of specific binding of Shiga-like toxin (verocytotoxin) and non-specific interaction of Shiga-like toxin 2 antibody with human polymorphonuclear leucocytes. Nephrol. Dial. Transplant. **22**, 749-755
22 Akira, S. and Takeda, K. (2004) Toll-like receptor signalling. Nat. Rev. Immunol. **4**, 499-511
23 Creagh, E. M. and O'Neill, L. A. (2006) TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. Trends Immunol. **27**, 352-357
24 Gazi, U. and Martinez-Pomares, L. (2009) Influence of the mannose receptor in host immune responses. Immunobiology. **214**, 554-561
25 Barbieri, L., Battelli, M. G. and Stirpe, F. (1993) Ribosome-inactivating proteins from plants. Biochim. Biophys. Acta. **1154**, 237-282
26 Peumans, W. J., Hao, Q. and Van Damme, E. J. (2001) Ribosome-inactivating proteins from plants: more than RNA N-glycosidases? FASEB J. **15**, 1493-1506
27 Calderwood, S. B., Auclair, F., Donohue-Rolfe, A., Keusch, G. T. and Mekalanos, J. J. (1987) Nucleotide sequence of the Shiga-like toxin genes of Escherichia coli. Proc. Natl. Acad. Sci. U S A. **84**, 4364-4368
28 O'Brien, A. D., Tesh, V. L., Donohue-Rolfe, A., Jackson, M. P., Olsnes, S., Sandvig, K., Lindberg, A. A. and Keusch, G. T. (1992) Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. Curr. Top. Microbiol. Immunol. **180**, 65-94
29 Simmons, B. M., Stahl, P. D. and Russell, J. H. (1986) Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. Multiple intracellular pathways for a chain translocation. J. Biol. Chem. **261**, 7912-7920
30 Skilleter, D. N., Paine, A. J. and Stirpe, F. (1981) A comparison of the accumulation of ricin by hepatic parenchymal and non-parenchymal cells and its inhibition of protein synthesis. Biochim. Biophys. Acta. **677**, 495-500
31 Valera, I., Vigo, A. G., Alonso, S., Barbolla, L., Crespo, M. S. and Fernandez, N. (2007) Peptidoglycan and mannose-based molecular patterns trigger the arachidonic acid cascade in human polymorphonuclear leukocytes. J. Leukoc. Biol. **81**, 925-933
32 Ryd, M., Alfredsson, H., Blomberg, L., Andersson, A. and Lindberg, A. A. (1989) Purification of Shiga toxin by alpha-D-galactose-(1----4)-beta-D-galactose-(1----4)-beta-D-glucose-(1- ---) receptor ligand-based chromatography. FEBS Lett. **258**, 320-322
33 Sperti, S., Montanaro, L. and Rambelli, F. (1986) Dye affinity chromatography of ricin subunits. Biosci. Rep. **6**, 1035-1040
34 Brigotti, M., Rambelli, F., Zamboni, M., Montanaro, L. and Sperti, S. (1989) Effect of alpha-sarcin and ribosome-inactivating proteins on the interaction of elongation factors with ribosomes. Biochem. J. **257**, 723-727

35 Pope, C. G. and Stevens, M. F. (1958) The purification of diphtheria toxin and the isolation of crystalline toxin-protein. Br. J. Exp. Pathol. **39**, 139-149
36 Cassatella, M. A., Cappelli, R., Della Bianca, V., Grzeskowiak, M., Dusi, S. and Berton, G. (1988) Interferon-gamma activates human neutrophil oxygen metabolism and exocytosis. Immunology. **63**, 499-506
37 van Setten, P. A., Monnens, L. A., Verstraten, R. G., van den Heuvel, L. P. and van Hinsbergh, V. W. (1996) Effects of verocytotoxin-1 on nonadherent human monocytes: binding characteristics, protein synthesis, and induction of cytokine release. Blood **88**, 174-183
38 Hedblom, M. L., Cawley, D. B., Boguslawski, S. and Houston, L. L. (1978) Binding of ricin A chain to rat liver ribosomes: relationship to ribosome inactivation. J. Supramol. Struct. **9**, 253-268
39 Bainton, D. F., Ullyot, J. L. and Farquhar, M. G. (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. J Exp Med. **134**, 907-934
40 Akgul, C., Moulding, D. A. and Edwards, S. W. (2001) Molecular control of neutrophil apoptosis. FEBS Lett. **487**, 318-322
41 Benatti, L., Nitti, G., Solinas, M., Valsasina, B., Vitale, A., Ceriotti, A. and Soria, M. R. (1991) A Saporin-6 cDNA containing a precursor sequence coding for a carboxyl-terminal extension. FEBS Lett. **291**, 285-288
42 Stirpe, F., Gasperi-Campani, A., Barbieri, L., Falasca, A., Abbondanza, A. and Stevens, W. A. (1983) Ribosome-inactivating proteins from the seeds of Saponaria officinalis L. (soapwort), of Agrostemma githago L. (corn cockle) and of Asparagus officinalis L. (asparagus), and from the latex of Hura crepitans L. (sandbox tree). Biochem. J. **216**, 617-625
43 Hartley, M. R. and Lord, J. M. (2004) Cytotoxic ribosome-inactivating lectins from plants. Biochim. Biophys. Acta. **1701**, 1-14
44 Macher, B. A. and Klock, J. C. (1980) Isolation and chemical characterization of neutral glycosphingolipids of human neutrophils. J. Biol. Chem. **255**, 2092-2096
45 Woo, B. H., Lee, J. T., Na, D. H. and Lee, K. C. (2001) Sepharose-unbinding ricin E as a source for ricin A chain immunotoxin. J. Immunol. Methods **249**, 91-98

Table 1 Binding of Stx1 to PMN in the presence of putative competing molecules

PMN were treated with 6 nM Stx1 with or without the indicated additions and the binding of the toxin to cells was measured by indirect flow cytometric analysis as described in Experimental. In this condition about 50% saturation of receptors was obtained (MCV = 1.54 ± 0.23, mean ± S.D., n = 3). $^{*}P < 0.001$ vs control.

	% PMN binding activity
No addition	100
Toxins	
Diphtheria toxin (0.5 μM)	81.1 ± 20.4
Ricin (0.5 μM)	5.4 ± 7.6*
Ricin (0.5 μM) reduced by 4% mercaptoethanol	8.7 ± 7.2*
Ricin B chain (0.02 μM)	12.7 ± 9.0*
Ricin A chain (0.02 μM)	3.9 ± 3.4*
NEM-treated ricin A chain (0.02 μM)	3.7 ± 1.0*
Mannose receptor agonists	
Mannosylated BSA (1.4 μM)	94.0 ± 6.4
Ovalbumin (0.5 μM)	85.1 ± 12.6
TLR agonists	
Pam2CSK4 (1 μM) (TLR 2 and 6)	87.5 ± 17.7
Pam3CSK4 (1 μM) (TLR 2 and 1)	92.4 ± 10.7
Peptidoglycan (10 μg/ml) (TLR 2)	100.0 ± 1.1
Lipoteichoic acid (10 μg/ml) (TLR 2)	100.1 ± 3.2
Flagellin (0.7 μM) (TLR 5)	96.7 ± 4.7

Biochemical Journal Immediate Publication. Published on 01 Sep 2010 as manuscript BJ20100455

Table 2 Inhibition by RIPs of the binding of Stx1 to PMN

PMN were treated with 50 nM native Stx1 or labelled [^{125}I]Stx1 in the absence and in the presence of RIPs and the binding of Stx1 to cells was measured as described in Experimental by indirect flow cytometric analysis or by detecting cell-associated radioactivity, respectively. In these conditions, full saturation of receptors was obtained either with fluorescent (MCV = 3.25 ± 0.49, mean ± S.D., n = 3) or radiolabelled Stx1 (0.365 ± 0.016 pmol [^{125}I]Stx1 bound/10^6 PMN, mean ± S.D., n = 3). $^{*}P < 0.005$ vs control.

	% PMN binding activity	
	Native Stx1	[^{125}I]Stx1
No addition	100	100
Ribosome-inactivating proteins (2 nM)		
Ricin A chain	13.5 ± 3.3*	26.6 ± 1.4*
Gelonin	38.7 ± 18.0*	35.1 ± 18.2*
Saporin S6	43.0 ± 12.0*	37.9 ± 9.5*

.

Table 3 Dissociation constant and number of binding sites of the interaction ricin A chain/PMN

	K_d		Number of binding sites	
	Scatchard plot	Non-linear regression	Scatchard plot	Non-linear regression
Donor 1	0.87×10^{-9}	1.26×10^{-9}	173 000	178 000
Donor 2	1.00×10^{-9}	1.40×10^{-9}	184 000	177 000

Figure 1 Flow cytometric analysis illustrating the effect of ricin A chain on the binding of Stx1 to PMN

(**A**) control PMN; (**B**) PMN treated with 6 nM Stx1 (90 min at 37°C); (**C**) PMN treated with 6 nM Stx1 in the presence of 20 nM ricin A chain (90 min at 37°C); (**D**) PMN pre-incubated with 2 nM ricin A chain (90 min at 37°C), and post-incubated after extensive washing with 6 nM Stx1 (90 min at 37°C)

Figure 2 Inhibition of protein synthesis and triggering of the apoptotic program in PMN treated with whole ricin or ricin A chain

(**A**) PMN were incubated for 90 min at the indicated concentrations of RIPs, extensively washed and processed to determine the rate of protein synthesis as described in Experimental. Values are the means ± S.D. from three different experiments. The [^{3}H]leucine incorporated by untreated cells was 483 ± 23 dpm and 160 ± 34 dpm, after 90 min and 15 h of incubation of freshly isolated PMN, respectively. ***** $P < 0.005$ vs control cells. (**B**) Nuclear shape of PMN in different conditions as assessed by DAPI-staining. PMN (2% non lobulated nuclei) resuspended in RPMI containing 10% FCS were either untreated or treated with ricin A chain or ricin at the indicated concentrations for 10 h and 24 h. After incubation, changes from lobulated to shrunken, ovoid and pyknotic nuclei (apoptotic nuclei) were determined. Values are the means ± S.D. from three different experiments. At time 0 control PMN showed 98 % lobulated nuclei. ***** $P < 0.001$ vs control cells.

Figure 3 Effect of whole ricin or ricin A chain on the survival of PMN after 10 h of culture

PMN (1 x 10^6) from healthy donors were incubated for 10 h in the absence and in the presence of ricin and ricin A chain at the indicated concentrations (see below) and analyzed by flow cytometry after staining with annexin V and PI. Quadrants C1, necrosis; quadrants C2, late apoptotic cells; quadrants C3, live cells; quadrants C4, early apoptotic cells. (**A**) untreated PMN; (**B**) PMN treated with 50 nM ricin; (**C**) PMN treated with 20 nM ricin A chain; (**D**) PMN treated with 2 nM ricin A chain.

Figure 4 Binding of fluorescent ricin A chain to isolated PMN

Saturation binding experiments with increasing concentrations of Alexa Fluor® 488-ricin A chain in the absence (filled squares) and in the presence (open squares) of 500 nM native ricin A chain. Values are the means ± S.D. from three different experiments performed as described in Experimental.

Figure 5 Flow cytometric analysis illustrating the effect of Stx1 on the binding of fluorescent ricin A chain to PMN

(**A**) control PMN; (**B**) PMN treated with 2 nM Alexa Fluor® 488-ricin A chain (90 min at 4°C); (**C**) PMN pre-incubated with 60 nM Stx1 (90 min at 37°C) and post-incubated with 2 nM Alexa Fluor® 488-ricin A chain (90 min at 4°C).

Figure 1

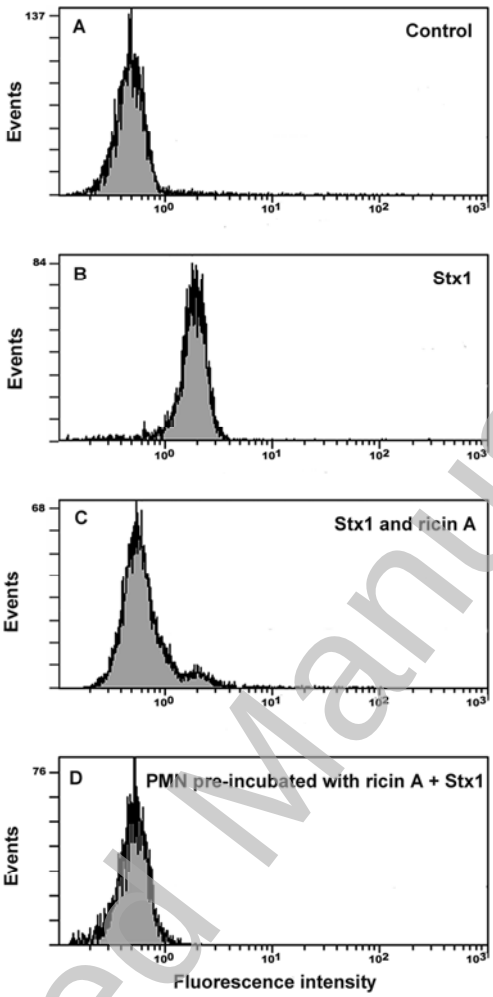

Figure 2

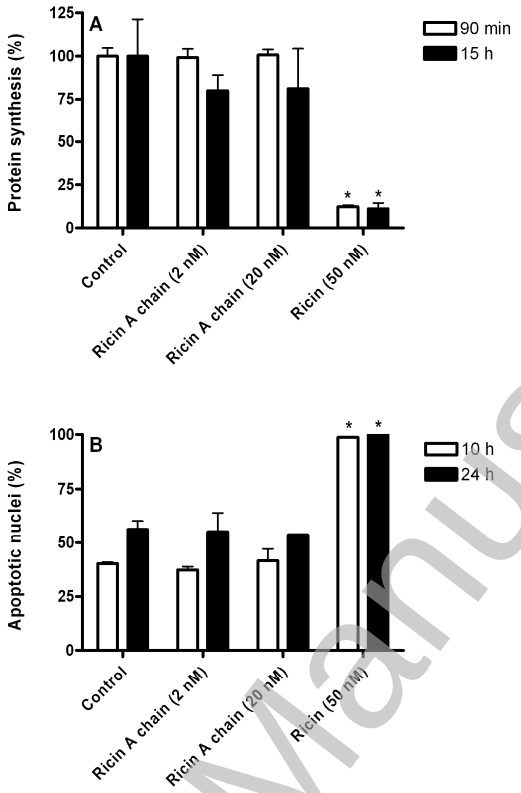

Figure 3

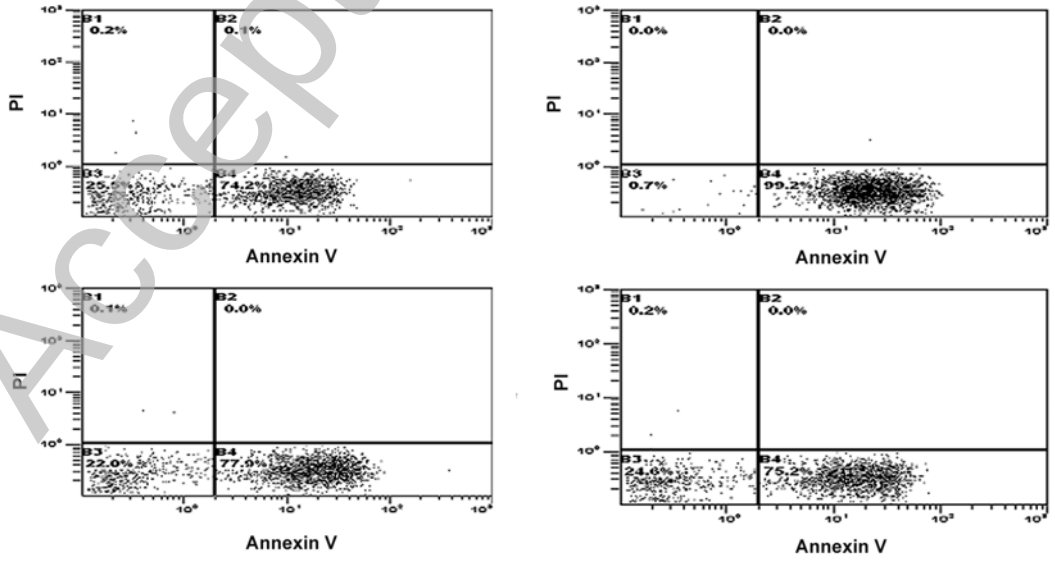

Figure 4

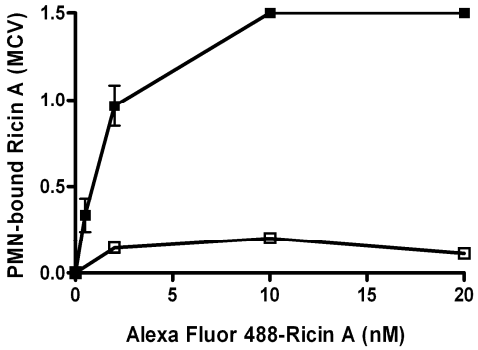

Figure 5