Production and Properties of Mouse Monoclonal Anti-Adriamycin Antibody

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Eight mouse IgG; monoclonal anti-Adriamycin antibodies were produced in culture and in ascites in BALB/c nude mice. The binding constant and specificity was measured by an inhibition ELISA method. The assay was an indirect method using horseradish peroxidase-labeled goat Fab' antibody and 2mM ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) containing 2.5 mM H2O2 as a color reaction reagent. The binding constant of the antibodies was 10^6 to 10^8 M^-1. The antibodies possessed different specificities and affinities to the adriamycin. Though adriamycin is a low molecular weight (M.W. 579.99) hapten antigen, it possesses several epitopes as antigenic determinant sites. The antigenic epitopes of the adriamycin, which until recently were unknown, were analyzed by the monoclonal antibodies.

(Key Words: monoclonal antibody, adriamycin)

INTRODUCTION

Adriamycin, an anthracyclc antibiotic isolated from Streptomyces peucetius is effective in treating several different types of neoplastic tissues (1, 2). Unfortunately, however, long-term administration of this drug to tumor bearing individuals results in cardiotoxicity (3, 9) as well as drug resistance (8). In order to overcome these side effects, many pharmacological derivatives of adriamycin have been developed. These derivatives were used to examine immunological cross reactivity against a monoclonal anti-adriamycin antibody. In addition, this anti-adriamycin antibody was used in an attempt another approach to overcome drug resistance. A polyclonal IgG antibody against adriamycin was produced in rabbits, and its specificity measured by an established ELISA test (14). The polyclonal anti adriamycin antibody was used to immunohistochemically localize adriamycin in paraffin embedded tissue sections (7). Also, the polyclonal antibody was used for the neutralization of an LD50 dose of adriamycin administered to mice in vivo, and to a HeLa cell line in vitro (15). In that study, a 50% lethal dose of adriamycin, injected in the tail vein of mice, was neutralized by an i.v. injection of rabbit polyclonal anti-adriamycin antibody. The mice survived the lethal dose in vivo: the HeLa cell line survival in vitro. These findings showed that the anti-cancer drug antibody effectively neutralized the administered drug in vivo and in vitro.

Although the polyclonal antibody was effective in neutralizing the drug, the rabbit immunoglobulins acted as foreign substances in mice immune system, and further injections were not possible. To circumvent this handicap, a mouse monoclonal antibody was produced. This study has two purposes; one is the production of mouse monoclonal antibodies; the second is whether the pharmacologically effective sites of adriamycin could be determined.

MATERIALS AND METHODS

All procedures were performed under sterile conditions.

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Immunogens

Adriamycin is a non-immunogenic hapten, but it has an amino functional group suitable for carrier protein conjugation. This amino functional group was used for coupling adriamycin covalently to a free carboxyl group in bovine serum albumin (BSA) as described previously (4, 11, 14).

Briefly, 1-ethyl-3 (dimethylaminopropyl) carbodiimide (EDCI): 50 mg in 1 ml of pyridine: water (0.6:1) was added with stirring to a solution of 50 mg adriamycin and 10 mg BSA in 1 ml water, pH 6.8. After 16 hours at 4 °C, the immunogen was separated from the small molecular components by dialysis against 0.01M PBS pH 7.2.

Immunization

Three male BALB/c mice, 8 weeks old (Japan CLEA, Tokyo, Japan) were immunized and boosted a total of three times, 21 days apart, by intraperitoneal and subcutaneous injections of 200μg adriamycin-BSA conjugate emulsified with an equal volume (0.5 ml) of complete Freund's adjuvant (Difco Laboratories, Detroit, Michigan, U.S.A.). Three days before spleen cells were harvested for cell fusions, each mouse was injected intravenously with 200μg of adriamycin in saline.

Fusion

Cell fusions were performed using a modification of established techniques (5, 6). Minced spleens were passed through a 100 mesh nylon filter and diluted to 3 x 10^8 cells 10 ml serum-free RPMI 1640 medium. To this, a volume (1.6 x 10^8 cells) of Ig non-secreting mouse myeloma cells (p3 x 63-Ag.8.653:a gift from the Japanese Cancer Research Resources Bank, Tokyo) was added. The cells were mixed, and centrifuged 2 times at 1200 r.p.m. for 3 minutes in serum-free medium. The supernatant was removed, and 2 ml of 50% PEG 4000 (GIBCO BRL, Life Technologies, Inc. Tokyo) was slowly added to the pelleted cells over 5 minutes. Next, 50 ml of serum free medium, at 37 °C, was added and mixed by pipeting several times followed by centrifugation for 5 minutes at room temperature. The pellet of cells was suspended in 150 ml of 10% serum (Daigo GF-1, WAKO Pure Chemicals Co. Ltd, Osaka) containing RPMI 1640 culture medium. Aliquots of 5 x 10^5 cells/well were added to the 96 wells of microtiter plates and incubated in a humidified 5% CO_2 atmosphere overnight at 37 °C. The next day, 0.1 ml of HAT medium, containing 10% serum, was added to each well. The HAT medium was changed everyday. After 14 to 20 days post-fusion, when the hybridomas reached near confluence, the supernatant in each well was screened for antibody against adriamycin by an ELISA method (14), using rabbit IgG anti-mouse IgG antiserum and horseradish peroxidase (HRP) conjugated goat Fab' anti-Rabbit F(ab')_2 antibody. The hybridoma cells in antibody positive wells were then cloned.

Hybridoma growth and cloning

Sixteen hybridomas were cloned by limiting dilution in 96-well microtiter plates, collagen coated as a feeder layer. Anti adriamycin hybridomas (detected by ELISA) were first expanded in 24-well culture plates, then in 12-well plates, and finally in larger culture bottles. Aliquots of medium were collected and pooled (containing about 10^6 to 10^7 cells), and frozen in the presence of 10% DMSO (Dimethyl sulfoxide, WAKO Pure Chemicals Co. Ltd, Osaka, Japan), in liquid nitrogen at each stage of expansion. One of the antibody-producing hybridomas was injected intraperitoneally into BALB/c nude mice (9 x 10^6 cells) without 2, 6, 10, 14-Tetramethylpentadecane (pristane, Aldrich Chemical Company, Inc., Milwaukee, Wis., U.S.A.). About 24 days later, ascitic fluid was collected and stored at -80 °C.

Adriamycin coated ELISA Plates

Plate coating procedures were performed by methods previously reported (12, 13, 14), with slight modifications. The wells of the plates were pre-coated with 100 μl of 100 μg/ml of gelatin, incubated overnight at room temperature, washed with 250 μl of water 3 times, then dried in desiccated containers for 3 to 7 days and kept dry until used. The coated gelatin plates were oxidized with 10mM NaIO_4 for 30 minutes. The wells were washed 3 times with 250 μl of 10 mM carbonate buffer pH 9.4. Next, 100 μl of 50μg/ml of adriamycin in 10 mM carbonate buffer were incubated for
90 minutes at room temperature, then the plates were washed 3 times with 250μl of 10 mM PBS containing 0.05% Tween 20 (Tween-PBS). The plates were stored in a desiccated chamber and kept at 4°C until used.

**Immunoglobulin subclass typing**

The isotype of the MAb in the culture medium was determined using an anti mouse immunoglobulin detection kit (SEROTC: Wellcome Foundation Limited) which is based on red cell agglutination. In this test, 30μl of 1:50 diluted cultured supernatant was incubated with 30μl of specific isotype antibody-coupled red cells by gentle shaking for 30 seconds. The plate was covered and left on a cool flat surface for one hour, then examined for red cell agglutination.

**ELISA for adriamycin**

Using the adriamycin coated plates, the binding affinity was measured (10). An aliquot of 50μl of serially diluted cultured medium, containing Tween/PBS or ascites fluid, was added to the wells of the plates and incubated for 90 minutes at room temperature. After the wells were washed 3 times with 250μl of Tween-PBS, 50μl of 1:50 diluted rabbit anti-mouse IgG was added for 90 minutes. After again washing the plates with Tween-PBS, 50μl of HRP conjugated anti-rabbit F(ab')2 antibody (1:500 dilution), were added for 90 minutes, washed again with Tween/PBS, then 100μl of color reagent ABTS containing H2O2 were added. After one hour, color intensity was measured by a Molecular Device Microtiter ELISA reader, at 405 and 490 nm wave lengths, without stopping the color reaction.

In the inhibition experiment, 125μl of serially diluted, known amounts of standard adriamycin and/or adriamycin's derivatives, aclorubicin (gift of Yamanouchi Pharmaceutical co., Ltd.), epirubicin (gift of Farmitalia Carlo Erba, Co., Ltd.), daunorubicin (gift of Meiji Seika Kaisha, Ltd.), and culture medium, were preincubated in round-bottomed 96 well culture plates over-night at 4°C. The 50μl of preincubated samples were transferred, in triplicate, to adriamycin coated wells and incubated overnight. The next day, after the wells were washed 3 times with Tween/PBS, 50μl of rabbit anti-mouse IgG antibody (1:50 dilution) were added for 90 minutes. After washing with Tween/PBS, 50μl of HRP conjugated anti rabbit F(ab')2 antibody (1:500 dilution), in Tween/PBS, was added for 90 minutes, after which 100μl of ABTS containing H2O2 was added. One hour later, color intensity was measured by a ELISA reader at wave length of 405 and 490 nm. The results were calculated on SOFTmax Computer Systems, and standard curves were prepared.

Neutralization of adriamycin on HeLa S3 cells in culture.

Monoclonal antibodies from the two cultures and one ascites were examined for neutralizing activity against adriamycin on HeLa S3 cells cultured at 5% CO2 and 95% air on controlled culture systems. Equal volumes of diluted ascites or culture medium monoclonal antibodies were mixed with 10^{-3} μM to 0.25 μM adriamycin solution for neutralization.

**RESULTS**

In the fusion of adriamycin spleen cells to the myeloma parent cells, 576 of 1440 wells (40%) were positive for cell growth, and 80 of the 576 wells produced IgG anti-adriamycin antibodies. The antibody screening was performed using anti-mouse IgG (γ chain specific) antibody, since no other type of immunoglobulin such as IgA or IgM were observed. The cloning of the immunoglobulin producing cells in one well was repeated and examined again for antibody producing ability. Eight clones of the anti-adriamycin antibody producer were removed to larger culture bottles, and finally, the culture medium was collected and kept at -80°C until the ELISA assay. A part of the cloned cells were frozen in 10% DMSO containing culture medium and stored in liquid nitrogen. One hybridized clone was transplanted into the abdominal cavity of pristane-unprimed BALB/c nude mice. Ascitic fluid was collected, pooled, and stored at -80°C until the ELISA assay.

Immunoglobulin subclass typing was carried out on 8 culture mediums, using a mouse immunoglobulin detection kit based on red cell agglutination. All the 8 clones were producing IgG1 type immunoglobulin.

The anti-adriamycin curves of 8 monoclonal
antibodies in culture medium are shown in Figure-1. Each curve had a different reaction pattern from the standard adriamycin. The reaction curves were drawn on a 4-parameter or Log-Logit on calculation formula in a Soft-
Max Computer System. These patterns indicate that some of the monoclonal antibodies are recognizing the different epitopes on the adriamycin molecule. Using concentrations ranging from 3.125 to 100 mg/ml, adriamycin and compounds similar in molecular structure (aclarubicin, epirubicin and daunorubicin), were examined for cross-reactivity by comparing their standard curves as shown in Figure 2. Monoclonal antibody No. 5 (MAb.N5) recognized the molecule on the side of the sugar chain, but did not recognize the doxorubicin ring, the glycoloyl group, or the acetyl group at - 9 position in the doxorubicin ring. In Figure-3, mono-clonal antibody No. 6 (MAb.N6) showed a similar standard curve to that of Figure 2, since both antibodies (N5 and N6) possessed similar characteristics. In Figure 4, antibody N1 (MAb.N1) recognized doxorubicin's ring structure. The other 5 antibodies had properties similar to these 3.

Since, speculated from these results, the antigenic epitope is, one is shape of sugar chain (epi type or normal type), the length of the sugar chains, and another is whole structure of adriamycin molecule. But in this experiment the glycoloyl group in the adriamycin or the acetyl group in the aclarubicin attached at positi on 9 on doxorubicin ring showed no antigenicity.

The monoclonal antibodies neutralized the activity of the adriamycin administered to HeLa S3 cells in culture.

DISCUSSION

MAbs were prepared using spleen cells from BALB/c mice that were immunized with adriamycin coupled to BSA through peptide bonding. Because adriamycin is a small molecule hapten, it was necessary to conjugate it to a carrier protein. In the present experiment, the functional amino group in the distal part of the adriamycin molecule was utilized for carrier protein conjugation, to bovine serum albumin. The screening of the antibody-producing hybridomas was carried out using adriamycin-conjugated gelatin-coated ELISA plates. In the production and screening of the antibody, it was necessary to choose different types of carrier proteins and different types of conjugation processes (16). The carbodiimide reagent was used for covalent bonding to adriamycin, BSA conjugation for the immunizing antigen preparation, and NaOCl oxidization for conjugating adriamycin to gelatin coated ELISA plates. If the same conjugation process was used for both steps, false positive antibody-producing clones would result. It seemed that both free-form and BSA-coupled adriamycin-recognizing antibodies were produced. At the antibody screening step, both antibodies were detected, but the protein-coupled adriamycin-recognizing antibody could be eliminated because the antibody's specificity was checked using free adriamycin as the competitor, in a competitive ELISA assay.

The antibodies produced were all IgG1 type immunoglobulins, whereas antibodies which recognize the sugar chain are commonly IgG2 (Tada, N., personal communication). The antibodies we obtained did not recognize the sugar chain exactly in adriamycin, but rather recognized the sugar chain including whole structure of the derivatives. Another recommendation was that we should use pristane for harvesting the ascites, because it is an inflammation-inducing reagent, effective for harvesting large amounts of ascites, but without undesirable immunoglobulins.

Another interesting phenomenon was despite the covalent coupling of the bovine serum albumin to the amino group on the sugar chain, this configuration not only could block the antigenicity of the hapten, but was able to recognize the sugar chain. We cannot explain this phenomenon at present. This phenomenon is important for the production of anti-hapen antibody with specific affinity. The pharmacological effects of adriamycin were neutralized by the polyclonal rabbit anti-adriamycin antibody both in vivo and in vitro, as previously reported (15). In that study, the 50% lethal dose of 0.34 mg of adriamycin in the mouse was neutralized by a single dose of 3.4 mg of IgG per 25 g body weight mouse. The antibody could be used in the neutralization of the drug. The monoclonal
antibody produced in the present study possessed adriamycin-neutralizing activity as shown by the HeLa S3 cells anti-proliferation effect (data not shown). Our results indicate that the monoclonal antibodies prevented the toxic effect of adriamycin in vitro. The monoclonal antibody would have wider application if we knew the site of the pharmacologically effective epitope. It might be possible to block the site by a very specific monoclonal antibody, thereby neutralizing the undesirable side effects. Mouse monoclonal antibodies are more advantageous than rabbit polyclonal antibodies, because numerous injections could be given to mice.

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