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PREPARATION OF A HYBRID OF FRAGMENT Fab' OF ANTIBODY AND FRAGMENT A OF DIPHTHERIA TOXIN AND ITS CYTOTOXICITY

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SUMMARY

The fragment A of diphtheria toxin was linked to the fragment Fab' of Ig from rabbit antiserum against L1210 cells by the reaction of Fab' with the S-sulfonated fragment A and purified by chromatography on Sephadex G 150. The hybrid thus prepared showed a high cytotoxicity *in vitro* to L1210 cells which are insensitive to diphtheria toxin itself.

Most antitumor agents exert cytotoxic action not only on tumor cells but also on proliferating normal cells, severely limiting their therapeutic usefulness. In order to circumvent this there have been several attempts (1-4) to obtain antitumor agents of selective toxicity by conjugating diphtheria toxin to antitumor antibodies. The extraordinary potency of diphtheria toxin should be beneficial because of the general paucity of the target antigens on the tumor cells. In these attempts, however, the antibody-toxin conjugates were prepared by using glutaraldehyde (3), toluene diisocyanate (1), diethyl malonimidate dihydrochloride (2), or chlorambucil (4) as the coupling agent. These agents have a strong tendency to cause intramolecular linking and/or linking between the molecules of one species as well as the desired antibody-toxin coupling. Moreover, since the whole molecule of diphtheria toxin has so far been coupled with antibody, part of its own ability to bind to toxin-sensitive cells are brought into the conjugate diminishing the cellular selectivity.

Diphtheria toxin are composed of two functionally different moieties, A and B (5). The B moiety binds to a cell-surface receptor enabling at least

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the A moiety to enter the cell, where it terminates protein synthesis by enzymatic inactivation of elongation factor 2 (EF-2) (6,7). Therefore, combining covalently antitumor antibodies only with the A moiety (fragment A) might afford conjugates which destroy tumor cells more selectively. On the other hand, since it is the Fab moiety of antibody that combines antigen, the Fab portion might suffice as the carrier ligand to be conjugated to cytotoxin. On the basis of the above consideration, we have prepared a novel hybrid molecule by combining fragment Fab' of rabbit anti-mouse leukemia L1210 antibody with fragment A of diphtheria toxin by a disulfide bond and examined the cytotoxicity of the hybrid to L1210 cells.

MATERIALS AND METHODS

Materials. Diphtheria toxin (a culture broth) and L1210 cells were generous gifts from Dr. N. Ohtomo, Chemo-Sero-Therapeutic Institute, Kumamoto and Dr. T. Kataoka, Cancer Chemotherapy Center, Japan Foundation for Cancer Research, Tokyo, respectively. Radioactive nicotinamide [U-^{14}C]adenine dinucleotide was purchased from Radiochemical Centre, Amersham; cold NAD from Nakarai Chemicals; goat antiserum against rabbit IgG from Seikagaku Kogyo; medium RPMI1640 from Nissui Seiyaku; Kanamycin sulfate from Banyu Pharmaceutical; and fetal calf serum from Gigco.

Fragment Fab' having one sulfhydryl group (Fab'-SH) of IgG from rabbit antiserum against L1210. Antiserum against mouse leukemia L1210 cells was produced in rabbits by 1 i.v. and then 3 s.c. injections of 10^6 leukemia cells emulsified in complete Freund's adjuvant at 7-day intervals. The precipiate obtained by adding ammonium sulfate (final 50% saturation) was dialyzed against 0.01 M phosphate buffer (pH 7.6) and chromatographed through DEAE cellulose equilibrated with the same buffer to afford IgG fractions. IgG was digested with pepsin (8) to give the $F(ab')_2$ fragment. $F(ab)_2$ (14.6 mg) in 2.0 ml of 0.01 M Tris·HCl—0.14 NaCl—2 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.9) was reduced with 2 mM 2-mercaptoethanol at 37°C for 1 h and throughly dialyzed against 5 mM CH_3CO_2Na—0.14 M NaCl (pH 5.6) to give Fab'-SH.

S-Sulfonated fragment A (Fragment A-SSO_3^-) of diphtheria toxin. Diphtheria toxin was obtained from a culture broth of Corynebacterium diphtheriae PW8 and scissored with trypsin to nicked toxin according to the procedure described by Collier et al. (9). To the solution of the nicked toxin thus obtained was added urea (final 6 M), Na_2SO_3 (final 0.168 M), and $Na_2S_4O_6$ (final 0.042 M), and the mixture was incubated at 37°C for 2 h. The resulting mixture was chromatographed on Sephadex G150 equilibrated with 0.03 M sodium acetate (pH 5.3) containing 6 M urea, and the fractions of Fragment A-SSO_3^- were collected and dialyzed against distilled water.

Hybrid of fragment Fab' of antibody and fragment A of diphtheria toxin (Fab'-S-S-Fragment A). One ml of 7.3 mg per ml of Fab' in 5 mM CH_3CO_2Na—0.14 M NaCl was mixed with 1.5 ml of 2.7 mg per ml of Fragment A-SSO_3^- in saline, and the mixture was dialyzed against 1 ℓ of 0.05 M glycine buffer-0.10 M NaCl-2 mM EDTA (pH 9.2) at 4°C for 72 h. The reaction mixture was chromatographed on Sephadex G 150 (superfine) in saline.

RESULTS AND DISUSSION

The fragment $F(ab')_2$ of IgG from rabbit antiserum against Ll210 cells was reductively cleaved only at the intra-heavy-chain disulfide bond with 2-mercaptoethanol to afford fragment Fab' having one sulfhydryl group (Fab'-SH). On the other hand, diphtheria toxin was scissored with trypsin to nicked toxin (9), which was treated with Na_2SO_3-$Na_2S_4O_6$ to give S-sulfonated fragment A (Fragment A-SSO_3^-) (6). Fab'-SH was sllowed to react with Fragment A-SSO_3^- under dialysis. The dialysis was performed to facilitate the reaction by eliminating the generated sulfite ion from the reaction mixture. The mixture was chromatographed on Sephadex G 150 to give four major protein peaks (Fig. 1). Fab' and fragment A in each fraction were analyzed by single radial

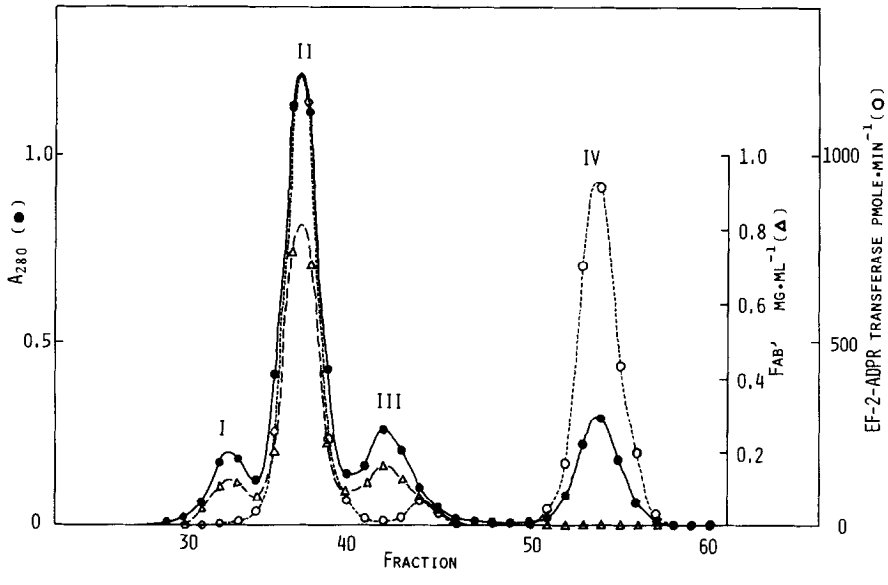

Fig. 1 Sephadex G 150 column chromatography of the products of reaction between Fab'-SH and Fragment A-SSO_3^-. The reaction mixture was chromatographed on Sephadex G 150 (superfine) (1.6 cm × 93 cm) in saline. The protein concentration of each fraction was measured by absorption at 280 nm (-●-). EF-2-ADPR transferase activity (-o-) was determined by the method of Honjo (7) as follows. EF-2 (80 pmol), NAD-(ademine)-^{14}C (45 pmol, 6550 cpm), and an aliquot of each fraction were incubated in 0.1 ml of 0.1 M Tris·HCl—20 mM dithiothreitol—2 mM EDTA at 37°C for 10 min. Protein was precipitated with 5% trichloroacetic acid and collected on a filter (Millipore HAWP 02500), and the radioactivity was measured. The content of rabbit Fab' (-Δ-) was determined as follows; to an aliquot of each fraction was added successively dithiothreitol (final 5 mM) and iodoacetamide (final 30 mM), the resulting solution was incubated for 48 h in a well on agarose plate containing 10% goat antiserum against rabbit IgG, and the diameter of the precipitation ring was measured.

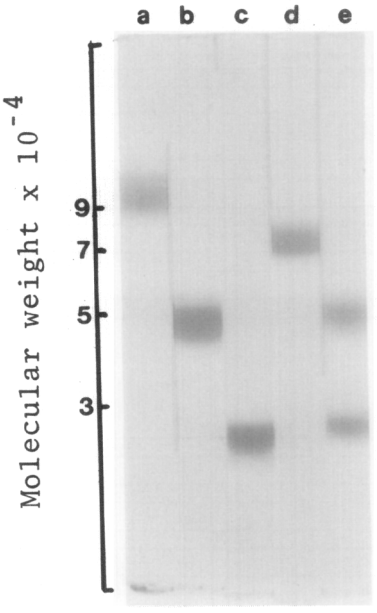

Fig. 2 Analysis by SDS-polyacrylamide gel electrophoresis. The electrophoresis was performed in 6% gel according to the procedure of Weber and Osborn (13). a, $F(ab')_2$; b, Fab'; c, fragment A; d, fractions 37 and 38 (peak II) of the Sephadex G 150 chromatography; e, protein of peak II reduced in 0.1 M Tris·HCl (pH 7.9) with 1 mM 2-mercaptoethanol at 37°C for 1 h followed by S-alkylation with 10 mM iodoacetamide. A linear relationship was observed between the log values of the molecular weights (5,8) of $F(ab')_2$, Fab', and fragment A (92,000, 46,000, and 24,000, respectively) and their mobilities.

immunodifusion using anti-rabbit IgG serum and by measurement of EF-2-adenosine diphosphate ribose (ADPR) transferase activity, respectively. An estimation was made on the contents of each of the four protein peaks from the above analyses and the known molecular weights of the components: peak I, $F(ab')_2$; peak II, the hybrid; peak III, Fab' and a small amount of fragment A dimer; and peak IV, fragment A. Peak II was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis to give only one band with the molecular weight of 7.2×10^4 as determined by its mobility relative to those of the marker proteins $F(ab')_2$, Fab', and fragment A (Fig. 2). Treatment of the substance of peak II with 2-mercaptoethanol gave Fab' and fragment A as judged by SDS-polyacrylamide gel electrophoresis. Based on the above data, it was concluded that the substance of peak II was the hybrid

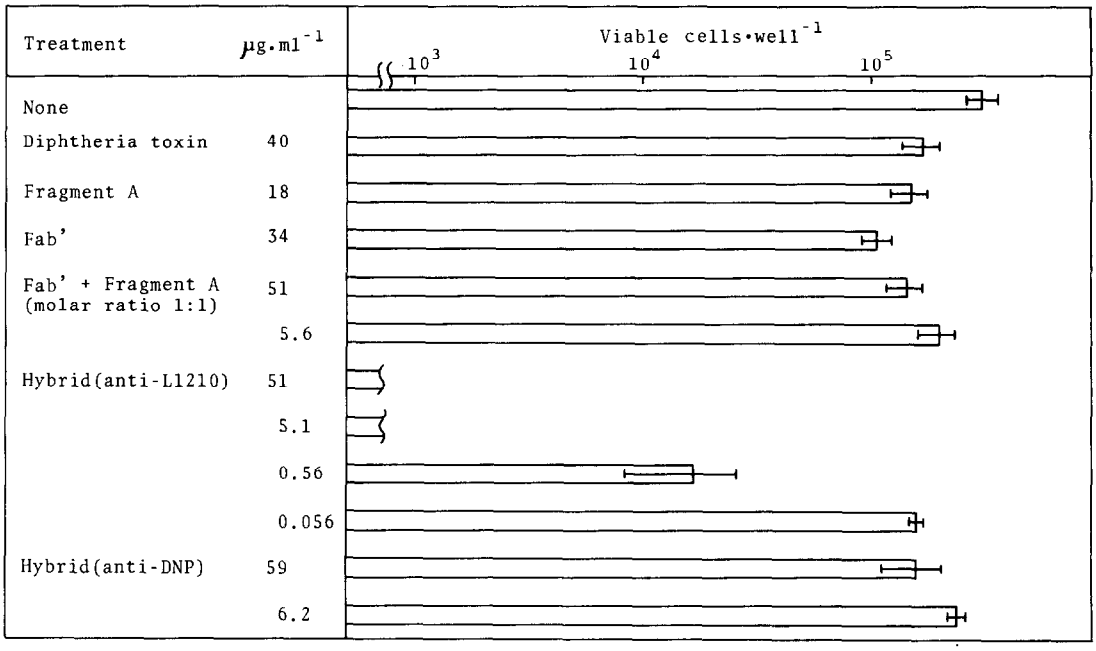

Fig. 3 Cytotoxicity of the hybrid Fab'-S-S-Fragment A against L1210 cells. L1210 cells were collected from ascites of a tumor-bearing CDF_1 mouse, and used after washing with the culture medium. In a multi-well plate (each well area 2.0 cm^2, Linbro) was placed 1.4 ml of RPMI1640—10% fetal calf serum—20 μM 2-mercaptoethanol—100 μg of kanamycin sulfate per ml, 0.1 ml of the cell suspension (2×10^5 cells/ml) and test samples of various concentrations. Beforehand the samples were dialyzed against saline, and the thiol group of Fab' was blocked with iodoacetamide. After 48-h incubation in a humidified atmosphere of 5% CO_2 in air, the cell suspension was centrifuged at 3000 rpm for 5 min. The cell pellets obtained were suspended in 0.5 or 2 ml of 0.3% trypan blue in phosphate buffered saline, and the cells which had not been dyed were counted. The bars and the lines indicate means and standard deviations of triplicate determinations. The hybrid directed against DNP was prepared similarly as that against L1210 from antibody purified by affinity chromatography (14) on 2,4,6-trinitrophenylated serum albumin-Sepharose 4B.

generated by the coupling of one molecule of Fab' and one molecule of fragment A *via* a disulfide bond (Fab'-S-S-Fragment A). The yield of the hybrid was approximately 4.5 and 52 times as large as those of $F(ab')_2$ and fragment A dimer, respectively, indicating that the disulfide bond was formed preferentially between Fab' and fragment A.

In order to examine the cytotoxicity of the hybrid, L1210 cells were incubated with the hybrid, its components, or intact toxin. After the 48-h incubation, the viable cells were counted by die exclusion with trypan blue (Fig. 3). When the concentration of the added hybrid was 51 μg/ml, the number

of the viable cells was almost constant over the first 10-h period and then decreased rapidly to become less than 1×10^3 (0.35% of the control) at the assay. At the lower concentration of the hybrid the lag time for the appearance of the cytotoxicity became longer, but the number of the viable cells was only 1.7×10^4 (5.5%) even at 0.56 μg of the hybrid per ml. This marked cytotoxicity was neitheir observed for Fab' nor fragment A. Moreover, the equimilar mixture of Fab' and fragment A did not affect the cell viability substantially. This indicates that the cytotoxicity of the hybrid is not due to the synergism of Fab' and fragment A. The 2,4-dinitrophenol (DNP)-specific hybrid, prepared similarly from rabbit anti-DNP antibody and fragment A of diphtheria toxin, affected the proliferation of L1210 cells only to a small extent, even at a high hybrid concentration, 59 μg/ml. Therefore, the cytotoxicity of the hybrid of fragment Fab' of the antibody and fragment A of diphtheria toxin has a clear immunological specificity.

L1210 cells are insensitive to diphtheria toxin probably due to their lack of the receptors for the B moiety of the toxin (10,11). The present study shows that the hybrid having Fab' of an anti-L1210 antibody as the carrier protein replacing the B moiety of the toxin binds to the cell surface antigens to enable fragment A to enter into the cytosol leading to the death of the cells. DePetris and co-workers (12) observed that a conjugate of Fab of an anti-immunoglobulin and ferritin, after diffusion on the lymphocyte surface without forming a patch or a cap, was gradually internalized as pinocytotic vesicles. Probably our monovalent hybrid enters the cell by the same mechanism. The hybrids prepared from antitumor antibodies and fragment A of diphtheria toxin may prove useful as antitumor agents of high potency and selectivity.

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