NEW BIOMEDICAL TECHNOLOGIES

Synthesis and Properties of the Immunotoxin CD5-Ricin

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Synthesis and properties of an immunotoxin produced by conjugating ricin with a novel monoclonal antibody (IgG3 of the ICO-104 class) are described. Cytolytic activity of the synthesized immunotoxin, determined by two independent methods and expressed in LD_{50}, is $0.3\text{-}0.6\times10^{-7}$ M. Its specificity for target cells containing the CD5 antigen is shown.

Key Words: *immunotoxin; monoclonal antibodies; T cells; Jurkat cells; K-562 cells; cytolytic activity*

Immunotoxins are hybrid proteins whose selective cytolytic activity against target cells is made possible through attachment of the toxin (an inhibitor of ribosomal synthesis) to a specific monoclonal antibody (MAb). Such immunotoxins find use during the purification of bone marrow intended for autografting to eliminate an undesirable cell population with strong bioincompatibility reactions.

Here we describe the synthesis and properties of an immunotoxin which is the conjugate of a ribosomal synthesis inhibitor (plant ricin) and a MAb to the CD5 antigen of T cells (ICO-104 MAb). The specific characteristics of this MAb have been described [1]. MAbs derived from different hybridomas are known to vary with regard to such variables as the number of oligosaccharide groups and the position and number of *S-S* bonds. MAbs may also show other structural anomalies that influence their solubility and their ability to aggregate and break down into structural domains. There is evidence that murine immunoglobulins G (IgG) of different isotypes may react in different ways to a change in pH [5,6]. One possible consequence of this is an altered microenvironment of the functional groups at the surface of the molecule, which in turn may affect the accessibility of these groups to a modifying agent. In view of this, we optimized the synthesis of the immunotoxin and examined factors by virtue of which the final product could be obtained with the highest possible yield and the conjugate would retain the ability to bind to the cellular antigen CD5 and inhibit ribosomal synthesis in the target cell.

MATERIALS AND METHODS

The materials used included the following: two transplantable cell lines - Jurkat (malignant non-Hodgkin's T-cell lymphoma) and K-562 (erythroblastic tumor cells) [8]; diethylaminoethyl cellulose (DEAE 52; Serva); Acrylex P-10 (Reanal); Sephadex G25-F (Pharmacia); Sephacryl S-300 (Pharmacia); Blue Sepharose CL-6B (Pharmacia); N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP;

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Serva); ricin (Lektinotest Company, Lvov), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Serva); dithiothreitol (Serva); and several Russian-made reagents of analytical grade.

IgG3 MAb of the ICO-104 class was isolated by concentration, on an Amicon PM-10 membrane, of the culture supernatant after separating hybridoma cells and by separation of low-molecular components of the medium in an Acrylex P-10 column in 0.075 M sodium-phosphate buffer, pH 7.4, followed by negative sorption on diethylaminoethyl cellulose in the same buffer.

Synthesis of ricin 2-pyridyldisulfide. To 9-10 mg of ricin in 0.05 M sodium-borate buffer, pH 7.5, 150 μg of SPDP dissolved in 25 μl of freshly distilled dimethyl formamide were added with stirring, and the mixture was kept for 30 min at room temperature with continuous agitation, care being taken to see that no foam formed. Thereafter, the reaction mixture was separated in a 1.8×22 cm column with Sephadex G25-F equilibrated with 0.1 M phosphate buffer, pH 7.5, to which 0.1 M NaCl, 1 M ethylenediamine tetraacetate (EDTA), and 0.02% sodium azide were added.

Synthesis of ICO-104 IgG3 2-pyridyldisulfide. To 8.6-21 mg of ICO-104 IgG3 in 2-2.5 ml of 0.05 M sodium-borate buffer, pH 7.5, 92-185 μg of SPDP dissolved in 20-30 μl of freshly distilled dimethyl formamide were added, and the mixture was incubated for 30 min at room temperature with stirring. The reaction mixture was then separated in a 1.8×30 cm column with Sephadex G25-F equilibrated with 0.1 M acetate buffer, pH 4.5, to which 0.1 M NaCl, 1 mM EDTA, and 0.02% sodium azide were added.

Reduction of ICO-104 IgG3 2-pyridyldisulfide with dithiothreitol. ICO-104 IgG3 2-pyridyldisulfide in a volume of 20-25 ml was concentrated to a volume of 2.6-6.8 ml on an Amicon PM-10 membrane, dithiothreitol was added, and the mixture was incubated for 30 min at room temperature with stirring. The reaction mixture was then separated in a 1.8×30 cm column with Sephadex G25-F equilibrated with 0.1 mM phosphate buffer (presaturated with nitrogen), pH 7.5, to which 0.1 M NaCl and 1 mM EDTA were added. The ICO-104 IgG3-thiol was collected in a volume of 20-25 ml.

Conjugation. To 2.6-11.6 mg of the ICO-104 IgG3-thiol collected from the column, 1.02-4.66 mg of ricin 2-pyridylsulfide was immediately added, and the mixture was incubated for 18 h at room temperature and then for 24 h at 8°C. The reaction mixture was concentrated on an Amicon PM-10 membrane and separated in a 1.0×90 cm column with Sephacryl S-300 in 0.01 M phosphate buffer, pH 7.5, to which 0.1 M NaCl and 1 mM EDTA were added (Fig. 1).

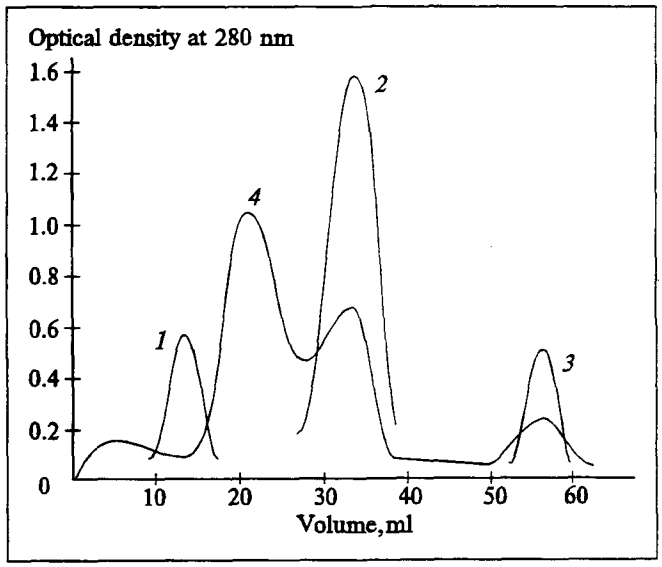

Fig. 1. Sephacryl S−300 chromatography in 0.1 M phosphate buffer, pH 7.5, of the thiol−disulfide exchange reaction products. *1*) blue dextran; *2*) IgG; *3*) ricin; *4*) immunotoxin.

The conjugate was subjected to further purification in a 0.5×90 cm Blue Sepharose CL-6B column as described by Martin *et al.* [8]. The column was pretreated with 1 mg of IgG, and 5.3 mg of the conjugate in 1 ml of 0.05 M phosphate buffer, pH 7.5, were applied to the column. The purified conjugate was eluted in 30 ml with a yield of 26% (Fig. 2).

The number of substituted 2-pyridyldisulfate groups was estimated as described previously [3]. The modified ricin or ICO-104 IgG3 was treated with 25 mM dithiothreitol, pH 4.5, for 30 min at room temperature.

Assays for cytolytic activity. Cytolytic activity of the ICO-104 IgG_3 conjugate was analyzed *in vitro* on the human T-lymphoblastoid cell line Jurkat and the human erythroblastic cell line K-562 using a solid-phase immunochemical assay procedure [9]. Cytolytic efficiency was evaluated from the intensity of staining by adding the vital dye MTT to Jurkat and K-562 cells after their incubation with the conjugate.

Briefly, to each well of a 96-well plate 12.5×10^3 cells in 50 μl of complete medium (RPMI-1640, 4 μM L-glutamine, 4mg/ml gentamicin, and 10% fetal calf serum) were pipetted and the test conjugate preparations were added in dilutions. Lactose was added to the appropriate wells.

As controls, cells in the complete medium and ricin and ICO-104 IgG3 in the complete medium in dilutions were used.

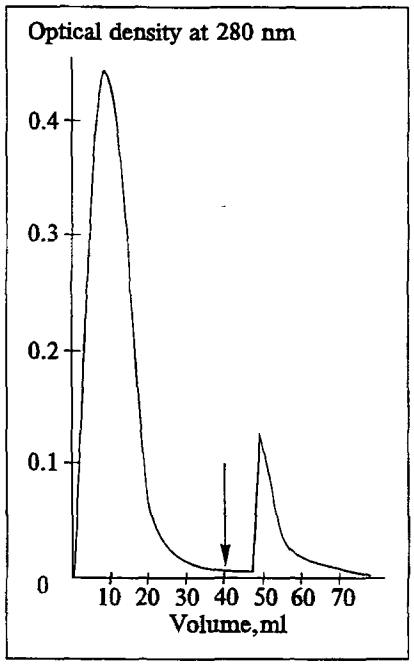

Fig. 2. Blue Sepharose CL-6B chromatography of immunotoxin-containing fractions. The site of immunotoxin recovery is arrowed.

After a 20-h incubation at 37°C, 10 μl of MTT (5 mg/ml in 0.01 M phosphate buffer, pH 7.4) were added to each well, and incubation was continued for 4 h at room temperature. After the incubation, the formazan that had formed during the reaction was extracted with 150 μl of 0.04 N HCl in mixture with isopropanol. Optical density (absorbance) was measured at 570 nm using a Multiscan photometer (Flow).

Cytolytic activity of the conjugate was also determined by counting live cells in a Kvantitsit apparatus designed for intravital cell morphometry at the Kavetskii Institute of Oncology in Ukraine. To the cell of this apparatus, tumor cells in the complete medium ($200\text{-}600\times10^3$ cells/ml) and a conjugate or ricin solution were placed under sterile conditions. In the control tests, tumor cells were incubated in the complete medium. Incubation time was 50 h. Evaluations were made on the basis of the "plateau" phase of the cell cycle.

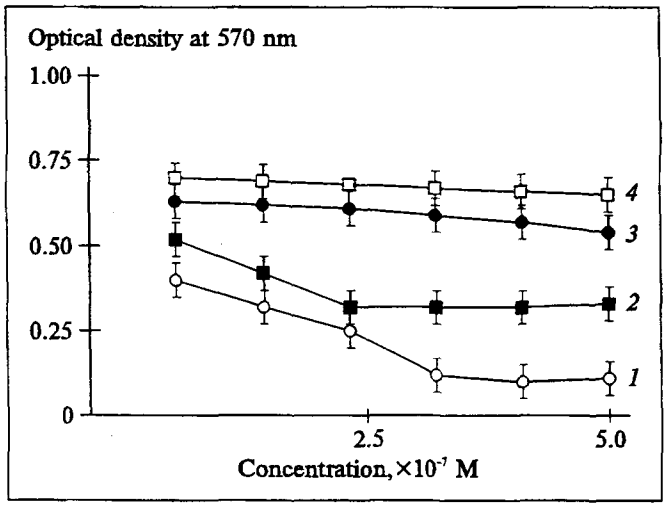

Fig. 3. Inhibition of ribosomal synthesis in Jurkat (*1*, *2*) and K-562 (*3*, *4*) cells as a function of immunotoxin concentration. *2* and *4*) cells were incubated with the immunotoxin in the presence of 50 mM lactose.

RESULTS

Synthesis of the ICO-104 IgG3-ricin conjugate was carried out by the method described by Cumber *et al.* [4]. 2-Pyridyldisulfide groups in the modified ricin molecule combined with thiol groups introduced into the IgG3 through pyridyldithiolation and subsequent reduction with dithiothreitol. Incubation of the proteins thus modified under mild conditions results in a conjugate in which the proteins are linked up by a disulfide bridge. The conjugate has the following structure:

IgG-NH-OC-CH_2-CH_2-S-S-CH_2-CH_2-CO-NH-ricin

The ICO-104 IgG3 and ricin were modified by the introduction of 2-pyridyldisulfide groups in an excess of SPDP. The reaction products were separated in a Sephadex G25-F column. At a ricin concentration of $1.5\text{-}1.6\times10^{-4}$ M and a ricin:SPDP ratio of 1:3, one 2-pyridyldisulfide group was introduced per ricin molecule. The recovery of the modified ricin was 70-90%, and there was a small peak of aggregates. At lower ricin concentrations in the reaction medium and the same ricin:SPDP ratio, the yield of modified ricin decreased to 55% and the peak of aggregates was larger.

The ICO-104 IgG3 was reacted with SPDP in concentrations of $0.25\text{-}0.68\times10^{-4}$ and IgG3:SPDP ratios of 1:9.5, 1:5.2, and 1:4.3. In all cases, three 2-pyridyldisulfide molecules were introduced per ICO-104 IgG3 molecule. A 95% recovery of the modified ICO-104 IgG3 was obtained when its concentration in the reaction mixture was 0.68×10^{-4} M and the IgG3:SPDP ratio was 1:4.3. The aggregation was then minimal.

The ICO-104 IgG3 2-pyridyldisulfide was reduced at concentrations of $0.68\text{-}3.38\times10^{-4}$ M in the presence of 17-42 mM dithiothreitol. The reduction of 2-pyridyldisulfide to thiol with a 94% yield of the final product was achieved at an ICO-104 IgG3 2-pyridyldisulfide concentration of 0.68×10^{-4} M in the presence of 17 mM dithiothreitol.

The reaction mixture contained polymeric conjugates and unreacted IgG3 and ricin in addition to the ICO-104 IgG3-ricin conjugate. The aggregated polymeric IgG3 and the unreacted ricin were readily separable in a Sephacryl S-300 column (Fig. 1). However, complete separation of the conjugate from

the unreacted IgG3 could not be achieved with Sephacryl S-300, and for this reason a stage of chromatography with Blue Sepharose CL-6B of the fractions corresponding to the conjugates was introduced. Separation of the conjugate and IgG3 is based on the property of the ricin A chain to bind to the dye F3GA Blue [7]. With Blue Sepharose CL-6B chromatography, the conjugate was separated from the unreacted IgG3 which does not combine with the F3GA Blue dye. Elution of the conjugates from Blue Sepharose CL-6B was effected with 0.5 M NaCl. The yield of purified conjugate was 26% with respect to the applied protein. Analysis of SDS electrophoresis in a 5-20% polyacrylamide gel gradient indicated the presence in the Blue Sepharose CL-6B-purified preparation of a single band corresponding in molecular weight to a hybrid molecule with a 1:1 IgG3 to ricin ratio in the complete absence of original components participating in the reaction of thiol-disulfide exchange.

The purified conjugate was stored, after passage through a 0.22 μ Millipore membrane, at -70°C for 2 months without noticeable loss of activity.

Cytolytic activity of the synthesized immunotoxin was evaluated in a solid-phase immunochemical assay on 96-well plates using the human T-cell line Jurkat (CD5-positive) and the human erythroblastic cell line K-562 (CD5-negative). The appropriate cell concentration for the assay was found (1.25×10^3 cells/ml), and the degree of ribosomal synthesis inhibition at different immunotoxin concentrations ($5\text{-}50\times10^{-7}$ M) was determined. CD5-negative K-562 cells were used for comparison. Parallel tests for ribosomal synthesis inhibition were run in the presence of lactose which, as shown previously, blocks binding sites of the lectin moiety of ricin (B chain).

Selectivity of the immunotoxin was assayed by the difference between its LD_{50} for cells expressing (Jurkat) and not expressing (K-562) the CD5 antigen. For Jurkat cells the LD_{50} was 0.6×10^{-7} M, whereas for K-562 cells no 50% inhibition of ribosomal synthesis could be achieved even at 10 times higher concentrations.

It should be noted that lactose safeguards both Jurkat and K-562 cells from the toxic action of the immunotoxin (Fig. 3). A similar finding was made in another study [2].

Cytolytic activity of the immunotoxin was also evaluated by direct enumeration of live cells using the Kvantitsit apparatus designed for intravital cell morphometry. Jurkat cells were incubated with various immunotoxin concentrations and 50% survival was observed at a concentration of 0.3×10^{-7} M.

The most important characteristic of an immunotoxin is its concentration at which ribosomal synthesis in the target cells is inhibited by 50%. For the immunotoxin synthesized by us on the basis of a novel MAb (ICO-104 IgG3), similar LD_{50} values have thus been obtained by two independent methods.

REFERENCES

1. A. Zh. Kashaeva, A. Yu. Baryshnikov, G. V. Vikha, *et al.*, *Biotekhnologiya*, № 11-12, 17-23 (1993).
2. A. G. Tonevitskii, A. Yu. Toptygin, W. Marx, *et al.*, *Dokl. Akad. Nauk SSSR*, **307**, № 6, 1507-1511 (1985).
3. J. Carlsson, H. Drevin, and R. Axen, *Biochem. J.*, **173**, 723-737 (1978).
4. J. A. Cumber, A. J. Forrester, and B. M. J. Foxwell, *Methods Enzymol.*, **112**, 207-225 (1985).
5. W. Jiskoot, M. Bloemendal, B. Haeringen, *et al.*, *Europ. J. Biochem.*, **201**, 223-227 (1991).
6. W. Jiskoot, A.-M. V. Hoven, A. A. M. De Koning, *et al.*, *J. Immunol. Methods*, **138**, 181-189 (1991).
7. P. P. Knowles and P. E. Thorpe, *Analyt. Biochem.*, **160**, 440-443 (1987).
8. P. J. Martin, E. R. Giblett, and J. A. Hansen, *Immunogenetics*, **15**, № 2, 385-388 (1982).
9. T. Mosmann and T. A. T. Fong, *J. Immunol. Methods*, **65**, 55-57 (1983).