A Neutralizing Antibody to the A Chain of Abrin Inhibits Abrin Toxicity both In Vitro and In Vivo†

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PLANT RIBOSOME-INACTIVATING PROTEINS (RIPs) ARE RNA N-GLYCOSIDASES THAT INHIBIT PROTEIN SYNTHESIS IN CELLS. ABRIN, A TYPE II RIP, IS AN AB TYPE TOXIN, WHICH IS ONE OF THE MOST LETHAL TYPES OF TOXIN KNOWN. THE A CHAIN FACILITATES THE ENTRY OF THE MOLECULE INTO THE CELL, IN WHEREAS THE A CHAIN EXERTS THE TOXIC EFFECT. WE HAVE GENERATED HYBRIDOMAS SECRETING ANTIBODIES OF THE IMMUNOGLOBULIN G CLASS SPECIFIC TO THE RECOMBINANT A CHAIN OF ABRIN. ONE MONOCLONAL ANTIBODY, NAMELY, D6F10, RESCUED CELLS FROM ABRIN TOXICITY. IMPORTANTIY, THE ANTIBODY ALSO PROTECTED MICE FROM LETHAL DOSAGES OF THE TOXIN. THE NEUTRALIZING EFFECT OF THE ANTIBODY WAS SHOWN TO BE DUE TO INTERFERENCE WITH ABRIN ATTACHMENT TO THE CELL SURFACE.

Ribosome-inactivating proteins (RIPs) are a family of protein toxins that bring about the inhibition of protein synthesis either directly by inactivating the ribosomes or indirectly by modifying factors involved in translation (27). They are distributed widely in nature and are found in bacteria, fungi, and plants (34). One of the most potent members of the RIP family is abrin produced by the subtropical climber Abrus precatorius (2). Abrin is an AB type toxin with a 30-kDa A chain, an RNA N-glycosidase that irreversibly inactivates the 28S rRNA of the mammalian 60S ribosomal subunit. Once in the cytosol, the A chain depurinates the adenine of the alpha-sarcin-ricin loop and thereby arrests host cell protein synthesis (7, 28). The B chain is a galactose-specific lectin and hence binds to cell surface glycosylated receptors, which allows the toxin entry (31, 16). Apart from inhibiting protein synthesis, RIPs induce apoptosis (23). Abrin shows significant similarities to ricin at the sequence level as well as the structural level, but abrin is several times more potent than ricin (28). There is much interest in understanding the bioactivities of these proteins, owing to their extreme toxicity (the 50% lethal dose [LD50] of abrin for mice is 0.04 μg/kg of body weight, and the LD50 of ricin is 3 μg/kg) (35), stability, and easy availability. Both proteins cause pulmonary edema, with acute destructive alveolitis and apoptosis and necrosis in the lower respiratory tract epithelium (10, 41). RIPs from different plants are potent elicitors of sensitization and immunoglobulin E (IgE) production (36). The use of these proteins as bioterrorist weapons is also of considerable concern (3, 4, 17). The passive administration of antibodies has proven to be a specific and effective mode of defense against poisoning by various biological toxins (29). Although both anti-A chain and anti-B chain antibodies are able to neutralize toxins in vitro and in vivo, antibodies against the A chain of ricin have better protective efficacy than anti-B chain antibodies (14, 19).

While many detection and treatment modalities for ricin toxicity have been developed previously, few methods for the treatment of abrin toxicity are known (3, 40, 15). No antidote or vaccine for abrin has been described before now. The aim of the present study was to identify high-affinity neutralizing antibodies capable of inhibiting the toxicity of abrin. Toward this end, a panel of monoclonal antibodies (MAbs) against the recombinant abrin A chain (rABA) were raised and the neutralizing potentials of the antibodies were tested. One MAb, D6F10, completely inhibited abrin toxicity when tested in a cell culture system as well as when tested in mice.

MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium, RPMI 1640, Iecove’s modified Dulbecco’s medium (IMDM), bovine serum albumin (BSA), acridine orange, ethidium bromide, fetal bovine serum (FBS), propidium iodide, RNase A, and protease K were purchased from Sigma (St. Louis, MO), Ni-nitrilotriacetic acid (NTA) beads were purchased from Qiagen (Germany), and Sephadex G-100 was purchased from Amersham Pharmacia Biotech (Sweden).

Cell lines. MCF-7 (human breast cancer cell line) and OVCAR-3 (human ovarian cancer cell line) cells were cultured in Dulbecco’s modified Eagle’s medium, Jeko Jurkat (human T-cell line) cells were cultured in RPMI 1640, and Sp20 myeloma and hybridoma cells were cultured in IMDM at 37°C in a humidified air-CO2 (19:1) atmosphere. The cell lines were obtained from the National Centre for Cell Sciences, Pune, India. All media were supplemented with 10% (vol/vol) FBS, 100 IU of penicillin/ml, and 100 μg of streptomycin/ml.

Purification of abrin and Abrus precatorius agglutinin I. Abrin and A. precatorius agglutinin I were purified from the seeds of A. precatorius as described previously (31). The seed kernels were soaked in 5% acetic acid overnight and homogenized. The crude extract was subjected to 30% ammonium sulfate precipitation, and the supernatant was subsequently subjected to 90% ammonium sulfate precipitation. The precipitate was dissolved in 20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl (PBS) and dialyzed extensively against the same buffer. The dialysate was centrifuged at 10,000 rpm at 4°C for 15 min (Beckman Coulter Avanti JE), and the supernatant was analyzed by chromatography on a lactamyl-Sepharose affinity column equilibrated with PBS. The bound proteins (abrin and A. precatorius agglutinin I) were then eluted with 0.4 M lactose and dialyzed against PBS. The dialysate was then loaded onto a Sephacore G-100 gel filtration column equilibrated with PBS. The fractions completing peak I (molecular mass of 120 kDa) and peak II (molecular mass of 60 kDa) were pooled separately, dialyzed extensively against water, and lyophilized.

Ricin and Ricinus communis agglutinin I were purified from the seeds of R. communis earlier in this laboratory, using procedures reported elsewhere (2; S. Bagaria and A. A. Karande, unpublished data).

Purification of rABA and native A chain of abrin. The rABA clone was a kind gift from J. Y. Lin, National Taiwan University, Taiwan, Republic of China. The
rABA was subcloned in the pRSETA vector and expressed in Escherichia coli BL21/pLysS cells upon induction with 400 μM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. The cells were then pelleted, sonicated, and centrifuged at 14,000 rpm for 30 min at 4°C (Beckman Coulter Avanti JE). The recombinant protein was purified from the cell lysate on Ni-NTA beads per the manufacturer’s protocol. The native A chain of abrin was prepared by subjecting abrin to reduction with 50 mM dithiothreitol in PBS for 1.5 h. Upon the dissociation of the disulfide bond, the B chain precipitated and the supernatant containing the A chain was subjected to lactamyl-Sepharose affinity chromatography to remove the unreduced abrin (26, 32).

Establishment of hybridomas. The protocol followed for the generation of hybridomas was essentially that reported by Kohler and Milstein (12) with a few modifications (6, 9). Twenty micrograms of rABA emulsified in Freund’s adjuvant was administered subcutaneously to 6- to 8-week-old female BALB/c mice. After two booster doses of rABA of 10 μg each, mice were rested for a month. Subsequently, mice were injected intraperitoneally with 100 μg of the antigen in saline, and on the fourth day after injection, the animals were sacrificed, the spleens were excised, and single-cell suspensions were prepared. Splenocytes were mixed with Sp2/0 mouse myeloma cells at a ratio of 5:1 and fused using polyethylene glycol 4000 (Merck, Rahway, NJ). The cells were then aliquoted into 96-well plates in IMDM supplemented with 20% FBS, 50 μM β-mercaptoethanol, and HAT (10 mM hypoxanthine, 40 μM aminopterin, 1.6 mM thymidine). After 12 to 14 days, when the hybridoma cells attained >50% confluence, the supernatant from each well was tested for the presence of antibodies by an enzyme-linked immunosorbent assay (ELISA). Clones secreting antibodies were selected based on their reactivities to rABA, the native A chain of abrin, and abrin; expanded; and subsequently subcloned to obtain monoclonality by the limiting dilution method. After two booster doses of rABA of 10 μg of antigen in the presence or absence of an ~100-fold molar excess of either anti-rABA MAbs or unlabeled abrin for 60 min on ice. After a brief spin at 3,000 rpm for 5 min, cells were washed twice with ice-cold Hanks balanced salt solution, resuspended in 200 μl of ice-cold PBS, and either analyzed by fluorescence-activated cell sorting (FACS) or visualized under a fluorescence microscope (Leica, Germany). A total of 10,000 events were analyzed, and the mean fluorescence was calculated. The FITC-labeled abrin retained 80% of its activity, as determined by polyclonal antibody synthesis inhibition assays.

RESULTS
All the experiments, except the in vivo toxicity experiments with mice, were carried out at least three times. Representative data are presented. Statistical analyses of data were carried out by using the Student t test.

Purification of toxins, abrin A chain, and rABA. Abrin and A. precatorius agglutinin I were purified as described above. The native A chain of abrin was prepared by subjecting the protein to reduction by 50 mM dithiothreitol. Owing to the presence of five intramolecular disulfide bonds, the B chain precipitates upon reduction, while the A chain remains in solution. The A chain was purified by passing through a lactamyl-Sepharose affinity column to remove any unreduced abrin. rABA was expressed as a His-tagged fusion protein in E. coli BL21/pLysS cells and purified by Ni-NTA affinity column chromatography. The Coomassie blue-stained-gel profile of the purified proteins is shown in Fig. 1. The larger size of the rABA than of the native A chain is because of 32 additional amino acids derived from the pRSETA vector during cloning. The native abrin A chain is 251 amino acids in length, and the rABA contains 283 amino acids. The A. precatorius agglutinin I A chain is 10 residues longer at the C terminus than the abrin A chain and contains a potential N glycosylation site at Asn250, which is not present in the abrin A chain (Fig. 1).

Reactivities of anti-rABA MAbs to the abrin A chain and other RPs. Hybridomas were established from splenocytes of mice immunized with rABA. Hybridomas were selected based on the abilities of the secreted antibodies to bind both the native A chain of abrin and rABA. All the antibodies were found to be of the IgG1 class as determined by isotyping. Studies pertaining to four of the MAbs, D6F10, F5B10, D5B8, and F12B11, are described in this report. The dilution curves for the binding of MAbs to immobilized rABA are shown in Fig. 2. It is clear from the figure that dilution curves for the MAbs F5B10, D5B8, and F12B11 exhibited identical slopes, while that of MAb D6F10 showed a different profile. All the
MAbs also bound to the A chain in the whole abrin molecule. However, the level of binding of MAb D6F10 was significantly higher than those of the other antibodies (Fig. 3). Abrin shows a good level of sequence homology to *A. precatorius* agglutinin I and, to some extent, also to the closely related type II RIPs *ricin* and *R. communis* agglutinin (1, 13, 28). When tested for binding in an ELISA (Fig. 3) or a Western blot analysis (Fig. 4; data not shown for ricin and *R. communis* agglutinin), none of the MAbs bound to the other RIPs, demonstrating that the MAbs recognize epitopes unique to abrin. All the MAbs bound to the A chain of abrin and the rABA in Western blots, showing that the MAbs recognize sequential epitopes (Fig. 4).

**Screening of rABA MAbs for neutralization of abrin cytotoxicity.** The abilities of the MAbs to neutralize abrin toxicity were tested by a protein synthesis assay. The 50% inhibitory concentrations (IC₅₀) for the inhibition of protein synthesis by abrin on MCF-7 cells (Fig. 5) and OVCAR-3 cells (data not shown) were estimated to be ~0.4 to 0.8 ng/ml. Abrin at concentrations >10-fold higher than the IC₅₀, 10 ng/ml, was mixed with 25 μg of each of the purified MAbs/ml, and the mixtures were added to MCF-7 cells grown in cell culture plates. Only the MAb D6F10 showed complete rescue of the cells from abrin-mediated inhibition of protein synthesis, while none of the other MAbs showed any detectable neutralization activity (Fig. 6a). In agreement with the observation that the MAb D6F10 did not bind RIPs other than abrin under native conditions (Fig. 3), we found no neutralizing activity of MAb D6F10 toward ricin (50 ng/ml) and *A. precatorius* agglutinin I (1 μg/ml) (Fig. 6b). MAb D6F10 at 2.5 μg was able to neutralize 100% of the toxicity induced by 12.5 ng of abrin/ml (Fig. 7). As abrin also induces apoptosis (22), cells cultured with abrin (10 ng/ml) in the presence of MAb D6F10 (25 μg/ml) were analyzed for apoptosis. There was complete inhibition of abrin-induced apoptosis of Jurkat cells (Fig. 8a). The apoptotic population was also quantified by using FACScan (Fig. 8b).

**MAb D6F10 prevents abrin binding on cells.** The antibody D6F10 was raised against the A chain of abrin. The mode of inhibition of the cytotoxic effect by many anti-A chain antibodies described earlier is still not clear (18). The binding of toxin on the cell surface is the first event in the intoxication process, and therefore, we analyzed the binding of abrin-FITC on
Jurkat cells in the presence and absence of MAb D6F10. Results obtained from FACS analysis (Fig. 9a) and fluorescence microscopy (Fig. 9b) indicated that MAb D6F10 inhibits the binding of abrin on the cell surface. Hence, it appears that the binding of the antibody to the toxic subunit prevents the binding of the toxin to the receptors and brings about the neutralization effect. Interestingly, cells incubated with only abrin-FITC separated into two peaks (Fig. 9a); the smaller one represents the population of cells with low-level abrin binding. The observation of only one peak when the cells were incubated with the labeled toxin in the presence of MAb F5B10 can be explained as follows: MAb F5B10 binding to abrin does not inhibit the binding of the toxin to the cell surface. As antibodies are bivalent and two molecules of abrin-FITC would be bound to each molecule of the MAb, there would be two molecules of abrin-FITC at the surfaces of the cells for every one molecule of cell-bound abrin-FITC. In such a case, the population of cells with fewer molecules bound to the galactose receptors would also acquire more fluorescence due to the recruitment of more abrin molecules through the bound antibody.

In vivo protection by MAb D6F10. We studied the in vivo protective efficacy of MAb D6F10. Mice (~6 months old) were injected intraperitoneally with either MAb D6F10 or MAb F5B10, and 60 min later, abrin was administered via the same route. Mice were observed for more than 2 months. A concentration of 250 μg of the D6F10 antibody provided complete protection against the lethality of 1.25 μg of abrin (Fig. 10), while no such effect was observed with either the MAb F5B10 or normal mouse IgG.

DISCUSSION

Abrin is one of the most powerful plant toxins known (28, 25). The reported IC₅₀ for protein synthesis by abrin in cultured cell lines is ~0.4 ng/ml (23), and the LD₅₀ for mice is ~0.04 μg/kg (35). Although not infectious, it is a probable agent for use as a bioweapon (4) because of its stability (it is partially active at temperatures up to 50°C [data not shown]) and the relatively easy purification procedure that yields milligram amounts of the toxin from the seeds. Though the mechanisms of toxicity of some RIPs at the cellular and molecular levels have been delineated previously, the development of an antidote has proven elusive (21). Chemical inhibitors targeting
the active site in the A chain or the lectin pockets of the B chain have been identified previously but have had limited application due to a lack of cell specificity and general toxicity (3, 30). Antibody-based therapies, on the other hand, for the treatment of poisoning by various toxins are more promising. Several neutralizing antibodies for biological toxins have been described earlier (5, 20, 24, 39). Antibodies to the galactose binding domain of the B chain have been shown to inhibit ricin binding to cells, thereby inhibiting toxin activity (19, 20). Antibodies to the A chain of ricin also protect cells effectively from ricin toxicity (18). However, there are no reports on neutralizing antibodies to the A chain or B chain of abrin.

Toward the objective of establishing antibodies that would neutralize abrin, a panel of MAbs against the rABA was established and characterized extensively. The MAbs recognized specifically the rABA as well as the abrin A chain under native as well as denaturing conditions. There is a divergence of more than 75% between plant and bacterial RIPs in primary structures, even though they have identical mechanisms of action. This large amount of sequence variability may result in the plant and bacterial RIPs’ having distinct neutralizing epitopes.

FIG. 8. (a) Jurkat cells were cultured with abrin (10 ng/ml) in the presence or absence of antibodies (25 μg/ml) for a period of 12 h, stained with acridine orange-ethidium bromide, and analyzed by fluorescence microscopy. Arrowheads point to apoptotic nuclei. (b) In another experiment, the cells were incubated with abrin, fixed in 70% ethanol, and stained with ethidium bromide. The percentage of apoptosis was quantified by using FACSscan. The experiment was carried out at least three times. Representative data are presented.
In agreement with the earlier predictions by other groups (13), in spite of the high level of sequence similarity observed among different RIPS (abrin, ricin, R. communis agglutinin I, and A. precatorius agglutinin I), mostly unique epitopes were recognized by the anti-rABA antibodies. The inhibition of protein synthesis and apoptosis are the two major effects of RIPS on cells (8, 33). MAb D6F10 but none of the other MAbs rescued MCF-7 cells from abrin-mediating inhibition of protein synthesis (Fig. 6a). We also observed that, in the presence of this antibody, there was a significant reduction in apoptosis induced by abrin among Jurkat cells (Fig. 8) and that a 200-fold molar excess of antibody was required to inhibit the abrin toxicity in OVCAR-3 cells (Fig. 7). It is pertinent to mention here that all the toxin binding and neutralization assays were carried out with all three cell lines, Jurkat, MCF-7, and OVCAR-3, and we obtained very comparable data.

Studies with mice were also initiated to validate the protective effect of the MAb D6F10 as determined by in vitro cell culture assays. A level of protection of 100% was seen in the mice that received this antibody either along with, or 1 h prior to, the administration of a lethal dose of the toxin, and the mice appeared to be normal as observed for up to 10 weeks. Further studies are in progress to understand the effects of the long-term administration of this MAb to mice and to identify the time spans within which the antibody would be effective if administered after abrin poisoning through various routes. Antibodies neutralize the cytotoxicity of toxins by different mechanisms: blocking the initial stages of toxin trafficking, like binding and internalization, or inhibiting the intracellular processing or inducing the degradation of the toxins (34). Binding assays performed with FITC-labeled abrin indicated that the neutralization effect exhibited by D6F10 is probably due to the prevention of toxin attachment on the cell surface by steric hindrance of the B chain binding. In conclusion, we have obtained a MAb, namely, D6F10, to the abrin A chain that neutralizes the toxicity of abrin both in vitro and in vivo. Experiments are under way in our laboratory to identify the epitopes of the anti-A chain antibodies and to characterize the mode of inhibition of toxin binding by MAb D6F10.

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