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Specific dose-dependent damage of Lieberkühn crypts promoted by large doses of type 2 ribosome-inactivating protein nigrin b intravenous injection to mice

M.J. Gayoso[a], R. Muñoz[b], Y. Arias[b], R. Villar[c], M.A. Rojo[b], P. Jiménez[b], J.M. Ferreras[b], I. Aranguez[d], T. Girbés[b,*]

[a]*Departamento de Biología Celular, Histología y Farmacología, Facultad de Medicina, Universidad de Valladolid, 47005 Valladolid, Spain*
[b]*Departamento de Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de Valladolid, 47005 Valladolid, Spain*
[c]*Departamento de Hematología, Hospital Pío del Rio Hortega, SACYL, 47010 Valladolid, Spain*
[d]*Departamento de Bioquímica y Biología Molecular, Facultad de Farmacia, Universidad Complutense, 28040 Madrid, Spain*

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Abstract

Nigrin b is a non-toxic type 2 ribosome-inactivating protein as active as ricin at ribosomal level but 10^5 and 5×10^3 times less toxic for animal cell cultures and mice, respectively, than ricin. The purpose of the present study was to analyze the effects of intravenous injection of large amounts of nigrin b to the mouse. Injection through the tail vein of 16 mg/kg body weight killed all mice studied before 2 days. Analysis of several major tissues by light microscopy did not reveal gross nigrin b-promoted changes, except in the intestines which appeared highly damaged. As a consequence of the injury, the villi and crypt structures of the small intestine disappeared, leading to profuse bleeding and death. In contrast, intravenous injection of 5 mg/kg body weight was not lethal to mice but did trigger reversible toxic effects. In both cases, lethal and sub-lethal doses, the target of nigrin b appeared to be the highly proliferating stem cells of the intestinal crypts, which had undergone apoptotic changes. In contrast to nigrin b, the injection of 3 μg/kg of ricin kills all mice in 5 days but does not trigger apoptosis in the crypts. Therefore, the effect seen with sub-lethal nigrin b concentrations seems to be specific. Nigrin b killed COLO 320 human colon adenocarcinoma cells with an IC_{50} of 3.1×10^{-8} M and the effect was parallel to the extent of DNA fragmentation of these cells. Accordingly, despite the low general toxicity exerted by nigrin b as compared with ricin, intravenous injection of large amounts of nigrin b is able to kill mouse intestinal stem cells without threatening the lives of the animals, thereby opening a door for its use for the targeting of intestinal stem cells.

Keywords: Nigrin b; Ricin; Ribosome-inactivating protein; Apoptosis; Small intestine; Stem cells

Introduction

Since the discovery of the highly poisonous protein toxin ricin a large number of attempts have been made to unravel the molecular mechanism of action and its toxicity at cellular level and in animals (Lord et al., 1994). Ricin intoxication promotes a broad variety of toxic effects. Thus, severe cardiac, renal, intestinal, and lung derangement have been described (Bradberry et al., 2003; Brown and White, 1997; Griffiths et al., 1987; Hughes et al., 1996; Leek et al., 1989). The intensity and reversibility of the toxic effects are dependent on the dose of ricin administered (Bradberry et al., 2003). For mice, the lethal dose (LD_{50}) is reached at 2.6 μg/kg body weight (Barbieri et al., 1993; Stirpe, 2004).

Concerning the molecular mechanism through which ricin triggers its toxic effects, it was found that ricin promotes the specific cleavage of the N-glycoside linkage

Abbreviations: TUNEL, terminal deoxyribonucleotidyl transferase-mediated dUTP-nick-end labeling; IC_{50}, inhibitory concentration 50%.

* Corresponding author. Fax: +34 983 423 082.
E-mail address: girbes@bio.uva.es (T. Girbés).

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between the A_{4324} and the phosphoribose backbone of 28 S rRNA from rat liver (reviewed in Girbes et al., 2004; Stirpe, 2004). Ricin is the head of a class of important enzymes that depurinate nucleic acids and are known as ribosome-inactivating proteins or RIPs. RIPs have been found in plants, bacteria, and fungi (Girbes et al., 2004) and, very recently, a RIP-like activity has also been found in mammals (Barbieri et al., 2001). Based on their ability to bind sugars, RIPs are classified in two categories (Barbieri et al., 1993; Girbes et al., 2003; Stirpe, 2004). Type 1 RIPs do not bind sugars and are composed of only one catalytic polypeptide chain that contains the N-glycosidase activity whose apparent Mr range is 25–30 kDa. Type 2 RIPs are able to bind sugars and are composed of two kinds of polypeptide chains: a catalytic A chain with N-glycosidase activity, which is equivalent to a type 1 RIP, linked by disulfide bonds to a B chain with sugar-binding ability that confers the whole RIP the nature of lectin. In recent years, interest in RIPs has increased due to their use as the toxic part of anti-cancer immunotoxins (Bolognesi and Polito, 2004; Fracasso et al., 2004; Frankel et al., 2000; Kreitman, 2000; von Mehren et al., 2003) and conjugates (Citores et al., 2002). Furthermore, it has been shown that some RIPs have a direct inhibitory action on HIV-1 replication (Barbieri et al., 1993; Stirpe, 2004).

Over the past 10 years, a new category of RIPs has been found; these are known as non-toxic type 2 RIPs and include nigrin b from elder (*S. nigra* L.) bark and ebulin l from dwarf elder (*S. ebulus* L.) leaves (Girbes et al., 2003). These proteins are structurally and enzymatically related to ricin and the other highly toxic type 2 RIPs (Barbieri et al., 2004; Girbes et al., 2003). However, in contrast to ricin, they are much less toxic to cells and animals, due to the reduced ability of the B chains to bind to the polysaccharide chains present at the surface of plasma membrane proteins (Pascal et al., 2001; Svinth et al., 1998). During our experimental work with nigrin b and mice, we found that when administered at very large concentration nigrin b promoted a serious intestinal derangement that led to death (Girbes et al., 2003). Here, we have investigated the effects of nigrin b at both lethal and sub-lethal doses on mouse gut and the mechanism through which the nigrin b effect seems to be exerted. For comparative purposes, we also analyzed the effects of nigrin b on COLO 320 (human colon adenocarcinoma) cells.

Methods

Materials. The chemicals and biochemicals were of the highest purity available and the sources were the same as those reported elsewhere (Citores et al., 2002; Girbes et al., 1993). Highly purified nigrin b was prepared as described previously (Girbes et al., 1993). The apoptosis detection kit-DeadEnd Fluorimetric TUNEL System was purchased from Promega (Madison, WI, USA).

Cells and animals. COLO 320 (human colon adenocarcinoma) cells were obtained from the European Collection of Cell Cultures (ECACC) and grown in RPMI 1640 medium (GIBCO BRL, Barcelona, Spain) supplemented with 10% FBS under 5% CO_2 at 37 °C and were collected before confluence. CD1 mice were obtained from the our university facilities, housed in plastic cages in a temperature-controlled room, and fed ad libitum with free access to water under a 12-h light–dark cycle. Management of the animals followed the guidelines of the European Communities Council (86/609/EEC) for laboratory animal care and experimentation.

Nigrin b treatment and tissue sample collection. For the toxicity analysis, either nigrin b or ricin was injected through the tail vein with 0.1 ml of a solution containing variable amounts of protein in 5 mM phosphate-buffered saline, pH 7.4. For in vivo comparisons of nigrin b and ricin toxicities, four groups of mice were injected with different doses of each one of the proteins and maintained with food ad libitum when possible for 30 days. Four groups (nine mice per group) were injected with 5, 7.5, 10, and 16 mg/kg body weight of nigrin b and four groups (five mice per group) received 1, 3, 5, and 8 μg/kg body weight of ricin. For histological studies, six groups of at least four adult male or female mice were used. Three groups of mice received 5 mg/kg of nigrin b and were allowed to survive for 5, 24, and 72 h. Another two groups of animals were injected with 16 mg/kg of nigrin b and were allowed to survive for 5 and 24 h. The mice from the sixth group were used as controls and were injected with the vehicle solution and were allowed to survive for 24 h. All animals were anesthetized and perfused transcardially with 4% paraformaldehyde in phosphate buffer. Samples of different organs – brain, eye, kidney, lung, stomach, intestines, pancreas, liver, spleen, skin, testis, and ovary – were post-fixed in the same fixative for 24 h and paraffin wax-embedded for light microscopy studies.

Light microscopy histological analysis. Histological sections of the organs chosen were stained with hematoxylin–eosin for histological analysis and selected zones were studied for apoptosis with the TUNEL method as indicated above. Additionally, propidium iodide was used for general fluorescent staining of nuclei. For bright-field and fluorescent microscopy, an Axiophot Zeiss photomicroscope with epifluorescence and a Spot digital camera were used.

Cell viability analyses. Cell viability was determined by a colorimetric assay based on cleavage of the tetrazolium salt WST-1 to formazan by mitochondrial dehydrogenases in viable cells. 3×10^3 COLO 320 cells in 0.1 ml of medium were seeded in 96-well plates and incubated at 37 °C under 5% CO_2 in the absence or the presence of either nigrin b or ricin, as described in the legends to the figures. Next, the cells were incubated for another 2 h with 10 μl/well of the

cell proliferation reagent WST-1 (Roche Diagnostics S.L., Barcelona, Spain) at 37 °C under 5% CO_2. After plate shaking for 1 min, the absorbance of the samples was measured using a microtitre plate reader set at 450 nm with 620 nm as reference. A background of cultures without cells was subtracted.

DNA fragmentation analysis. COLO 320 cells (2×10^6/T-25 flask) were incubated for 48 h in the absence or the presence of nigrin b at the concentrations indicated in the text. After treatment, cells were harvested by centrifugation at 200 $\times$ *g* for 10 min. The pellets were lysed and the DNA was isolated following the instruction of the genomicPrep for cells and tissue DNA isolation Kit (Amersham Biosciences Europe GmbH, Barcelona, Spain). DNA electrophoresis was carried out in 1.5% agarose gels using TBE buffer (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA, pH 8.0). DNA was visualized with an ultraviolet lamp.

Results

Effects of intravenous injection of nigrin b and ricin on mice

As shown in Fig. 1, nigrin b at 5 mg/kg body weight did not kill any animal (none of the animals had died at 30 days after the injection) and no external signs of gross derangement were evident. Nigrin b at 7.5 mg/kg body weight killed some of the animals but at 7 days after the injection. In contrast, nigrin b at 10 mg/kg killed all the animals within 72 h after injection, and at 16 mg/kg did so during the first 36 h. The animals that were killed by nigrin b showed a dark area in the small intestine, apparently caused by massive bleeding. In clear contrast to nigrin b, ricin requires much lower concentrations to kill mice. Thus, ricin at 3 μg/kg body weight killed all the animals in the first 5 days after injection, and at 8 μg/kg killed all the animals in the first 36 h. In all cases of lethality, a physical depression was observed in the animals at least 12 h before death.

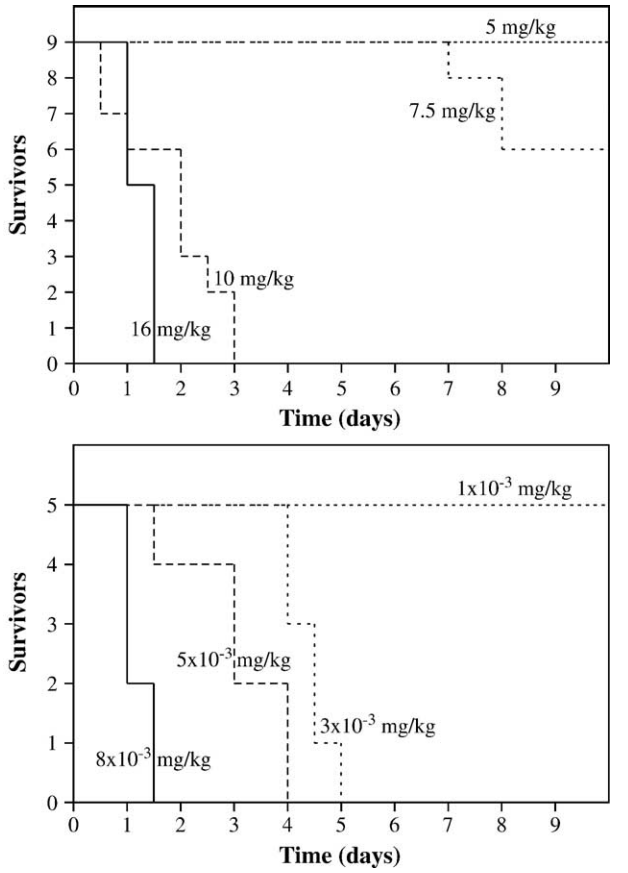

Fig. 1. Kaplan–Meier plots of nigrin b and ricin effects on mice. Groups of animals were treated with different doses of either nigrin b (upper panel) or ricin (lower panel) as indicated in Methods. Survival was assessed at the indicated times upon death the animals the abdominal cavity was opened for visual inspection.

Histological study of the organs of mice treated with a sub-lethal concentration of nigrin b

The external appearance of the animal treated with 5 mg/kg of nigrin b was unaltered and no changes were visible. Histological study of the organs of the mice treated with sub-lethal doses of nigrin b only revealed signs of tissue damage in the intestines. Although individual variations were observed, the general and more characteristic histological features will be described.

At 5 h after nigrin b administration, the mucosa of the small intestine showed histological alterations, mainly in the Lieberkühn crypts and to a lesser extent in the intestinal villi (Figs. 2A and B). The epithelium of the crypts displayed a large number of injured cells, which were mainly located in the middle third. These dying cells stained with haematoxylin–eosin showed an apoptotic-like morphology, with cell shrinkage, an increase in cytoplasmic eosinophilia, condensation of the chromatin with aggregation in peripheral masses, and the final formation of apoptotic bodies (Fig. 2A). The apoptotic-like cells had sloughed off into the crypt and intestinal lumina. Using the TUNEL method, the nuclei of these apoptotic-like cells appeared labeled with fluorescein, demonstrating the DNA fragmentation by endogenous nucleases and the apoptotic type of this cellular death (Figs. 2 D–F). No apoptotic cells were found in the intestinal crypts of the control group. The general structure of the intestinal villi was normal and their covering epithelium only showed minor shrinkage in the basal cytoplasm (Fig. 2B).

In the large intestine, the histological structure was well preserved, with only some scattered apoptotic cells on the wall of the intestinal crypts. The apoptotic cells shed into the gland and intestinal lumen (Fig. 2C). A slight inflammatory infiltrate was observed in the lamina propria but not in the submucosa.

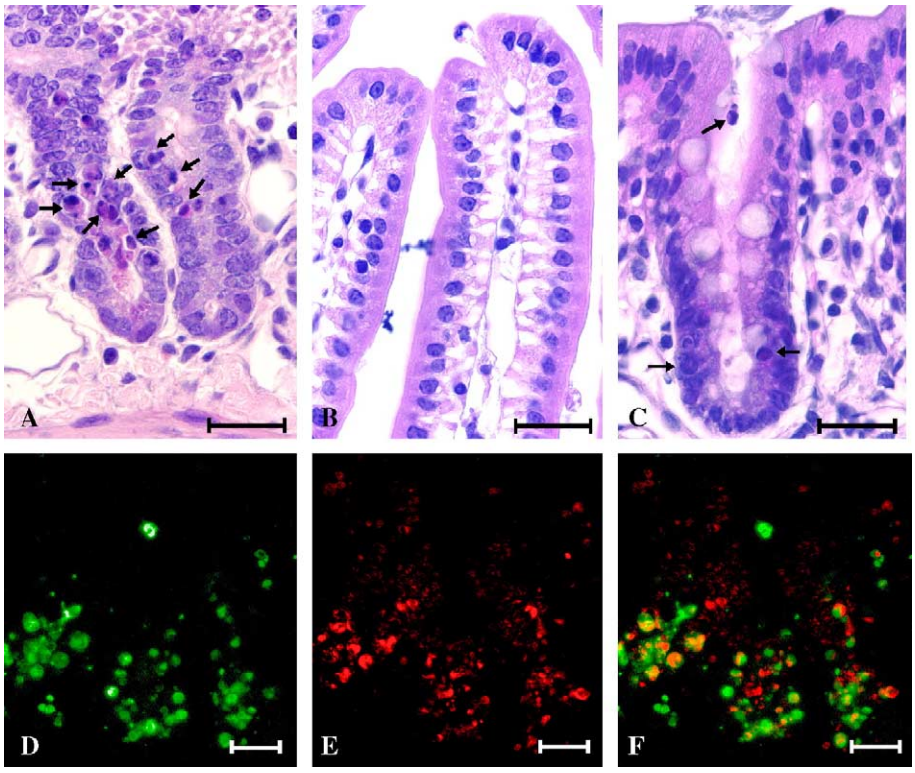

Fig. 2. Histological sections of small and large intestines stained with haematoxylin–eosin (A, B, and C) and using the TUNEL method (D, E, and F) 5 h after administration of 5 mg/kg of body weight of nigrin b. The crypts of the small intestine (A) show injured cells mainly in the middle third (arrows), while the epithelium of the villi is not affected (B). In the crypts of the large intestine, the injured cells (arrows) were scarce (C). The apoptotic cells in the small intestine crypts were fluorescein-labeled (D) and propidium iodide was used as nuclear general stain (E). The merged image is shown in (F). Scale bar = 25 μm.

The lesions caused 24 h after the administration of 5 mg of nigrin b were more important and were also restricted to the intestine. In the small intestine, the crypts were atrophied, very shortened, and their epithelial covering was formed by flattened undifferentiated cells (Fig. 3A). Some of these flattened cells and also the cellular debris found in the glands and intestinal lumen showed the histological and histochemical characteristics of apoptosis. The villi generally appeared slightly edematous, with some inflammatory infiltration in the lamina propria (Fig. 3B).

In the large intestine, the histological structure was well preserved with only a few scattered apoptotic cells on the wall of the intestinal crypts. The apoptotic cells shed into the gland and intestinal lumina (Fig. 3C). An inflammatory infiltrate was observed in the lamina propria but not in the submucosa.

Three days after a sublethal dose of nigrin b, lesions in the small intestine began to recover in the Lieberkühn crypts (Fig. 3D). At this time, the intestinal villi was still lesioned (Fig. 3E). In the crypts of the small intestine, few apoptotic cells were observed but, in contrast, a relatively high number of mitoses was observed (Fig. 3D), apparently higher than in the control animals (data not shown). The aspect of the cells covering the crypts indicated that they were regenerated cells. The intestinal villi were edematous, with enterocytes displaying different degrees of necrosis and disruption of the covering epithelium (Fig. 3E). At the base of villi, the epithelium was made up of cells that seemed to have been regenerated from the crypts. The inflammatory infiltrate seen at 24 h was not evident. In contrast with the small intestine, the lesions in the large intestine were still in progression. The epithelium of the crypts of the large intestine was very damaged and a large proportion of apoptotic cells shed into the intestinal lumen (Fig. 3F). This epithelium recovered later and after 9 days was completely recovered. Hemorrhage in the lamina propria and edema with an inflammatory infiltrate in the submucosa were also observed. In the lumen of the large intestine, large accumulations of apoptotic and inflammatory cells sloughed off from the intestinal epithelium were found. In some animals, this cellular debris occupied most of the intestinal lumen.

In order to assess whether the preferential specificity of nigrin b on the intestinal crypts is a feature of this special kind of type 2 ribosome-inactivating protein or rather it is a general property of all of type 2 ribosome-inactivating proteins, we studied the effects of the injection of 3 μg/kg of ricin, a concentration which kills all mice in 5 days. No signs of apoptosis were detected in the intestinal crypts (data not shown). This fact contrasts with the effects of the injection of 5 mg/kg of nigrin b, which does not kill any animal after 10 days. Also in contrast with nigrin b, 3 μg/kg

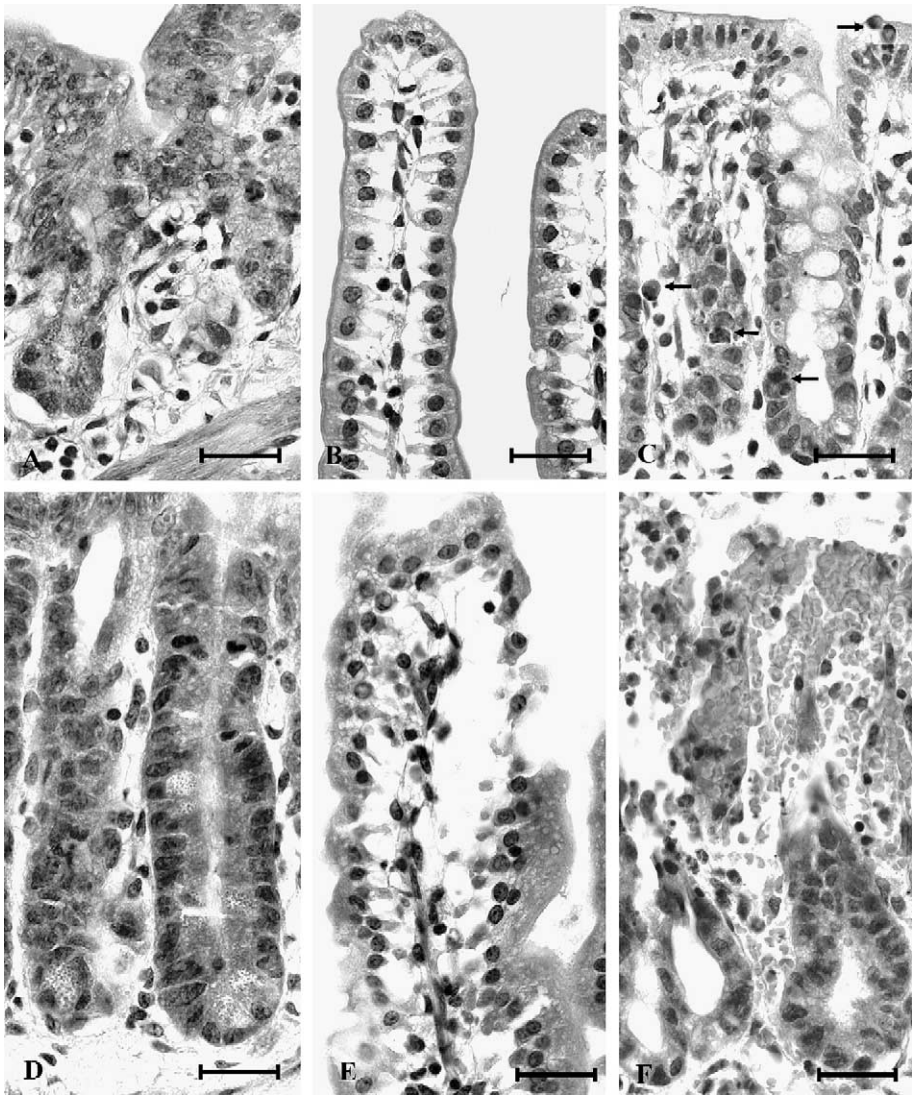

Fig. 3. Histological sections of small and large intestines stained with haematoxylin–eosin for 24 h (A, B, and C) and 3 days (D, E, and F) after 5 mg/kg of nigrin b administration. After 24 h, the small intestine crypts were atrophied (A) but the epithelium of the villi did not show histological lesions (B). The large intestine crypts (C) had some apoptotic cells (arrows). After 3 days, the small intestine crypts (D) were recovering their epithelium and mitoses were abundant (D). The villi were losing their epithelium and recovering it at the base (E). The large intestine is very damaged and most of its epithelium has sloughed off (F). Scale bar = 25 μm.

of ricin promotes a serious derangement of liver cells as reported elsewhere (reviewed in Doan, 2004). This points out that the sub-lethal effects of nigrin b are specific for the intestinal crypts.

Histological analysis of organs of mice treated with a lethal dose of nigrin b

Histological study of the organs of mice treated with lethal doses of nigrin b revealed no apparent signs of tissue damage except in the intestines, where very important lesions were observed. Five hours after treatment with 16 mg/kg of nigrin b the general architecture of the intestinal wall was altered, with more important lesions in the Lieberkühn crypts and intestinal villi of the small intestine. The large intestine was also lesioned but less intensely.

In the small intestine, the crypts were atrophied and some of them had disappeared. The rest were constituted by an atrophied epithelium and dying cells with an apoptotic-like morphology (Fig. 4A). The apoptotic-like cells had sloughed off into the crypt and intestinal lumen. Using the TUNEL method, we confirmed the apoptotic type of this cellular death (data not shown). The intestinal villi were edematous and frequently their epithelium was partially lost at the base of the villi, resulting in their rupture and elimination (Fig. 4B). We did not observe apoptotic cells in the villus epithelium. The lamina propria showed an inflammatory infiltrate with leucocytes and plasma cells.

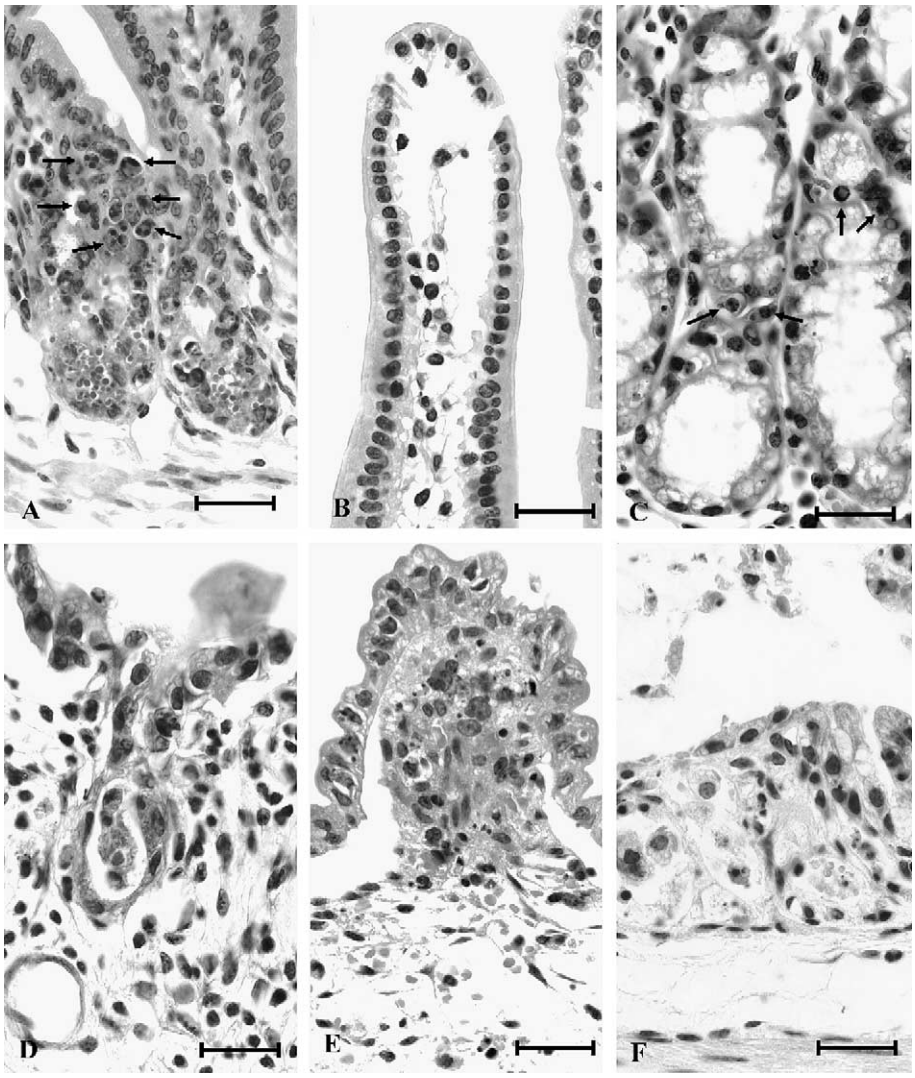

Fig. 4. Histological sections of small and large intestines stained with haematoxylin–eosin 5 h (A, B, and C) and 24 h (D, E, and F) after 16 mg/kg of nigrin b administration. After 5 h, the small intestine showed a large amount of apoptotic cells (arrows) in the crypts (A) and rupture and elimination of the villi (B). The large intestine crypts (C) also presented numerous apoptotic cells (arrows). After 24 h only the remains of the histological structure of the small intestine crypts (D) and villi (E) could be recognized. Also, the large intestine was covered by an incomplete epithelial layer (F). Scale bar = 25 μm.

In the crypts of the large intestine, the damaged cells were mainly distributed in the middle and the lower third of the crypt. From morphological and histochemical analyses, it seems that they had undergone apoptosis (Fig. 4C). The lamina propria in the large intestine was also infiltrated by numerous leucocytes and plasma cells.

After 24 h of this treatment, the mucosa of the small and large intestines was severely damaged, with loss of its histological organization and almost total elimination of the epithelium. In the small intestine, the crypts had almost completely disappeared, leaving only some remains of cellular debris and some flat epithelial cells (Fig. 4D). The villi had also been eliminated and a few structures comprising some epithelial cells covering connective tissue formed the rest of these structures (Fig. 4E). The lamina propria lost its organization containing the remains of epithelial cells and connective and inflammatory cells (Figs. 4D and E). In some intestinal areas, an important degree of hemorrhage was observed in the lamina propria.

In the large intestine, the destruction of the mucosa was similar, and it was reduced to an unstructured mixture of connective and inflammatory cells incompletely covered by flat epithelial cells (Fig. 4F). The lumen of the large intestine was frequently occupied by the remains of the mucosa and inflammatory cells (Fig. 4F).

Cytotoxicity of nigrin b on COLO 320 cells

COLO 20 cells were preincubated with different concentrations of either ricin or nigrin b for 3 h, after which the RIP was removed by washing the cells with fresh medium. Then, the cultures were incubated for another 48 h and viability was further determined. As

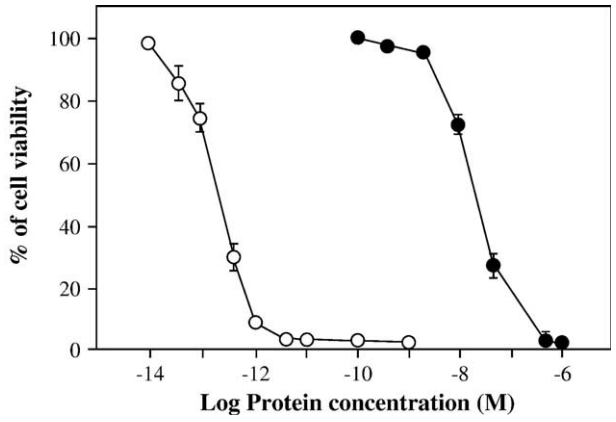

Fig. 5. Effects of ricin and nigrin b on COLO 320 cell viability. Cells were incubated with different concentrations of either ricin (○) or nigrin b (●) for 3 h to allow the internalization of the protein and after they were washed and fresh medium was added to the cultures and incubated for 48 h and the cell viability was determined as indicated in Methods. Bars represent the SEM of seven experiments.

shown in Fig. 5, under these conditions, the LC_{50} for nigrin b was 3.1×10^{-8} M. This indicated that nigrin b was efficiently bound and internalized during the 3 h of preincubation. Further incubation with nigrin b-free medium allowed us to follow its intracellular fate and reach its target, resulting in translocation to the cytosol and the inhibition of protein synthesis, which in turns led to the loss of cell viability (Citores et al., 2003; Girbes et al., 2003). In contrast to nigrin b, ricin reduced cell viability with an LC_{50} value of 2×10^{-13} M.

COLO 320 DNA fragmentation promoted by nigrin b

Since the injection of nigrin b promoted a large number of cell deaths, we wished to determine whether sublethal concentrations of nigrin b would be able to trigger apoptosis in vitro, as they did in vivo in stem cells present in the intestinal Lieberkühn crypts. As a first approach, we selected a model of intestinal cell with a high proliferating rate such as the COLO 320 cell line derived from a human colon adenocarcinoma. Fig. 6 shows the effects of the incubation of COLO 320 cells with different concentrations of nigrin b. Nigrin b at 10^{-9} M, a concentration which did not exerted cytotoxicity at all, did not promote the internucleosomal DNA fragmentation characteristic of apoptosis. In contrast, nigrin b at 10^{-8} M, which reduced cell viability by 35%, promoted a clear DNA degradation consistent with apoptosis. At 10^{-7} M, which reduced viability by 75%, nigrin b also promoted a large extent of DNA degradation. It seems that the loss of cell viability and internucleosomal degradation run in parallel.

Another approach was to analyze the DNA of the cecal contents of animals after 3 days of treatment with nigrin b at 5 mg/ml. Such material mainly comprises cell debris derived from the splitting of damaged intestinal cells and a highly degraded DNA was found (data not shown).

Discussion

Nigrin b has been described as a non-toxic type 2 ribosome-inactivating protein based on the fact that it has approximately 4000 times less in vivo toxicity to mice than ricin (Battelli, 2004; Battelli et al., 1997; Girbes et al., 1993, 2003; Girbes, 2004). In cultured animal cells, nigrin b shows also a noticeably lower cytotoxicity than ricin, lying in the range of 1600- to 108,000-fold, depending on cell types although with the exception of melanoma cells, which are resistant to ricin (Muñoz et al., 2001). The different cytotoxicity of nigrin b and ricin seems to be related to the different intracellular pathway used by both RIPs (Battelli, 2004; Battelli et al., 1997, 2004; Citores et al., 2003). On the other hand, the intraperitoneal injection of large amounts of nigrin b kills mice (i.p. LD_{50} 12 mg/kg body weight; Batelli et al., 1997; Girbes et al., 2003). In contrast, it has been reported that in vivo ricin at concentrations in the range of few μg/kg body weight promotes a general derangement consisting of strong multi-organ pathological effects that also include effects on several tissues and cell types of the intestine (Bradberry et al., 2003). In our hand, after 5 h of intravenous injection of 3 μg/kg ricin, no evident signs of gut alteration like that promoted by nigrin b at sub-lethal concentrations were seen. In contrast, and according to previous work (Bradberry et al., 2003), ricin promoted extensive liver derangement (data not shown).

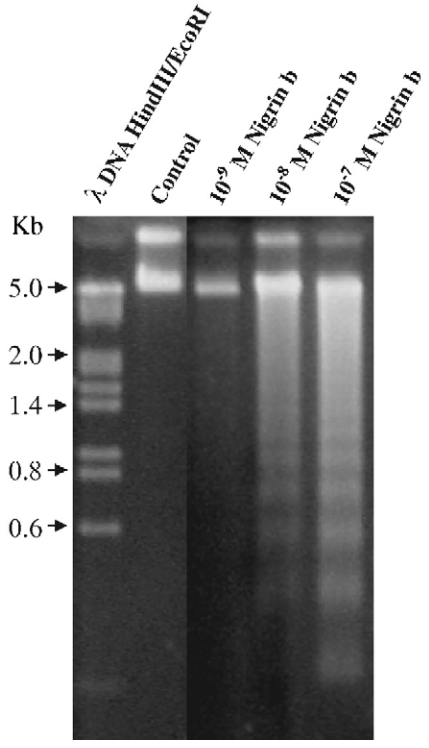

Fig. 6. Effects of nigrin b on the internucleosomal DNA fragmentation. COLO 320 cells were incubated in the absence or the presence of variable nigrin b concentrations for 48 h. Then, the DNA was isolated and 3 μg were electrophoresed as indicated in Methods. The arrows indicate the corresponding size of the standards (λDNA *Hin*dIII/*Eco*RI) in kb.

In our experiments, the lethality of ricin was exerted at concentrations 4000-fold lower than those required for nigrin b (Fig. 1). Independent of the lethality of the dose, the nigrin b-dependent damage was specifically exerted on the intestine, indicating that this section of the gastrointestinal tract has target cells that seem to be intestinal stem cells or a slightly programmed set of stem cells, also with a high proliferation rate. The specific destruction of these cells by nigrin b might perhaps be due to the presence of some kind of specific receptor for nigrin b in the target cells, in contrast with their neighboring cells and, in general, with other tissues and organs that apparently remain unaltered following nigrin b treatment. Since other cells with high turnover rates in other organs, for example, the stomach, remain unaffected by the treatment with nigrin b, the argument of proliferation being the only reason for the sensitivity to nigrin b does not seem to be sufficiently robust to explain the observed effects on the small and large intestines. An alternative hypothesis is that the intestinal target cells would express the receptor target for nigrin b only in the undifferentiated or poorly differentiated stages characteristic of stem cells, and also perhaps in cancer cells. In support of this interpretation are our results on the effects of nigrin b on the viability of undifferentiated colon adenocarcinoma cell line COLO 320. Further supports comes from the fact that injection of 5 mg/kg body weight of nigrin b to 25 g mice gave an approximate plasma concentration of the protein ranging from 10^{-6} to 10^{-7} M, depending on which fluid volume is considered (either only the blood volume or the total water content of the animal). However, this estimation is arbitrary and most probably overestimated since it does not take into account the binding of nigrin b to the cells, its possible degradation, excretion and other phenomena that could alter the free nigrin b concentration in plasma.

Since the LC_{50} of nigrin b on COLO 320 cells is 3×10^{-8} M, it seems fairly likely that the target intestinal cells will share the same molecular target as the cancer COLO 320 cells and in this sense they could be closely related. On the other hand, a recent work has linked mutations in stem cells to the predisposition to development of late-life cancers, such as those in the skin and colon (Frank and Nowak, 2003).

Preliminary experiments indicated that the oral ingestion of large amounts of nigrin b such as those that trigger intravenous toxicity does not promote any visible derangement in mice. This could partly be due to digestion-associated intestinal degradation processes performed by the microbial flora. Nonetheless, nigrin b structurally-related agglutinins, which also display D-galactose-binding lectin activity, such as SNA I and SNA II, do not seem to be completely degraded and promote a significant dysplasia of the intestinal crypts but no lethal derangement of the mouse gut (Pusztai et al., 1990). The feeding of a diet containing protein from raw kidney bean which contains phytohemagglutinin (PHA) led to a time-dependent hyperplastic growth of the intestinal crypts (de Oliveira et al., 1988; Jordinson et al., 1999). Additionally, the oral administration of ML-1 hemagglutinin from mistletoe which display a type 2 ribosome-inactivating protein activity have growth stimulatory effects (Pryme et al., 2002). Therefore, agglutinins do not trigger effects like nigrin b.

Thus, the lack of oral effects of nigrin b could be interpreted in the sense that the nigrin b receptor molecules would in fact only be accessible through the circulation: for instance, the basal surface of the highly polarized intestinal epithelial cells. Further work will address this hypothesis.

From a pharmacological point of view, the very low toxicity of nigrin b (Batelli et al., 1997; Girbes et al., 1993; present results) as compared with ricin (Lord et al., 1994) affords nigrin b an important advantage over ricin and other toxins for the construction of immunotoxins and conjugates targeting sick cells, that is, cancer cells. In keeping with this, we have recently reported the suitability of nigrin b for the construction of conjugates with transferrin, which display specific cytotoxicity on cells over-expressing transferrin receptors, such as HeLa cells (Citores et al., 2002). From a toxicological point of view, its usefulness makes nigrin b much safer to handle than ricin, thereby reducing biohazard risks.

In conclusion, our results on the systemic injection of nigrin b at sublethal concentrations open a new door for studying the conditions of the specific toxicity of nigrin b on intestinal stem cells or poorly-differentiated stem cell-derived cells that could be very interesting from the pharmacological point of view since it may allow to nigrin b to kill these cells and perhaps stem-cell-derived intestinal cancers.

Acknowledgments

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