Arch. Pharm. Res. Vol. 17, No. 6, pp. 452-457, 1994

Ricin A Immunotoxins of IgG and Fab of Anti-CALLA Monoclonal Antibody: Effect of Water Soluble Long-chain SPDP on Conjugate Yield, Immunoselectivity and Cytotoxicity

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(Received October 1, 1994)

The water soluble long-chain crosslinker, sulfo-succinimidyl-6-[3'-(2-pyridyldithio)-propionamido]hexanoate (S-LC-SPDP) was used to prepare ricin A chain (RTA) immunotoxins constructed with whole IgG and Fab fragments of the anti-common acute lymphoblastic leukemia antigen (CALLA) monoclonal antibody. In this study, a) S-LC-SPDP modification efficiencies of whole IgG and Fab, b) conjugation yields of the immunotoxins prepared and c) *in vitro* immunoreactivity and cytotoxicity of immunotoxins constructed were examined. IgG-RTA and Fab-RTA immunotoxins were prepared with 67.3% and 57.0% conjugation yields, respectively. These long spacer intermolecular linked immunotoxins were selectively immunoreactive and cytotoxic aganinst to immunogenic Daudi cells but little or no-binding and cytotoxic against to antigen K562 cells. Both IgG-RTA and Fab-RTA immunotoxins were 210- and 45-fold more active than intact RTA *in vitro*, respectively.

Key words: Immunotoxin, Ricin A chain, CALLA, Monoclonal antibody, Fab fragment, Crosslinking, Sulfo-LC-SPDP, Drug targeting

INTRODUCTION

Immunotoxins are cytotoxic agents constructed by covalently linking monoclonal antibody (MoAb) directed against tumor-associated antigens with peptide toxins. The crosslinking agent must be capable of holding the toxin and MoAb together in the extracelluar environment and release the toxin from the MoAb in the proper intracellular vesicle to permit protein synthesis-inactivating activity of the toxins.

The commonly used toxins in the preparation of immunotoxins are Pseudomonas exotoxin A (Fitzgerald *et al.*, 1983), diphtheria toxin (Ross *et al.*, 1980), ricin (Marsh *et al.*, 1988), abrin (Thorpe *et al.*, 1981), gelonin (Stirpe *et al.*, 1980), or A chains of diphtheria toxin, ricin, and abrin (Byers and Baldwin, 1992). The most frequently used toxic agent is the ricin A chain (RTA), a ribosome inactivating protein (RIP) that inactivates protein synthesis in eukaryotic cells by specific covalent modification of 60S ribosomal subunit (Lord *et al.*, 1991).

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The immunotoxins constructed with whole IgG might lead to nonspecific binding with normal cells due to the Fc portion. Therefore Fab fragment immunotoxins of similar antigen binding activity were more interesting in *in vivo* use (Fulton *et al.*, 1986). To prepare ricin A chain containing immunotoxins, a heterobifuncitional crosslinking agent, N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (Carlsson *et al.*, 1978) was widely used but this SPDP has limited water solubility, therefore it must be dissolved in a small amount of organic solvent (ethanol, dimethyformamide, dimethylsulfoxide etc.) before crosslinking reaction. The spacer bridge component of SPDP that connects the two reactive ends of the crosslinker is relactively short (6.8 Å) and this short bridge can make steric hindrance effects which may occur during the conjugation of antibody to toxin (Derocq *et al.*, 1988). To reduce the steric hindrance effects of crosslinking agent and to avoid organic solvent incorporation, a water soluble long chained (15.6 Å) crosslinker, sulfo-succinimidyl-6-[3'-(2-pyridyldithio)-propionamido]hexanoate(S-LC-SPDP) was developed recently (Uckun *et al*, 1993).

In this study, ricin A chain conjugated immunotoxins of IgG and Fab fragment of monoclonal antibody against anti-common acute lymphoblastic leukemia

antigen (CALLA) were prepared using S-LC-SPDP as a crosslinker and their immunological characteristics and cytotoxic effect were evaluated. The human leukemia cell lines of Daudi and K-562 were adopted to examine immunoreactivity and *in vitro* cytotoxicity of prepared immunotoxins.

MATERIALS AND METHODS

Monoclonal Antibody and Its Fab Fragments

Monoclonal antibody 269-65 (IgG_1) was obtained in ascites form from the hybridoma cell line secreting anti-CALLA monoclonal antibody in Balb/c mice as described previously (Lee *et al.*, 1990). The IgG containing ascitic fluid was one-step fractionated by affinity chromatography on Hydroxylapatite (Bio-Rad, USA) as previously described (Stanker *et al.*, 1985).

The whole IgG antibody were digested with papain to produce Fab fragments and the Fab was isolated by chromatography on Protein-A Sepharose CL-4B (Pharmacia, Sweden) and purified on DEAE-Sephacel (Pharmacia, Sweden).

The purified IgG and Fab were shown to undergo binding reactions with Daudi, NALM-6, and RPMI 8402 cells, whereas no reactivity was observed with the K562 and MOLT4 cells as previously reported (Chun and Lee, 1993). In this study, Daudi and K562 were employed as antigen-positive and antigen-negative cells, respectively.

Ricin-A Chain

After cleavage of the disulfide bond of ricin (Toxin RCA_{60}, Sigma, USA) with 2-mercaptoethanol (Sigma, USA), the ricin A chain was isolated by ion-exchange chromatography on DEAE-Sephacel (Pharmacia, Sweden) and concentrated on CM-Sepharose (Pharmacia, Sweden) as previously described (Olsnes and Pihl, 1973). Purified RTA were analyzed by 10% SDS-PAGE under nonreducing conditions on a vertical slab gel apparatus (EC185, E-C, USA) at constant ampere (EC570, E-C, USA). Protein bands were visualized by Coomassie Blue staining.

Modification of IgG and Fab with S-LC-SPDP

Two milliliters of the purified IgG (5 mg/ml) or Fab fragments (5 mg/ml) in phosphate buffered saline solution (PBS, pH 7.4) were mixed with excess of S-LC-SPDP (Pierce, USA), according to previous reported procedure (Carlsson *et al.*, 1978). All modification reactions were carried out at room temperature for 30 min with gentle rocking and excess of S-LC-SPDP was removed by passage over Sephadex G-25 (Pharmacia, Sweden).

The extent of amino group modifications of the IgG and Fab were determined by measuring the released amount of pyridine-2-thione groups following treatment with dithiothreitol (DTT, Sigma, USA) by UV-Visible spectrophotmeter (Shimadzu, Japan). The concentration of these liberated groups was calculated from the molar extinction coefficient of $E_{343nm}=8.08\times10^3$ (Uckun *et al.*, 1993).

The effect of S-LC-SPDP modification on the specific immunoreactivties of IgG and Fab were examined by the enzyme-linked immunosorbent assay (ELISA) method as described below.

Preparation of Immunotoxins

Immunotoxins were prepared by crosslinking RTA to the S-LC-SPDP modified IgG or Fab via isulfide linkage. In brief, IgG or Fab were allowed to react with each 10 molar excess of S-LC-SPDP for 30 min at room temperature. Excess reagent was removed by gel-filtration on Sephadex G-25 and the modified IgG or Fab were coupled with at 5 molar excess of freshly activated RTA for 16 hr at room temperature. The reaction mixtures were purified from RTA by chromatography on Sephadex G-200 (Sigma, USA). The final immunotoxin concentrations were adjusted to 1 mg /ml in PBS, pH 7.4 and stored at 4℃.

The IgG-RTA and Fab-RTA immunotoxins were analyzed by 5% and 10% SDS-PAGE, respectively, under nonreducing conditions to preserve disulfide bond between IgG or Fab and the attached RTA.

Immunoreactivity Studies

To examine the immunoreactivities of IgG, Fab, modified IgG and Fab and immunotoxins with the target human leukemia cells, ELISA method was performed as descried previously. Briefly, the target cells were fixed on 96-well microtiter plates at a density of 4×10^4 cells/well and the wells were incubated 2 hr at 37℃ with various concentrations of samples. Plates were then washed with PBS and incubated for 90 min with anti-mouse IgG peroxide conjugate (Sigma, USA), then plates were incubated for 30 min enzyme substrate (o-phenylenediamine/0.05% hydrogen peroxide). The reaction was stopped by adding 2M sulfuric acid and plates were read using microtiter plate at wavelength of 492 nm (Multiskan MCC/340 MK II, Flow Lab., Switherland).

In vitro Cytotoxicity Test

To study the cytotoxicity of the immunotoxins, Daudi and K562 cells in 96-well microtiter plates at a concentrations of 1×10^4 cells/well were treated with various concentrations of IgG-RTA or Fab-RTA immunotoxins in complete RPMI 1640 medium (GIBCO, USA) supplemented with 10% heat-inactivated fetal calf se-

rum (GIBCO, USA) for 48 hr at 37℃ in a humidified atmospheric 5% CO_2. Following treatment, the number of viable cells was determined by MTT assay as previously described (Ford *et al.*, 1989). The living cells converted the yellow water soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrolium bromide (MTT, Sigma, USA) into a dark blue formazan product and the amount of formazan produced was determined using microtiter plate reader at test wavelength of 540 nm and reference wavelength of 620 nm.

RESULTS AND DISCUSSION

The anti-CALLA MoAb was one-step purified from mouse ascites fluid using hydroxylapatite chromatography and it showed selective immunoreactivity against CALLA-positive Daudi lymphoblastoid cells and little or no-binding for antigen negative K562 cells. The Fab fragment of IgG was highly purified by chromatography on Protein-A Sepharose CL-4B and DEAE-Sephacel and showed a single band at 46 kDa under 10% SDS-PAGE as shown in Fig. 3.

The Fab fragment was 15 to 20-fold less immunoreactive against Daudi cells than its parental IgG (Fig. 5A). This accords with previous finding (Derocq *et al.*, 1988) and the lower binding affinity of the Fab was probably due to slight denaturation of the antibodies during the papain digestion process of chromatographic processes.

Derivatizing ratio of pyridyl disufide group into IgG and Fab fragment have a great role for the immunological stability of parent molecules and reaction activity with RTA. Average 2.2 and 1.4 pyridyl disulfide groups of S-LC-SPDP were derivatized to a mole of IgG and Fab, respectively, when each 10 molar excess of S-LC-SPDP was added to antibodies as shown in Table I. The large excess of S-LC-SPDP (50-fold) and longer reaction time (up to 2 hr) could not enhance the modification yields as shown in Fig. 1A. S-LC-SPDP modification might slightly decreased the immunoreactivity of antibodies. Immunoreactivity of IgG was little or not affected by S-LC-SPDP modification but S-LC-SPDP modified Fab was relatively less immunoreactive than that of modified IgG as shown In Fig. 1B, probably due to the increased hydrophobicity as a result of the introduction of long-chained aromatic groups into the small Fab (Carlsson *et al.*, 1978).

IgG-RTA and Fab-RTA immunotoxins were formed by conjugation the S-LC-SPDP modified antibodies and the reduced RTA, allowing disulfide exchange to occur. Excess of RTA was then separated from the conjugation mixture by chromatography on Sephadex G-200 as shown in Fig. 2.

Each peak was determined by 5% SDS-PAGE. Fig. 3 shows a typical heterogeneous mixtures of RTA conjugated IgG. The IgG-RTA mixture contains several bands of immunotoxins. Based on the molecular weight of the bands, they represent IgG immunotoxins containing mono-(IgG-RTA, 180 kDa), di-(IgG-2RTA, 210 kDa), tri-(IgG-3RTA, 240 kDa), tetra-(IgG-4RTA, 270 kDa) RTA molecules and some higher oligomeric hydrids. Almost equimolar mono- and di-RTA immunotoxins were detected with traces of higher oligomeric conjugates. In this study, the pooled fraction from 9 to 17 of Fig. 2a was used without further separation.

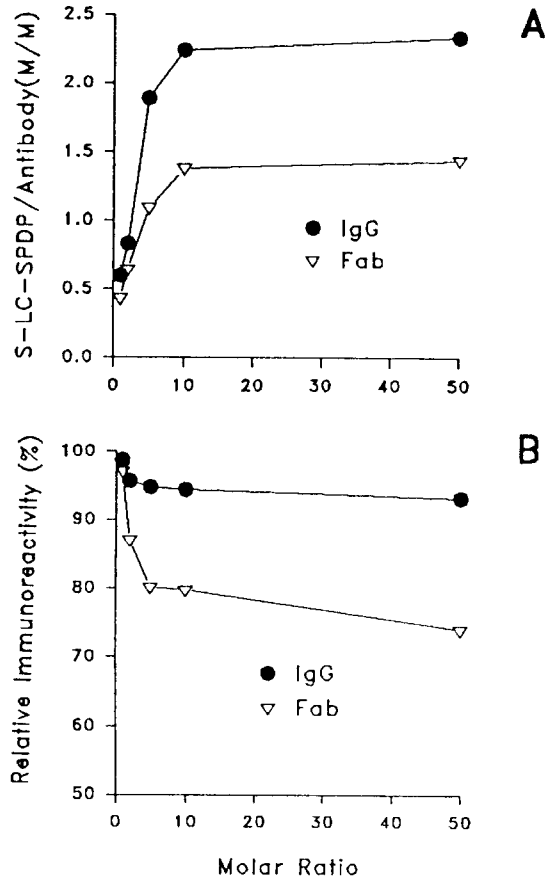

Fig. 1. Effect of reaction concentration ratio of S-LC-SPDP to IgG and Fab for their modification yield (A) and corresponding relative immunoreactivity (B).

Contrary to the conjugating reaction for IgG-RTA immunotoxin, Fig. 3b shows that dimeric Fab-RTA (76 kDa) is a major product with trace amount of trimeric (Fab-2RTA, 106 kDa) and tetrameric (Fab-3RTA, 136 kDa) in the Fab-RTA conjugation using S-LC-SPDP.

The RTA conjugation yields were calculated from the amounts of unconjugated RTA and free IgG or Fab when each 5 molar excess of RTA was added to S-LC-SPDP modified antibodies. The degree of RTA conjugated to S-LC-SPDP modified IgG and Fab were 67.3% and 57.0%, respectively as shown in Table I.

The conjugation reactions were very sensitive against

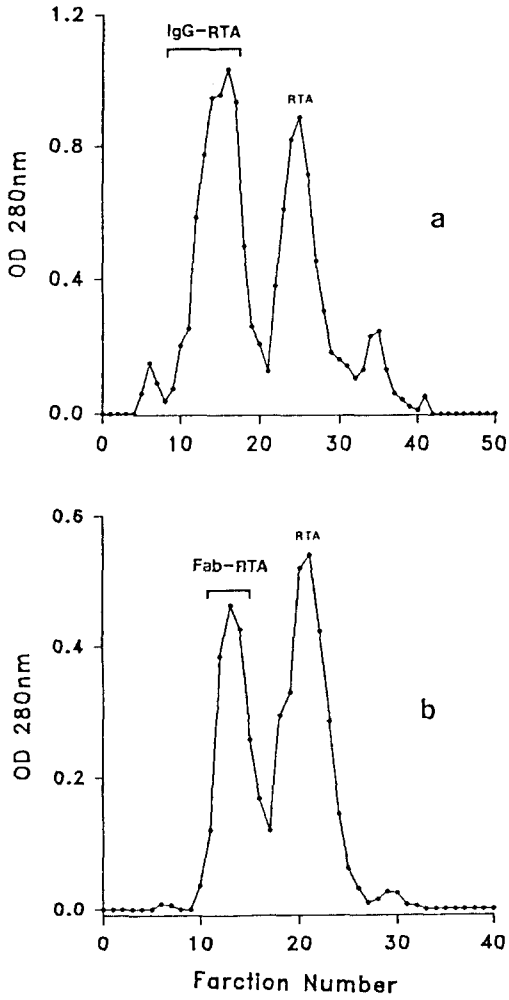

Fig. 2. Elution profiles of the IgG-RTA (a) and Fab-RTA (b) immunotoxins on Sephadex G-200 gel filtration chromatography.

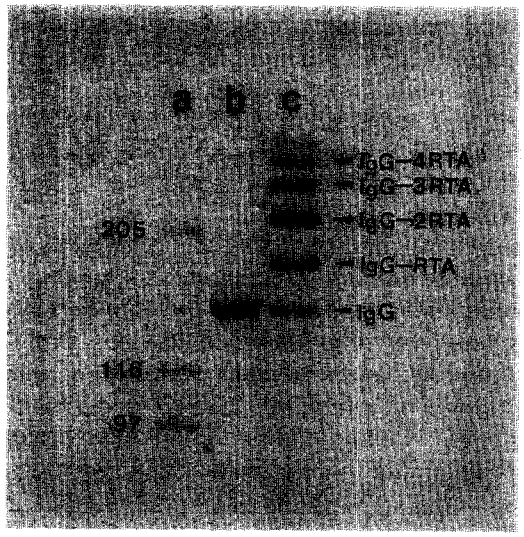

Fig. 3. 5% SDS-PAGE analysis of IgG and IgG-RTA. Each lane stands for; a: molecular weight standard marker, b: IgG, c: IgG-RTA.

Table 1. Modification of S-LC-SPDP into the antibodies and RTA conjugation yields

Antibody	S-LC-SPDP modification[a] (S-LC-SPDP/Antibodies, M/M)	RTA conjugation yield[b]
IgG	2.2± 0.5	67.3%
Fab	1.4± 0.3	57.0%

[a] Degree of S-LC-SPDP modification obtained when 10 molar excess of S-LC-SPDP added to IgG or Fab. The numbers of 2-pyridyl disulfide structures introduced were calculated from amount of pyridine-2-thione released on treatment of the modified IgG or Fab with a large excess of DTT. [b] RTA conjugation to S-LC-SPDP modified IgG or Fab, 5 molar excess of RTA added to antibodies.

a) the S-LC-SPDP modification, b) conjugation reaction time, c) RTA/antibody molar ratio and d) total protein concentration. The RTA conjugation yields were proportional to degree of cross-linker modification of antibody. The RTA conjugation yields of IgG-RTA was higher than that of Fab and it was considered that higher modification yields might lead higher RTA conjugation.

Long conjugation reaction times (36 to 72 hr) and large excess of RTA (10 to 20-fold to antibody) could not enhance the RTA conjugation yields, and furthermore long reaction times (up to 72 hr) and large excess of RTA caused serious protein precipitation and formation of high molecular weight aggregates. Total protein concentration of conjugation mixtures were important in conjugation, at the low concentration of under 1 mg/ml, the reactions were very slow or unreacted and high concentration of protein of over 5 mg/ml lead rapid reaction but formed high molecular aggregates and RTA precipitation.

Immunoreactivity of antibodies is generally reduced by conjugation with toxins (Youle and Neville, 1982; Fulton *et al.*, 1986). Decreasing immunoreactivity of IgG and Fab against target Daudi cells were also observed in this study. By conjugation with RTA, IgG-RTA and Fab-RTA showed 5- to 7-fold and 3- to 5-fold lower binding affinities than those of intact IgG and Fab fragment, respectively. The Fab-RTA showed 15- to 20-fold less immunoreactivity than IgG-RTA as shown in Fig. 5B. This may be attributed to the loss of avidity during the papain digestion of IgG and slight denaturation in conjugation procedure. Another reason for the lower immunoreactivity of Fab immunotoxin assembled with SPDP method may be the statistical distribution of A-chain around the small Fab molecule leading to a steric hindrance of single binding site (Derocq *et al.*, 1988).

The *in vitro* cytotoxicity of IgG-RTA, Fab-RTA and intact RTA on Daudi cells were measured for the anti-

genic and non-antigenic cells by MTT assay method. The concentrations required to killing 50% of target cells (LD_{50}) were shown Table II. The IgG and Fab-RTA showed as a mean value 210-fold and 45-fold potent than intact RTA, resepctively, and the IgG-RTA were 4.5-fold more potent than Fab-RTA in killing target cells as shown Fig. 6.

It is generally accepted that the cytotoxic potencies of immunotoxins are proportional to binding affinity of immunotoxin to target cells. The Fab immunotoxins having relatively low immunoreactivities over $F(ab')_2$ and whole IgG immunotoxins showed 4- to 70-fold lower potency comparing $F(ab')_2$ immunotoxins or whole IgG immunotoxins when conjugated by short-chained (6.8 Å) crosslinker SPDP (Derocq *et al.*, 1988, Fulton *et al.*, 1986). In cace of employing long-chained cross-linker (100 Å) to enhance their immunoreactivity, these long peptide spacer immunotoxins showed 10-fold higher cytotoxicity than conventional

Table II. Cytoxicity of RTA immunotoxins on Daudi cells

Immunotoxin	LC_{50} ± SD (M)	Number of experiments
IgG-RTA	$1.24 \pm 0.65 \times 10^{-10}$	6
Fab-RTA	$5.58 \pm 0.58 \times 10^{-10}$	6
RTA[a]	$2.61 \pm 0.79 \times 10^{-8}$	9

[a] Intact ricin A-chain.

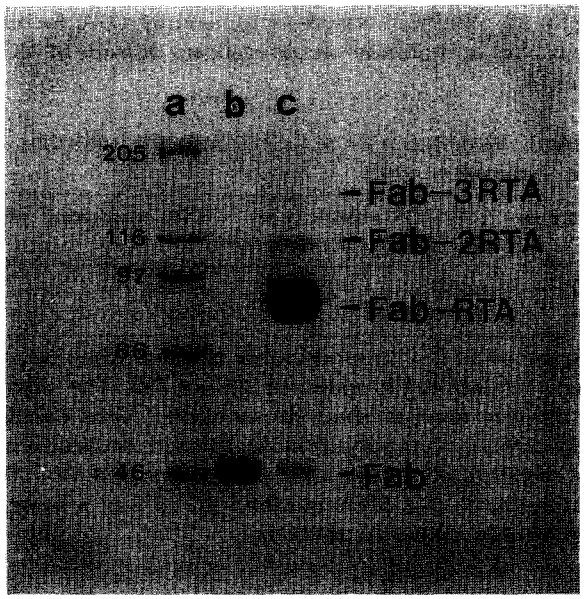

Fig. 4. 10% SDS-PAGE analysis of Fab and Fab-RTA. Each lane stands for; a: molecular weight standard marker, b: Fab and c: Fab-RTa.

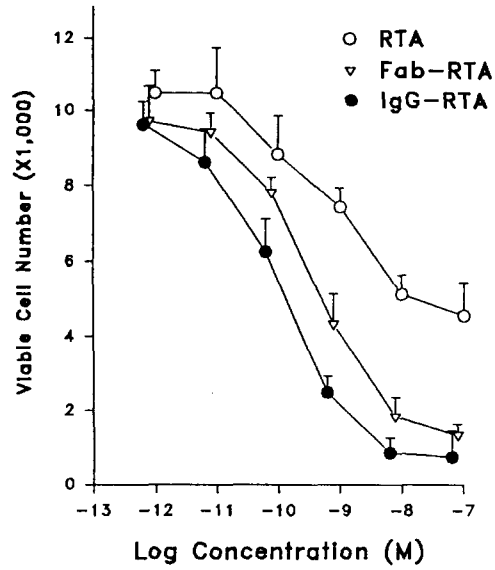

Fig. 6. Cytotoxic effect IgG-RTA, Fab-RTA and intact RTA upon Daudi cells in tissue culture. Daudi cells were cultured for 48 hr in the presence of IgG-RTA, Fab-RTA and RTA. The viable cells were measured by MTT assay. Points indicates the mean of measurements of viable cells.

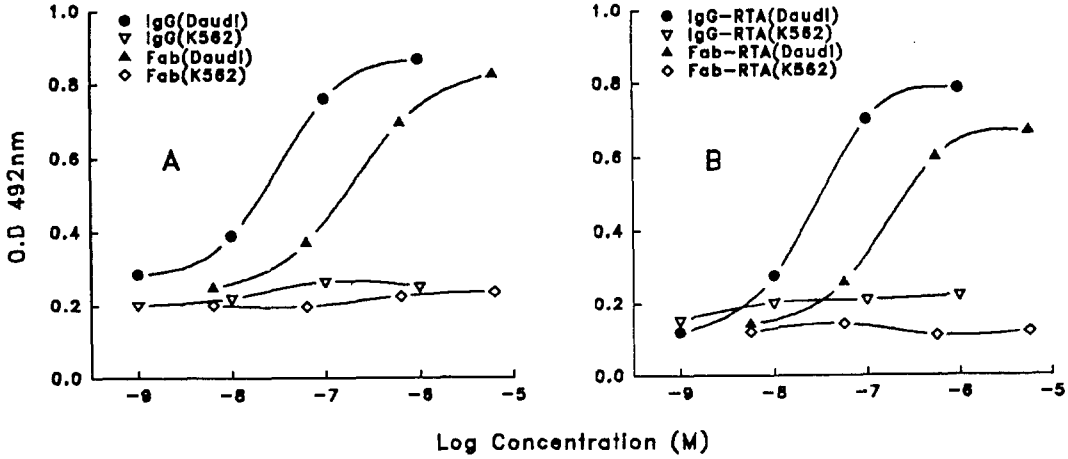

Fig. 5. Immunoreactivity of Immunotoxins on immunogenic Daudi and non-immunogenic K562 cells.

short-chained immunotoxins (Marsh and Neville, 1988). In this study, despite 15- to 20-fold lower immunoreactivity of Fab-RTA comparing IgG-RTA, Fab-RTA showed relatively high potent cytotoxicity to Daudi cells. This relatively higher cytotoxicity of Fab immunotoxins might be explained due to the long spacer bridge (15. 6 Å) of Fab-RTA conjugated by S-LC-SPDP which could reduce the steric hindrance of binding site and different geometric parameter between Fab and whole IgG immunotoxin in which RTA is statistically nearer to the plasma membrane in Fab immunotoxin as compared with whole IgG immunotoxin (Derocq *et al.*, 1988).

In conclusion, the choice of a suitable cross-linker is importance for preparation of immunotoxins with highest cytotoxicity and specificity. The RTA immunotoxins of whole IgG and Fab fragments of anti-CALLA MoAb using a water soluble long-chained crosslinker S-LC-SPDP were prepared with high conjugation yield and they exhibited selective immunoreactivity and cytotoxicity on the antigenic cell lines *in vitro*. Relative high cytotoxic effect of Fab-RTA immunotoxin was also confirmed over whole IgG-RTA in terms of their immunoreactivities and this finding suggests that Fab immunotoxins seem to be more prospective for *in vivo* studies.

ACKNOWLEDGMENT

This work was partially supported by research grants from the Ministry of Education, Ministry of Health and Social Affais of Korea and the Korea Science and Engineering Foundation.

REFERENCES CITED

Byers, V. S. and Baldwin, R. W., Targeted kill: From umbrellas to monoclonal antibodies. *J. Clin. Immunol.*, 12, 391-405 (1992).

Carlsson, J., Drevin, H., and Axen, R., Protein thiolation and reversible protein-protein conjugation: N-succinimidyl 3-(2-pyridyldithio)propionate, a new hterobifuncational reagent. *Biochem. J.*, 173, 723-737 (1978).

Chun, C. J. and Lee, K.C. Cytotoxicity of anti-CALLA monoclonal antibody conjugates to methtotrexate. *Arch. Pharm. Res.*, 16, 347-348 (1993).

Derocq, J. M., Casellans, P., Laurent, G., Ravel, S., Vidal, H. and Jansen, F., Comparison of the cytotoxic potency of T101 Fab, $F(ab')_2$ and whole IgG immunotoxins. *J. Immunol.*, 141, 2837-2843 (1988).

Fitzgerald, D. J. P., Trowbride, I. S., Pastan, I. and Willingham, M. C., Enhancement of toxicity of anti-trasferrin receptor antibody-Pseudomonas exotoxin conjugates by adenovirus. *Proc. Natl. Acad. Sci.* USA, 80, 4134-4138 (1983).

Ford, C. H. J., Richardson, V. J. and Tsaltas, G., Comparison of tetrazolium colorimetric and [^{3}H]-uridine assays for *in vitro* chemosensitivity testing. *Cancer Chemo. Pharmacol.*, 24, 295-301 (1989).

Fulton, R. J., Uhr, J. W., Vitetta, E. S., The effect of antibody valency and lysosomotropic amines on the synergy between ricin A chain- and ricin B chain-containing immunotoxins. *J. Immunol.*, 136, 3103-3109 (1986).

Lee, K. C., Lee, Y. J., Kim, W. B. and Cha, C. Y., Monoclonal antibody-based targeting of methotrexate-loaded microspheres. *Inter. J. Pharm.*, 59, 27-33 (1990).

Lord, J. M., Hartley, M. R. and Roberts, L. M., Ribosome inactivating proteins of plants. In Lord, J. M. (ed.), *Seminars in Cell Biology*, Saunders Scientific, Philadelphia, 1991, pp. 15-22.

Marsh, J. W. and Neville, D. M. Jr., A flexible peptide spacer increase the efficacy of holoricin anti-T cell immunotoxins. *J. Immunol.*, 140, 3674-3678 (1988).

Olsnes, S., Pihl, Different biological properties of the two constituent peptide chains of ricin. *Biochemistry*, 12, 3131-3126 (1973).

Ross, W. C. J., Thorpe, P. E., Cumber, A. J., Edwards, D. C., Hinson, C. A. and Davies, J. S., Increased toxicity of Diphtheria toxin for human lymphoblastoid cells following covalent linkage to anti-(human lymphocyte) globulin or its $F(ab')_2$ fragment. *Eur. J. Biochem.*, 104, 381-390 (1980).

Stanker, L. H., Vanderlaan, M. and Juarez-Salinas, H., One-step purification of mouse monoclonal antibodies from ascites fluid by Hydroxylapatite chromatography. *J. Immunol. Methods.*, 76, 157-169 (1985).

Stirpe, F., Olsnes, S. and Pihl, A., Gelonin, a new inhibitor of protein synthesis, nontoxic to intact cells, isolation, chracterization, and preparation of cytotoxic complexes with concanavalin A. *J. Biol. Chem.*, 255, 6949-6953 (1980).

Thorpe, P. E., Cumber, A. J., Williams, N., Edwards, D. C., Ross, W. C. J. and Davies, A. J. S., Abrogation of the non-specific toxicity of abrin conjugated to anti-lymphocyte globulin. *Clin. Exp. Immunol.*, 43, 195-200 (1981).

Uckun, F. M., Myers, D. E., Irvin, J. D., Kuebelbeck, V. M., Finnegan, D., Chelstrom, L. M. and Houston, L. L., Effect of the intermolecular toxin-monoclonal antibody linkage on the *in vivo* stability, immunogenicity and anti-leukemic activity of B43 (Anti-CD19) pokeweed antiviral protein immunotoxin. *Leukemia and Lymphoma*, 9, 459-476 (1993).

Youle, R. J., and Neville, D. M. Jr., Kinetics of protein synthesis inhibition by ricin anti-Thy 1.1 monoclonal antibody hybrids. *J. Biol. Chem.*, 257, 1598-1601 (1982).