

INTRODUCTION OF B-CHAIN-INACTIVATED RICIN INTO MOUSE MACROPHAGES AND RAT KUPFFER CELLS VIA THEIR MEMBRANE F_c RECEPTORS

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Ricin is a glycoprotein which is present in the seeds of *Ricinus communis* and is extremely toxic to animals and man (1, 2). After intravenous administration the LD₅₀ dose in mice is about 65 ng. The toxin inhibits the protein synthesis by inactivating the ribosomes. Ricin, which has a mol wt of 65,000 consists of two polypeptide chains joined together by an SS bond. The A chain or "effectomer" is an enzyme capable of inactivating specifically the 60S ribosomal subunit (2-8). The B chain or "haptomer" is responsible for the binding of the toxin to receptors on the cell surface, which is a prerequisite for toxic effect on intact cells. The binding is inhibited by lactose, and there is now good evidence that the B chain of the toxin interacts with membrane glycoproteins containing nonreducing terminal galactose residues (1, 9-13).

The B chain of the toxin binds to animal cells in general including erythrocytes. Thus, to make the toxic effect selective against certain kinds of cells such as tumor cells, a number of points related to the introduction of the toxin into cells have to be clarified. In this study we have asked the following questions: (a) Can the toxin be introduced into cells through other cell membrane receptors than via the B-chain receptor? (b) Does internalization through endocytosis (most likely including exposure of the toxin to lysosomal enzymes) neutralize the toxic effect?

In attempts to throw light on these questions we have studied the effect of [ricin·antiricin B] complexes on mouse macrophages and rat Kupffer cells. The results indicate that such complexes, subsequent to binding to F_c receptors of cells, are indeed internalized and lead to inhibition of protein synthesis and cell death.

Materials and Methods

Isolation of Ricin. Ricin was extracted from the seeds of *Ricinus communis* (a batch of Castor beans obtained from Deutsche Rizinus Oelfabrik, Boley & Co, Krefeld-Ürdingen, West Germany) and purified by chromatography using DE-52, CM-52, and Sepharose 4B columns (1, 9, 10). To prepare its constituent polypeptide chains, ricin was treated with 5% 2-mercaptoethanol in the presence of 0.5 M D-galactose, and the A and the B chain were separated on DE-52 and CM-52 columns as described elsewhere (1, 9, 10). Dilution of the toxin used in these experiments was always done in cell culture medium (containing 0.02% fetal calf serum).

Preparation of Antitoxin. Antiserum against ricin was raised in rabbits. Ricin was first treated for 3 days with 5% formaldehyde at room temperature in 0.05 M sodium phosphate buffer

at pH 7.5 (1). After removal of excess formaldehyde by dialysis, rabbits were given a primary injection of 0.2 mg formalized protein in phosphate-buffered saline (PBS, 0.14 NaCl, 0.01 M phosphate, pH 7.4) followed by six injections (10-days intervals) of 0.5 mg of the same material. Serum was collected 1 wk after the last injection. Booster injections were later given at 10-day intervals during which time weekly bleedings were made.

Purification of Antibodies to the B Chain of Ricin. Ricin B chain was linked covalently to CNBr-activated Sepharose 4B, as described by Axen et al. (14). Small columns (2–3 ml) containing the particular Sepharose-linked antigen were equilibrated with PBS containing 20 mM lactose, and antiserum was passed through the column. The column was then washed with the same buffer, and finally the bound antibodies were eluted with 0.2 M glycine-HCl (pH 2.8) in 0.5 M NaCl (11).

Cells

HELA CELLS. HeLa cells were maintained in shaker cultures at 37°C in Gibco minimum essential medium containing 10% calf serum (Biocult, Grand Island Biological Co., Grand Island, N. Y.) at a concentration of $1-4 \times 10^6$ cells/ml. Growing cells were harvested by centrifugation for 5 min at 1,000 g, washed twice with Hanks' balanced salt solution, pH 7.4, and then resuspended to a concentration of about 3×10^5 cells/ml in medium containing one-tenth of the normal concentration of leucine.

MACROPHAGES. The experiments were carried out with male and female mice weighing 25–30 g. Macrophages were obtained by peritoneal washings as described by Cohn and Benson (15). The cells were suspended at a concentration of approximately 0.5×10^6 cells per ml in medium 199 (Biocult, Grand Island Biological Co.), pH 7.4, with penicillin and streptomycin (100 U of each per ml), supplemented with 20% newborn calf serum (Grand Island Biological Co.). In all cases the serum was inactivated at 56°C for 30 min. Samples of cell suspension (1 ml) were left to sediment in Linbro tissue culture plates with 16-mm troughs (FB-16-24-TC, Linbro Chemicals Co., Ltd., New Haven, Conn.), and after 1–2 h nonadhering cells were removed by washing with serum-free medium, and then new culture medium was added. The cells were always cultured in a CO₂ incubator at 37°C. All experiments were performed on macrophages cultured for 20–40 h.

RAT KUPFFER CELLS. Adult male hybrid rats from two inbred lines (male A/GUS from Chester Beatty Cancer Institute, London, England and female hooded rats from Radiumhospitalet, Oslo, Norway) weighing between 250 and 320 g were used. The rats were fed the standard pellet diet and water ad libitum. Kupffer cells were obtained from rat livers by the two-step technique of collagenase perfusion and pronase digestion of hepatocytes as described in detail by Munthe-Kaas et al. (16). The Kupffer cells were cultured in the same manner as described above for the mouse peritoneal macrophages, except that rinsing of unattached cells was carried out after overnight culture of the total nonparenchymal cell suspension. The concentration of Kupffer cells was about $2.5-3 \times 10^5$ cells per ml culture.

Neutralization of Ricin by Antisera. The amount of antibody required to neutralize the toxic effect of ricin on HeLa cells was determined in the following way: Increasing amounts of ricin and a certain amount of antibody specific for the B chain (later called antiricin B) were added to a series of roller tubes containing 2 ml of HeLa cells in low leucine medium, supplemented with 0.5% heat-inactivated calf serum (Grand Island Biological Co.). In all experiments the toxin and antitoxin were incubated for 15 min at room temperature and then for 30 min at 0°C before being added to cell cultures. Duplicate tubes were made at each toxin dilution. After 3 h rotation at 37°C, 0.5 μ Ci of [¹⁴C]leucine was added. 1 h later the cells were collected by centrifugation (1,000 g, 5 min) the supernate was discarded, and the cells were dissolved in 2 ml of 0.1 N KOH. After 15 min incubation trichloroacetic acid was added to a final concentration of 10% (wt/wt). The precipitated protein was collected on Gelman glass filters, type A, and the radioactivity was measured in a Beckman scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The incorporation of leucine fell sharply when there was a slight excess of ricin over antiricin B. The amount of antibody required to completely prevent the toxic effect of ricin was taken as the end point.

Effect of Ricin on Protein Synthesis. Before measuring the protein synthesis in macrophages and Kupffer cells, the cells were washed once with serum-free medium and then incubated in low leucine medium containing 2% fetal calf serum. Increasing amounts of ricin or [ricin · antiricin B] complex were added to a series of 1-ml cultures. Duplicate cultures were made at each toxin dilution. After incubation at 37°C for the period of time indicated in legend to figures, 0.5 μ Ci of

[¹⁴C]leucine was added to each culture, and the incubation was continued. The incubations were terminated by removing the medium from the troughs and adding 1 ml of 0.1 N KOH to each culture. After 30 min incubation at room temperature the material was transferred to tubes, and the troughs were washed twice with 0.1 N KOH. Trichloroacetic acid was then added to a final concentration of 10% (wt/wt), and the radioactivity of the precipitated protein was measured. HeLa cells were incubated in the same way except that the cells were in suspension and were centrifuged when the incubation was terminated.

Soluble Immune Complexes. Soluble immune complexes of rabbit IgG and antirabbit IgG were prepared in antiserum excess. Rabbit IgG was isolated from rabbit serum by using DEAE cellulose chromatography (17). Antirabbit IgG serum was purchased from Hyland Division, Travenol Laboratories AB, Bromma, Sweden.

Iodination of Ricin. Ricin was iodinated with ¹²⁵I by the lactoperoxidase method of Marchalonis (18) as modified by Sandvig et al.¹

Preparation of [Red Cell-Ricin-Antiricin B] Complexes. Complexes consisting of red blood cells, ricin, and antiricin B were prepared in the following way: Human red blood cells (O⁺) were washed three times with PBS and suspended in PBS to a final concentration of 5% (vol/vol of packed cells), and a sample was added to solutions of ricin to give final concentrations of 0.025% and 0.01 mg per ml. Control experiments carried out with [¹²⁵I]ricin showed that after incubation for 15 min at room temperature the binding of toxin to the red cell surfaces had reached equilibrium. Under these conditions about 50% of the toxin was bound to the red cells.¹ Then a neutralizing amount of antiricin B was added, and the mixture was incubated for 15 min at room temperature and for 30 min at 0°C. The [red cell-ricin-antiricin B] complexes formed were added to cell cultures in amounts corresponding to a final concentration of 1 μg ricin per ml.

The toxicity of the [red cell-ricin-antiricin B] complex was tested by measuring the protein synthesis in Kupffer cells. Concurrently, an identical series of cultures was prepared for phase contrast microscopy. The cultures were set up with fitted glass cover slips and the same number of cells per ml. The cells were incubated for 1 h at 37°C with the [red cell-ricin-antiricin B] complex. At the end of the incubation the cultures were rinsed with medium and fixed in 2% buffered glutaraldehyde for 15 min at 4°C. They were then rinsed with distilled water and mounted for examination on a Leitz phase contrast microscope fitted with a camera. Pictures were taken with Kodak panatomic X film (Eastman Kodak Co., Rochester, N. Y.).

Results

Toxicity of Ricin on Mouse Macrophages and the Effect of Lactose. Mouse macrophages were incubated with increasing concentrations of ricin for 5 h and then with [¹⁴C]leucine for 3 more h. The results in Fig. 1 show that in the presence of 0.1 ng of ricin per ml the rate of protein synthesis was reduced to 20% of that of the control containing no toxin. When the medium contained 10 mM lactose about 200 times as much ricin was required to produce an equivalent reduction in the rate of protein synthesis. It is thus clear that the macrophages are very sensitive to ricin, and that the toxicity of ricin on macrophages can be reduced by lactose as earlier observed in Ehrlich ascites cells and HeLa cells (6, 11). This indicates that the binding of ricin involves similar receptor sites on these cell types.

Effect of [Ricin-Antiricin B] Complex on Macrophages. To study if the toxin can enter the cell even when the B chain is inactivated advantage was taken of the fact that macrophages have receptors for the Fc part of immunoglobulins (19). Ricin was mixed with neutralizing amounts of antibody specifically directed against the B chain of the toxin, to give a toxin-antitoxin complex where the B chain could not bind to cell surface receptors. The complex is schemati-

¹ Sandvig, K., S. Olsnes, and A. Pihl. 1976. Chain of events in the toxic action of abrin and ricin. I. Kinetics of binding to surface receptors of human cells. *J. Biol. Chem.* In press.

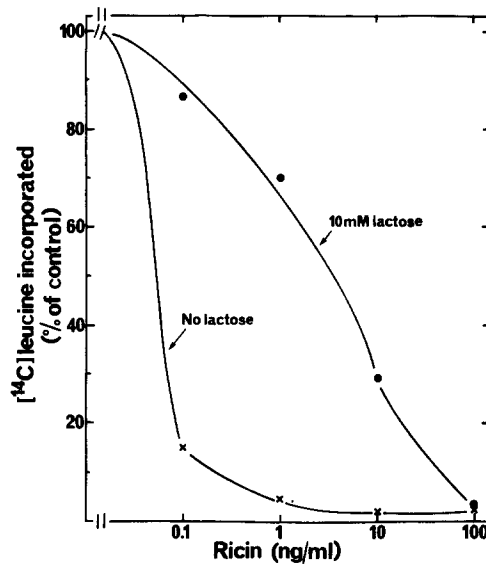


FIG. 1. Effect of ricin on protein synthesis in macrophages in the presence and absence of lactose. After 5-h exposure to the indicated concentrations of ricin, [¹⁴C]leucine incorporation into cell protein was measured over a 3-h period. The ordinate shows leucine uptake as percentage of control with no ricin.

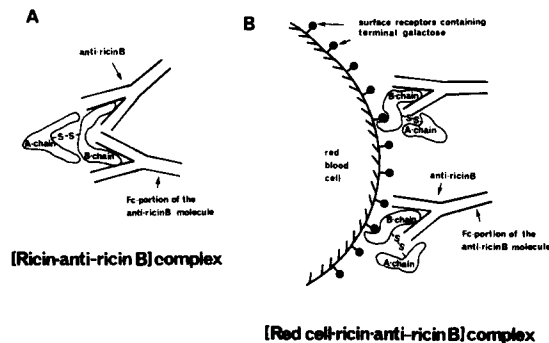


FIG. 2. Schematic drawing of the [ricin·antiricin B] complex and the [red cell·ricin·antiricin B] complex.

cally drawn in Fig. 2 A. Increasing concentrations of the complex were added to mouse macrophages, and then the protein synthesis was measured after 26 h of incubation. The data in Fig. 3 show that in the presence of the [ricin·antiricin B] complex protein synthesis was reduced, indicating that the complex is indeed taken up by the macrophages. Furthermore, the toxic effect of the [ricin·antiricin B] complex could not be reduced by 10 mM lactose in the medium. However, the toxic effect was prevented by soluble antigen-antibody complexes (rabbit IgG antirabbit IgG) which on the other hand did not prevent the toxic effect of free ricin. The results indicate that different binding mechanisms are involved in the uptake of free ricin and of [ricin·antiricin B] complexes, and that the A chain of the complex had retained at least part of its biological activity. To rule out the possibility that the toxic effect could be due to some free toxin in the

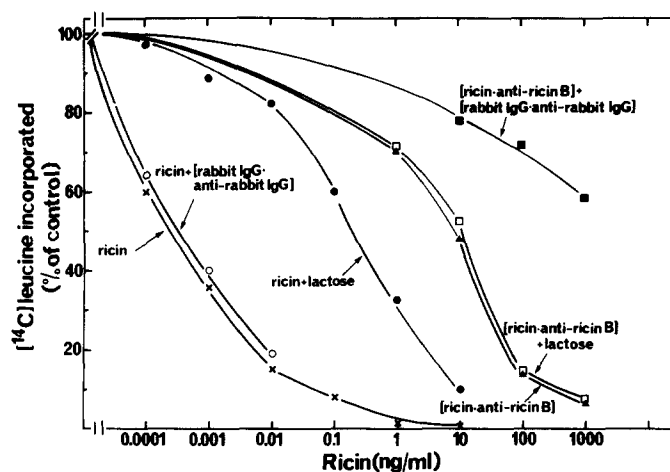


FIG. 3. Effect of ricin and [ricin·antiricin B] complex on macrophages. After 17-h exposure to the indicated concentrations of ricin, [^{14}C]leucine incorporation into cell protein was measured over a 9-h period. The effect of 10 mM lactose and of soluble immune complexes on toxicity is also shown. The ordinate shows leucine uptake as percentage of control with no ricin.

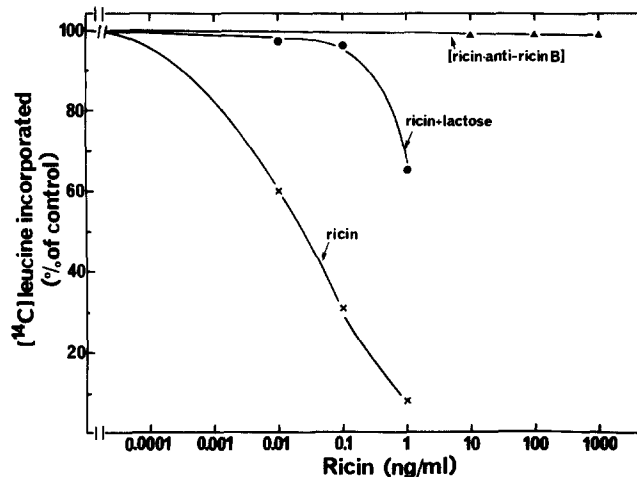


FIG. 4. Effect of ricin and [ricin·antiricin B] complex on HeLa cells. After 17-h exposure to the indicated concentrations of ricin [^{14}C]leucine incorporation into cell protein was measured over a 9-h period. The effect of 10 mM lactose and of [ricin·antiricin B] complexes is also shown. The ordinate shows leucine uptake as percentage of control with no ricin.

medium, HeLa cells (which have no Fc receptor) were incubated for the same period of time with the [ricin·antiricin B] complex. As shown in Fig. 4 these cells were not affected by the complex.

Effect of [Red Blood Cell·Ricin·Antiricin B] Complexes on Protein Synthesis in Rat Kupffer Cells. In attempts to demonstrate more clearly the toxic effect of the [ricin·antiricin B] complex, this was attached to red cells to form a large complex (Fig. 2 B). Human red cells were used rather than sheep red blood cells, since they bind more ricin under the conditions here used. Inasmuch as human

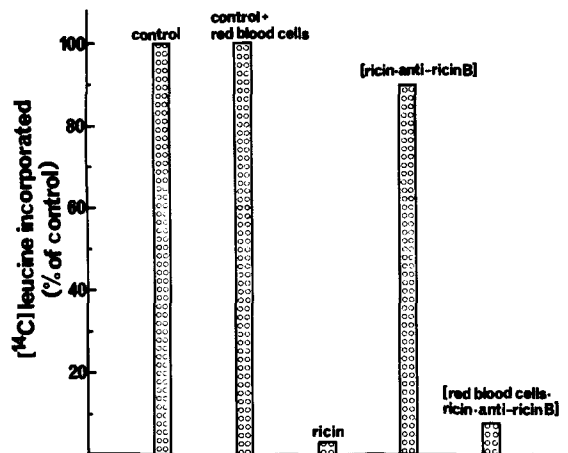


FIG. 5. Effect of [ricin-antiricin B] complex and of [red blood cell·ricin-antiricin B] complex of rat Kupffer cells. After 3-h exposure to ricin and the different complexes [¹⁴C]leucine incorporation in the cell protein was measured over a 3-h period. The ordinate shows leucine uptake as percentage of control with no ricin. The complexes were added to cell cultures in amounts corresponding to a final concentration of 1 μ g ricin per ml.

red cells are often phagocytosed directly by mouse macrophages we used instead rat Kupffer cells, another type of mononuclear phagocytes, which usually do not react spontaneously with human erythrocytes. The results in Fig. 5 show that after incubation for 6 h protein synthesis was slightly inhibited by the [ricin-antiricin B] complex, whereas the complex of [red cell·ricin-antiricin B] inhibited protein synthesis almost completely. To ascertain that this effect was not due to the presence of free toxin in the medium, HeLa cells were again incubated with the same complex for the same period of time. These cells were not affected by the complex (data not shown). Furthermore, red blood cells themselves had no effect on protein synthesis in Kupffer cells.

Attempts were made to follow the events by phase microscopy. It is well known that sheep red blood cells coated with IgG antibodies attach to the Fc receptors of the macrophage membrane and are subsequently internalized. It might be expected that the [red cell·ricin-antiricin B] complex would behave in the same manner. The results are shown in Fig. 6. It can be seen (picture *a*) that more than 95% of the Kupffer cells appear to be more or less covered by red cells, indicating some type of specific binding. In order to see whether some of the red cells associated with the Kupffer cells had been internalized, the Kupffer cells were exposed to distilled water for 15 s before fixing with glutaraldehyde. This hypotonic shock will lyse all the red cells on the surface of the Kupffer cells. The picture obtained after hypotonic shock (picture *b*) shows that many red cells are inside the Kupffer cells, indicating that the [red cell·ricin-antiricin B] complex had indeed been phagocytosed. In a control experiment untreated red cells were added to Kupffer cells. It can be seen (picture *c*) that some of the red cells were unspecifically attached to the Kupffer cells. After hypotonic shock (picture *d*) only a few ghosts could be seen on the cell surface, and no red cells could be seen inside the cell. When red blood cells preincubated with ricin were added to Kupffer cells (Picture *e*), more red blood cells were attached to the cells than

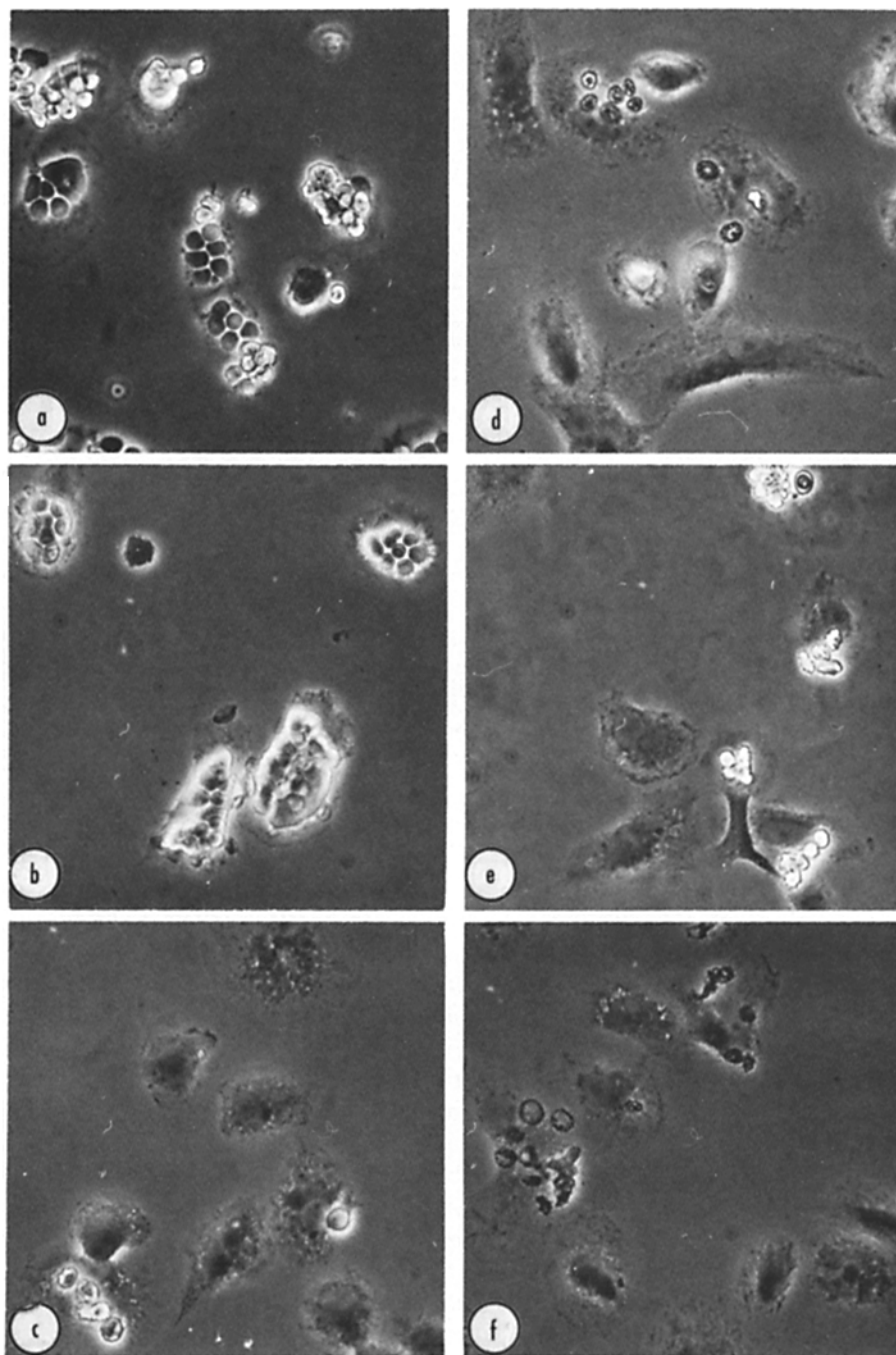


FIG. 6. Phase microscopy studies of Kupffer cells incubated with the [red cell-ricin-antiricin B] complex. (a) Kupffer cells plus the [red cell-ricin-antiricin B] complexes. (b) Kupffer cells plus [red cell-ricin-antiricin B] complex, after hypotonic shock. (c) Kupffer cells plus untreated red cells. (d) Kupffer cells plus untreated red cells, after hypotonic shock. (e) Kupffer cells plus red cells preincubated with ricin. (f) Kupffer cells plus red cells preincubated with ricin, after hypotonic shock.

when untreated red cells were used, presumably due to the presence of some agglutinin and/or dimer of ricin in the toxin preparation. After hypotonic shock (picture *f*) no red cells appeared to reside inside the cell. As a final control, Kupffer cells were incubated with red blood cells plus the [ricin·antiricin B] complex (i.e., the B chain of the toxin was inactivated before red cells were added). Although some of the red cells again were unspecifically attached, no cells could be seen inside the cell after hypotonic shock (pictures not shown).

Effect of [Ricin·Antiricin A] Complexes On Macrophages. Previous studies on a cell-free system from rabbit reticulocytes have shown that only the free A chain, as such, is capable of inhibiting protein synthesis and that antiricin A completely inhibits the toxic effect.² The above experiments with macrophages indicated that A chain can be liberated from a complex of ricin with antiricin B. The question arises whether in the case of these cells the toxic A chain could be liberated also from a complex with antiricin A, since the complex is presumably taken up by endocytosis and may be transported to the lysosomes where conceivably the immunoglobulin part could be digested more easily than the A chain. To test this possibility the cells were incubated with complexes consisting of ricin and antiricin A. The results showed that this [ricin·antiricin A] complex had no inhibitory effect on protein synthesis in macrophages.

Discussion

The present studies with peritoneal and liver macrophages indicate that ricin can be attached to cells by some other binding mechanism than via the B chain, the natural binding moiety of the toxin. Macrophages, which in contrast to HeLa cells, have surface receptors for the Fc portion of the immunoglobulin molecule, bind, internalize, and are subsequently killed by a complex consisting of ricin and antiricin B. The binding is not inhibited by lactose, but by soluble antigen-antibody complexes which presumably compete for the Fc receptors. All these data indicate that it is the Fc receptor on the macrophage membrane which is involved in the binding and uptake of the [ricin·antiricin B] complex. It is likely that the uptake occurs by endocytosis.

The toxic effect of [ricin·antiricin B] complex is very low compared to that of free ricin. Thus, whereas only about 0.0005 ng/ml of free ricin was sufficient to inhibit protein synthesis by 50% after 26 h, 10 ng of ricin in the complex with antiricin B was required for the same effect. There may be several reasons for this difference. Firstly, the number of ricin receptors on the surface of macrophages is probably of the same order of magnitude as that of HeLa cells, i.e. about 3×10^7 per cell,¹ whereas the number of Fc receptors on the macrophage membrane is only about 2×10^6 (20). Secondly, the affinity of ricin and of [ricin·antiricin B] complex for the two types of receptors may be different. A third explanation for the low toxicity of the [ricin·antiricin B] complex is that vesicles formed during the internalization of the complex may fuse with lysosomes (21). This will expose the toxin to lysosomal enzymes, and it is possible

² Olsnes, S., K. Sandvig, K. Refsnes, and A. Pihl. 1976. Chain of events in the toxic action of abrin and ricin. II. Rates of different steps involved in the inhibition of protein synthesis. *J. Biol. Chem.* In press.

that only part of the toxin will escape digestion and be able to act on the ribosomes.

At this point it is interesting to note that macrophages are much more sensitive to free ricin than are HeLa cells. Thus, after 26 h of incubation the protein synthesis in macrophages is inhibited 50% by 0.0005 ng ricin per ml, whereas 0.01 ng ricin per ml is required on HeLa cells for the same effect. Furthermore, previous studies have showed that Ehrlich ascites cells are about 10 times as sensitive to ricin as HeLa cells. The reason for this difference is not understood.

Soluble immune complexes bound to macrophages are not necessarily internalized, since cross-linking of the receptors may be required. When red cells are used as carriers for the [ricin·antiricin B] complex, as shown in the experiment with rat Kupffer cells, protein synthesis was completely inhibited after only 7 h. This relatively rapid inhibition of protein synthesis compared to the case when [ricin·antiricin B] complex was used may be explained by the fact that [red cell·ricin·antiricin B] complexes are large and easily phagocytosed by the Kupffer cells, as seen in the phase contrast microscope, leading to a much greater number of toxin molecules inside the cells. The situation with ricin may be analogous to that which appears to exist in the case of diphtheria toxin, which in many respects is similar to ricin. Thus, Bonventre et al. (22) have obtained evidence that diphtheria toxin taken into cells by pinocytosis is usually not toxic, as it is degraded by lysosomal enzymes before it can reach the cytoplasm. However, when large quantities of diphtheria toxin were pinocytosed, as was the case when it had been adsorbed to latex particles before being added to macrophages, toxicity was expressed, and they assumed that some of the internalized toxin may then escape proteolytic digestion. A similar situation may occur when [red cell·ricin·antiricin B] complexes are phagocytosed by the Kupffer cells.

In the case of free ricin, the uptake mechanism in the cells is not yet understood. Although evidence has accumulated that free ricin may also be taken up by pinocytosis or some related process, (for review see reference 23) it is not proved that this uptake mechanism is primarily responsible for the intoxication process. It is conceivable that the toxicity expressed in cells exposed to ricin is caused largely by toxin taken up by a different mechanism not involving pinocytosis. The possibility must be considered that when the B chain binds to galactose-containing receptors, the traversal of the A chain or the intact toxin across the membrane is in some way facilitated. The very high toxicity of free ricin compared to that of the [ricin·antiricin B] complex accords with this view.

Ricin and the related toxin abrin have been reported to possess anticancer activity (for review see reference 23). However, the usefulness of these agents is strongly reduced by their general toxicity. The possibility has therefore been discussed that abrin and ricin conjugated to tumor-specific antibodies may intoxicate selectively cancer cells (23), provided that the binding capacity of the B chain is inactivated. The present demonstration that ricin can be introduced selectively into cells under controlled conditions causing destruction of only these target cells is promising in this regard. Further work along this line is in progress in this laboratory.

Summary

Experiments have been made to test whether the toxic lectin ricin can be bound to and introduced into cells by some other mechanism than via its B chain, the natural binding moiety of the toxin, without its toxic effect being neutralized.

Complexes consisting of ricin and antibodies specifically directed against ricin B chain were incubated with mouse peritoneal macrophages and rat Kupffer cells, which are known to possess surface receptors for the Fc portion of the immunoglobulin molecule. After incubation for 26 h, cellular protein synthesis, as measured by incorporation of labeled leucine into acid-insoluble material, was completely inhibited. HeLa cells, which do not possess Fc receptors, were unaffected by the complex. The effect of the complex on protein synthesis of macrophages was prevented by soluble antigen-antibody complexes, but not by the presence of lactose which prevents attachment of the ricin B chain to the cell membrane.

The [ricin·antiricin B] complex was attached to red cells, and the resulting complex was incubated with rat Kupffer cells. Cellular protein synthesis ceased after 6 h, and phase contrast microscopy studies showed that the complexes were taken up by the Kupffer cells. The data indicate that ricin, when present in the complex with antiricin B, can be introduced into cells through cell membrane receptors other than the B chain receptor, in this case the Fc receptor, and that the internalized toxin retains a least part of its activity.

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