Stability and Cytotoxicity of Fab-Ricin A Immunotoxins Prepared with Water Soluble Long Chain Heterobifunctional Crosslinking Agents

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The effects of the hindered and non-hindered water soluble long-chain disulfide bonds on the stability and cytotoxicity of the ricin A chain (RTA) immunotoxin were examined. The RTA immunotoxins were prepared with the Fab fragments of anti-common acute lymphoblastic leukemia antigen (CALLA) monoclonal antibody (Fab-RTA) using sulfosuccinimidyl-6-[(-methyl-(-(2-pyridyldithio)toluamido]hexanoate (S-LC-SMPT) and sulfosuccinimidyl-6-[3-(2-pyridyldithio)-propionamido]hexanoate (S-LC-SPDP). The prepared Fab-RTA immunotoxins were evaluated for their conjugation yield, immunoreactivity, thermal and disulfide bond stability and cytotoxicity. The conjugation yield of the Fab-RTA immunotoxin from the water soluble long chain crosslinking agents, S-LC-SMPT and S-LC-SPDP, were comparable. Both Fab-RTA immunotoxins exhibited a similar immunoreactivity and thermal stability in aqueous solution. However, S-LC-SMPT -mediated Fab-RTA, sterically hindered, showed an enhanced disulfide bond stability in vitro over S-LC-SPDP mediated one. In the cytotoxicity against antigenic cell Daudi, the S-LC-SMPT -mediated RTA immunotoxin maintained a comparable cytotoxicity, compared with S-LC-SPDP mediated Fab-RTA immunotoxin.

Key words: Immunotoxin, Ricin A chain, Fab, Monoclonal antibody, S-LC-SMPT, S-LC-SPDP

INTRODUCTION

The most common approach for the preparation of immunotoxins, hybrid molecules formed by coupling a polypeptide toxin to an monoclonal antibody (MoAb), is to delete the non-specific binding B-chain from the toxin and linking the protein inhibitory portion of the A chain (RTA) to a specific Fab fragments of MoAb (Thrush *et al.*, 1996). Heterobifunctional crosslinking agents such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and 4-succinimidyl-oxycarbonyl-a-(2-pyridyldithio) toluene (SMPT) (Carlsson *et al.*, 1978) have been widely used to synthesize reducible disulfide bonded Fab-RTA immunotoxins. SPDP mediated RTA immunotoxins possess a potent in vitro cytotoxicity on the target tumor cells but their disulfide bonds become unstable in vivo (Wawarzynczac *et al.*, 1991). As a result, the antibody may be separated from the toxin, competing with the intact immunotoxin for the antigen sites on the cell (Thorpe *et al.*, 1987). Alternatively, SMPT has been used in the preparation of more stable immunotoxins (Thorpe *et al.*, 1987). It has a benzene ring and a methyl group adjacent to a disulfide bond and these functional groups hinder the disulfide linkage, thus protecting from reduction by thiolate anions. SMPT has been used to prepare the immunotoxins with deglycosylated RTA (Ghetie *et al.*, 1993).

Both SPDP and SMPT possess low water solubility and, therefore, are needed to be dissolved in small amounts of organic solvent before crosslinking (Fulton *et al.*, 1986). Also, the spacer bridge component that connects the two reactive ends of these crosslinkers is relatively short (SPDP: 6.8 Å, SMPT: 11.2 Å), which may lead to the steric hindrance of the binding site of antibody that is closely surrounded by conjugated toxins. With Fab-based RTA immunotoxins, the steric hindrance may occur at the single binding site of the small Fab molecules due to the statistical distribution of RTA conjugated to the antibody molecules (Derocq *et al.*, 1988). To reduce the steric hindrance effect of the crosslinking agents and to avoid the organic solvent incorporation, water soluble long chained crosslinkers, sulfosuccinimidyl-6-[3-(2-pyridyldithio)-

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propionamido]hexanoate (S-LC-SPDP,15.6 Å) and sulfo-succinimidyl-6-[ö-methyl-ö-(2-pyridyldithio)-toluamido]hexanoate (S-LC-SMPT, 20.0 Å), have been introduced. However, a limited information is available (Uckun *et al.*, 1993; Woo *et al.*, 1994).

In this study, we compared the Fab-RTA immunotoxins (Fig. 1) prepared with the water soluble long chain crosslinkers, S-LC-SMPT and S-LC-SPDP, for their in vitro stability and cytotoxicity. Fab fragments of the monoclonal antibody against common acute lymphoblastic leukemia antigen (CALLA) and RTA were adopted as the antibody and toxin, respectively, and human leukemia cell lines of Daudi and K562 were adopted to examine the immunoreactivity and in vitro cytotoxicity of RTA immunotoxins.

MATERIALS AND METHODS

Materials

Anti-CALLA 269-65 monoclonal antibody (IgG_1, MoAbs) was obtained in ascites form from the hybridoma cell line secreting anti-CALLA MoAb in Balb/c mice as described previously (Chun and Lee, 1993). Ricin A chain and Fab fragments of anti-CALLA MoAbs were prepared as described previously (Woo *et al.*, 1994). Daudi and K562 were employed as the antigen-positive and antigen-negative cells, respectively.

Preparation of Fab-RTA immunotoxins

Fab-RTA immunotoxins were prepared as described previously with a slight modification (Woo *et al.*, 1994). Fab fragments (1 mg/ml in PBS containing 10 mM EDTA, pH 7.4) were allowed to react with each 10 molar excess S-LC-SMPT or S-LC-SPDP (Pierce, Rockford, IL, USA, 20 mM in PBS) for 30 min at room temperature. Excess reagents were removed by gel-filtration on Sephadex G-25 (Pharmacia, Sweden) and S-LC-SMPT-and S-LC-SPDP-derivatized Fab fragment solutions were concentrated to 2 mg/ml using the Amicon 30 centrifugal concentrator (Amicon, Beverly, MA). Then, each 5 molar excess of freshly activated RTA with dithiothreitol (Sigma, St. Louis, MO) was added to the derivatized Fab fragment solutions. Conjugation reactions were performed for 16 h at room temperature followed by filtration with a 0.22 μm membrane filter. The solution was then subjected to Superose HR 10/30 column (Pharmacia, Sweden) with 50 mM PBS (containing 10 mM EDTA, pH 7.4) to separate the RTA immunotoxins from the unconjugated Fab fragments and RTA. Fractions of the Fab-RTA immunotoxins (M.W., 76 kDa) were pooled, concentrated to a final concentration of 1 mg/ml and stored at 4°C. The Fab-RTA immunotoxins were analyzed by 10% SDS-PAGE under the nonreducing conditions.

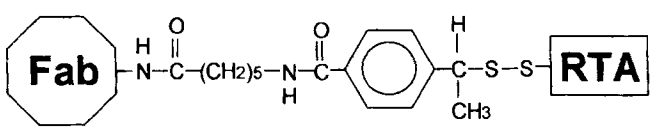

Fig. 1. General structure of S-LC-SMPT mediated Fab-RTA immunotoxin.

Thermal stability

The Fab-RTAs (0.2 mg/ml in PBS containing 10 mM EDTA and 0.02% Na-azide, pH 7.4) were stored at -20, 4, 25 or 37°C for 1, 5 and 10 days. Concentrations of the intact Fab-RTA remaining were analyzed by Superose HR 10/30 column chromatography at 280 nm. The initial concentration of each Fab-RTA stored at -70°C was designated 100% and all subsequent concentrations were expressed as the percentage of the initial concentrations.

Stability in glutathione

The Fab-RTAs were treated with 2 mM glutathione (Sigma, St. Louis, MO, USA) at 37°C over an 18 h period, with the glutathione-free RTA immunotoxins used as the control. The release rate of RTA was determined by using the Superose HR 10/30 column chromatography. The reaction rate constants were calculated from the amount of Fab-RTAs unreduced over time.

Immunoreactivity and cytotoxicity

ELISA was used to examine the immunoreactivity of Fab fragments and Fab-RTAs against the target human leukemia Daudi and K562 cells (Woo *et al.*, 1994). To determine the cytotoxicity, Daudi cells in 96-well microtiter plates at a concentrations of 1×10^4 cells/well were treated with various concentrations of Fab-RTAs in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (GIBCO, USA) for 48 h at 37°C in a humidified atmospheric 5% CO_2. The number of viable cells was determined by MTT assay as previously described (Woo *et al.*, 1998).

RESULTS AND DISCUSSION

The cytotoxic potency and *in vivo* stability of RTA immunotoxins are important in immunotoxin studies (Thorpe *et al.*, 1988). In this study, anti-CALLA Fab-RTA immunotoxins were prepared using the water soluble long-chain disulfide crosslinker, S-LC-SMPT and S-LC-SPDP, and were separated from Fab and RTA conjugation mixtures by size-exclusion HPLC on Superose HR 10/30 column. The derivatization ratio of the pyridyl disulfide group into Fab fragment plays an important role in the conjugation yield of immunotoxins. The extent of amino

group modification of the Fab fragment was determined on the basis of the liberation of pydine-2-thione upon dithiothreitol treatment as described previously (Na *et al.*, 1999). The degree of RTA conjugation was calculated from the peak area of the prepared intact RTA immunotoxins and free Fab fragments when each 5 molar excess of RTA was added to the activated Fab fragments. The mean derivatization ratio of the pyridyl disulfide group into Fab fragment was 1.4 for both S-LC-SMPT and S-LC-SPDP (n=6 each). The RTA conjugation yields to the activated Fab fragments averaged 54% for S-LC-SMPT and 57% for S-LC-SPDP (Woo et al., 1994).

One of major weak points of SMPT despite its higher stability in the biological fluids has been its lower conjugation yield (13%) of immunotoxin than that (25%) of SPDP (Vallera *et al.*, 1996). In this study, the conjugation yield of immunotoxin from S-LC-SMPT became similar to that from S-LC-SPDP. Fab-S-LC-SMPT-RTA show-ed no significant difference in the affinity for the antigenic Daudi cells compared with Fab-S-LC-SPDP-RTA. Also, both immunotoxins showed no reactivity with nonantigenic K562 cells (Fig. 2). The lower immunoreactivity of *Fab-RTA* immunotoxins may be attributed by the statistical distribution of A-chain around the small Fab molecule leading to a steric hindrance of a single binding site (Derocq *et al.*,1988) or by the loss of avidity during the conjugation reaction. The degradation of Fab-RTA was studied in aqueous solution

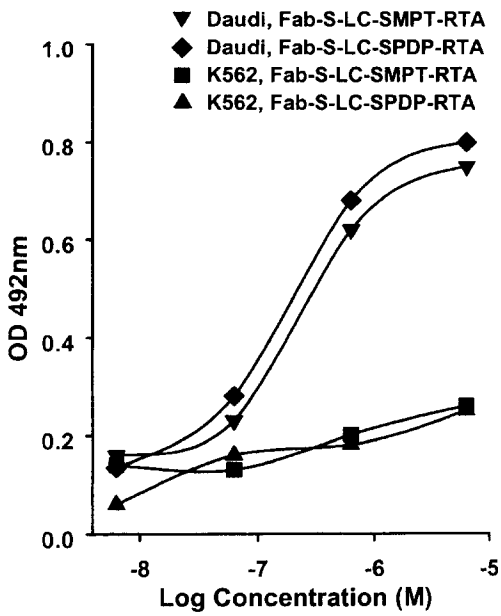

Fig. 2. Binding affinity of Fab-RTA immunotoxins to cell surface. The target cell of 1×10^4 cells/well were treated with various concentrations of samples for 2 h at 37°C. in a humidified atmospheric 5% CO_2 and the number of viable cells was determined by MTT assay method. The results are shown as mean values (±SD) of three experiments.

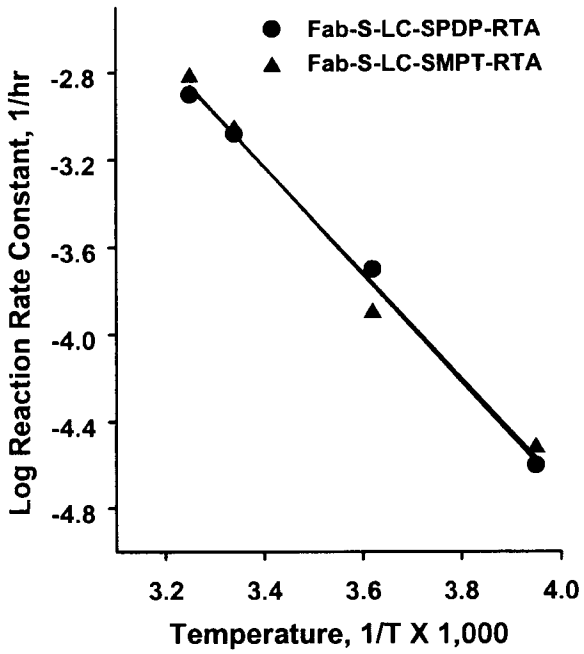

Fig. 3. Thermal degradation rates of Fab-RTA immunotoxins at pH 7.4. Samples (0.2 mg/ml) were stored at different temperature for 1, 5 and 10 days and concentrations of the intact Fab-RTA remaining were analyzed by Superose HR 10/30 column chromatography at 280 nm.

ranging from -20°C to 37°C for 10 days. The degradation reactions at 37°C obeyed pseudo-first-order kinetics. Fig. 3 shows the first-order plot of the rate data obtained at pH 7.4. The bond type of linkages between the Fab and RTA had no significant influence on the stability of Fab-RTA in the aqueous solution.

The stability of ricin A immunotoxins *in vivo* depends on their susceptibility to proteolytic degradation and catabolism. The disulfide bond used to link the antibody to toxin may be cleaved by the enzyme-catalyzed thiol exchange reaction or in the presence of glutathione. It has been suggested that RTA immunotoxins do not break down significantly when incubated in mouse plasma or whole blood in vitro and the splitting may occur within one of the body organs, possibly the liver. The concentration of glutathione in blood is low, but higher concentrations (0.5 to 10 mM) are found intracellularly. The breakdown of RTA immunotoxins with 2 mM glutathione at 37°C was assessed by measuring the amount of unreduced RTA immunotoxins using size-exclusion HPLC (Fig. 4) The Fab-S-LC-SPDP-RTA was easily reduced by glutathione, with over 50% of free Fab fragments being released within 18 h in the presence of 2 mM glutathione. Steric hindered, disulfide bonded Fab-S-LC-SMPT-RTA was more stable than Fab-S-LC-SPDP-RTA in the presence of the reducing agent *in vitro*. About 73% of Fab-S-LC-SMPT-RTA remained intact at 18 h of incubation (2 mM glutathione). This increased stability of Fab-S-LC-SMPT-

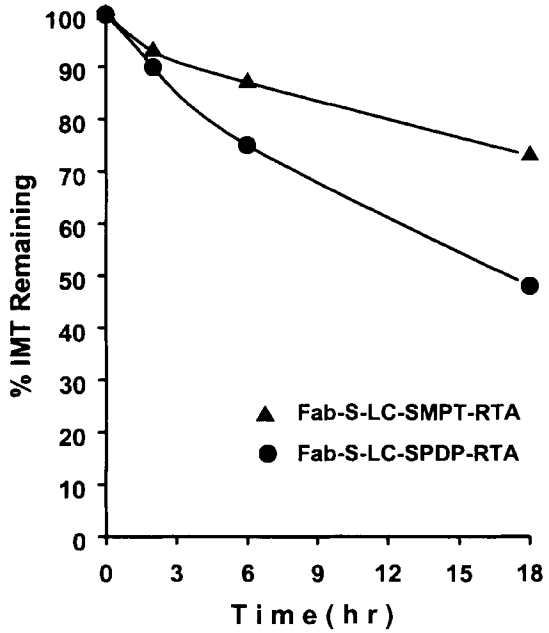

Fig. 4. Breakdown rates of Fab-RTA immunotoxins in glutathione. Samples were treated with 2 mM glutathione at 37°C over an 18 h period and concentrations of the intact Fab-RTA remaining were analyzed by Superose HR 10/30 column chromatography at 280 nm.

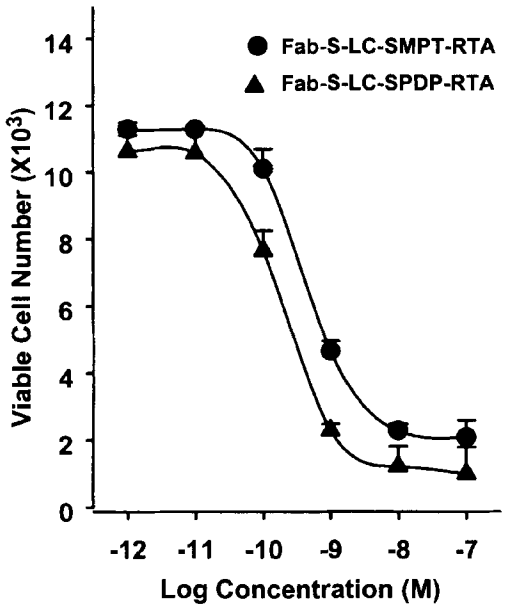

Fig. 5. Cytotoxicity of Fab-RTA immunotoxins on Daudi cells. The target cell of 1×10^4 cells/well were treated with various concentrations of samples for 48 h at 37°C in a humidified atmospheric 5% CO_2 and the number of viable cells was determined by MTT assay method. The results are shown as mean values (±SD) of three experiments.

RTA over Fab-S-LC-SPDP-RTA was similar to that of Fab-SMPT-RTA over Fab-SPDP-RTA previously reported (Carroll *et al.*, 1994). Thus, it is possible that the disulfide bond of RTA immunotoxins may be slowly split by glutathione in the blood *in vivo* and that the placement of the hindering groups around the disulfide bond may protect it from attack. Alternatively, the splitting may be due to the action of disulfide reductase and the hindering groups may interfere with the enzymatic attack.

Next, the *in vitro* cytotoxicity of Fab-RTA immunotoxins to antigenic B-lineage lymphostoid Daudi cells was measured and are shown in Fig. 5. The LD_{50} values calculated for the S-LC-SMPT and S-LC-SPDP mediated RTA immunotoxins were 8.4×10^{-10} M and 4.1×10^{-10} M, respectively. With the higher *in vitro* stability, the S-LC-SMPT mediated RTA immunotoxin was 1.8 times less potent than the S-LC-SPDP mediated Fab-RTA immunotoxin. The S-LC-SPDP and S-LC-SMPT mediated RTA immun-toxins exhibited 64-and 31-fold increased cytotoxicity over their intact RTA, respectively.

In summary, the steric hindered Fab-RTA immunotoxin prepared with S-LC-SMPT was compared with the Fab-RTA immunotoxin prepared with S-LC-SPDP. Unlike the intact SMPT, the conjugation yield of immunotoxins from SMPT became similar to that from SPDP by using the water soluble long chain crosslinking agents. Immunoreactivities of both RTA immunotoxins did not differ significantly but the S-LC-SMPT mediated, steric hindered Fab-RTA exhibited an enhanced disulfide bond stability in vitro over the Fab-RTA immunotoxin prepared with the use of S-LC-SPDP. The S-LC-SMPT mediated RTA immunotoxin maintained a comparable cytotoxicity but a higher in vitro stability against antigenic cell Daudi, compared with the S-LC-SPDP mediated Fab-RTA immunotoxin.

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