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TARGETING OF THE ANTIVIRAL PROTEIN FROM PHYTOLACCA AMERICANA WITH AN ANTIBODY

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The antiviral protein (PAP) of Phytolacca americana was conjugated with the Fab' fragment of IgG from a rabbit antiserum against murine leukemia L1210 cells via a disulfide bond employing N-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) as the coupling agent. The conjugate showed a potent in vitro cytotoxicity against L1210 cells which was competitively blocked by $F(ab')_2$ directed against L1210 cells. PAP itself did not exhibit the cytotoxicity at the concentration corresponding to the PAP content in the conjugate concurrently tested.

INTRODUCTION

It is known that Phytolacca americana (pokeweed) contains an antiviral protein (1). This protein (PAP) has an action of inhibiting the mechanical transmission of RNA viruses to other plant hosts (2,3) and was purified as a single peptide chain of a molecular weight of 27,000 dalton by Irvin (4). Although the mechanism of the antiviral activity has not yet been throughly elucidated, PAP has an activity of terminating protein systhesis in a cell-free system (4,5), and a suggestion has been made that PAP carried into infected cells inhibits host and viral protein syntheses there (6).

In an approach to develop a highly selective antitumor agent, we previously prepared a conjugate by coupling intracellularly active, anzymic A-chain of ricin, a plant toxin, with an antibody and demonstrated that the conjugate thus prepared showed a cytotoxic activity against the target cells exhibited through the binding of the antibody portion of the conjugate to the cells (7,8).

Abbreviations used: DTT, dithiothreitol; 2-ME, 2-mercaptoethanol; PAP, Phytolacca americana antiviral protein; PBS, phosphate buffered saline; PDP, 3-(2-pyridyldithio)propionyl group; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SPDP, N-succinimidyl 3-(2-pyridyldithio)-propionate.

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In this paper we report that a conjugate of PAP with the Fab' fragment of an antibody manifests a potent cytotoxicity against the target cells. PAP is a ricin A-chain-like protein devoid of the cell-binding moiety, and there is not any chance of contamination of PAP with a cytotoxic protein, contrary to the case of ricin A-chain whose complete separation from parent cytotoxic ricin is very difficult to accomplish. This is an advantage of using PAP as the toxic component of the antibody conjugates. Antibody–PAP conjugates may not only be potential, highly selective antitumor agents, but also serve as a tool to study the mechanism of the antiviral activity of PAP.

MATERIALS AND METHODS

Materials. The leaves of the plant Phytolacca americana were collected in Hino, Tokyo, Japan in September, 1980. Pepsin from porcine stomach mucosa, hemin, and bovine serum albumin were purchased from Sigma Chemical Co., Saint Louis, Mo.; N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) from Pharmacia Fine Chemicals AB, Uppsala, Sweden; amino acid mixtures for protein synthesis assay, 2-mercaptoethanol (2-ME), dithiothreitol (DTT) and N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid from Nakarai Chemicals Ltd., Kyoto, Japan; ATP, GTP, creatine phosphate, and creatine kinase from rabbit muscle from Boehringer Manheim GmbH, Manheim, Germany; L-[4,5-^{3}H]leucine (60 Ci/mmol) from Radiochemical Centre, Amersham, England; culture medium RPMI1640 from Nissui Seiyaku Co., Tokyo, Japan, fetal calf serum from Grand Island Biological Co., Grand Island, N. Y.; kanamycin sulfate from Banyu Pharmaceutical Co., Tokyo, Japan; Falcon tissue culture plate (96 wells) from Becton, Dickinson and Co., Sunnyvale, Calif.; and Freund's complete adjuvant from Iatron Laboratories, Inc., Tokyo, Japan.

L1210 cells. Murine leukemia L1210 cells were provided by Dr. T. Kataoka, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, Japan and maintained serially by intraperitoneal passage at weekly invervals of 1×10^5 cells in a DBA/2 mouse. Five or 6 days after inoculation, ascites L1210 cells were collected in phosphate buffered saline (PBS) and used for cytotoxicity test or immunization after being washed three times with the culture medium employed or PBS, respectively.

Preparation of 3-(2-pyridyldithio)propionylated PAP (PAP–PDP). PAP was obtained from leaves of Phytolacca americana according to the method of Irvin (4). Namely, homogenized leaves were filtered through cheese cloth. Material obtained as precipitate by adding ammonium sulfate to the filtrate was subjected to DEAE cellulose and then phosphocellulose column chromatographies to give pure PAP which gave one band on sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGA) as shown in Fig 2 (disc 2). To 1.6 ml of a PAP solution in 20 mM Na phosphate buffer–0.14 M NaCl–1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 (3.91 mg protein/ml) was added 0.09 ml of 9 mM ethanolic solution of SPDP, and the reaction was allowed to proceed at room temperature for 30 min. The excess SPDP and generated N-hydroxysuccinimide were removed by gel filtration on Sephadex G-25 (0.8×43 cm) in the same buffer as above to give PAP–PDP. The average number of PDP introduced to one molecule of PAP was determined to be 2 by treating an aliquot of PAP–PDP with 2-ME followed by the measurement of absorbance at 343 nm due to liberated pyridine-2-thione (9).

Preparation of F(ab')$_2$ and Fab'-SH. These fragments of IgG were prepared as previously described (7). Briefly, an antiserum against L1210 cells was produced in rabbits by four weekly subcutaneous injection of 5×10^6

cells emulsified in Freund's complete adjuvant. The IgG fraction of the antiserum obtained by ammonium sulfate precipitation followed by DEAE cellulose column chromatography was treated with pepsin followed by Sephadex G-200 superfine column chromatography to give $F(ab')_2$. The reduction of $F(ab')_2$ with 2 mM 2-ME and dialysis against 5 mM Na acetate buffer—0.14 M NaCl—1 mM EDTA, pH 5.6 afforded Fab'—SH. Non-specific $F(ab')_2$ was similarly prepared from serum from a non-immunized rabbit.

Ricin A-chain. Preparation of ricin from Ricinus communis beans and isolation of its A-chain were performed as described by Olsnes and Pihl (10).

Preparation of the conjugate Fab'—S—S—PAP. To 2.0 ml of PAP—PDP in 20 mM Na phosphate buffer—0.14 M NaCl—1 mM EDTA, pH 7.5 (2.99 mg protein/ml) was added 1.0 ml of Fab'—SH in the same buffer (4.1 mg protein/ml), and the reaction was allowed to proceed at room temperature overnight. The mixture was chromatographed on Sephadex G-150 superfine (1.6×93 cm) in 0.9% NaCl to give the conjugate in which Fab' is linked to PAP via a disulfide bond, Fab'—S—S—PAP.

SDS—PAGE. SDS—PAGE was performed in 5% gel by the method of Weber and Osborn (11) to analyze the conjugate.

Inhibition of protein synthesis in a cell-free system. Rabbit reticulocyte lysate used for the test was prepared according to the method of Pelham and Jackson (12). Serially diluted test samples were added to 50 µl of an assay mixture consisting of the lysate (20 µl), N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (15 mM, pH 7.6), CH_3COOK (0.08 M), $Mg(CH_3COO)_2$ (1 mM), ATP (1 mM), GTP (0.4 mM), creatine phosphate (8 mM), creatine kinase (0.15 mg/ml), hemin (0.02 mM), L-[4,5-^{3}H]leucine (30 µCi/nmol, 5 µCi/ml), and cold amino acid mixture without leucine (3 µM), and the mixture was incubated at 37°C for 3 min. An aliquot (0.04 ml) of each assay mixture was placed on a paper disk (Whatman 3 MM, 2.5 cm), and radioactivity of the fraction insoluble in hot trichloroacetic acid was determined on a liquid scintillation counter (Packard 3255) according to the method of Igarashi, et al. (13).

Cytotoxicity assay of the conjugate. L1210 cells in medium RPMI1640 (2×10^4 cells/ml) containing 10% fetal calf serum, 20 µM 2-ME, kanamycin sulfate (0.1 mg/ml), and a test sample were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C for 48 h, and 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

As the carrier to transfer PAP into the cell we employed the Fab' fragment of IgG from a rabbit antiserum against murine leukemia L1210 cells, and we chose the disulfide bond for conjugating PAP covalently with the Fab', considering that in plant toxins such as ricin (10), abrin (14), and modecin (15), the two subunits, the binding and the toxic subunits, are linked together via a disulfide bond. Mild reduction of $F(ab')_2$ prepared from the rabbit IgG by pepsin cleavage afforded Fab' having one sulfhydryl group (Fab'—SH). On the other hand, the reaction of the lysine residues of PAP with SPDP gave PAP in which, on average, two active disulfide groups were introduced per one molecule of PAP (PAP—PDP). The final conjugation reaction was carried

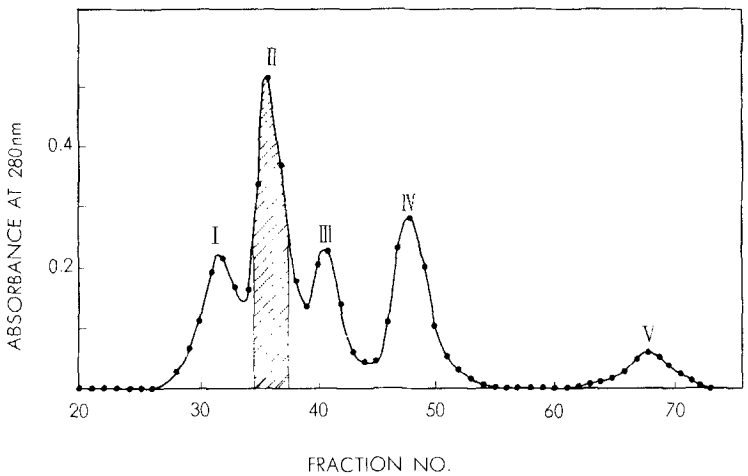
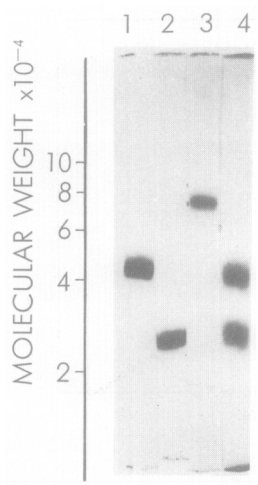


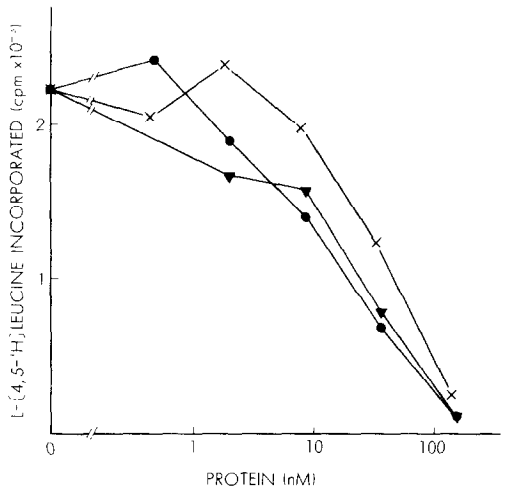
Fig. 1 Elution profile of Sephadex G-150 superfine column chromatography of the reaction mixture of Fab'-SH and PAP-PDP. The protein concentration of each fraction was measured by absorbance at 280 nm. The protein of the shaded area was pooled and employed as the conjugate Fab'-S-S-PAP.

out by mixing Fab'-SH and PAP-PDP in a molar ratio of 1:1. Fig. 1 shows the elution profile of chromatography of the reaction mixture on Sephadex G-150 superfine. There were two protein peaks (peaks I and II) at the positions of molecular weights larger than those of Fab' and PAP whose peaks were supporsed to be peaks III and IV, respectively from the elution volumes--peak V was supporsed to be of pyridine-2-thione generated from the reaction. These peak I and II proteins were analyzed by SDS-PAGE (Fig. 2). The peak II protein, which gave one band at a position of a molecular weight of 75,000 dalton (disc 3), was cleaved to Fab' and PAP on reduction with 2-ME (disc 4). Therefore, this protein proved to be the conjugate Fab'-S-S-PAP generated by the coupling of one molecule of Fab' and one molecule of PAP via a disulfide bond. Protein of the shaded area of Fig. 1 was pooled (40% of the total protein) and employed for the subsequent study. The peak I protein had a molecular weight of ca. 100,000 dalton and was cleaved to Fab' and PAP on reduction with 2-ME (date not shown), indicating that it was a conjugate generated by coupling of one molecule of PAP with two molecule of Fab'.

The inhibitory activities on eukaryotic protein synthesis of PAP and of PAP-SH liberated from Fab'-S-S-PAP with DTT were assayed employing a cell-free system derived from rabbit reticulocytes (Fig. 3). The activity of PAP-SH



(2)



(3)

Fig. 2 Analysis of Fab'—S—S—PAP by SDS—PAGE. a, Fab'—SH; b, PAP; c, peak II protein (Fab'—S—S—PAP) of Fig. 1; d, peak II protein reduced with 2-ME. The reduction was performed by treating the conjugate with 2 mM 2-ME in 0.1 M Tris·HCl, pH 8.3 at 37°C for 1 h, and the reduced proteins were alkylated with 50 mM iodoacetamide at room temperature for 30 min.

Fig. 3 Inhibition of protein synthesis in cell-free system. A lysate from rabbit reticulocytes was used as the cell-free, protein synthesis system. The samples (100 μg protein per ml) were preincubated with 9.1 mM DTT in 20 mM Tris·HCl, pH 8.2 containing 1 μg/ml of bovine serum albumin at 37°C for 30 min. The reticulocyte lysate in an assay mixture containing L-[4,5-^{3}H]-leucine was incubated at 37°C for 3 min with serially diluted test samples, and the incorporated L-[4,5-^{3}H]leucine was determined. ▼, PAP; ●, PAP-SH liberated from Fab'—S—S—PAP with DTT; ×, ricin A-chain.

was of the same magnitude as that of PAP indicating that the introduction of two (on average) N-3-mercaptopropionyl groups into one molecule of PAP did not decrease the activity of PAP, though it was reported that succinylation of the lysine residues of PAP inactivated the protein (1,16).

The cytotoxicity of the conjugate against target cells was determined by cell culture of L1210 cells with the conjugate added to the medium (Fig. 4). Fab' did not affect the cell viability. PAP alone decreased the viability only to a small extent at its high concentration (3×10^{-6} M). Ussery et al. (17) described that PAP did not affect the protein synthesis of cultured HeLa cells even at a concentration of 1×10^{-5} M. Contrary to these observations, Fab'—S—S—PAP exhibited an extremely strong cytotoxicity, and no viable cells were found at a concentration of 1×10^{-7} M. Since the cytotoxicity was not exhibited by an equimolar mixture of Fab' and PAP, the exhibition of a potent cytotoxicity by the conjugate was not due to a

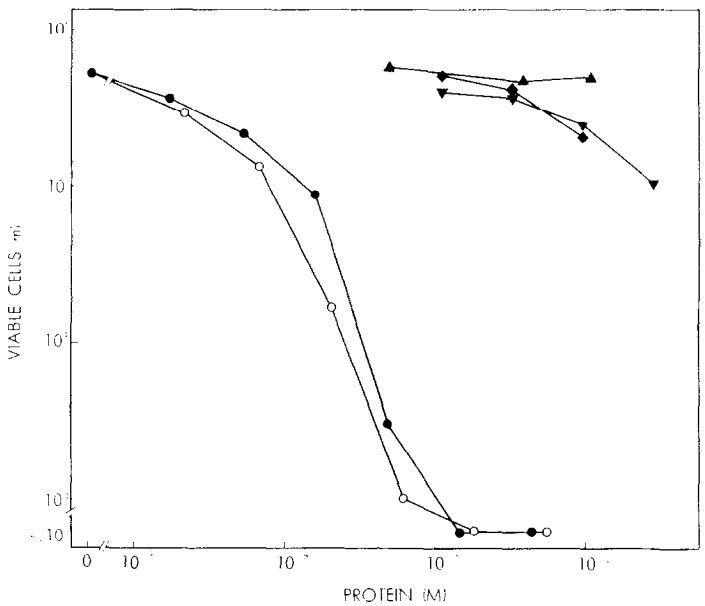


Fig. 4 Cytotoxicity of Fab'—S—S—PAP against L1210 cells. L1210 cells (2×10^4 cells/ml) were cultured at 37°C for 48 h with serially diluted test samples added to the medium, and the numbers of the viable cells were determined by trypan blue dye exclusion method. ●, Fab'—S-S PAP; ▲, Fab'; ▼, PAP; ◆, an equimolar mixture of Fab' and PAP; ○, Fab'—S—S—ricin A-chain.

synergistic effect of the two components but by virtue of the covalent coupling of the two.

It was examined if the cytotoxicity of Fab'—S—S—PAP was competitively inhibited by $F(ab')_2$. L1210 cells were cultured in RPMI1640 medium containing a constant contration (7×10^{-8} M) of Fab'—S—S—PAP together with increasing concentration of $F(ab')_2$ prepared from anti-L1210 IgG or normal mouse IgG, and the cell viability was determined (Fig. 5). Anti-L1210 $F(ab')_2$ clearly inhibited the cytotoxicity of Fab'—S—S—PAP, though at its high concentrations it decreased the cell viability by agglutinating the cells. These effects were not observed with non-specific $F(ab')_2$. Therefore, Fab'—S—S—PAP exhibits the cytotoxicity against L1210 cells through the binding of its Fab' moiety to the target antigens on the cell surface.

Fab'—S—S—PAP has the almost same magnitude of cytotoxicity against the target cells as Fab'—S—S—ricin A-chain. In the possible development of the ricin A-chain conjugate as an antitumor agent, it may be an obstacle that it is considerably difficult to obtain pure A-chain free from whole molecule of

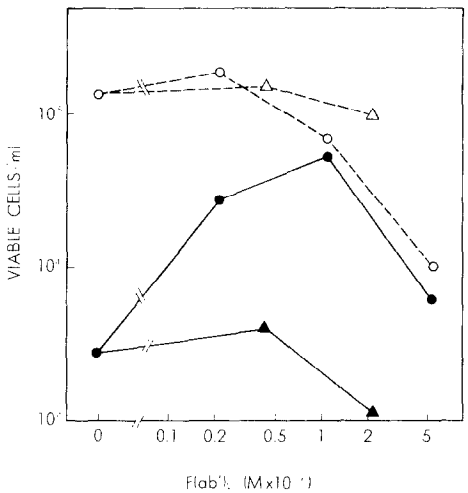


Fig. 5 Inhibition of the cytotoxicity of Fab'-S-S-PAP by $F(ab')_2$. L1210 cells (2×10^4 cells/ml) were cultured at 37° for 48 h with Fab'-S-S-PAP (7×10^{-8} M) and serially diluted anti-L1210 or non-specific $F(ab')_2$ added to the medium, and the numbers of the viable cells were determined by trypan blue dye exclusion method. ●, Fab'-S-S-PAP plus anti-L1210 $F(ab')_2$; ○, anti-L1210 $F(ab')_2$; ▲, Fab'-S-S-PAP plus non-specific $F(ab')_2$; △, non-specific $F(ab')_2$.

ricin which has non-specific, extremely potent cytotoxicity. There have been a considerable number of reports on ricin A-chain conjugates with various antibodies including monoclonal ones prepared by the hybridoma method (18-20), and these conjugates including Fab'-S-S-ricin A-chain generally showed only 1/100-1000 of the cytotoxicity of ricin. Therefore, a minute amount of ricin contaminating the A-chain conjugates leads to the detrimental effect on the non-target cells. In this regards, PAP has an advantage because it is originally devoid of the carrier moiety.

PAP has been shown to inhibit the mechanical transmission of several RNA plant viruses to plant hosts other than pokeweek (2,3). Ussery et al. demonstrated that PAP inhibits polio virus replication in HeLa cells, and studied its mechanism (6,17). According to their study, PAP does not affect the protein synthesis of the intact cells and the cells preinfected with the virus, but inhibits the protein synthesis when the cells were infected with the virus mixed with PAP. These data indicate two possibilities. Firstly, the binding of the virus to the cell brings about a change on the cell surface, and as a result PAP can enter into the cell. Secondly, the virus might become the carrier of PAP for its entry to the cell by the endocytosis mechanism.

Ussery et al. (6) could not show the binding of the virus to PAP. However, since the present study has demonstrated that PAP leads cells to death very efficiently when conjugated with Fab', a carrier which binds to the cell surface, the possible attachment of PAP to the viruses deserves further detailed examination.

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