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Purified Immunotoxins That Are Reactive with Human Lymphoid Cells

MONOCLONAL ANTIBODIES CONJUGATED TO THE RIBOSOME-INACTIVATING PROTEINS GELONIN AND THE POKEWEED ANTIVIRAL PROTEINS*

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Seven different monoclonal antibodies of the IgG class that are reactive with four different antigens on human lymphoid cells were utilized to form immunotoxins with the ribosome-inactivating proteins gelonin and the three known pokeweed antiviral proteins. Thirteen different immunotoxin combinations were prepared. The ribosome-inactivating proteins were modified with 2-iminothiolane. The sulfhydryl groups so introduced were reacted with maleimido groups or with dithiopyridyl groups that had been introduced into the antibodies. The toxin-antibody conjugates so formed were purified by affinity chromatography on protein A-Sepharose CL-4B, ion exchange chromatography, and by gel filtration and were characterized by polyacrylamide-dodecyl sulfate gel electrophoresis. The purified immunotoxins were free of nonconjugated monomeric proteins and aggregates of very high molecular weight. All the immunotoxins showed the *specific* binding of the component antibody as measured by indirect immunofluorescence binding assays. The activities of the ribosome-inactivating proteins were unaffected by conjugation where the cross-link to the antibody contained a disulfide bond and when assayed after reductive cleavage of the linker. Disulfide-linked immunotoxins with six of the antibodies were highly cytotoxic for the target cells. However, immunotoxins containing an anti-B1 antibody showed no cytotoxicity.

The possible use of antibodies to target pharmacologic agents, such as toxins, was first proposed by Ehrlich (1). Research to exploit this idea has developed rapidly in the last decade, owing much to the ability to produce pure highly specific monoclonal antibodies using the hybridoma technology (2). Recently, monoclonal antibodies have been developed that recognize tumor-associated antigens (3, 4, 11, 12), and it is the hope that such antibodies can be exploited to deliver toxic agents to particular types of tumor cells in order to kill them selectively. The ribosome-inactivating proteins (5, 6) seem to be ideal toxic agents for this purpose. Most effort has been directed toward using ricin (extracted from castor beans, *Ricinus communis*) which consists of two nonidentical subunits (A- and B-chains) that are joined by a disulfide bond (6). The B-chain has the property of binding to cell-surface carbohydrates and promotes the uptake of the A-chain into cells (6). Entry of the A-chain into the cytoplasm of a cell then results in the death of the cell by catalytic inactivation of its ribosomes. Immunotoxins have been made by conjugating intact ricin to antibodies (7–9). Such immunotoxins exhibit *specific* toxicity only in the presence of lactose which at high concentration competes with the cell surface carbohydrates for the ricin B-chain binding site(s). *In vivo*, these immunotoxins are expected to be nonspecifically toxic, as is ricin itself, and are, therefore, unlikely to be of therapeutic value, although they may have limited use in the *in vitro* treatment of bone marrow for transplantation (9, 10).

There is a class of ribosome-inactivating proteins that have properties and characteristics similar to those of ricin A-chain alone (5). Gelonin (20) and the three known pokeweed antiviral proteins (21) are examples of such proteins. They are basic proteins, of M_r about 30,000 (5). These proteins have several advantages over ricin A-chain in the preparation of immunotoxins: they are extremely stable proteins, they do not bind to cells and so are nontoxic to intact cells (except at very high concentrations), and they are safe to purify and manipulate in the laboratory without the extreme precautions necessary for work with ricin (5). These proteins are good candidates for the preparation of immunotoxins since, at least in principle, such immunotoxins will only bind to the cells selected by the antibody. Immunotoxins have been made using gelonin and PAP,[1] and in general they showed specific cytotoxicity similar to immunotoxins prepared with ricin A-chain (13–19, 22–25).

There is an important caveat in the interpretation of the experiments that have been reported using immunotoxins made with ricin A-chain, gelonin, or PAP. There is not a single example of an immunotoxin that was completely purified from nonconjugated antibody. This is an important obstacle to the proper interpretation of these reports and hampers understanding of the mechanisms involved in the cytotoxicity of such immunotoxins. We describe here the preparation of highly purified immunotoxin conjugates using seven different monoclonal antibodies and using the ribosome-in-

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[1] The abbreviations used are: PAP, pokeweed antiviral protein; PAP II, pokeweed antiviral protein type II; PAP-S, pokeweed antiviral protein from seeds; SPDP, *n*-succinimidyl 3-(2-pyridyldithio)propionate; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; bis-tris, 2-[bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-propane-1,3-diol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CALLA, common acute lymphoblastic leukemia antigen; RPMI, Roswell Park Memorial Institute.

activating proteins gelonin, PAP, PAP II, and PAP-S. The principle of the purification methods should be generally applicable. The immunotoxins reported here were prepared both with a cleavable linker containing a disulfide bond and with a noncleavable linker, in order to evaluate the importance of release of the toxic agent from the antibody. All the immunotoxins were analyzed for their ribosome-inactivating capacity, their ability to bind to cell-surface antigens, and their *in vitro* cytotoxic potency toward lymphoid cell lines.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

DISCUSSION

We have developed methods for the preparation of purified immunotoxin conjugates in high yield, utilizing the techniques of affinity chromatography, gel filtration, and ion exchange chromatography with buffers of carefully defined composition. Thirteen different immunotoxin conjugates were made using seven different monoclonal antibodies and four different ribosome-inactivating proteins. The immunotoxin preparations contain no nonconjugated antibody, no nonconjugated ribosome-inactivating proteins, and no aggregates of very high molecular weight. These purified and well defined immunotoxins made it possible for the first time to perform quantitative binding studies and to perform cytotoxicity tests without fear of the effect of competition by nonconjugated antibody on the experimental result, for example, by blocking antigens or saturating the internalization pathways. Also, the purity of the immunotoxin preparations permitted a careful comparison to be made of the biological activities of the component proteins with their nonconjugated counterparts.

Gelonin and the pokeweed antiviral proteins were not affected by modification with 2-iminothiolane in their ability to inhibit protein synthesis. The ability of the modified toxins to inactivate ribosomes in a cell-free system of protein synthesis was indistinguishable from that of the native proteins. This is in contrast with the results of earlier work using *N*-succinimidyl 3-(2-pyridyldithio)propionate to modify gelonin (22); the gelonin was inactivated by about 90%. It is possible that 2-iminothiolane and *N*-succinimidyl 3-(2-pyridyldithio)propionate each react preferentially with different amino groups of the gelonin molecule. Another factor is the preservation of the positive charge at amino groups upon reaction with 2-iminothiolane that may account for this difference. Our results suggest that 2-iminothiolane is the reagent of choice for modifying ribosome-inactivating proteins, in order to introduce sulfhydryl groups while preserving their toxic activity.

When the modified toxins were covalently linked to an antibody, the ribosome-inactivating activity of the toxins was reduced by about 70%. When the covalent link between the toxin and the antibody included a disulfide bond, then the full ribosome-inactivating activity as measured in a cell-free system could be restored by reductive cleavage of the linker. This may account, in part, for the observation that the immunotoxins J5-gelonin and J5-PAP-S exhibited greater cytotoxicity (about 10^3-fold) on cultured CALLA-positive cell lines when the linker included a disulfide bond than when the linker was noncleavable. There is one claim in the literature that immunotoxins containing pokeweed antiviral proteins are more cytotoxic when linked by a noncleavable linker than when linked by a disulfide linker (25). However, we could not confirm this result, and our observations with purified immunotoxins were consistent with previous findings with immunotoxins containing ricin A-chain made with cleavable and noncleavable linkers (17). A cleavable linkage between the toxin and the antibody presumably allows the toxin to escape more easily into the cytoplasm from the membrane-bound antibody/antigen complex (48).

Binding studies showed that the immunotoxins made with all seven antibodies showed specific binding to antigen-bearing cells. Cytotoxicity experiments *in vitro* showed that immunotoxins made with six of the seven antibodies showed considerable enhancement of the toxic effect of the ribosome-inactivating proteins, with ID_{50} concentrations that were about 10^4-fold lower than that of the native ribosome-inactivating protein. The enhanced toxicity was completely specific for cells bearing the cognate antigen. Immunotoxins made with gelonin or with the pokeweed antiviral proteins gave qualitatively similar results. However, none of the immunotoxins made with anti-B1 showed any more toxicity than that exhibited by the native ribosome-inactivating protein, even at concentrations that were near the saturation of antibody/antigen binding. This result was obtained on several B1 antigen-positive cell lines, including some that were also positive for CALLA and for Ia antigens, and which were sensitive to immunotoxins made with the antibodies J5 or I-2, respectively.

Receptor-mediated endocytosis of an antigen/immunotoxin complex may be essential for cytotoxicity. This has been suggested on the basis of experiments with lysosomotropic agents which raise the pH of intracellular acidic vesicles (51) and which increase the potency of immunotoxins (52, 53). There is some evidence to suggest that the T11 surface antigen (29) and the CALLA (49) can be internalized, and perhaps these antigens utilize the mechanisms involving coated pits, coated vesicles, and endosomes (48). Our preliminary results suggest that the Ia antigens can also be internalized carrying the I-2 antibody. However, the current evidence suggests that the B1 antigen (50) remains firmly on the cell surface and shows no tendency to be internalized.[3] Thus, the B1 antigen may be excluded from the coated pits involved in receptor-mediated endocytosis (48). This suggests a possible explanation for the lack of cytotoxicity of immunotoxins made with anti-B1; that is, that the B1 antigen does not transport the immunotoxin complex inside the cell. Further evidence will be required to assess the relationship between endocytosis and the cytotoxicity of the immunotoxins made with ribosome-inactivating proteins. However, it is clear from our present results that the properties of the target antigen require careful consideration when designing immunotoxin conjugates.

The availability of pure immunotoxin conjugates will allow us to examine in greater detail than hitherto the effect of various parameters on the cytotoxicity exhibited by the immunotoxins. For example, the antibody-binding affinity of the immunotoxins, the number of cell-surface antigens capable of binding the immunotoxins, and the properties of different antigens once bound by an immunotoxin such as in receptor-mediated endocytosis may all influence the degree of cytotoxicity of the immunotoxins. We are now employing

[2] Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–5, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-546, cite the authors, and include a check or money order for $9.20 per set of photocopies. Full size

several different *in vitro* cytotoxicity assays, including those that directly measure cell survival, to investigate these parameters and to study potentiators of cytotoxicity such as adenovirus (54) and lysosomotropic agents (53). These experiments may help to determine how to improve the efficacy of the immunotoxins. Also, little is known about the biological properties of immunotoxins *in vivo*. Purified conjugates using different anti-T11 antibodies, three of which are described here, may be useful biological reagents for this purpose since the antibodies react with a surface antigen found on T cells of various species of monkey that is analogous to the human T11 surface antigen (34).

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cocktail was from National Diagnostics, Somerville, NJ. Fluorescein-labelled goat anti-mouse IgG and fluorescein-labelled goat anti-rabbit IgG were from Meloy Laboratories, Springfield, VA.

Murine Monoclonal Antibodies - Monoclonal antibodies were produced by hybridoma cells grown as ascites tumors in Balb/c mice. Seven different antibodies, each of the IgG class, were used in this work. J5 is an IgG2a antibody that is specific for the CALLA (3). J30 is an IgG2 antibody that is specific for gp26, a cell surface glycoprotein expressed on non-T acute lymphoblastic leukemia cells (26). Anti-B1 is an IgG2a antibody that identifies the B1 differentiation antigen expressed on B cells (27). I-2 is an IgG2 antibody that reacts with Ia antigens (28). Anti-T11$_{1A}$, anti-T11$_{1B}$ and anti-T11$_{1C}$, of isotype IgG1, IgG2a and IgG2b respectively, are antibodies that react with the T11 surface antigen which is found on all resting human T cells (29). Samples of ascites fluid containing the monoclonal antibodies were kindly provided by Drs. J. Ritz (J5, J30), L.M. Nadler (anti-B1, I-2) and E.L. Reinherz (anti-T11 antibodies) of the Dana-Farber Cancer Institute, Division of Tumor Immunology.

Purification of Monoclonal Antibodies - All steps were done at 4° C. All of the antibodies, except for anti-T11$_{1A}$ (an IgG1), were purified from ascites fluid by affinity chromatography on protein A-Sepharose CL-4B using the method described previously (30). Antibodies bound by the protein A were eluted with 0.1 M acetic acid containing NaCl (0.15 M). Antibody-containing fractions were immediately neutralized by the addition of one-tenth volume of 1.0 M NaHCO$_3$, and were then dialyzed against 10 mM NaP$_i$ buffer, pH 6.0, containing glycine (50 mM) and NaN$_3$ (0.4 mM), for further purification by ion-exchange chromatography on columns of Cm-cellulose (Whatman, CM-52) equilibrated in the same buffer. The columns (30 ml bed volume for 120 mg of protein) were developed with gradients of NaCl in the same buffer; 0-100 mM for J5, 0-200 mM for I-2, anti-B1, anti-T11$_{1B}$ and anti-T11$_{1C}$, and 0-300 mM for J30. The purified antibodies were finally dialyzed against 10 mM KP$_i$ buffer, pH 7.2, containing NaCl (145 mM) and stored at -70° C.

Anti-T11$_{1A}$ does not bind to protein A. Ascites fluid containing this antibody was passed through protein A-Sepharose CL-4B to remove traces of murine immunoglobulins that do bind to protein A. The eluate was then fractionated by adding $(NH_4)_2SO_4$ to 50% saturation. The precipitated protein was dissolved in 10 mM KP$_i$ buffer, pH 7.2, containing NaCl (145 mM), and then dialyzed into the pH 6.0 buffer for chromatography on a column of Cm-cellulose as described above: the column was developed with a gradient of 0-300 mM NaCl. Fractions containing anti-T11$_{1A}$ were pooled, concentrated and submitted to gel filtration on a column (99 cm x 2.6 cm) of Sephacryl S-300 equilibrated in 10 mM KP$_i$, pH 7.2, containing NaCl (145 mM).

All the antibodies were judged pure by polyacrylamide/dodecyl sulfate gel electrophoresis and by isoelectrofocussing (pH range 3.5 to 9.5) on polyacrylamide gels. The isoelectrofocussing gels showed that the purified monoclonal antibodies gave the typical pattern of 3 to 5 closely spaced bands that has been described previously (31). The average final yield of each antibody, per ml of ascites fluid, were 3.0 mg for J5, 0.06 mg for J30, 2.4 mg for anti-B1, 1.6 mg for I-2, 3.2 mg for anti-T11$_{1A}$, 3.0 mg for anti-T11$_{1B}$ and 3.2 mg for anti-T11$_{1C}$.

Source and Purification of Ribosome-Inactivating Proteins - Seeds from Gelonium multiflorum were from United Chemical and Allied Products, 10 Clive Row, Calcutta-1, India, and were obtained through the Meer Corporation, North Bergen, NJ. Gelonin was purified by the method previously described (20). Seeds and leaves of Phytolacca americana (pokeweed) were collected in Brookline, MA. The pokeweed antiviral proteins, PAP and PAP II, were purified from leaves as described previously (21), and PAP-S was purified from the seeds by the method described previously (32).

Cell Lines - Seventeen different human lymphoblastoid cell lines available at the Dana-Farber Cancer Institute, and one monkey lymphoblastoid cell line obtained from Dr. N. L. Letvin, N. E. Regional Primate Center, Southboro, MA, were used in the course of this work. Representative experiments described in the text employed the nine cell lines described below. CALLA-positive human cell lines included Nalm-6 (37), BJAB (35), Namalwa (ATCC CRL 1432), and Raji (35). Nalm-6, Namalwa and Raji were also used as positive cell lines in tests with antibody I-2, and Nalm-6 was used as a positive line with the antibody J30. BJAB, Raji and Ramos (35) were used as positive cell lines in tests with anti-B1. The anti-T11 antibodies, which were produced against the T11 antigen found on human T cells, also react with the homologous molecule on T cells from other primates (34): the experiments with the anti-T11 antibodies described in this work utilized the cell line 1022 derived from lymphocytes from a cotton-topped marmoset (36). The human cell lines HOLT-4 (ATCC CRL 1582), HSB-2 (38) and HL-60 (39) were used as negative cell lines in experiments with various antibodies. Cells were grown at 37° C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY) supplemented with heat-inactivated (56° C for 30 min) fetal calf serum (10%) (Flow Laboratories, McLean, VA), L-glutamine (2 mM), pyruvate (1 mM), penicillin (50 units/ml) and streptomycin (50 μg/ml). Cells were maintained in asynchronous exponential growth by dilution twice per week to about 1-2 x 10^5 cells/ml.

METHODS

Experiments with the antibody J5 and with gelonin are described in detail. Unless otherwise described in the text, the experimental procedures for the other antibodies, and for the pokeweed antiviral proteins, were done in the same way.

Modification of J5 with SMCC - J5 (1 mg/ml) in 100 mM NaP$_i$ buffer, pH 7.0, containing EDTA (0.5 mM) was mixed with SMCC added from a 10 mM solution in dioxane, and then incubated at 30° C for 30 min. Reactions were terminated by gel filtration at 4° C through columns of Sephadex G-25 equilibrated with the pH 7.0 buffer, or by dialysis at 4° C against the pH 7.0 buffer. The level of incorporation of maleimido groups was measured by incubating samples of modified J5 with excess [^{14}C]-cysteine at 25° C for 60 min, after which 1 vol of 20% (v/v) 2-mercaptoethanol containing cysteine (1 mg/ml) was added and incubation at 25° C continued for 30 min. Bovine serum albumin (100 μg) was added as a carrier, and the protein was precipitated by the addition of 0.25 vol of 50% (w/v) trichloroacetic acid. After incubation at 0° C for 30 min, the protein precipitate was collected onto glass fiber discs (Whatman, GF/C) and the radioactivity retained on the discs was measured by scintillation counting using a Packard Tri-Carb Model 4530 counter (efficiency for ^{14}C was 75%). There was a linear relationship between the incorporation of maleimido groups and the initial concentration of reagent (1 group introduced per molecule of antibody at about 40 μM reagent) under these conditions. The maleimido groups introduced into J5 were stable for at least 24 h at 4° C in the pH 7.0 buffer (47).

Modification of J5 with SPDP - Portions of a stock solution (10 mM) of SPDP in ethanol were added to J5 (1 mg/ml) in 100 mM NaP$_i$ buffer, pH 7.0, containing EDTA (0.5 mM), and the mixture was incubated at 30° C for 30 min. Reactions were terminated by gel filtration or by dialysis as described above for SMCC modifications. The level of incorporation of dithiopyridyl groups was linear with respect to the initial concentration of reagent (about 1 group introduced per molecule of antibody at 20 μM reagent under these conditions) and was measured as described previously (40). Dithiopyridyl groups were stable for many days at pH 7.0 (40).

0.1 M acetic acid containing NaCl (0.15 M) and 0.1 vol of 1.0 M KP$_i$ buffer, pH 7.5, was added to each fraction immediately after collection. The protein was dialyzed against 5 NaP$_i$ buffer, pH 6.5, containing NaCl (35 mM) and NaN$_3$ (0.4 mM) and was then applied to a column of Cm-cellulose (Whatman, CM-52; 30 ml column for 100 mg antibody) which had been equilibrated with the same buffer. Non-conjugated J5 does not bind to Cm-cellulose under these precise conditions of ionic strength and pH, and was removed from the column by washing with buffer. The gelonin-containing conjugate was bound by the column and was eluted in a small volume with 100 mM NaP$_i$ buffer, pH 6.5, containing NaCl (1.0 M). The conjugated protein, now free of non-conjugated J5 and gelonin, was submitted to gel filtration on a column of Sephacryl S-300 (99 cm x 2.6 cm) equilibrated with 10 mM KP$_i$ buffer, pH 7.0, containing NaCl (145 mM) in order to remove aggregates of high molecular weight. The J5-gelonin conjugate was finally sterilized by passage of the solution throu a 0.22 μm filtration membrane (Millex-GV, Millipore Corporation, Bedford, MA).

Purification of Conjugates with Other Antibodies, and Conjugates with Pokeweed Antiviral Proteins - The procedure for the conjugation of gelonin with anti-B1, and the purificatio of the conjugate, were exactly as described for J5. The methods for making and purifying conjugates of gelonin with the antibodies I-2, J30, anti-T11$_{1B}$ and anti-T11$_{1C}$ differed from the example of J5 only in the solutions used for the separation of non-conjugated antibody from conjugate using Cm-cellulose. All four buffers contained NaP$_i$ (5 mM) and NaN$_3$ (0.4 mM) and were adjusted to pH 6.5, except for the buffer for J30 which was pH 7.0. The concentration of NaCl was 40 mM in the solutions for I-2 and J30, 34 mM in t solution for anti-T11$_{1C}$, and 25 mM in the solution for anti-T11$_{1B}$. Anti-T11$_{1A}$ does not bind to protein A. Following conjugation of this antibody to gelonin as described fo J5, the reaction mixture was concentrated by ultrafiltration, and the excess non-conjugat gelonin and aggregates of high molecular weight were separated by gel filtration on a col (99 cm x 2.5 cm, for a 12 ml sample containing 100 mg of antibody) of Sephacryl S-300 equilibrated with 10 mM KP$_i$ buffer, pH 7.0, containing NaCl (145 mM). Non-conjugated anti-T11$_{1A}$ was separated from the conjugate by Cm-cellulose fractionation as described fo J5 except that the buffer was 5 mM NaP$_i$ buffer, pH 6.5, containing NaCl (21.5 mM) and NaN$_3$ (0.4 mM). The purified conjugate was eluted from the column as described above and dialyzed into 10 mM KP$_i$ buffer, pH 7.0, containing NaCl (145 mM). PAP, PAP II and PAP-S were all conjugated with J5 and with anti-B1. PAP-S was also conjugated with anti-T11$_{1A}$ and anti-T11$_{1B}$. All these conjugates were prepared and purified using methods identical to those for the corresponding gelonin conjugates.

Measurement of Protein Concentration - Protein concentrations of solutions of purified proteins were determined from their A$_{280}$, assuming $E^{1\%}_{1cm}$ values of 14.0 for IgG, 6.7 for gelonin (20), and 8.3, 8.9 and 8.9 for PAP, PAP II (21), and PAP-S (43), respectively. T protein concentrations of purified conjugates were also estimated from A$_{280}$ values: for example, antibody-gelonin conjugates of molar ratio 1:1 or 1:2 were calculated to have $E^{1\%}$ values of 12.8 and 12.0, respectively. The actual ratio may be estimated from polyacrylamide/dodecyl sulfate gels.

Polyacrylamide gel electrophoresis - Cross-linking reactions and conjugate purification w analyzed by polyacrylamide/dodecyl sulfate gel electrophoresis in gel slabs (14.5 cm x 90 x 0.75 cm) cast with acrylamide gradients (5-10% (w/v) for gels run under non-reducing conditions) prepared as described previously (44). Sample buffers for gels run under non-reducing conditions contained 10 mg/ml iodoacetamide (41). Gels for samples under reducing conditions were cast with 12.5% (w/v) acrylamide, or with 10-20% (w/v) acrylamid gradients. Some gels were dried onto dialysis membranes for scanning with a Quick-Scan g scanner from Helena Laboratories. Isoelectrofocussing gels were run on a multiphor electrophoresis apparatus from LKB using PAG plates (LKB) containing ampholines of a pH range from 3.5 to 9.5.

Antigen-Binding Activity of Antibodies or Conjugates - The binding activity of antibodie conjugates was measured by indirect immunofluorescence (45). Cells (1 x 10^6) were incubated at 0° C for 30 min with serial dilutions of antibody or conjugate in 100 μl of Eagle's Minimum Essential Medium for suspension cultures (Gibco Laboratories) supplement with 2.5% (v/v) pooled human serum of AB-type and 1% (v/v) 1 M HEPES buffer, pH 7.2, containing NaCl (0.9% w/v). The cells were then washed three times with ice-cold medium before they were stained with fluorescein-labelled goat anti-mouse IgG antibody for 30 m at 0° C, using 100 μl of a 1:25 dilution of the stock solution (Meloy Laboratories) with medium. The cells were again washed three times with ice-cold medium. The fluorescent antibody-coated cells were finally analyzed on an EPICS IV fluorescence-activated cell sorter (Coulter Electronics, Hialeah, FL).

The binding activity of conjugates that contained gelonin was also determined by incubating cells, which had been treated with conjugate, with a rabbit anti-gelonin antiserum before staining with fluorescein-labelled goat anti-rabbit Ig antibody, using procedures similar to those described above. The rabbit anti-gelonin antiserum was kind prepared by Dr. Jerome Ritz. New Zealand white rabbits were injected (s.c. and i.m.) wi gelonin in complete Freund's adjuvant 3 times per week for 2 weeks using 1 mg of gelonin each time. The rabbits were bled following 1 or 2 booster injections of gelonin (1 mg) incomplete adjuvant.

Assay of Protein Synthesis in a Cell-Free System - The inhibitory activity of gelonin or pokeweed antiviral protein, towards protein synthesis was measured in a rabbit reticuloc lysate system. The assay was based on that of Pelham and Jackson (46), using materials provided by New England Nuclear supplemented with additional reagents. One microliter samples of gelonin or conjugates, diluted to 0.02 μg/ml of gelonin with 10 mM KP$_i$ buffer, pH 7.4, containing NaCl (20 mM) and bovine serum albumin (0.2 mg/ml), were added to the reticulocyte lysate (10 μl) in 0.5 ml Eppendorf centrifuge tubes at 0° C. The reactions were started by the addition of 16 μl of a mixture containing salts and buffer cocktail (England Nuclear), a mixture of 19 amino acids as described previously (46), creatine phosphate (0.15 μmol), creatine phosphokinase (2.5 μg), mRNA (80 ng), and [^{3}H]-leucine (μCi) diluted to a specific radioactivity of 57 mCi/μmol. After rapid mixing, the tubes w incubated at 30° C. Samples (3 μl) were taken at different times and the incorporation [^{3}H]-leucine into protein was quenched by dilution into distilled water (0.4 ml). Radiolabelled protein was quantified as described previously (46).

Cytotoxicity Assay - Cells (0.1 ml of medium, containing 5 x 10^4 cells) were plated into 96-well (flat bottom) polystyrene microtiter plates (Microtest III, Becton Dickinson). Equal volumes (0.1 ml) of medium, containing serial dilutions of the proteins being teste for cytotoxicity, were added to each well and the cells were then incubated at 37° C in a humidified atmosphere containing 5% CO_2. After the required incubation time, the cells were pulsed for 2 h with [^{3}H]-thymidine (0.8 μCi/well), and then harvested and lysed onto glass fiber discs using a PHD cell harvester (Cambridge Technology, Inc., Cambridge, MA). The radioactivity that was retained on the filters after washing with water and ethanol w measured in 2 ml of Betafluor using a Packard Tri-Carb 4530 scintillation counter. All assays were done in triplicate and each experiment was repeated at least 3 times. Values ID$_{50}$ were estimated as the concentration of immunotoxin that caused 50% inhibition of [^{3}H]-thymidine incorporation.

gel filtration.

We then experimented with ion-exchange chromatography using Cm-cellulose. The results of initial experiments led us to define the composition of a buffer that allowed all the non-conjugated J5 to pass through the column while all the gelonin-containing complexes were retained, consistent with gelonin being a more basic protein than J5. As described in the Methods section, the conjugate species could then be eluted by solutions of high salt concentration. Purified J5-gelonin species with exactly one, or exactly two, molecules of gelonin per J5, could be prepared by a second fractionation on Cm-cellulose, eluting the conjugates with shallow gradients of NaCl. However, for most biological tests described in this paper, this further fractionation was found unnecessary.

Figure 2 (panels a and b) illustrates from polyacrylamide gels the degree of purity achieved after each step in the purification procedure for a J5-gelonin conjugate containing a disulfide link (panel a) and an analogous immunotoxin with a thioether link (panel b). In both cases, the three-step purification yielded J5-gelonin conjugates that were free of non-conjugated J5, of non-conjugated gelonin, and of aggregates of high molecular weight (Lanes 4 and 8, Fig. 2 (a,b)). When the purified conjugates were analyzed on gels under reducing conditions (Figure 2, panel c), the conjugate that contained a disulfide link showed only the expected 3 bands (lane 10) corresponding to gelonin and to the heavy chain (M_r 53,000) and the light chain (M_r 27,000) of the antibody. The conjugate that contained the thioether link showed no free gelonin on the gels as expected (lane 9), but instead showed several bands of higher molecular weight, the strongest of which (M_r 84,000) likely corresponds to gelonin cross-linked to a heavy chain of J5.

The purification scheme for J5-gelonin conjugates was used successfully on a small scale (1 mg of J5) and a large scale (100 mg of J5). To maximize the yield of conjugate with respect to antibody while keeping the formation of aggregates of very high molecular weight to a minimum, J5 was modified to introduce about 2.5 functional groups per molecule, and this was mixed with a 5-fold molar excess of modified gelonin that contained only 0.6-0.7 sulfhydryl groups per molecule. The yield of conjugate with respect to antibody after the three steps of purification was 40-50%.

Preparation and Purification of Conjugates Between Gelonin and Other Antibodies - Conjugates between gelonin and IgG2 antibodies (J30, I-2, anti-B1, anti-Tll_{1B}, anti-Tll_{1C}) were prepared using the methods detailed for J5. The only variation in the procedures was in the exact ionic strength and pH of the buffer used for the Cm-cellulose step (see Methods) that provides the complete separation of non-conjugated antibody from the conjugates as judged by polyacrylamide/dodecyl sulfate gel electrophoresis. The conjugates contained antibody linked to one or two molecules of gelonin, and the mixtures did not contain non-conjugated antibody or free gelonin.

Anti-Tll_{1A} is an antibody of isotype IgG1 and does not bind to protein A. Consequently, the first step in our standard procedure described above was replaced by a gel filtration step, which is capable of separating the conjugate from non-conjugated gelonin as described in Methods. Gel filtration was used as the first step of purification only when necessary, since the protein A-Sepharose CL-4B affinity step was quick to perform and was better suited to the processing of large amounts of protein in large volumes. Further, if there is any aggregation of non-antibody proteins, which may occur under some conditions, these are more effectively removed from antibody-conjugates by the affinity chromatography step.

Preparation and Purification of Conjugates Between Antibodies and Pokeweed Antiviral Proteins - PAP, PAP II and PAP-S were each conjugated to J5 using SPDP to modify the J5, thus forming conjugates that contained a disulfide bond. We found that exactly the same conditions could be used for the preparation and purification of each of the conjugates with the three different pokeweed antiviral proteins as described for the corresponding gelonin conjugate. PAP-S has also been conjugated to anti-B1, anti-Tll_{1A} and anti-Tll_{1B} using the disulfide-linker, and has been conjugated to J5 using the linker containing a thioether bond. Again, their preparation and purification followed exactly that of the corresponding gelonin conjugate.

Ribosome-Inactivating Activity of the Conjugates - Figure 3 (a) shows that 20 pg of gelonin completely inhibited protein synthesis in the rabbit reticulocyte lysate system. Assays of gelonin and of gelonin modified with 2-iminothiolane (1.4 sulfhydryl groups/mol) after prior reduction with dithioerythritol (20 mM, 30° C for 30 min), showed exactly the same inhibition (Fig. 3 (a)). Up to 4 sulfhydryl groups per molecule could be introduced into gelonin by reaction with 2-iminothiolane, without impairing its capacity to inhibit protein synthesis (data not shown). When gelonin was linked to J5 using the non-cleavable linker, the rate of inhibition of protein synthesis was slower than that of native gelonin and, as expected, this could not be affected by prior reduction with dithioerythritol (Fig. 3 (a)). Assays done with further dilutions of native gelonin suggested that the activity of the gelonin linked to J5 in this way was about 20-30% of that of native gelonin. The gelonin within a J5-gelonin conjugate formed with the disulfide-linker was also less active than native gelonin when assayed without prior reduction (Fig. 3(b)). However, pre-incubation of the conjugate with dithioerythritol released fully active gelonin that was indistinguishable from native gelonin in its ability to inhibit protein synthesis in these assays (Fig. 3 (b)). Figure 3 (c) shows that identical results were obtained with the anti-B1-gelonin conjugates formed with the disulfide-linker. These experiments with J5 and anti-B1 are representative of the results obtained with the other antibodies used in this work. Analogous results were obtained with all the conjugates using the pokeweed antiviral proteins: that is, the inhibitory activity of these toxins when conjugated to an antibody was reduced to about 20-30% of that of the native proteins, and reduction of the conjugates containing a disulfide linker resulted in release of proteins that were fully functional (for example, Figure 3 (d)).

make it safe to say that the conjugation of gelonin or the pokeweed proteins to any of the monoclonal antibodies did not block the specific binding of the antibody.

Indirect immunofluorescence was also used to detect the conjugated gelonin bound to the cell surface *via* the antibody component of the conjugate. The technique utilized a rabbit anti-gelonin antiserum and subsequent visualization with fluorescently-labelled goat anti-rabbit Ig. This method yielded binding curves identical to those shown in Figure 4. In addition, this technique allowed us to confirm that native gelonin does not bind to cells. Further, it enabled us to examine the stability of the disulfide linker in a J5-gelonin conjugate when incubated in the growth medium for cells at 37° C. Duplicate samples were withdrawn at intervals and used in cell-surface binding assays, staining either for mouse IgG or for gelonin. The ratio of the fluorescence values (staining for gelonin/staining for mouse IgG) after 100 h of incubation of the conjugate (0.1 μM) was 93% of that determined prior to incubation. Thus, the rate of cleavage of the disulfide-linker in the growth medium was slow, which suggests that dissociation of the gelonin from the antibody is unlikely to be a significant problem in cytotoxicity experiments *in vitro* of 48-72 h duration.

Cytotoxicity of the Immunotoxin Conjugates - The cytotoxicity of the conjugates was tested *in vitro* with several different cell lines by measuring the relative level of DNA replication by the incorporation of [^{3}H]-thymidine as an index of cytotoxicity. Figure 5 shows representative examples of these experiments. The conjugate between J5 and gelonin containing a disulfide linker, was a potent inhibitor of the growth of Nalm-6 cells which bear the CALLA (Fig. 5 (a)). The ID_{50} of the conjugate, 30 pM, was about 10^4 times lower than the ID_{50} of 0.4 μM for native gelonin. Cells which lack the CALLA (HSB-2; MOLT-4) were unaffected by the J5-gelonin conjugate (data not shown). The conjugate between J5 and gelonin with the non-cleavable thioether-linker caused less toxicity than that of the disulfide-linked conjugate (Fig. 5 (a)). The ID_{50} of the non-cleavable conjugate, 0.06 μM, was only about 10-fold lower than that of native gelonin. These results with J5-gelonin conjugates were supported by experiments with J5-PAP-S conjugates. Cytotoxicity experiments in which Namalwa cells were exposed to J5-PAP-S conjugates for 3 days gave ID_{50} values of 84 pM, 0.1 μM and 4.5 μM for the conjugate containing a disulfide bond, the conjugate containing a thioether bond, and for native PAP-S, respectively. These experiments showed that the use of a linker containing a disulfide bond resulted in greatly enhanced toxicity (about 10^3-fold) over equivalent immunotoxins containing a non-cleavable bond. Further experiments described below refer only to conjugates made with the disulfide linker.

Figure 5 (b) shows a cytotoxicity experiment with a gelonin-conjugate made from the antibody I-2, in which Nalm-6 cells were exposed to the conjugate for 1, 2 or 3 days before they were pulsed for 2 h with [^{3}H]-thymidine. Two or three days exposure led to high toxicity, showing an ID_{50} of 10 pM after 3 days, which is in the same range as the toxicity exhibited by the J5-gelonin conjugate. However, after 24 h exposure to the I-2-gelonin conjugate, inhibition of [^{3}H]-thymidine incorporation did not exceed 50% at concentrations of immunotoxin as high as 0.1 μM. This experiment illustrates an important point common to all the purified immunotoxins with a disulfide linker described in this work: they are highly toxic towards target cells *in vitro*, but the development of the toxic effect requires 2 or 3 days of exposure. This phenomenon is in marked contrast to the very rapid onset of toxicity upon exposure of Nalm-6 cells to ricin. A few hours of exposure to this toxin results in complete inhibition of [^{3}H]-thymidine incorporation (Fig. 5 (c)).

Figure 5 (d) shows the result of a cytotoxicity test with BJAB cells using conjugates between gelonin and the antibodies J5 and anti-B1. Even though BJAB cells express the B1 antigen, the anti-B1-gelonin conjugate was not toxic at 0.1 μM after 3 days of exposure. Under identical conditions, J5-gelonin was highly cytotoxic towards these cells (which are CALLA-positive) with an ID_{50} of 80 pM (Figure 5 (d)), which shows that this cell line is not inherently resistant to an immunotoxin containing gelonin. This experiment is one representative example of tests performed using five different cell lines that expressed the B1 antigen; the only toxicity exhibited by anti-B1-gelonin conjugates was the slight (if any) toxicity of gelonin alone.

Table 1 summarizes the ID_{50} values of all seven different antibody-gelonin conjugates with disulfide-linkers, on a group of seven cell lines. It shows that the anti-B1-gelonin conjugate is an exception, in that no additional toxicity towards cell lines that expressed the B1 antigen was conferred by conjugation of the gelonin to anti-B1. The purified gelonin-conjugates with the other six monoclonal antibodies all showed high toxicity towards cell lines that expressed the relevant antigens, with ID_{50} values in the range of 8 nM to 1 pM. When immunotoxins were tested for cytotoxicity on cells that lack the relevant antigen, the conjugates showed only the slight toxicity (if any) of free gelonin. The seven non-conjugated antibodies were non-toxic in the concentration range tested (up to 1 μM), and none of them influenced the toxicity of non-conjugated gelonin.

Cytotoxicity experiments performed with the conjugates between the pokeweed antiviral proteins and the antibodies J5, anti-B1, anti-Tll_{1A} and anti-Tll_{1B} were consistent with the results obtained with the conjugates containing gelonin (Table 1). Suffice it to say that all the conjugates containing the disulfide linker, except for conjugates with anti-B1, showed high specific cytotoxicity towards cells that expressed the relevant antigen after exposure of the cells for two or more days to the immunotoxin. ID_{50} values ranged from 3 nM to 0.1 pM, which were 10^4 to 10^6-fold lower than the ID_{50} concentration of the non-conjugated pokeweed antiviral proteins. Like the corresponding gelonin-conjugate, a conjugate between PAP-S and anti-B1 was no more toxic than non-conjugated PAP-S towards all the cell lines tested, irrespective of whether the cell line expressed the B1 antigen.

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