Production and Immunochemical Feature for Syngeneic Monoclonal Anti-Idiotypic Antibodies to Morphine

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Three syngeneic monoclonal anti-idiotypic antibodies for morphine were produced by using a murine morphine-specific monoclonal antibody (MO-3) as an idiotype. The resulting anti-idiotypic antibodies and morphine were competitively reacted with MO-3 in a dose-dependent manner. The reactivity of one molecule of the anti-idiotypic antibody was found to correspond with two molecules of morphine based on a competitive idiotype–anti-idiotype immunoassay.

Regarding the administration of narcotic analgesics, of which morphine has been used as the main drug for the relief of pain in terminal cancer patients for a century, we have recently established some hybridomas-secreting murine monoclonal antibodies that are specific for morphine.[1] The hybridomas showed a small cross-reactivity against codeine. A quantitative analysis to detect morphine in sera was accomplished by using MO-3 and MO-5 monoclonal antibodies in a competitive inhibition ELISA (enzyme linked immunosorbent assay). The molecular steric configuration of the antigen recognition site of an anti-idiotypic antibody is considered to resemble that of the original antigen. Therefore, the preparation of an anti-idiotypic antibody and an analysis of the recognition site are important for not only the development of new drugs but also for the design of mutated molecules.

It has been proposed that an anti-idiotypic antibody has the potency of a powerful vaccine.[2—5] Furthermore, some antigens can be replaced by their anti-idiotypic antibodies. Recently, many investigations have been carried out with respect to not only a xenogeneic monoclonal anti-idiotypic antibody,[6—8] but also a syngeneic monoclonal anti-idiotypic antibody.[9—12] However, there has been few reports concerning applications to a practical immunoassay system or discussions on the molecular basis concerning the reaction between an antigen and an anti-idiotypic antibody.

In this report, the production of syngeneic anti-idiotypic monoclonal antibodies and applications to competitive idiotype-anti-idiotype immunoassay for morphine are described. The characterization of anti-idiotypic antibodies is also discussed.

Experimental

Preparation of the Immunogen: Murine monoclonal antibody MO-3, which is specific for morphine and does not cross-react with codeine, was purified from the acsites fluid by dialysis against a 0.1 M Tris-HCl buffer (pH=7.4) (1 M=1 mol dm^{-3}) and *o*-(diethylaminoethyl) cellulose (DEAE) ion-exchange chromatography. One mg ml^{-1} of purified MO-3 antibody in Dulbecco's PBS (PBS) was mixed with 1 mg ml^{-1} of keyhole limpet hemocyanin (KLH: Boehringer Mannheim GmbH, Mannheim, Germany) in PBS, followed by the addition of 28 μl of 10% glutaraldehyde. The mixture was stirred for one hour at room temperature and submitted to dialysis against PBS twice. The resulting MO-3 monoclonal antibody which conjugated with KLH (MO-3–KLH) was injected into BALB/c mice as an immunogen for obtaining a syngeneic anti-idiotypic monoclonal antibody.

Immunization: Female BALB/c mice were subcutaneously immunized twice with 100 μg/mouse of MO-3-KLH, which had been emulsified with the equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.) at 2-week intervals. Further immunizations were subcutaneously administered by the injection of 100 μg/mouse of MO-3–KLH emulsified in Freund's incomplete adjuvant (Difco Laboratories) at intervals of 2—4 weeks. The sera taken from the mice were examined concerning the degree of the immune response with respect to the anti-idiotypic antibody by the sandwich ELISA method[9,13] described later in detail.

Cell Fusions: In each respondent mouse, a final dose of 100 μg of MO-3–KLH in 200 μl of PBS was intravenously injected through the tail vein three days before fusion. The immunized spleen cells removed from the mice were fused with myeloma P3 X63-Ag8-U1 cells (Fusion I) or myeloma X63-Ag8.6.5.3 cells (Fusion II) at a ratio of 5 : 1 using 50% PEG 1500 (Boehringer Mannheim GmbH), respectively. The fused cells were placed onto the wells of 96 well-culture plates (Becton Dickinson and Co., Oxnard, CA, U.S.A.) and cultivated in a HAT medium. Fused cells were screened in order to find the anti-idiotype antibodies secretion cells by means of the modified sandwich ELISA method. For positive wells, the inhibitive sandwich ELISA was performed in order to examine whether the secreted antibody has the ability to react with the morphine-binding sites of the MO-3 antibody. Hybrids which were found to secrete antibodies specific for the paratope of MO-3 antibody when cloned by the limiting dilution method. The isotypes of the resulting monoclonal anti-idiotypic antibodies were determined using a mouse monoclonal antibody isotyping kit (Amersham International plc, Amersham, U.K.). Ascites fluid was obtained by intraperitoneal injection of the hybridoma cell lines in pristane-primed female BALB/c mice.

Modified Sandwich ELISA for the Screening of Anti-Idiotypic Antibody: For measuring anti-idiotypic antibody production in an immunized mice sera and in supernatants of hybridoma cells, sandwich ELISA was carried out as follows: The MO-3 antibody (10 μg ml^{-1}) was first coated on ELISA plates. Then, the serially diluted antisera or the culture supernatants were put into the wells. The mixture was allowed to stand for 2 h at room temperature. A

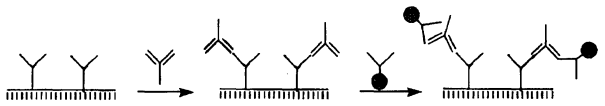

Fig. 1. Sandwich ELISA for the screening of anti-idiotypic antibody. Y; MO-3 antibody Y; anti-idiotypic antibody Y; POD labeled MO-3 antibody.

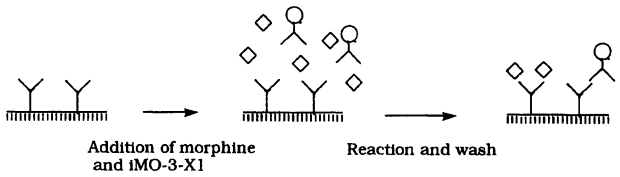

Fig. 2. Competitive idiotype anti-idiotype ELISA for morphine. Y; MO-3 antibody (iMO-3-X1 idiotypic antibody) Y; POD labeled iMO-3-X1 (POD labeled MO-3) ◇; morphine.

horseradish peroxidase (POD) labeled MO-3 antibody was added into the wells of the plate, followed by incubation for 1 h at room temperature. Finally, the enzymatic reaction by POD was performed by adding a substrate, H_2O_2, and a chromogen, *o*-phenylenediamine. In this method, immunoreaction may take place in accordance with the scheme described in Fig. 1.

Competitive Idiotype–Anti-Idiotype Immunoassay for Morphine: iMO-3-X1 is one of the anti-idiotypic monoclonal antibodies obtained in this experiment. Both iMO-3-X1 and MO-3 antibodies were purified from ascites fluid by affinity chromatography using a protein G-Sepharose column (Pierce, Rockford, IL, USA). The purity of antibodies was monitored by SDS–polyacrylamide gel electrophoresis.

Fab' fragments of MO-5 and iMO-3-X1 antibodies were obtained by digesting the antibodies with pepsin, followed by reduction with 2-aminoethanediol. Horseradish peroxidase was labeled to those Fab' fragments according to the method of Ishikawa et al.[14]

In the case of inhibitive sandwich ELISA, morphine and POD labeled Fab' of the anti-idiotypic monoclonal antibody were simultaneously added into the immunoplate on which MO-3 antibody had already been fixed (Fig. 2). In the assay, the morphine concentrations (Takeda Chemical Industries, Osaka, Japan) were varied, while that of POD-labeled Fab' of anti idiotypic antibody was kept constant. A similar experiment, in which the fixed iMO-3-X1 and the POD labeled Fab' of MO-3 were used, was also carried out. In this case, an enzymatic reaction of peroxidase was performed using hydroperoxide as a substrate and 2,2′-azinobis(3-ethyl-2-benzothiazoline-6-sulfonate) as a chromogen. The absorbance at 405 nm was measured for each well using a microplate spectrophotometer (MTP-32, Corona Electric Co., Katsuta, Japan).

Results and Discussion

Screening for Anti-Idiotypic Antibodies: Only two out of 555 wells from culture supernatants of the tissue culture plates were positive in the modified sandwich ELISA in Fusion I, and one out of 265 wells

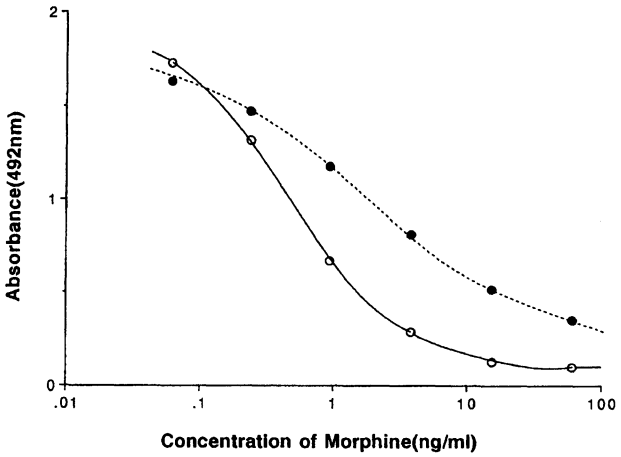

Fig. 3. Competitive inhibition ELISA when iMO-3-X1 was immobilized on ELISA plate. POD-Fab' (MO-5) was used as a free antibody which is competitively reacted against the fixed iMO-3-X1 and free morphine (open circle). POD-MO-3 was used as a free antibody (closed circle).

in Fusion II. The antibodies from the three positive wells completely inhibited the reaction between morphine and the MO-3 antibody in the sandwich ELISA. Namely, the hybrids of three positive wells should produce an anti-idiotypic antibody against MO-3. The probability of the hybrids secreting an anti-idiotypic antibody was extremely low compared with the case of normal cell fusion to obtain the idiotype monoclonal antibody. Hybrids in three positive wells were cloned three times in 96 well-tissue culture plates by the limiting dilution method, and three stable hybridoma strains, (named iMO-3-P1, iMO-3-P2 and iMO-3-X1), were obtained. The isotypes were IgG_1 (k) for all anti-idiotype antibodies.

Competitive Idiotype-Anti-Idiotype Immunoassay for Morphine: The immunological feature of the iMO-3-X1 antibody was examined by a competitive idiotype–anti-idiotype immunoassay. In this assay, the anti-idiotypic antibody and morphine competitively reacted with the idiotype MO-3 (or MO-5) antibody. The binding of a POD-labeled Fab' fragment of the MO-3-(-5) antibody to the immobilized iMO-3-X1 antibody was inhibited along with an increase in the morphine concentration (Fig. 3). In Fig. 3, the MO-3 and -5 antibodies labeled with POD were reacted with iMO-3-X1 fixed on an immunoplate under the inhibition of morphine. Both antibodies gave good calibration curves. The curve for the iMO-3-X1 and POD-MO-5 system was very similar to that previously obtained by the authors concerning the MO-3, MO-BSA and morphine system.[1] In the case of the iMO-3-X1 and POD-MO-3 system, the slope became slightly flat. The difference in the slope may be due to the characteristics of the monoclonal antibodies used. In any case, it has been revealed that the anti-idiotypic monoclonal antibody, iMO-3-X1,

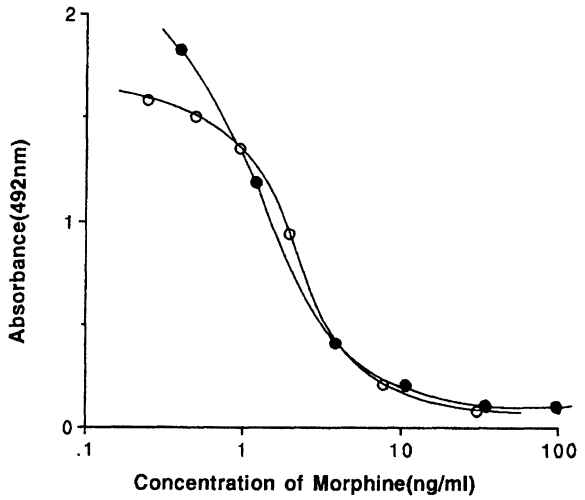

Fig. 4. Competitive inhibition ELISA when POD-Fab' (iMO-3-X1) was used as a free antigen. Antibody, MO-3 (open circle) and MO-5 (closed circle), was respectively coated as one antibody and iMO-3-X1 labeled with peroxidase was competitively reacted along with another antigen, morphine.

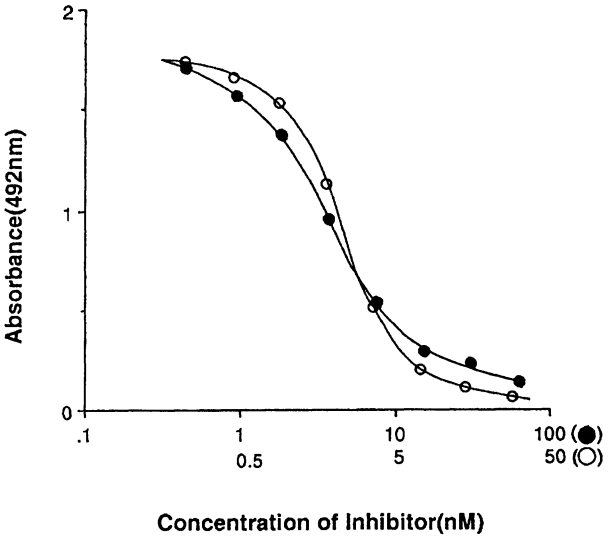

Fig. 5. Stoichiometrical comparison between morphine and anti-idiotype in ELISA. Both morphine and iMO-3-X1 antibody were employed as an inhibitor and then compared in the competitive immunoassay on MO-3 antibody-coated ELISA plates. In the assay, the Fab' fragment of iMO-3-X1 antibody was used as the peroxidase labeled form. Serially diluted morphine (closed circle) or iMO-3-X1 antibody (open circle) solutions were added to each well of the MO-3 antibody-coated ELISA plate and then the solution of peroxidase-labeled Fab's of iMO-3-X1 was added for the competitive immunoreactions against the inhibitor, morphine or iMO-3-X1.

could behave the same as a morphine molecule, and that the sensitivity for morphine reached as low as 0.1 ng ml^{-1}.

In reverse, similar results were observed in the binding of a MO-3 (or MO-5) antibody to a free iMO-3-X1 antibody (Fig. 4). In Fig. 4, an anti-morphine monoclonal antibody, MO-3 or MO-5, was first coated, and then POD-labeled iMO-3-X1 was reacted as a competitor to morphine. Both calibration curves were similar and very close to each other. The sensitivity to morphine decreased by a factor of about one-tenth compared with that in Fig. 3. The reason may be explained as follows. Because the iMO-3-X1 antibody was fixed in the case of Fig. 3, and thus its quantity fixed on the immunoplate should be small, the possibility of MO-3 or -5 to bind with iMO-3X1 might decrease. Therefore, even at a low concentration of morphine inhibition was observed. On the other hand, since iMO-3-X1 and morphine were dissolved in similar concentrations as free antigens, a high concentration of morphine may be required for inhibition. The three-component system, i.e., anti-morphine antibody, anti-idiotypic antibody and morphine, satisfactory worked in all four cases as if the anti-idiotypic antibody was the morphine molecule. These results demonstrate that the iMO-3-X1 antibody definitely interacts with the morphine binding sites of both the MO-3 and MO-5 antibodies.

Combinations of Idiotype and Anti-Idiotypic Antibodies in a Competitive Immunoassay: Four combinations of the coating antibodies and peroxidase-labeled antibodies were tested in a competitive immunoassay in which MO-3 and MO-5 monoclonal antibodies were used as idiotype antibodies, and iMO-3-X1 was used as an anti-idiotype. In these combinations, the most preferable one was that iMO-3-X1 was coated and Fab' of MO-5 was labeled with POD.

Characterization of the Anti-Idiotypic Antibodies: The next experiment focused on a determination of the stoichiometry of the anti-idiotypic antibody to morphine. Since all resulting anti-idiotypic antibodies are of the IgG_1 subclass, it is anticipated that one anti-idiotypic antibody should act as two morphine molecules from the viewpoint of the immunological feature of the antibody. It is also interesting that the sensitivity for morphine was the highest when the iMO-3-X1 antibody was employed as the coating antibody, and MO-3 was used as the labeled antibody among the four combinations tested. The sensitivity is almost the same as those obtained by Gramsh et al.[10] and Cacalano et al.,[12] who carried out an assay using the idiotypic antibodies to opioid receptors and cyclosporine A, respectively.

On the other hand, it was our purpose to determine the immunological stoichiometry between iMO-3-X1 and morphine. Therefore, the immunological reactivities between them were examined by the competitive idiotype-anti-idiotype immunoassay. In the experiment, both morphine and the iMO-3-X1 antibody were used as inhibitors in a competitive immunoassay using MO-3 coated ELISA plates. Serially diluted morphine or

iMO-3-X1 antibody solutions were added to each well of the MO-3 antibody-coated ELISA plate along with a solution of peroxidase-labeled Fab' of iMO-3-X1. The two obtained calibration curves are also shown in Fig. 5. The curves of one mole of the iMO-3-X1 antibody and two moles of morphine almost overlapped over a wide concentration range, as expected. From these results, it is concluded that one molecule of the iMO3-X1 antibody corresponds to two molecules of morphine in the antigen–antibody reaction. Namely, even a large molecule, such as a monoclonal antibody, can behave the same as a small molecule, such as morphine, in the immunological reaction system.

The steric configuration of an amino acid sequence consisting of a complementary determining region, (CDR)-1, -2, and -3, of the resulting anti-idiotypic antibody may mostly resemble the chemical structure of morphine. Analyses of the amino acid sequences of CDR-1, -2, and -3 of the MO-3, MO-5, and iMO-3-X1 antibodies are now being planned in order to obtain a better understanding of the immunoreaction and for designing new biochemical molecules as morphine substitutes. These extensive studies will contributed to a clarification of the immunochemistry of antibodies.

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