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IMMUNOTOXINS OF PSEUDOMONAS EXOTOXIN A (PE): EFFECT OF LINKAGE ON CONJUGATE YIELD, POTENCY, SELECTIVITY AND TOXICITY

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Abstract—Conjugates of monoclonal antibodies and Pseudomonas exotoxin A (PE) were formed with disulfide or thioether bonds. Thioether conjugates which formed with succinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) modified PE and reduced antibody formed with an 80% yield of equimolar conjugate within 30 min with an offering of one to one (toxin:antibody). The efficiency and kinetics of thioether formation were much higher with SMCC than with other maleimide reagents as well as more efficient than disulfide linkers. Thioether linkage resulted in immunotoxin consistently more potent and more selective *in vitro* than disulfide bonded conjugate. Thioether bonded conjugates also proved to have other favorable *in vivo* properties compared to disulfide conjugates: (1) a longer half-life in serum; (2) increased tumor localization; and (3) reduced toxicity.

Toxicity of thioether linked holotoxin conjugates was directed at the liver hepatocyte but was easily monitored by serum liver enzymes. The conjugates are currently undergoing clinical evaluation for treatment of ovarian cancer with intraperitoneal administration. Research is ongoing to further decrease residual toxicity without reducing the potency of the conjugate.

INTRODUCTION

Conjugates of monoclonal antibodies with toxins offer considerable potential for localizing therapy to tumor sites while sparing normal tissues of toxicity typically encountered with unconjugated cytotoxic agents. Molecules too toxic for use as free drugs can be delivered selectively to tumor cells with reduced toxicity as conjugates.

Several strategies to create highly potent and selective immunotoxins have been explored. The most common approach is to link the protein inhibitory portion of plant toxins, namely A-chains or hemitoxins, or naturally occurring A-chain-like molecules called ribosomal inactivating proteins (RIPs), to monoclonal antibodies via disulfide linkages (Thorpe *et al.*, 1981, 1982, 1985; Hwang *et al.*, 1983; Ramakrishnan and Houston, 1984). Although these conjugates contain the enzymatic (toxic) portion of the protein, they have less potency than the holotoxin. This appears to be due to the lack of the B-chain which facilitates insertion and translocation of the A-chain across membranes (McIntosh *et al.*, 1983; Youle and Neville, 1982; Vittetta *et al.*, 1984).

An alternative strategy is to employ intact toxins (holotoxins) such as ricin or abrin or bacterial holotoxins like Pseudomonas exotoxin (PE) conjugated to monoclonal antibody (Thorpe *et al.*, 1984; Fitzgerald *et al.*, 1984). This results in agents of increased potency compared to the A-chain conjugates. This has been demonstrated *in vitro* by comparing conjugates formed with the holotoxin PE or recombinant ricin A-chain and antibodies against both breast and ovarian cancer cell lines (Bjorn *et al.*, 1985; Pirker *et al.*, 1985). Similarly, comparisons have been made with whole ricin and ricin A-chain conjugated to the same antibody (Vallera *et al.*, 1984).

The clinical application of holotoxin conjugates is, however, compromised by toxicity, since the conjugated holotoxin still retains some cell binding properties. Several avenues have been explored to reduce toxicity while retaining potency. Ricin B-chain binding can be inhibited by incubation with lactose or galactose or by covalent incorporation of these saccharides into the B-chain lectin site (Quinones *et al.*, 1984; Houston, 1983). Alternatively, holotoxins, with conjugation to antibody, can have reduced toxicity due to steric occlusion of cell binding (Thorpe *et al.*,

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[2]Abbreviations used: RIP, ribosomal inactivating protein; PE, Pseudomonas exotoxin A; FPLC, fast protein liquid chromatography; SMCC, succinimidyl 4-(maleimidomethyl)cyclohexane-1-carboxylate; TAC, IL-2 receptor; PBS/BSA, phosphate buffered saline with 1% bovine serum albumin; DTT, dithiothreitol; PIP, paraiodophenyl; $T_{1/2}$, serum half-life; ADP, adenosine diphosphate; LD_{100}, lethal dose 100; LDH, lactate dehydrogenase; SGOT, serum glutamic oxalate transaminase; SGPT, serum glutamic pyruvic transaminase; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); MBS, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; SMPB, succinimidyl 4-(*p*-maleimidophenyl)butyrate.

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1984; Godal *et al.*, 1986). In our hands, even the last approach produces conjugates with some antibodies which still have a high level of non-specific toxicity (Godal *et al.*, 1986; Morgan *et al.*, 1985). Non-conjugated PE is less toxic to cells than abrin, ricin or diphtheria toxin. In addition, conjugation of PE further decreases its toxicity. The reduction in toxicity, together with high *in vitro* potency (ID_{50} 10^{-10} to 10^{-12} *M*) and selectivity (3–4 logs) when linked to antibody, indicates the potential of this agent for clinical use. However, when conjugated via a disulfide linkage, PE conjugates still exhibit lethal toxicity at relatively low doses (300 μg/kg) when injected into primates (this publication).

In this study, we examined holotoxin conjugates constructed with thioether linkages rather than conventional disulfide bonds. Contrary to prior reports, demonstrating reduced potency of toxin conjugates with stable linkages (Edwards *et al.*, 1983), thioether linked PE conjugates were equally potent on antigen positive cells and more selective than disulfide bonded conjugates. In addition, higher doses of the thioether conjugates could be safely administered to primates. Thus, potency was preserved and toxicity reduced simultaneously. The particular method of constructing the thioether immunoconjugate was also shown to provide a markedly improved yield, thereby improving the eventual efficiency and cost effectiveness of therapy with these agents.

MATERIALS AND METHODS

Monoclonal antibodies and fragmentation

Two murine IgG2a antibodies were used in these studies: anti-TAC (antibody recognizing IL-2 receptor, kindly provided by Dr Tom Waldmann, National Cancer Institute, Bethesda, Maryland) (Uchiyama *et al.*, 1981a; Uchiyama *et al.*, 1981b); and 9.2.27, antibody recognizing a human melanoma-associated glycoprotein/proteoglycan (Morgan *et al.*, 1981). Three murine IgG2b antibodies were also used: NR-LU-10, directed to a pan-carcinoma antigen (Okabe *et al.*, 1984), OVB-3 (Willingham *et al.*, 1987) and NR-ML-05, directed to a different epitope than 9.2.27 on the human melanoma associated glycoprotein/proteoglycan (Woodhouse *et al.*, 1990).

F(ab)$'_2$ fragments of 9.2.27 were prepared from whole antibody by digestion with immobilized pepsin. The digest was fractionated by ion exchange chromatography to remove peptides and undigested antibody and then concentrated by ultrafiltration. F(ab)$'_2$ fragments were homogeneous as assessed by both SDS–PAGE and FPLC gel filtration.

Conjugation and fractionation

Disulfide bonded conjugates were produced according to previous published methodology (Pirker *et al.*, 1985) with some modification. Briefly, whole antibody and PE were reacted with 2-iminothiolane (2-IT) (molar ratio 5:1) at pH 9.5 in sodium bicarbonate buffer (0.1 *M*, 0.15 *M* NaCl). Unreacted reagent was removed by gel filtration and derivatized antibody then reacted with DTNB and excess reagent removed. DTNB activated, derivatized antibody was then mixed with 2-IT treated PE at room temperature for up to 4 hr. The conjugate mixture was then fractionated by FPLC gel filtration on a TSK 3000 column in PBS pH 7.2 to remove unreacted PE. Conjugate corresponding in size to a 1:1 molar ratio of PE to antibody was pooled for subsequent testing.

Thioether linked conjugates were prepared by first reacting PE with succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), succinimidyl 4-(*p*-maleimidophenyl)butyrate (SMPB), or *m*-maleimidobenzoyl-*N*-hydroxysuccinimide (MBS) at a molar ratio of 10:1 in pH 9.0 sodium bicarbonate buffer and unreacted reagent removed by gel filtration. Whole antibody was reacted with 25 m*M* dithiothreitol in 0.1 *M* phosphate buffered saline (PBS) pH 7.5 and excess reducing agent removed by gel filtration. The two conjugate components were then admixed and incubated at room temperature for up to two hours. Disulfide and thioether (SMCC) linked conjugates were also prepared with fragmented antibody. For this, the above procedure was followed except that F(ab)$'_2$ was substituted for whole antibody. Under the same reducing conditions as above, F(ab)$'_2$ was reduced to F(ab)′ before conjugation.

At least 2 batches of each type conjugate were produced with the batch size varying between 1 and 5 mg. In the case of OVB-3 conjugates the batch size was 800 mg as this was produced for a clinical trial (Morgan *et al.*, submitted for publication).

Crude conjugate mixtures were first separated by FPLC gel filtration. Fractions corresponding to a 1:1 molar ratio of conjugate and unconjugated antibody were pooled. Unconjugated antibody was removed by anion exchange chromatography on a Mono Q column (Pharmacia Fine Chemicals, Piscataway, NJ). Conjugate was eluted with a gradient of sodium phosphate starting with 5 m*M* sodium phosphate pH 7.6 and ending with 0.5 *M* sodium phosphate buffer pH 7.6 in 30 min (flow rate 0.5 ml/min).

Biochemical analysis of conjugates

Conjugate species separated by FPLC gel sieving or anion exchange chromatography were analyzed on SDS slab gels (10%), both reduced and non-reduced. Conjugates were also analyzed by analytical isolectric focusing using a pH 3–10 gradient (Pharmacia, Piscataway, NJ). Unmodified 9.2.27 focused in multiple bands with isoelectric points of 7.4, 7.3, 7.1, 6.9, 6.8 and 6.7 with an average pI of 7.2. Conjugate focused at an average pI of 7.46. Anti-TAC focused in a series of more basic isoelectric points, but following conjugation to PE focused at a pI similar to that of 9.2.27 conjugate. Both NR-LU-10 and NR-ML-05 had slightly acidic pI (6.5–7.05) and showed little change after conjugation to derivatized PE.

Tumor cell lines and cytotoxicity testing

In vitro cytotoxicity testing was performed as previously described using ^{3}H-leucine incorporation to measure surviving cells (Pavanasasivam *et al.*, 1987; Morgan *et al.*, 1987). 9.2.27 and NR-ML-05 (anti-melanoma) conjugates were tested on the human melanoma cell lines, A375 met mix (A375 M/M) which was antigen positive, A375 primary which was antigen negative and the HT-29 colon carcinoma cell line which was also antigen negative. For anti-TAC (anti-IL-2 receptor) conjugates, HUT 102 cells were the antigen positive, and CEM the antigen negative targets, respectively (Fitzgerald *et al.*, 1984). For NR-LU-10 (anti-carcinoma) conjugates, HT-29 was the relevant target and A375 M/M the irrelevant target. All cell lines were pretested for inherent sensitivity to unconjugated PE and showed similar sensitivity (ID_{50} = 100 ng/ml). ID_{50} values for conjugates on cells. The ID_{50} determinations represent the average of at least three separate determinations.

Conjugates were tested in two formats, short exposure and long exposure. For short exposure, conjugate was incubated with cells for 1 hr at 37°C, the cells gently washed and cultures continued for up to 72 hr before addition of ^{3}H-leucine. For long exposure, cells were exposed to conjugate for the entire 72 hr of the culture period.

Immunoreactivity

PE conjugates of 9.2.27 and NR-ML-05 were compared to unconjugated antibody for binding to target A375 M/M melanoma using flow cytometry as previously described (Pavanasasivam *et al.*, 1987). Similarly, conjugates of TAC were compared to unconjugated TAC antibody on HUT 102 cells and NR-LU-10 conjugate to unconjugated NR-LU-10 on HT-29 cells. OVB-3 antibody and conjugates were analyzed on ALAB breast carcinoma cells. Briefly, target cells were suspended in PBS/BSA at 1×10^5 cells/ml and incubated at 4°C for 30 min with titrated doses of antibody or conjugate including saturating or subsaturating doses (1 to 0.01 μg/ml). Cells were then washed twice with PBS/BSA, and incubated with 100 ml (1 μg/ml) of fluorescein labeled goat anti-mouse IgG for 30 min. Washed cells were then resuspended in PBS/BSA and analyzed for bound fluoresceinated secondary antibody using a cytofluorograph (Coulter Diagnostics, Hialeah, Florida). Immunoreactivity was assessed by comparison of the mean fluorescence index over 1000 channels for positive cells and then compared to a standard curve generated with fluoresceinated beads (Ortho Diagnostics) and expressed as fluorescein equivalents (FE). The percent of FE displayed by the conjugate was then expressed as a percent of the unmodified antibody at a subsaturation level. This type of assessment measures both changes in the percent of immunoreactive antibody molecules as well as alterations in affinity.

ADP ribosylation

PE as well as disulfide or thioether linked PE-conjugates were compared in a cell-free system as previously described (Vanness *et al.*, 1980). ADP-ribosylation measures the ability of toxins such as Pseudomonas exotoxin A and diphtheria toxin to transfer labeled ADP-ribose to an intracellular acceptor molecule which in the case of PE is elongation factor-2. Thus, the assay is a measure of enzymatic activity not dependent on cell binding or translocation of the toxin as is required for cell killing. Both disulfide and thioether conjugates were titrated in the assay after treatment with 8 *M* urea and 1 *M* DTT.

Biodistribution, serum half-life and toxicology

Thioether and disulfide linked conjugates of 9.2.27 were compared in a nude mouse xenograft model of human melanoma for tumor localization and biodistribution (Hwang *et al.*, 1985). PE was first radiolabeled with ^{125}I-para-iodophenyl (PIP) (Wilbur *et al.*, 1986). By this method, the radiolabel is not subject to dehalogenation and thereby more accurately reflects the biodistribution of conjugates. The labeled PE was then incorporated into conjugates and tested for retention of potency. Animals were administered 2–5 μg of labeled conjugate (2–5 μCi) intravenously and sacrificed at 20 hr post injection, organs blotted, weighed and counted as previously described (Wilbur *et al.*, 1986), and per cent dose per gram calculated for each tissue. In addition, serum half-life of radiolabeled conjugate was estimated in non-tumor bearing Balb/c mice by retro-orbital sampling of whole blood (Wilbur *et al.*, 1986). Groups of four mice were used for both biodistribution and serum half-life determinations.

Toxicity of disulfide and thioether conjugates was assessed both in mice and in cynomogolus monkeys. Both the 9.2.27 and anti-TAC PE conjugates were assessed in mice; only anti-TAC conjugates were assessed in monkeys. In both cases, comparisons were made to the corresponding disulfide conjugate. Groups of five mice were observed for death or survival as a final endpoint, whereas monkeys were monitored by liver enzyme levels and observation of other relevant symptoms including changes in behavior, appetite, nausea/vomiting and temperature. Lactate dehydrogenase (LDH) levels proved to be the most sensitive monitor of hepatic toxicity in monkeys. LDH levels of treated monkeys were compared to averages obtained from normal monkeys tested at Microbiological Associates (Rockville, MD) over a period of years.

RESULTS

Production and analysis of conjugates

Representative FPLC gel filtration profiles of corresponding batches of disulfide and thioether linked conjugates of anti-TAC and PE are shown in Fig. 1.

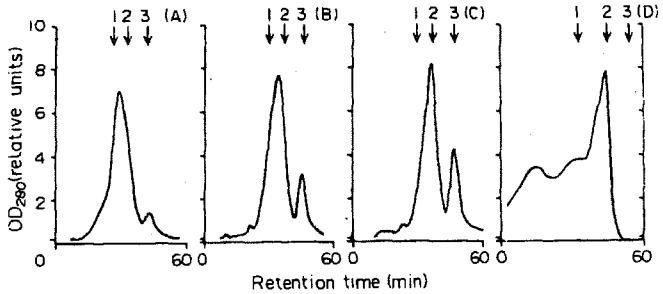

Fig. 1. Comparison of Pseudomonas exotoxin immunoconjugates by FPLC gel filtration. Disulfide and thioether linked conjugates were analyzed on a Superose 12 column at 1 ml/min (Pharmacia). (A) Thioether reaction (SMCC). (B) Thioether reaction (SMPB). (C) Thioether reaction (MBS). (D) Disulfide reaction. Thioether reactions were analyzed 15 min post initiation. Disulfide reaction was analyzed after 4 hr. 1, migration position of 1:1 molar ratio conjugate; 2, migration position of unconjugated antibody; 3, migration position of unconjugated PE.

Disulfide linkage employing iminothiolane (Panel D) produced conjugate species of varying sizes reflecting a range of molar ratios as reported previously. SDS–PAGE analysis of these fraction pools revealed size ranges corresponding to 2:1 and higher, and 1:1 molar ratios of PE:antibody. In contrast, thioether linkage (Panels A and B) primarily formed conjugate in a single size range which corresponded by SDS–PAGE analysis to a 1:1 molar ratio of PE to antibody (peak 1). Thioether conjugation efficiencies varied according to the reagent used for PE derivatization (Panels A through C). SMCC derivatized PE reacted with reduced antibody resulted in the best yields, e.g. 80% 1:1 conjugate with an offering ratio of 1:1 (PE to antibody). In comparison, disulfide conjugation typically gave a 30% yield of 1:1 conjugate, with an offering of 10:1 (PE to antibody). In addition, the best thioether conjugation required only a short incubation, with conjugation complete in 15 to 30 min whereas the disulfide linkage required greater than 4 hours for optimal yields. Interestingly, the elution position of 1:1 conjugate differed between thioether and disulfide linked conjugate. One to one conjugate with disulfide linkage was well discriminated from unconjugated antibody (compare peaks 1 and 2, Panel D) while 1:1 conjugate from thioether linkage migrated in the front edge of the antibody peak (compare peaks 1 and 2, Panel A). In fact, conjugation via thioether linkage was more easily monitored by disappearance of the unconjugated PE (peak 3) than formation of conjugate (compare Panel A, essentially complete conjugation, to Panel C, essentially no conjugate formation). Forming thioether linked conjugate with reduced antibody and SMCC-derivatized PE was the most efficient method for forming 1:1 conjugate. The combination of iminothiolane-derivatized antibody and SMCC-derivatized PE resulted in slower kinetics of formation as well as the formation of higher molecular weight conjugate (not shown).

Due to the difficulty in separating thioether linked conjugate from unconjugated antibody by gel filtration, anion exchange chromotography was employed to further purify conjugate containing fractions (Fig. 2). Conjugate but not antibody was bound to the Mono Q column under the conditions used and eluted with a hypertonic sodium phosphate buffer. Isolated 1:1 ratio thioether (SMCC) and disulfide conjugate as analyzed on SDS–PAGE under reducing conditions verified the nature of the bond (Fig. 3). As expected, reduction of disulfide conjugates gave three bands corresponding to PE and antibody heavy and light chains. Reduction of thioether conjugate gave bands corresponding to covalent adducts of PE with heavy and light chains with most of the adducts with the heavy chain as well as free heavy and light chain presumably from unconjugated antibody half molecules. Free PE was not apparent following reduction of thioether conjugate.

Immunoreactivity, potency and selectivity

Immunoreactivity of antibody was consistently reduced by conjugation with PE (Table 1). The loss of immunoreactivity was found regardless of which antibody and which linkage was used. This contrasted to our previous experience with conjugation of A-chains or ribosomal inactivating proteins which did not alter immunoreactivity (Pavanasasivam *et al.*, 1987).

Potency and selectivity of thioether and disulfide bonded conjugates were compared *in vitro*. Titration of ADP-ribosylating activity for disulfide and thioether conjugates of anti-TAC is shown in Fig. 4. In this cell-free system, PE requires unfolding by reducing and denaturing agents to expose the cofactor (NAO) binding site. Conjugates formed with the two types of linkages were equi-potent for enzymatic activity despite the demonstration by SDS–PAGE (above) that the thioether conjugate did not reduce to yield free PE.

We also evaluated thioether and disulfide conjugates of 9.2.27 in an *in vitro* cytotoxicity assay (Table 2). 9.2.27 (whole antibody) thioether conjugate was consistently more potent (and more selective) than its disulfide counterpart. Similar results were obtained in comparing thioether and disulfide linked TAC conju-

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