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Production of Monoclonal Antibody Specific for Human Hemoglobin

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Abstract: Hemoglobin is not normally present in sputum, urine or feces except in minute amounts. Demonstration of the presence of hemoglobin in these materials is an important diagnostic clue that usually implies bleeding within the respiratory, urinary or gastroentestinal tract, respectively.

In this work, a hybridoma line secreting monoclonal antibody (Mab) highly specific for human hemoglobin A (hHbA) was generated by fusion of immunized BALB/c mouse splenic lymphocytes with non-secreting mouse myeloma cells followed by selection in hypoxanthine-aminopterin-thymidine (HAT) medium and the screening of culture fluids for hHb A affinity in ELISA.

The Mab produced by MAM 2/3B6 is highly specific to hHbA and did not react with heterologous hemoglobin species. The sensitivity of the immune test systems based on the produced Mab was found to be 0.1 µg/of hHb per ml.

Key Words: Immunodiagnosis, monoclonal antibody, human hemoglobin, ELISA

Introduction

The development of hybridoma technology by Köhler and Milstein (1) provided an important opportunity for the examination of a range of difficult issues. Mabs are used in in-vitro diagnosis, in radioimmunoassays (RIA), enzyme-linked sorbent assays (ELISA), immunocytopathology, and flow cytometry in in-vivo diagnosis and immunotherapy.

Hemoglobin is the oxygen binding molecule of vertebrate red blood cells. It is also found in some invertebrates and in the root nodules of legumes (2).

Hemoglobin is not normally present in sputum, urine, or feces. The demonstration of the presence of hemoglobin in these materials is an important diagnostic clue that usually implies bleeding within the respiratory, urinary, or gastroentestinal tract. The biochemical tests for the detection of hemoglobin are dependent on the fact that heme proteins act as a peroxidase, catalyzing the reduction of H_2O_2 to water. This reaction requires a hydrogen donor (3).

Although benzidine derivatives are presumed to be carcinogenic, o-tolidine is used in a commercially available dipstick method (3). The o-tolidine tests can detect as little as 2 mg of hemoglobin per deciliter of urine and 100 mg of hemoglobin per 100 g feces, the equivalent of about 10 red blood cells per µl of urine or 1 ml of blood per 100 g of feces, respectively (2,3).

A guaiac-based commercial test for fecal occult blood is less sensitive than the o-tolidine based tests; false-positive results are therefore very infrequent. In a more sensitive test for blood in feces, heme-derived dicarboxyl-porphyrins are extracted from feces and measured fluorometrically. The procedure is more time consuming than o-tolidine or guaiac tests and more expensive, but it is much more specific than other tests for fecal blood. It does not distinguish between porphyrins derived from hemoglobin as opposed to those derived from myoglobin. Consequently, a diet containing no red meat must be adhered to for three days before the specimen is collected (2,3).

In this paper, we report the development of a hybrid cell (MAM/2-3B6) producing a monoclonal antibody specific to hHbA (β globin chain) for use in specific and sensitive detection of hHbA avoiding false results due to cross reactions of the antibody with non-human hemoglobins.

Materials and Methods

Preparation of hemolysate

Hemoglobin fractions (hemolysate) from normal individuals were prepared as described (4) and used for immunization of BALB/c mice. Red cells obtained from normal adults were washed three times in normal saline and lysed by shaking in two volumes of distilled water. The stroma was removed by vigorously mixing the lysed cells with one volume of carbon tetrachloride and centrifuging at 3000xg for 20 min. The clear hemoglobin solution above the stromal layer, which forms at the interface with the carbon tetrachloride, had a concentration of 8-10 gr/dl (4).

Immunization

Eight-week-old BALB/c mice were immunized subcutaneously with 1 mg hemoglobin emulsified in complete Freund's adjuvant (CFA). Two and five weeks after the initial injection, the animals received subcutaneous injections of 1 mg hemoglobin emulsified in incomplete Freund's adjuvant (IFA). After three weeks' rest, the animals were given three intravenous injections of 0.1 mg of hemoglobin on three consecutive days (5).

Fusion

The spleen cells were isolated from the mouse with the highest anti-hemoglobin antibody titer on day 59 and were fused with mouse myeloma cells, P3 X 63-Ag8.653 (ATCC CRL 1580), at a ratio of 1:6. The standard fusion, cloning and subcloning protocols were performed as described previously (6,7). Polyethylene glycol 4000 (Fluka) was used as the fusion agent.

ELISA

The indirect ELISA method was used to detect antibody activity in the mouse sera and hybridoma supernatant (7,8).

In the direct ELISA system, anti-hHbA monoclonal antibody conjugated to AP was used (9).

The competitive ELISA system was used to detect hemoglobin in feces. The stool specimen was dipped into extraction vials containing 1 ng-100 µg human hemoglobin in 1ml PBS. The vials were closed and shaken well. The mixtures were incubated for 10 minutes at room temperature. 100-µl supernatants were mixed with 200 ng MAM-2/3B6 - AP conjugate and transferred to ELISA microwells coated with 500 ng human hemoglobin. The inhibition of coated hemoglobin binding to MAM/2-3B6 was determined from the results (10). For the control, 1 ng-100 µg human hemoglobin in 1 ml PBS was used without the addition of a stool specimen.

Determination of Ig Type

The heavy-chain type of the monoclonal antibody was determined with an Ig subisotyping kit (Behring diagnostic).

Purification of Monoclonal Antibody

The antibody was precipitated from the hybridoma culture supernatant at 40 percent $(NH_4)_2SO_4$ saturation, in PBS (150mM K-phospate, pH 7.2) and dialyzed against the same buffer (11). The mouse monoclonal antibody was eluted from a Protein G Mab Trap Kit with elution buffer containing 1M glycine after immediate neutralization. Monoclonal antibody anti-hemoglobin was affinity -purified on Eupergit C (12).

Conjugation

0.35 mg of AP (Boehringer Manheim) in 0.035 ml volume was treated with 0.001ml of 25% glutaraldehyde solution. After incubation for 50 min at 25ºC, 0.15 mg of IgG (affinity purified) in potassium phosphate buffer was added. The mixture was gently stirred for 75 min at 25ºC, then transferred onto a sephacryl 300 (S 300) (SIGMA) column and the conjugate was eluted with Tris-HCl buffer (50 mM pH: 8.0 containing 0.1M NaCl, 1mM $MgCl_2$, 0.1% Na-azide (w/v)) in fractions of 1ml.

Preparation of Globin Chains

The heme moiety was separated from the globin in acid-acetone solution. The globin chains were separated by CM cellulose (CM 52) column chromatography using urea-phosphate buffer at pH 6-7, the optimum condition for the separation of globin chains. (4).

Electrophoresis

The hemoglobin was analyzed by SDS-PAGE (13) and native PAGE (14), followed by transblotting on polyvinylidene difluoride (PVDF) membrane (15). The immunoreactivity of the transferred peptides with the monoclonal antibody MAM-2/3B6 was assayed by western blotting (16).

Results

Preparation of anti-hHb

The mice with the highest titer of anti-hHbA antibody were sacrificed and the spleen cells were used in the fusion. On the 16th day, the culture fluids were tested for the presence of anti-hHbA by ELISA. Of the original 480 wells, 143 exibited macroscopically visible hybridoma clones in culture. Only 27 of the 143 produced anti-hHbA antibodies and MAM-2/3B6 was subjected to three subcloning steps by limiting dilution.

Characterization of anti-hHbA

The supernatant of the MAM-2/3B6 cells, propagated in normal culture flasks, and the secreted antibody were tested for possible cross-reaction with closely related antigens and also for reaction with globin chains separated by CM 52 chromatography. The results in Table 1 show that the mab produced by MAM-2/3B6 did not react with heterologous hemoglobin species, nor with the closely related homologous hemoglobin types fetal hemoglobin (hHbF) and human hemoglobin A_2 ($hHbA_2$). Its reaction with human transferrin was negligible. The antibody reacted with β-globin as well as (or even slightly better than) it did with hHbA.

The Mab was purified from MAM-2/3B6 cell culture supernatant by ammonium sulphate precipitation and chromatography on a Protein G mab trap column. (Fig.1).

The subisotype of MAM-2/3B6 mab was found to be IgG_1 (Table 2).

Mab MAM 2/3B6 was antigen-specific affinity-purified on Eupergit C; 25 mg of hHb was incubated with 200 mg of Eupergit C for 5 days at room temperature with stirring, and was then washed and mixed with mab MAM-2/3B6 (1.5 mg/ml). The monoclonal antibodies were eluted with 3.5 M sodium thiocyanate (Fig. 2). The functions under the A_{280} and A_{405} peaks were pooled.

Table 1. Reactivity of the monoclonal antibody MAM-2/3B6 with different hemoglobin species and globin chains.

Sample	A_{405}
No Antigen (-Ag) control (+Ab)	0.18
No antibody (-Ab) control (+Ag)	0.19
Mouse hemolysate	0.24
Rabbit hemolysate	0.21
Horse hemolysate	0.28
Bovine hemolysate	0.19
Sheep hemolysate	0.19
hHbF	0.30
$hHbA_2$	0.26
Human α globin	0.20
Human β globin	1.71
hHbA	1.65
Human apotransferrin	0.22
Human Serum Albumin	0.24

Table 2. Subisotype of MAM-2/3B6 antibody.

Subisotype	A_{405}
IgG_1	0.65
IgG_{2a}	0.17
.IgG_{2b}	0.18
IgG_3	0.2
IgM	0.16
IgA	0.15

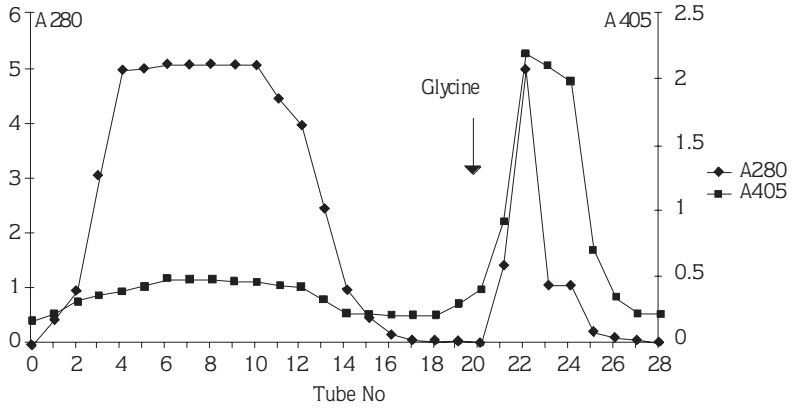

Fig.1. Affinity chromatography elution profile of the Mab MAM-2/3B6 from Protein G affinity column (1X1cm). First peak was obtained using binding buffer (20 mM Na-phosphate pH 7.0). IgG fractions eluted from column with 0.1 M Glycine HCl pH 2.7 (1ml/10 min/tube). Fractions were neutralized with 1M Tris HCl pH 9.

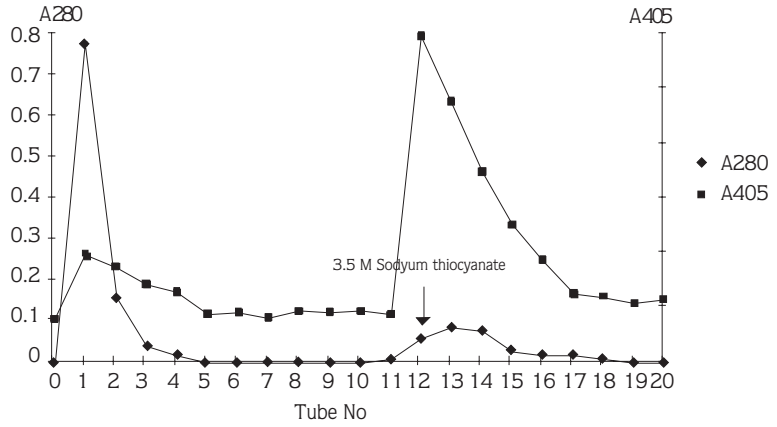

Fig. 2. Affinity purification of mab MAM-2/3B6 on C. 11^{th}-15^{th} fractions were collected.

Monoclonal antibody MAM-2/3B6 was conjugated to alkaline phosphatase as previously reported (16). The MAM-2/3B6-AP conjugate was purified on a Sephacryl 300 column. The main conjugate peak fractions were pooled and compared wih regard to specific activity by ELISA (Fig.3).

Fig.4 shows that the binding of anti-hHbA-AP to hHbA is directly proportional to the amount added in the ELISA system. The purified conjugate did not contain free IgG and AP and did not react.

The hemoglobin in the stool samples was determined according to competition with the free Hb for binding to MAM 2/3B6. As shown in Fig. 5, inhibition increased with hemoglobin concentration.

The immunoreactivity of MAM-2/3B6 with human hemoglobin was shown with SDS-PAGE (12%) (Fig. 6). Fig.7 shows the immunoreactivity of MAM-2/3B6 on native gel electrophoresis (12%).

Discussion

The aim of this study was to develop a functional antibody against hHb. The detection of occult blood in stool can be most useful in the early diagnosis and treatment of patients with colonic cancer. (17). The early stages of colorectal polyps and carcinomas are characterized by the intermittent release of small amounts of blood. The monitoring of high risk patients and systematic screening of the population over the age of 50 using a sensitive method is thus recommended.

Most commercial tests are based on the pseudoperoxidase activity of blood. Unless a strict diet has been observed during the days before testing, these tests are affected by a large proportion of false positive results. Thus, sensitivity is reduced, yielding many false negative results (17).

In this study, we developed a hybridoma clone, MAM-2/3B6, producing antibody highly specific to the human

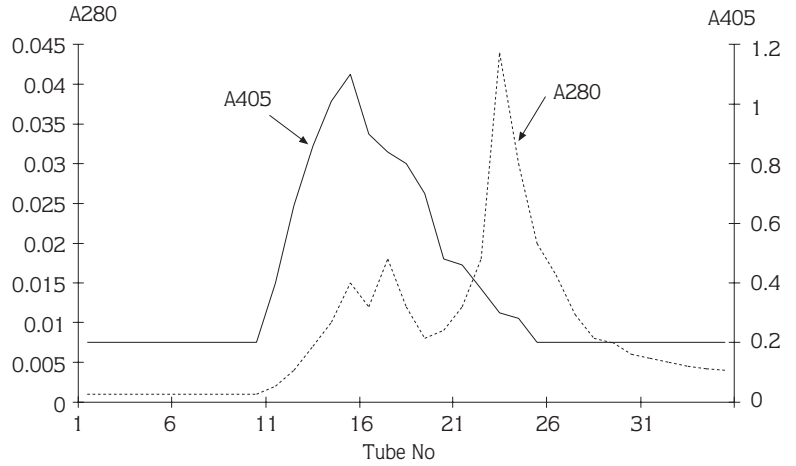

Fig. 3. Determination of main conjugate peak on S 300 column and its ELISA activity. The first protein peak containing fractions (A_{280}) which contain the purified conjugate was combined.

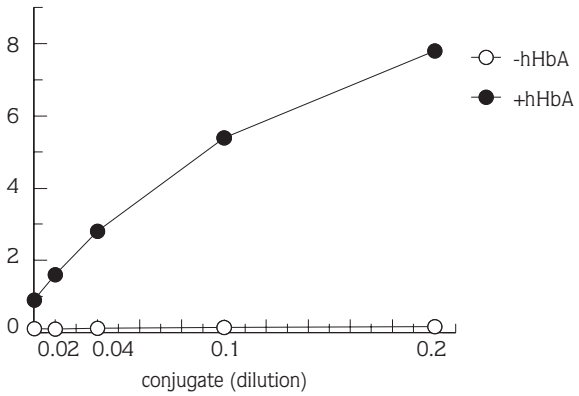

Fig. 4. Determination of purified anti-hHb-AP conjugate activity at hHb or PBS-coated plate (with direct ELISA).

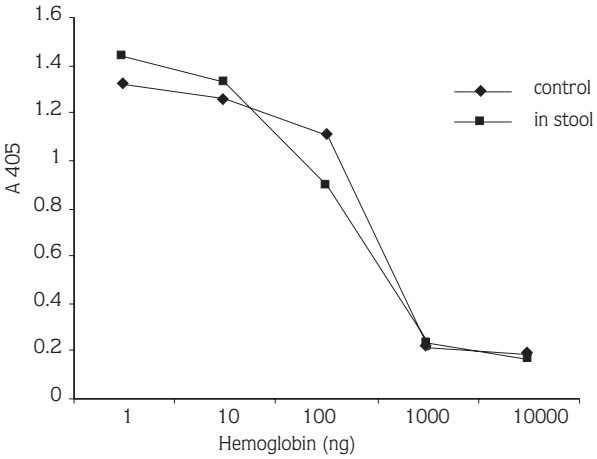

Fig. 5. Determination of free hemoglobin inhibition in stool.

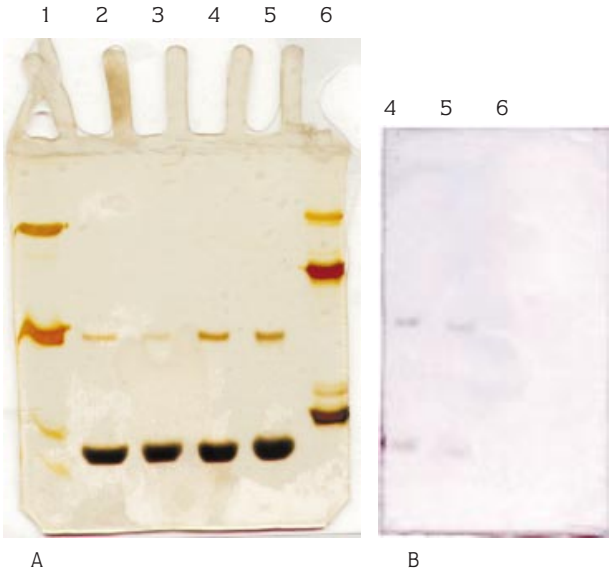

Fig. 6. Immunoreactivity of MAM-2/3B6 with human hemoglobin. A. SDS-PAGE: 1. Marker (67 kD and 25 kD), 2. Hemoglobin (reduced with βmet and nondenatured), 3. Hemoglobin (nonreduced with βmet and nondenatured), 4. Hemoglobin (reduced with βmet and denatured), 5. Hemoglobin (nonreduced with βmet and denatured). 6. Marker (44 kD and 13.7 kD). B. Analysis of western blotting.

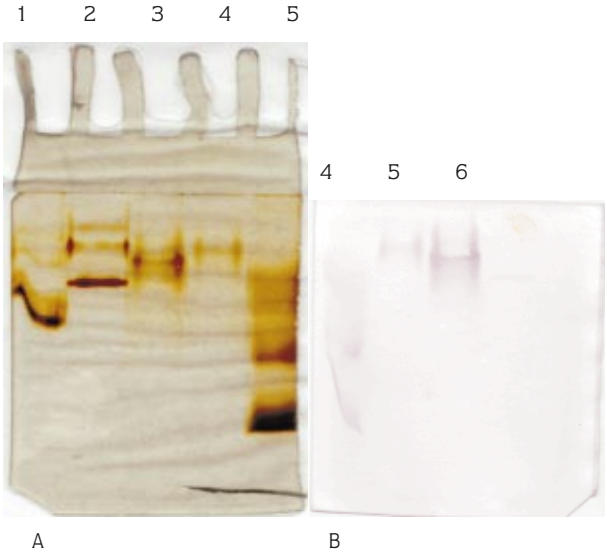

Fig. 7. Immunoreactivity of MAM-2/3B6 with human hemoglobin on native gel. A. Native-PAGE: 1. Marker (44 kD) 2. Marker (67 kD), 3. Hemoglobin (nondenatured), 4. Globin (nondenatured) 5. Hemoglobin (denatured).

hemoglobin β globin chain. The tests performed showed that the monoclonal antibody MAM-2/3B6 obtained by hybridoma technology has a high specificity and affinity (10^{-11}) for human hemoglobin. The results suggest that this antibody can be used for the immune diagnosis of occult blood in body fluids and feces by systems like ELISA as other hemoglobin variants do not have the β globin chain.

In the hHb test of the feces, the subjects did not need to follow a special diet since MAM-2/3B6 does not cross-react with non-human hemoglobins.

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