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CYTOTOXICITY OF A HYBRID PREPARED BY COUPLING DIPHTHERIA TOXIN A-CHAIN WITH IMMUNOGLOBULIN Fab' WITH N,N'-o-PHENYLENEDIMALEIMIDE

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SUMMARY

A hybrid protein was prepared by coupling the A-chain of diphtheria toxin with the Fab' fragment of immunoglobulin with N,N'-o-phenylenedimaleimide (PDM). Although in this hybrid, the two components were linked with each other with bonds which could not be reductively cleaved with 2-mercaptoethanol as in a hybrid cross-linked with a disulfide bond (e.g. Fab'-S-S-A-chain), it exhibited a potent cytotoxicity in vitro, one-third of that of Fab'-S-S-A-chain, against the target L1210 cells.

INTRODUCTION

As an approach to the development of new antitumor agents of a selective cytotoxicity, we previously prepared a hybrid protein by covalently coupling A-chain of diphtheria toxin, the intracellularly active enzymic portion of the toxin molecule with the antigen-binding site-containing Fab' fragment of an immunoglobulin via a disulfide bond and showed that the hybrid (Fab'-S-S-A-chain) exhibited a potent in vitro cytotoxic activity against its target cells through binding of its Fab' moiety to the cell-surface antigens (1). The same type of hybrid protein was also prepared using ricin A-chain as the toxic component by us (2) and Raso and Griffin (3). Diphtheria toxin or ricin A-chain hybrids prepared by coupling it with immunoglobulins (4-6), a lectin (7), or a peptide hormone (8) were also studied in recent years by several

Abbreviations used: DNP = 2,4-dinitrophenol; EDTA = ethylenediaminetetraacetic acid; PBS = phosphate buffered saline; PDM = N,N'-o-phenylenedimaleimide; SDS-PAGE = sodium dodecylsufate-polyacrylamide gel electrophoresis.

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other laboratories. In all of these previously prepared hybrids, the homing component and the toxic component were coupled by a disulfide bond, and no studies have been reported concerning the hybrids in which the two components are linked together by bond(s) other than the disulfide bond. We now wish to report the preparation of a hybrid by combining the A-chain of diphtheria toxin with the Fab' fragment of rabbit anti-murine leukemia L1210 IgG with N,N'-o-phenylenedimaleimide (PDM) and its cytotoxicity exhibited against L1210 cells. This is the first case ever reported that a toxin A-chain hybrid with antibody, an artificial toxin, from which A-chain cannot be reductively liberated exhibits a potent cytotoxicity.

MATERIALS AND METHODS

Materials. Diphtheria toxin was obtained from a culture broth of Corynebacterium diphtheriae PW8 according to the procedure of Collier et al. (9). Guinea pig antiserum against diphtheria toxin A-chain was obtained by the standard procedure employing A-chain emulsified in complete Freund's adjuvant for immunization. Goat antiserum against rabbit IgG was purchased from Seikagaku Kogyo, Tokyo; culture medium RPMI1640 from Nissui Seiyaku, Tokyo; fetal calf serum from Gibco, Grand Island; kanamycin sulfate from Banyu Pharmaceutical, Tokyo; and PDM from Nakarai Chemicals, Kyoto.

L1210 cells. Murine leukemia L1210 cells were kindly provided by Dr. T. Kataoka, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo and maintained serially by intraperitoneal passage at weekly intervals of 1×10^5 cells in a DBA/2 mouse (purchased from Charles River Japan, Atsugi). For cytotoxicity test, five days after inoculation, ascites L1210 cells were collected in phosphate buffered saline (PBS) and washed three times with the culture medium before use.

The Fab' fragment (Fab'-SH), the S-sulfonated diphtheria toxin A-chain (A-chain-SSO_3^-), and the reduced form of A-chain (A-chain-SH). Fab'-SH and A-chain-SSO_3^- were prepared as previously described (1,2). Briefly, Fab'-SH of anti-L1210 IgG was prepared by pepsin digestion and 2-mercaptoethanol treatment of the IgG fraction of antiserum obtained from a rabbit immunized with L1210 cells. Fab'-SH of anti-2,4-dinitrophenol (DNP) IgG was similarly prepared from affinity-purified, rabbit pure antibody against DNP. A-chain-SSO_3^- was prepared by treatment of diphtheria toxin with trypsin (9) followed by sulfitolysis. A-chain-SSO_3^- in 0.1 M Tris·HCl, pH 8.4 (5 mg protein/ml) was reduced with 10 mM 2-mercaptoethanol at 37°C for 1 h and subjected to Sephadex G-25 gel filtration in 5 mM CH_3COONa-0.14 M NaCl-1 mM ethylenediaminetetraacetic acid (EDTA), pH 5.5 (buffer A) to give A-chain-SH.

The Fab'-S-S-A-chain hybrid. This type of hybrid was prepared as previously described by the reaction of Fab'-SH with A-chain-SSO_3^- in 1:1 molar ratio followed by Sephadex G-150 superfine column chromatography (1).

The Fab'-PDM-A-chain hybrid. The hybrid cross-linked with PDM was prepared by a method similar to that used to couple β-D-galactosidase with IgG by Kato et al. (10). The reaction mixture resulting from the reduction (1,2) of $F(ab')_2$ with 2-mercaptoethanol was subjected to Sephadex G-25 gel filtration (0.8×43 cm) in buffer A. One ml of Fab'-SH in buffer A (7.4 mg protein/

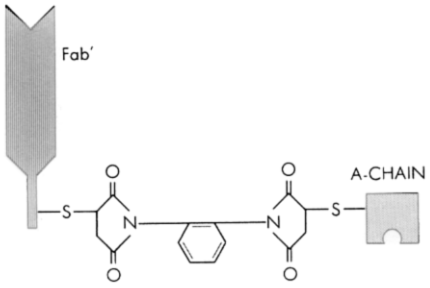

Fig. 1 Structure of Fab'-PDM-A-chain.

ml) thus obtained was treated with 0.05 ml of 20 mM PDM in N,N-dimethylformamide at 30°C for 30 min, and the mixture was subjected to the above Sephadex G-25 gel filtration to give Fab' having one maleimido group, Fab'-PDM. To 1.0 ml of Fab'-PDM thus obtained (4.3 mg protein/ml) were added 1.0 ml of A-chain-SH in buffer A (2.2 mg protein/ml) and 0.2 ml of 0.3 M sodium phosphate buffer, pH 6.5, and the reaction was allowed to proceed at 4°C overnight. The mixture was chromatographed on Sephadex G-150 superfine (1.6×93 cm) in 0.9% NaCl to give the pure hybrid Fab'-PDM-A-chain.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed in 5% gel according to the procedure of Weber and Osborn (11). Protein concentration of bands was monitored by scanning of the coomassie brilliant blue-stained gels at 552 nm (reference at 700 nm) in a dual wavelength TLC scanner (Shimazu CS-900).

Cytotoxicity assay of the hybrids. The assay was carried out as previously described (1,2). Namely, serially diluted hybrids were added to culture medium (RPMI1640-10% fetal calf serum-0.02 mM 2-mercaptoethanol-0.1 mg of kanamycin sulfate per ml) containing L1210 cells (2×10^4/ml), and after incubation in a humidified atmosphere of 5% CO_2 in air at 37°C for 72 h, the number of viable cells was determined by counting undyed cells after addition of one-tenth volume of 3% trypan blue in PBS.

RESULTS AND DISCUSSION

The Fab' fragment (Fab'-SH) of rabbit anti-murine leukemia L1210 IgG and the A-chain of diphtheria toxin (A-chain-SH) were coupled with PDM at the sulfhydryl groups of the cystein residues of the two components (Fig. 1). Treatment of Fab'-SH with excess PDM followed by removal of the unreacted PDM afforded Fab'-PDM resulting from the reaction of Fab'-SH with one of the two maleimido groups of PDM. Then, Fab'-PDM was treated with an equimolar amount of A-chain, and the mixture was chromatographed on Sephadex G-150 superfine to give three protein peaks, I, II, and III (Fig. 2). In Ouchterlony tests, the peak I protein formed a precipitin line with both goat anti-rabbit IgG and guinea pig anti-A-chain antisera, the peak II protein only with anti-IgG

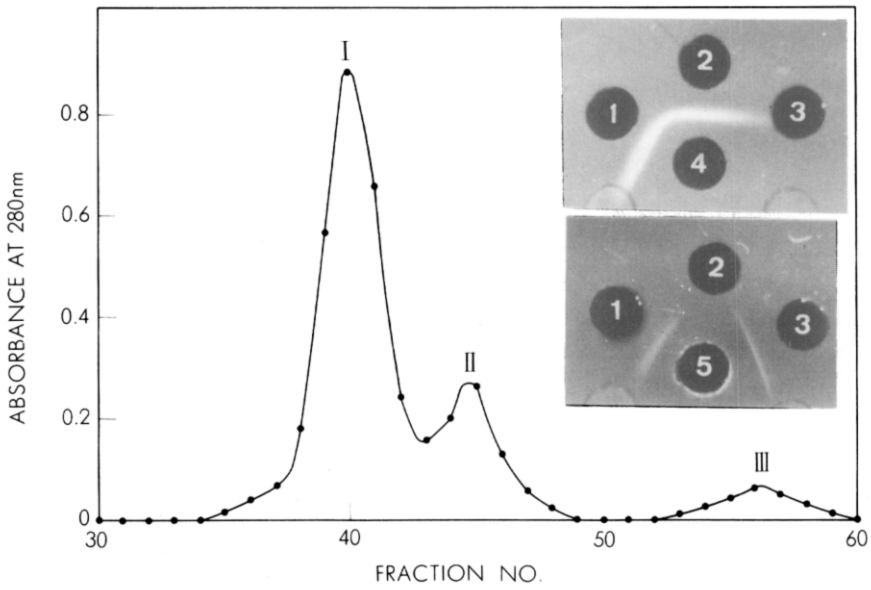

Fig. 2 Elution profile on Sephadex G-150 superfine chromatography of a reaction mixture of Fab'-PDM and A-chain-SH together with analysis of each protein peak by the Ouchterlony method. The protein concentration of each fraction was measured by absorption at 280 nm. The proteins of peaks I, II, and III in the wells 1, 2, and 3, respectively were tested against goat anti-rabbit IgG antiserum (well 4) and guinea-pig anti-A-chain antiserum (well 5).

antiserum, and the peak III only with anti-A-chain antiserum. In addition, the peak I protein migrated to the position of molecular weight 70,000 (Fig. 3b). This value is equal to the sum of the molecular weights of Fab' and A-chain (46,000 and 24,000, respectively). Although Fab'-S-S-A-chain, when treated with 2-mercaptoethanol, was cleaved to Fab' and A-chain as determined by SDS-PAGE (Fig. 3c), the peak I protein was not: although analysis of the peak I protein by SDS-PAGE after treatment with 2-mercaptoethanol detected a minute amount of materials at the positions of Fab' and A-chain, they were only 3.3% of the total (Fig. 3d). Therefore, the peak I protein is the Fab'-PDM-A-chain hybrid, and its yield from Fab'-PDM was 77%.

The cytotoxicity of the hybrid proteins against L1210 cells was studied by cell culture with hybrids added to the medium (Fig. 4). Although Fab' (anti-DNP)-PDM-A-chain showed no effect on the cell growth even at its high concentration, 30 μg/ml, Fab'(anti-L1210)-PDM-A-chain exhibited a potent

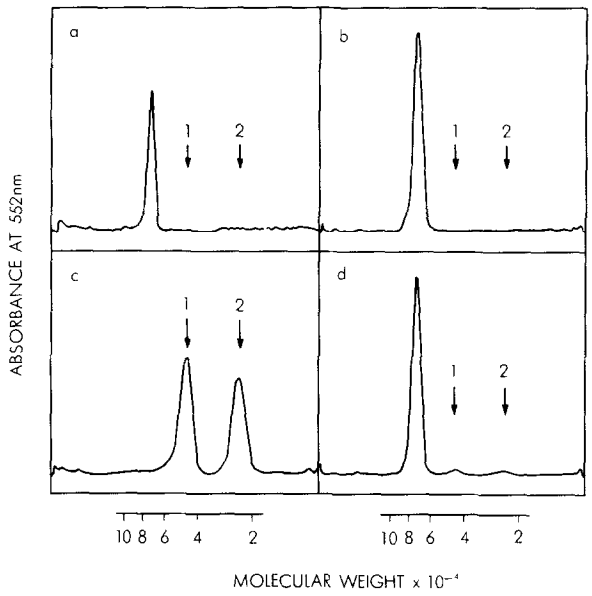

Fig. 3 Analysis by SDS-PAGE. a, Fab'-S-S-A-chain; b, Fab'-PDM-A-chain; c, reduced Fab'-S-S-A-chain; d, reduced Fab'-PDM-A-chain. The arrows 1 and 2 indicate the positions of Fab' and A-chain, respectively. Treatment of Fab'-S-S-A-chain and Fab'-PDM-A-chain with 2-mercaptoethanol was performed by incubating 60 µl of a hybrid solution in 0.083 M Tris·HCl-0.14 M NaCl, pH 8.2 (0.5 or 1 mg protein/ml) with 2-mercaptoethanol (final 2 mM) at 37°C for 1 h, and the mixture was, after treatment with excess iodoacetamide, subjected to electrophoresis.

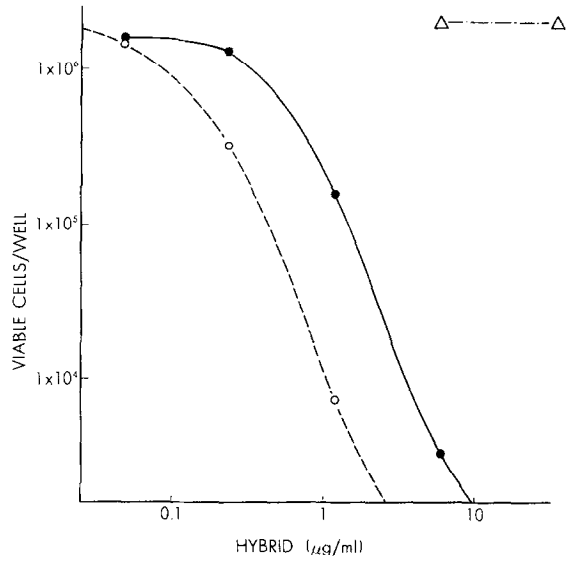

Fig. 4 Cytotoxicity of the hybrids against L1210 cells. Fab'(anti-L1210)-PDM-A-chain (●), Fab'(anti-DNP)-PDM-A-chain (Δ), and Fab'(anti-L1210)-S-S-A-chain (o).

cytotoxic activity against L1210 cells and decreased the number of the viable cells to less than 10^3/ml at concentrations above 10 μg/ml. This magnitude of activity was one-third of that of Fab'—S—S—A-chain. Previously we showed that an equimolar mixture of Fab' and A-chain exerted no effect on the cell proliferation (1). Therefore the cytotoxic activity of the Fab'(anti-L1210)—PDM—A-chain hybrid was manifested through the carrier effect of the Fab' moiety.

In natural toxins such as ricin (12), abrin (13), modeccin (14), and diphtheria toxin (15), their carrier moiety B and enzymic moiety A are linked with each other by a disulfide bond (in diphtheria toxin the two are sometimes bound together also by a peptide bond). In a cell-free system, the A moiety of diphtheria toxin did not exhibit its enzymatic activity when linked to the carrier B moiety, but manifested the activity when tested after liberated from the carrier moiety (15). A similar observation has also been made with respect to ricin (16). Therefore, the disulfide bond cross-linking the two moieties should be cleaved, for these toxins to inhibit protein synthesis enzymatically within the cell after the binding of the carrier moiety to the cell surface. It was contrary to our expectation that Fab'(anti-L1210)—PDM—A-chain from which A-chain could not be reductively liberated exhibited a potent cytotoxicity. The cystein residue of Fab' employed for the cross-linking is situated in the hinge region which is susceptible to cleavage by lysosomal proteases (17). The cytotoxicity of Fab'(anti-L1210)—PDM—A-chain may have been manifested by A-chain liberated by the cleavage in the hinge region of Fab' by a cell protease after binding of the hybrid to the cell surface.

As a next study for the application of toxin A-chain hybrids with antibody for antitumor agents, we plan to investigate which of the two types of hybrids, Fab'—PDM—A-chain or Fab'—S—S—A-chain, shows a larger *in vivo* antitumor effect.

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