On the Toxic Proteins Abrin and Ricin

STUDIES OF THEIR BINDING TO AND ENTRY INTO EHRlich ASCITES CELLS

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SUMMARY

The mechanism of penetration of the toxic plant proteins abrin and ricin into Ehrlich ascites cells has been studied by measuring, under different experimental conditions, the lag occurring from the time the cells have been incubated with the toxins until protein synthesis in the cells is inhibited.

When Ehrlich ascites cells were incubated with abrin or ricin at 37°, a lag time of at least 30 min occurred before protein synthesis, as measured by the incorporation of labeled leucine into acid-precipitable material, was reduced. At 20° the lag time was 6 hr. In a cell-free system from rabbit reticulocytes, the inhibition occurred immediately, indicating that the lag time observed in whole cells is due to slow penetration of the toxic principle into the cells.

When antisera against the toxins were added to the cell suspension after preincubation with the toxins, the toxic effects could be prevented for different lengths of time, depending on the incubation temperature and the nature of the antitoxin used. With antisera prepared against the whole toxins, the toxic effects were partially prevented when the antitoxins were added after less than 30 min of preincubation with the cells at 20°. Specific antisera against the isolated A chains (the toxic principle of the toxin) were able to rescue the cells after a longer preincubation with toxins than specific antisera against the B chains which are involved in the binding of the toxins to the cells. Abrin and ricin did not inhibit protein synthesis in reticulocytes, even though the toxins do bind readily to the surface membrane of these cells.

The results are consistent with our two-step model for the entry of abrin and ricin into the cells; in the first step of this model the toxins bind to receptors on the cell surface by their B chain (here denoted the haplomer), whereas in the second step the toxic A chain (here denoted the effectomer) penetrates into the cytoplasm, where it exerts its action. The latter process appears to be the slower one, and the data indicate that endocytosis may be involved in this process.

Abrin and ricin are two extremely toxic proteins present in the seeds of *Abrus precatorius* L. and *Ricinus communis* L. (1, 2). These two toxins have been reported to have cancerostatic properties (3, 4). Recently evidence has accumulated that abrin and ricin exert their effects in eucaryotic systems by inhibiting protein synthesis. Thus, they strongly reduce incorporation of labeled amino acids into protein in intact cells (3-5), as well as in cell-free systems (6-12). This effect has been shown to be due to inactivation of the ribosomes (9), apparently by an enzymatic process. Structural studies have shown that each toxin consists of two polypeptide chains, bound by disulfide bonds (Fig. 1). The smaller polypeptide (the A chain) inhibits protein synthesis in cell free systems and represents the toxic moiety of the molecule (11, 12). The B chain is necessary for the toxic action in living animals and the evidence indicates that it functions by binding the toxins to galactose-containing receptors on the cell surface (11, 12). Since several proteins in their biologically active form seem to have a similar structure (see "Discussion"), we propose here to denote that chain which carries the specific biological action (here the A chain) the "effectomer," and to call the chain responsible for the binding to the cell surface (here the B chain), the "haplomer."

Previously we have proposed a two-step model for the entry of abrin and ricin into cells (11, 12). In this model, the first step involves the binding of the toxin through the haptomer to the surface of the cells. In the second step the effectomer, or possibly the whole toxin, penetrates into the cytoplasm where the effectomer reaches its site of action.

The purpose of the present investigation has been to examine in more detail the binding of abrin and ricin to cells and their subsequent entry into the cytoplasm. To elucidate the underlying mechanisms, we have used antibodies directed specifically against the A and B chains and measured for how long after incubation of the cells with the toxins these specific antitoxins are able to protect the cells. The data support the two-step model previously proposed and suggest that endocytotic processes are involved in bringing the toxins into the interior of the cells.

EXPERIMENTAL PROCEDURES

Materials—Semen jequiriti (the seeds of *A. precatorius* L.) was obtained from Norsk Medicinskdepot, Oslo. Castor beans, (the seeds of *R. communis* L.) were kindly supplied by Deutsche Rizinus Oelfabrik Rolley & Co., 415 Krefeld-Herdingen, Germany. L-[U-14C]leucine (specific activity 331 Ci per mole) was obtained from the Radiochemical Centre, Amersham, England.

Preparation of Abrin and Ricin—Abrin was extracted with 5% acetic acid from semen jequiriti and purified to homogeneity by chromatography on a DEAE-cellulose column and affinity chromatography on a Sepharose 4B column (11). Ricin was extracted at pH 4.0 from defatted castor beans and purified by chromatog-
raphy on a carboxymethylcellulose column and by affinity chromatography on a Sepharose 4B column (12).

The L.D₅₀ doses in mice were 0.07 and 0.2 μg for abrin and ricin, respectively. The constituent peptide chains of abrin and ricin were isolated by reduction of the toxins with 5% 2-mercaptoethanol in the presence of 0.5 M (±)-galactose followed by chromatography on DEAE- and carboxymethylcellulose columns as earlier described (11, 12).

Cell Technique—The Ehrlich ascites tumor cells were obtained from mice 0 to 9 days after tumor transplantation. Ascites tumor fluid containing about 5 × 10⁶ cells was centrifuged at 300 × g for 5 min at 4°C and the supernatant was discarded. The pellet was resuspended and washed twice with 10 ml of the incubation medium (E 2a growth medium (13), containing 5% Hanks' salts without serum. The washed cells were resuspended in leucine-free incubation medium containing 5% calf serum to a final concentration of 5 × 10⁶ cells per ml and then aliquots of 1 ml were transferred to incubation tubes. Radioactively labeled isotopes as well as toxins were added as described in legends to figures and the samples were incubated in a shaking waterbath at 37°C. The pH of the cultures was maintained between 7.0 and 7.2 by gassing for 1 min per hour with a mixture of 95% O₂ and 5% CO₂.

At the times indicated, 25-μl samples were removed, poured into 1 ml of 0.1 M KOH, and incubated at room temperature for 30 min. Then trichloroacetic acid was added to a final concentration of 10% (w/v). The precipitated material was collected on Gelman glass fiber filters type A and the radioactivity was counted in a Beckman LS-130 scintillation system as earlier described (6).

Cell-free Protein Synthesis—The cell-free protein synthesizing system was an unfractionated rabbit reticulocyte lysate prepared as described by Lingrel (14). After incubation for different periods of time, 10-μl samples were removed, poured into 1 ml of 0.1 M KOH, and incubated at room temperature for 60 min. Trichloroacetic acid was then added to a final concentration of 10% (w/v) and the heme was converted into a colorless compound by the addition of 1% H₂O₂. The precipitated protein was collected on Gelman glass fiber filters and the radioactivity was counted.

Preparation of Antisera against Abri, Ricin, and Their Isolated Peptide Chains—Due to the high toxicity of the toxins the immunization was carried out with formaldehyde-treated toxins as earlier described (11, 12). The same procedure was used for the preparation of antisera against the isolated A and B chains. The protein (0.5 mg) was injected together with Freund's complete adjuvant. After 3 and 6 weeks, booster doses (without adjuvant) were given and the rabbits were bled 1 week after the last injection. Gel precipitation tests (Fig. 2, A and B) showed that the antisera obtained by immunization with whole toxins contained antibodies against both the A and the B chains. The sera from rabbits immunized with the isolated A chains contained traces of antibody against the corresponding B chains and vice versa. These traces were specifically removed by quantitative precipitation with the appropriate isolated peptide chains (15). Immunodiffusion studies indicated that after this treatment the antisera contained antibodies only against a single peptide chain (Fig. 2, C to F).

RESULTS

The Lag Time for Inhibition of Protein Synthesis in Ehrlich Ascites Cells—The Ehrlich ascites cells were metabolically highly active in vitro, synthesizing protein, DNA, and RNA, at a linear rate for several hours. When 1 μg per ml of abrin or ricin was added to a suspension of Ehrlich ascites cells, the incorporation of radioactively labeled leucine declined after a lag time of about 30 min (Fig. 3). Approximately the same lag time was observed even when the toxin concentration was increased to 100 μg per ml. In contrast, in a cell-free system from rabbit reticulocytes, protein synthesis was promptly reduced upon addition of 1 μg of abrin (7) or ricin (6). When isolated A chains were used, as little as 20 ng per ml gave an immediate inhibition of protein synthesis (Fig. 4). It is therefore probable that the lag time observed in whole cells reflects the time needed for the toxins to be transported to their site of action (see "Discussion").

The lag time for the toxic effect of abrin and ricin in intact cells was considerably increased with reduction in temperature. Thus, when the incubation was carried out at 20°C rather than at 37°C, inhibition of protein synthesis was not observed until after about 6 hours (Fig. 5).

Effect of Antitoxins Added During Lag Time—If the entry of the toxins into the cells involves distinct, separate steps, this
might be expected to be revealed by the use of specific antisera against the toxins and their constituent peptide chains. Since there is good evidence that the function of the B chains is to bind the toxins to receptors on the cell surface (11, 12), specific antisera against the B chains should be able to prevent toxic effects only until the toxins had become bound to the cell surface (Fig. 6). However, if the toxins after being bound to the cell surface, reside there for some time until they penetrate into the interior of the cells, specific antisera against the A chains should be capable of preventing the effect of the toxins for some time after antisera against the B chains had ceased to be effective. The results in Figs. 7 and 8 bear out this expectation. When antisera against isolated B chains were used (Figs. 7C and 8C), a small, but definite, protection was observed when the antisera were added after 5 to 10 min of preincubation of the cells with the toxin, whereas little or no protection was observed when the antitoxins were added after 30 min. The results indicate that at 20° most of the toxins are bound in less than 5 min. When antisera against A chains were used, strong protection was obtained when the antisera were added after 5 and 10 min of incubation of the cells with the toxins, and definite protection was also obtained when the antitoxins were added after 30 min. Almost identical results were obtained when antisera prepared against the intact toxins were used (Figs. 7A and 8A). This is in agreement with expectation since such antisera react both with the B and the A chains (Fig. 2, A and B). In separate control experiments, the amounts of antisera against A and B chains necessary to neutralize the toxins were determined. In the experiments presented, twice these amounts of antisera were used. It is therefore clear that the difference of anti-A and anti-B activity (Figs. 7 and 8) is not a dosis effect. The results indicate that the binding of the toxins is a distinct step which can be distinguished from the subsequent entry of the toxin into the interior of the cells.

If the preincubation was carried out at 0°, protection was achieved by addition of anti-B chain serum, even after 30 to 60 min of interaction with the toxins (Figs. 7D and 8D).

Effect of Abrin and Ricin on Reticulocytes—The data in Fig. 5 showed that at 20° about 6 hours elapse until protein synthesis in Ehrlich ascites cells was inhibited by abrin and ricin. Since at this temperature it was possible to protect the cells against abrin and ricin only by addition of antitoxins within the first 30 min of interaction, it follows that more than 5 hours proceeded after the toxins were added, as indicated, to 1-ml suspensions of Ehrlich ascites cells and protein synthesis at 20° was measured. Otherwise, conditions are as in Fig. 3.
FIG. 6. Hypothetical scheme of the interaction of abrin and ricin with cells.

synthesis was not reduced in the reticulocytes even when they were incubated for 5 hours at 37°C with a high concentration of the toxins. On the other hand, antibiotics of low molecular weight, such as cycloheximide and fusidic acid, exerted a strong inhibitory effect.

FIG. 7. Ability of specific antisera against abrin and its constituent chains to protect Ehrlich ascites cells from the toxic effect of abrin. Suspensions of Ehrlich ascites cells (1 ml) were preincubated with 1 μg of abrin at 20°C (A to C) or at 0°C (D). After the periods of time indicated on the figures, a neutralizing dose of anti-abrin, anti-abrin A chain, or anti-abrin B chain was added.

The data presented in this paper indicate that the lag time following exposure of cells to abrin or ricin until protein synthesis is inhibited, is strongly temperature dependent and that it can be subdivided into separate, distinct periods. During the first few minutes of exposure to the toxins at 20°C, addition of antisera specifically directed against the B chains can prevent the action of the toxins. Presumably this reflects the time needed for the toxin molecules to bind through their B chains to receptors on the cell surface. Then follows a period of up to 30 min when anti-B chain serum is ineffective, whereas addition of anti-A chain serum can still partially prevent the action of the toxins on the protein synthesis in the cells. During this time, the toxin molecules probably remain on the cell surface, attached in such a way that the A chains are still accessible to the anti-A chain serum. A third period then follows during which time antisera are ineffective. At 20°C this period is more than 5 hours. Most probably this period reflects the time required to transport the toxic A chains from the cell surface to their site of action, which is known to be the ribosome (9).

Previously we have obtained evidence that the B chains of the toxins bind to galactose-containing receptors on the cell surface. Thus it was found that the binding of the toxins to human erythrocytes could be inhibited by galactose or galactose-containing carbohydrates like lactose (17) and that each toxin molecule binds one molecule of lactose. Moreover, addition of
FIG. 8. Ability of specific antisera against ricin and its constituent chains to protect Ehrlich ascites cells from the toxic effect of ricin. Conditions as in Fig. 7, except that ricin and the appropriate antisera were used. A, anti-ricin added, preincubation temperature 20°C; B, anti-ricin A chain added, preincubation temperature 20°C; C, anti-ricin B chain added, preincubation temperature 20°C; D, anti-ricin B chain added, preincubation temperature 0°C.

FIG. 9. The effect of abrin and ricin on protein synthesis in rabbit reticulocytes. Samples (1 ml) of a suspension of washed reticulocytes (10
6 cells per ml) were incubated at 37°C under conditions as given in Fig. 3. To the samples was added: ●, 1 μg of abrin; ○, 1 μg of ricin; ▲, 10
-3 M cycloheximide. One sample, X, was used as control. Aliquots were removed as indicated and the acid-precipitable radioactivity was measured.

Lactose to the medium prevents the inhibiting effect of abrin and ricin on protein synthesis in Ehrlich ascites cells. Since abrin and ricin bind rapidly at 0°C to galactose-containing residues (11, 12), the present finding that the toxic effects of abrin and ricin could be prevented by anti-B chain serum for a longer period of time at 0°C than at 20°C, suggests that after rapid initial binding to the surface receptors, the B chain becomes more firmly attached to the cell surface by a temperature-dependent process.

The mechanism of the transport of the toxins into the cells is not clear. Ehrlich ascites cells are known to have a high endocytotic activity (18), and one obvious possibility is that macro-molecules sticking to the cell surface, are taken up by endocytosis. Indeed, this is known to be the case in the uptake of some viruses (19). This possibility is supported by the present finding that the transport was found to be strongly temperature-dependent which is also the case with endocytosis. Moreover, protein synthesis was not inhibited in intact reticulocytes, even by high concentrations of the toxins, in spite of the fact that the toxins readily bind to the reticulocyte surface membrane (17). Reticulocytes, like erythrocytes, have little or no endocytic activity (16). If endocytosis is involved in the uptake of the toxins, the lag time should largely reflect the time required for a molecule to be engulfed and to be liberated from an endocytotic vacuole into the cytoplasm.

If toxins are bound to many surface receptors, and if endocytotic processes occur at random, the probability will be high that an endocytotic process occurs sufficiently close to a toxin molecule to engulf it within a short time. On the other hand, when only a few toxin molecules are bound to the cell surface, a longer time would be expected to elapse until an endocytotic process takes place close enough to a bound toxin molecule to permit its entry into the cytoplasm. The fact that a longer lag time was indeed found at low toxin concentrations (data not shown) is consistent with such a mechanism. On the basis of earlier experiments (Ref. 8, and unpublished data), it may be estimated that the entry of only a few toxin molecules (perhaps a single one) is sufficient to inhibit protein synthesis in a cell.

It has recently been found that different cell types bind 1 to 2 × 10
7 molecules of ricin per cell (20, 21). On this basis it can be calculated that if all binding sites were occupied by ricin molecules about 5 to 10 μg of toxin would be bound per ml of reaction mixture under our experimental conditions. The fact that at 37°C the lag time for the inhibition of protein synthesis reached its minimum value (30 min) already in the presence of 1 μg of toxin per ml provides strong evidence that the number of receptor sites is not a limiting factor in the uptake of the toxin and cannot account for the observed lag time.

We have earlier shown that treatment of the toxins with 2-mercaptoethanol, which splits the disulfide bridges and separates the two chains, increases the inhibitory effect on protein synthesis in the cell-free system by a factor of 50 to 100 (8). The much

higher activity of the free A chains than of intact toxin in a cell-free system, suggests that the intact toxins as such may be unable to inhibit protein synthesis and that their apparent inhibitory effect in a cell-free system is actually due to contaminating traces of the reduced toxins.

It is not possible to decide from the present data whether only the A chains as such or the whole toxin molecules enter the cytoplasm. The cells contain disulfide reducing systems, and it is conceivable that the toxic A chain is liberated in the cytoplasm. However, it is also possible that the A chain is liberated from the B chain on the cell surface. The results of Onozaki et al. (5) suggest the latter possibility. These authors have found that ricin bound to Sepharose particles have a toxic effect on cells in culture. Since the diameter of the Sepharose particles is about 20 times that of the cells, it is clear that the toxic action does not require that the whole toxin is taken up by the cells. Since the toxin may be linked to the Sepharose particles by the A chain, the B chain, or both, it is possible that, in those cases where only the B chain is bound, the A chain may be liberated into the cell after reduction of the disulfide bridge.

It is well known that diphtheria toxin, like abrin and ricin, consists of an effector moiety and a carrier moiety which binds the toxin to receptors on the cell surface (22, 23). Recent evidence suggests that botulinus toxin (24) and tetanus toxin (25, 26) may have similar structures. It is an intriguing possibility that also some nontoxic, biologically active proteins like nerve growth factor and epidermal growth factor (27) may consist of an effector moiety and a carrier moiety.

Addendum—After the submission of this paper, Dr. G. L. Nicolson (The Salk Institute for Biological Studies, San Diego), using radioactively labeled ricin, has demonstrated by autoradiography that the toxin is first bound to the surface of cells, then it is taken up into a pinocytotic vesicle, and finally released into the cytoplasm (G. L. Nicolson, personal communication).

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