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Abstract

Immunotoxins composed of monoclonal antibodies (mAbs) and various toxins have been developed for the treatment of malignancies. We investigated the efficacy of three ricin toxin A-chain (RTA)-containing immunotoxins (ITs) conjugated from mAbs which recognize glycolipid asialo-GM2 and glycoprotein H-2d. These ITs retained the same immunoreactivity with mAbs. We evaluated the cytotoxicity of these ITs against mouse lymphoma cells L5178Y variants showing high (AA12,CC9) and low (27AV) expression of asialo-GM2. Anti-H-2d-RTA IT had the strongest cytotoxicity for all cell lines. Anti-asialo-GM2 (IgM)-RTA IT had stronger cytotoxicity than anti-asialo-GM2 (IgG3)-RTA IT. Anti-asialo-GM2-RTA ITs had different cytotoxicity against AA12 and CC9 cells. The establishment of appropriate anti-glycolipid mAbs may lead to effective immunotargeting therapy.

KEYWORDS: immunotoxin, glycolipid, glycoprotein

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Evaluation of Ricin A Chain-Containing Immunotoxins Directed Against Glycolipid and Glycoprotein on Mouse Lymphoma Cells

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Immunotoxins composed of monoclonal antibodies (mAbs) and various toxins have been developed for the treatment of malignancies. We investigated the efficacy of three ricin toxin A-chain (RTA)-containing immunotoxins (ITs) conjugated from mAbs which recognize glycolipid asialo-GM2 and glycoprotein H-2^d. These ITs retained the same immunoreactivity with mAbs. We evaluated the cytotoxicity of these ITs against mouse lymphoma cell L5178Y variants showing high (AA12,CC9) and low (27AV) expression of asialo-GM2. Anti-H-2^d-RTA IT had the strongest cytotoxicity for all cell lines. Anti-asialo-GM2 (IgM)-RTA IT had stronger cytotoxicity than anti-asialo-GM2 (IgG3)-RTA IT. Anti-asialo-GM2-RTA ITs had different cytotoxicity against AA12 and CC9 cells. The establishment of appropriate anti-glycolipid mAbs may lead to effective immunotargeting therapy.

Key words: immunotoxin, glycolipid, glycoprotein

For purposes of selective therapy, many researchers have attempted to conjugate monoclonal antibodies (mAbs) to toxins or drugs (1). The resulting immunotoxins showed significant cytotoxicity in *in vitro* experiments, and phase 1 clinical trials have been performed (2, 3). However, the effects of immunotoxins are variable, depending on the type of target cell and the nature of the antibody and antigen (4, 5).

There have been a few reports of immunotoxins against a glycolipid antigen. These reports revealed that immunotoxins against glycolipids exhibit sufficient cytotoxicity. The mouse lymphoma cell line L5178Y has variants differing in the quantity of ganglio-*N*-triosylceramide (asialo-GM2), and these variants are all H-2^d-positive (6). To evaluate the differential effects of immunotoxins directed against glycolipid and glycoprotein on the same cell, we used this cell line as target. We made immunotoxins from ricin toxin A (RTA)-chain and mAbs against asialo-GM2 and H-2^d, and evaluated their cytotoxicity.

Materials and Methods

Cell lines. Three clones of mouse lymphoma L5178Y were used (7). Clones AA12 and CC9 express high levels of asialo-GM2, while clone 27AV expresses low levels. All three clones express major histocompatibility complex (MHC) H-2^d. Cells were cultured in complete RPMI-1640 medium, supplemented with 15 % fetal bovine serum, at 37°C in 5 % CO_2.

Monoclonal antibodies. Three mAbs were used. Two were murine mAbs directed against asialo-GM2 (6, 8), subtype IgM and IgG. The third was a murine mAb directed against H-2^d, subtype IgG2a, donated by Dr. David Urdal (Immunex Inc., Seattle, WA, USA). IgG antibodies were purified from ascitic fluid by affinity chromatography on Sepharose-Staphylococcal protein A (Pharmacia Fine Chemicals, Piscataway, NJ, USA). IgM antibody was purified by precipitation with boric acid.

Ricin toxin A-chain. RTA was from EY Laboratories, Inc. (San Mateo, CA, USA). Purification was judged by SDS/PAGE. RTA retained the same ability as whole ricin to inhibit protein synthesis in a cell-free reticulocyte lysate assay. RTA was dialyzed against 0.01 M cysteine hydrochloride buffer (pH 5.0)

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before use.

Preparation of immunotoxin. MAbs and RTA were conjugated as follows (9, 10). Purified mAb (2.5-3 mg/ml) was dialyzed against Dulbecco's PBS (pH 7.3), and an 8-fold molar excess of SPDP (Pharmacia Fine Chemicals) in absolute ethanol was added with vigorous mixing. The solution was allowed to react for 30 min at room temperature, then dialyzed twice against PBS containing 2 mM EDTA. RTA (2 mg/ml) was reduced by the addition of 1 M dithiothreitol to a final concentration of 50 mM for 30 min at room temperature. After reduction, RTA was desalted on Sephadex G-25 equilibrated with PBS containing 1 mM EDTA. A 2.5- to 3-fold molar excess of reduced RTA was reacted with SPDP-substituted antibodies for 48 h at 4°C. The crude conjugate preparations were purified by chromatography on Sephacryl S-200 (1.5×50 cm). The quality of the conjugates was evaluated by SDS/PAGE, according to the method of Laemmli on an 8 % polyacrylamide gel (11).

Binding of antibodies and immunotoxins to cell lines. Immunoreactivity of antibodies and immunotoxins was examined by indirect immunofluorescence assay. Each cell line (1×10^5 cells) was incubated with saturating and limiting doses of primary antibody (mAbs and immunotoxins) for 45 min at 4°C. After washing three times with PBS, cells were incubated with secondary antibody (150 μl of 40-fold diluted fluorescein isothiocyanate-labelled rabbit anti-mouse antibody) for 60 min at 4°C. Cell staining was analyzed using a fluorescence-activated cell sorter (FACS) 440 (Becton-Dickinson FACS Systems, Sunnyvale, CA, USA).

In vitro cytotoxicity of immunotoxins. Cytotoxicity of immunotoxins was tested as follows. Cells in culture medium were distributed into wells of a flat-bottom 96-well microtiter plate at 5×10^4 per well. Immunotoxins were added at concentrations of 10^{-9} – 10^{-7} M to the wells in triplicate, and plates were incubated for 3 days at 37°C and then stained with 0.4 % trypan blue (20 μl) for 10 min at room temperature. Undyed cells were counted by a Bürker-Türk hemocytometer.

Results

Characterization of immunotoxins. Purified immunotoxins were analyzed by SDS/PAGE under nonreducing and reducing conditions. Immunotoxins showed a band having a molecular weight of > 150,000, suggesting the addition of A chains into the antibody. Upon reduction of conjugate with 2-mercaptoethanol treatment, free A-chain bands reappeared in the gel, confirming that the immunoconjugate was chemically linked through a disulfide bond.

Binding of immunotoxins and antibodies to target cells. This was evaluated by indirect immunofluorescence on FACS (Fig. 1). Anti-H-2^d mAb produced the highest fluorescence intensity in the three cell lines. Anti-asialo-GM2 (IgM) mAb produced higher fluorescence intensity than anti-asialo-GM2 (IgG) mAb in two cell lines. H-2^d may be distributed more densely than asialo-GM2 on the cell surface. AA12 and CC9 cells exhibited some difference in fluorescence intensity by the same mAb. Anti-H-2^d immunotoxin produced identical fluorescence profiles with unconjugated antibody on all three cell lines (data not shown). Anti-asialo-GM2 (IgM) immunotoxin produced somewhat higher fluorescence intensity than unconjugated antibody on two cell lines. Conjugation with RTA did not reduce the immunoreactivity of antibodies.

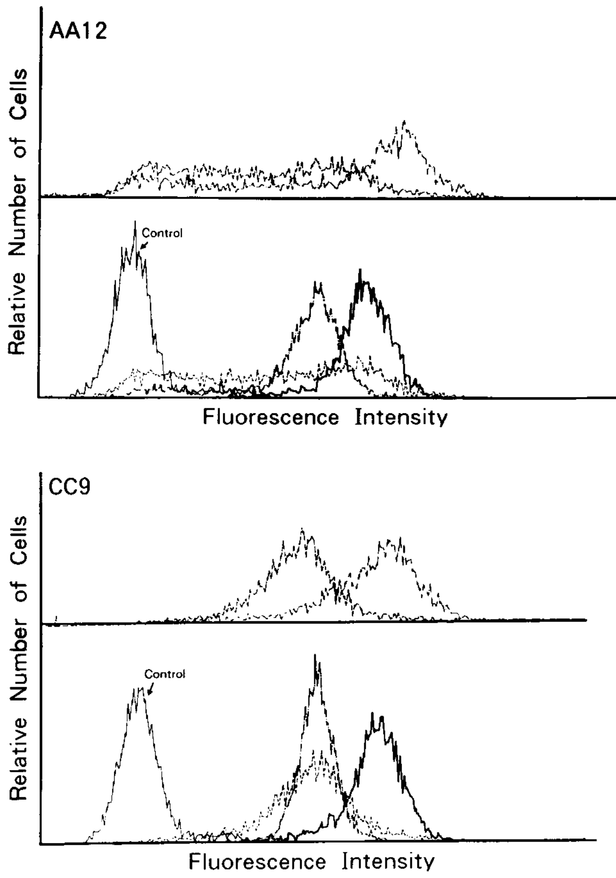
Cytotoxic activity of immunotoxins. *In vitro* cytotoxic effects of immunotoxins against target cells are summarized in Fig. 2. AA12 and CC9 cells express high levels of asialo-GM2. Anti-asialo-GM2 (IgM)-RTA immunotoxin was more cytotoxic to CC9 cells than anti-asialo-GM2 (IgG3)-RTA immunotoxin. These immunotoxins (IgM, IgG3) were less cytotoxic to AA12 cells. Anti-H-2^d-RTA immunotoxin was more cytotoxic than anti-asialo-GM2-RTA immunotoxins against two cell lines.

27AV cells express low levels of asialo-GM2. Anti-H-2^d-RTA immunotoxin showed strong cytotoxicity against 27AV cells, whereas anti-asialo-GM2-RTA immunotoxins showed no cytotoxicity.

There was no cytotoxicity in L5178Y cells cultured with the same amounts of RTA and mAbs as used in immunotoxins, nor in other antigen-negative cell lines (*e.g.*, mouse myeloma cell line SP2) cultured with same amounts of immunotoxins.

Discussion

Over the past decade, immunotoxins composed of mAbs covalently coupled to toxins or their subunits have been developed for the treatment of malignancies (12, 13), and several immunotoxins have been studied in clinical trials (14, 15).



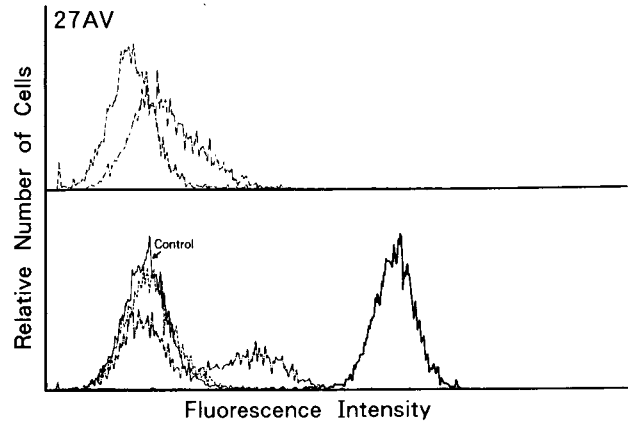


Fig. 1 Cytofluorometric analysis by indirect immunofluorescence of antibodies and immunotoxins bound to target cells. For control experiments, cells were directly incubated with fluoresceinated rabbit anti-mouse IgG.
Anti-asialo-GM2 IgG3 (------), anti-asialo-GM2 IgM (•—•—), anti-H-2^d IgG2a (——), anti-asialo-GM2 IgG3: RTA conjugate (– – –), anti-asialo-GM2 IgM:RTA conjugate (—••—)

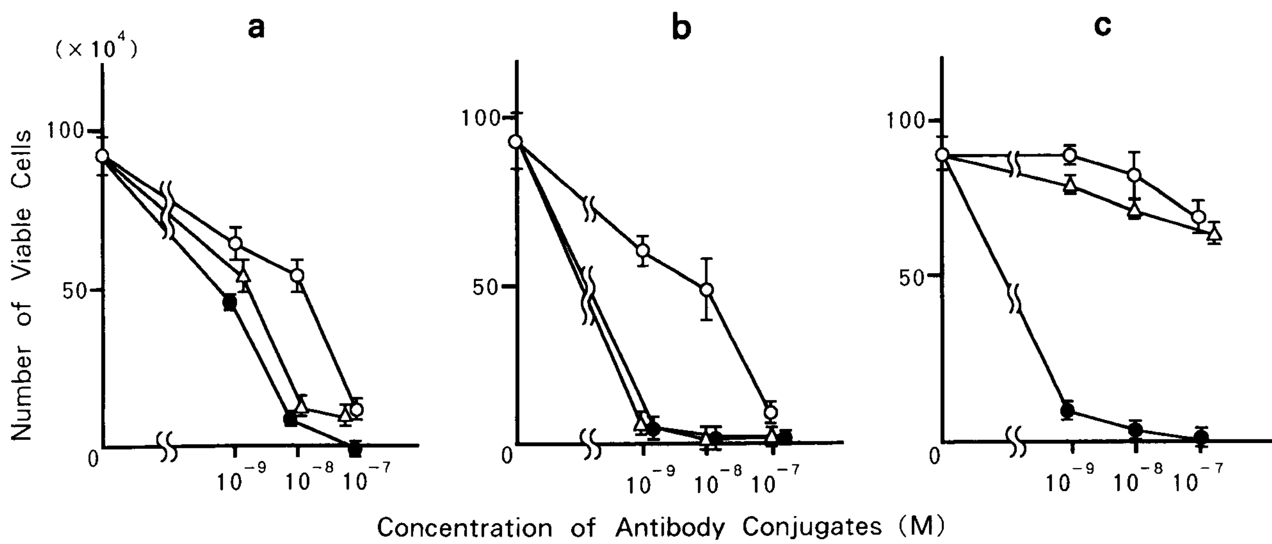


Fig. 2 Cytotoxicity of immunotoxins against L5178Y cell variants. Data are shown as mean ± S.D. (n = 3). AA12 cells (**a**), CC9 cells (**b**), and 27AV cells (**c**) were incubated with anti-asialo-GM2 IgG3 (○), anti-asialo-GM2 IgM (△) and anti-H-2d IgG2a (●): RTA immunotoxins.

We analyzed the efficacy of three RTA-containing immunotoxins, conjugated from mAbs directed against asialo-GM2 and H-2^d, on L5178Y mouse lymphoma cell variants. To evaluate immunoreactivity, cells were stained with immunotoxins and appropriate fluorescein isothiocyanate-labeled secondary antibodies. Conjugation with toxins was reported to have little or no effect on immunoreactivity of the antibodies (9, 16). In our experiments, the coupling procedure had no apparent effect on antigen-binding properties of three mAbs.

Cytotoxicity of immunotoxins is influenced by such factors as type of target cell, nature of the mAb, and nature and density of the cell surface antigen (3, 4). In this study, the cytotoxic effects of immunotoxins (anti-asialo-GM2 [IgM], anti-H-2^d) were different against AA12 and CC9. These cell lines are subclones of L5178Y, and showed almost identical mAb staining on FACS. The heterogeneity of these cells helps explain the differential cytotoxic effects of immunotoxins.

The toxicity of anti-asialo-GM2 (IgM)-RTA immunotoxin was stronger than that of anti-asialo-GM2 (IgG3)-RTA immunotoxin against AA12 and CC9 cells. These anti-asialo-GM2 mAbs are directed against the terminal galactosamine and carbohydrate of asialo-GM2 (8), and have different fluorescence intensities against cells. Reports on immunotoxins conjugated with IgM mAbs are rare, but indicate that these immunotoxins have high cytotoxicity (17, 18). The antibody subclass does not seem to affect the efficiency of immunotoxins. On the other hand, an epitope recognized by antibody was reported to play an important role in the internalization and efficiency of immunotoxins, and an mAb directed against the distal part of its antigen on the cell membrane was reported to exhibit weak cytotoxicity (4, 19). In this study, the anti-asialo-GM2 mAbs presumably recognized different parts of the same terminal molecule (8). The differential cytotoxic effects of the two anti-asialo-GM2-RTA immunotoxins may be due to differences of the epitope recognized by the antibody, and the nature of the antibody.

Anti-H-2^d-RTA immunotoxin showed stronger cytotoxicity than anti-asialo-GM2 (IgM, IgG3)-RTA immunotoxins. H-2^d may be distributed more densely than asialo-GM2 on the cell surface, judging from FACS analysis, and H-2^d antigen as glycoprotein is found both in the cytoplasm and on the external surface of the cell membrane. Although the density of cell surface antigen does not necessarily regulate cytotoxicity of immunotoxin (17), the rate of internalization and post-membrane processing of immunotoxin do influence cytotoxicity (20, 21). H-2^d may be effective for this reason. In this study, immunotoxin made from anti-asialo-GM2 mAb showed strong cytotoxicity, and different mAbs recognizing different epitopes of the same molecule showed different cytotoxicity. Immunotoxins conjugated from mAbs against glycolipid show sufficient cytotoxicity to target antigen-positive cells (17, 22). Production of better anti-glycolipid mAbs should lead to more effective immunotargeting therapies.

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