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Production and Immunological Characterization for Anti Hemin Monoclonal Antibody

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By using three kinds of antigens for immunization, screening and confirmation, a monoclonal antibody for hemin was produced. The probability of the antibody secreting fused cells was extremely low compared with the normal cell fusion. The antibody could also react some other metal substituted protoporphyrins such as Mn, Pd, Zn and Ga instead of Fe. The monoclonal antibody may loosely recognize the porphyrin ring unlike hemoglobin.

Many monoclonal antibodies against synthetic porphyrin [1-3] and partially modified natural type porphyrin[4] have been produced so far. However, no reports are seen for the production of the monoclonal antibody to natural type porphyrin, hemin, which belongs to a prosthetic group of heme proteins such as hemoglobin, myoglobin, cytochrome, peroxidase and so on. It has been said for a long time that the monoclonal antibody to hemin can hardly be obtained because hemin maintains the same chemical structure over various species including mammals and plants. Namely, immunological response would be small in the mouse even when it immunized with hemin haptenized-with proteins. Practically, the immunoresponse for the mouse was definitely low. Nonetheless, there are numerous reports for the preparations for monoclonal antibodies against small molecules.

It is considered that the monoclonal antibody to hemin could be produced provided that we prepare the appropriate hapten for hemin, which is suitable for immunization, screening and confirmation of the antibody. Because hemin is hydrophobic, it is hardly soluble in aqueous solution so that the assay system for screening and confirmation of the hybridoma must be taken into

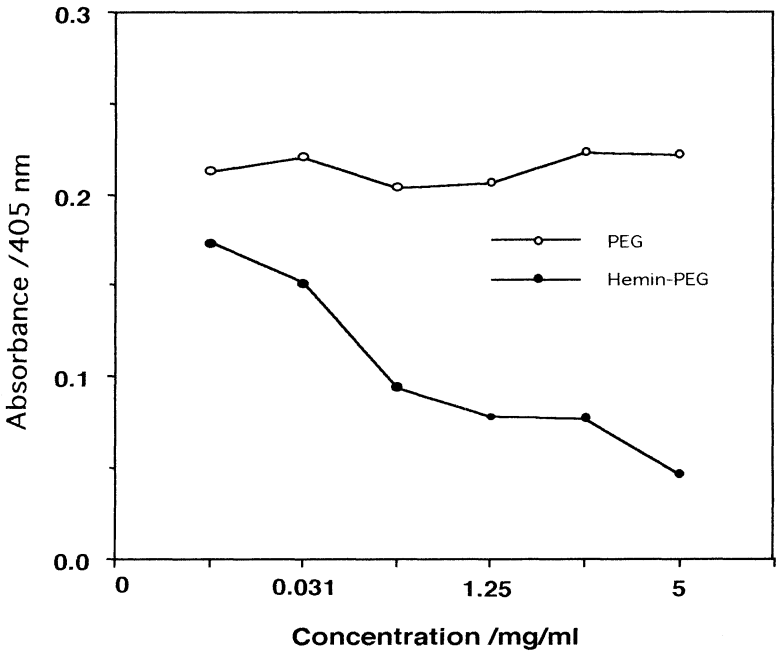

Fig.1. Competitive inhibition ELISA for PEG and hemin PEG

account from this point on planning the strategy.

Three types of hemin-conjugate were herein synthesized. First one is the hemin-human immunogloblin (hIgG) which is used for the immunogen, for hIgG mounts strong immunoresponse in Balb/c mouse from our empirical experiment for the production of monoclonal antibodies against small molecules.[5,6] Second is the hemin-bovine serum albumin(BSA) which was used as the fixed antigen at screening process of monoclonal antibody. Third is the hemin-polyethylene glycol(PEG) conjugate which was synthesized in order to carry out the inhibition test for the confirmation of the antibody at the final screening stage.

Through six times immunizations of hemin-hIgG conjugate, the titer for hemin in the serum of the immunized mice increased only up to 100-500 while that for hIgG reached to thousands or more. The mouse having higher titer being 470 was sacrificed for first cell fusion experiment. The lower titer mouse, 320, was submitted to second fusion. In the first cell fusion, 3.6×10^5 of spleen cells were seeded on 368 wells of culture plates. On the other hand, in second, 3.0×10^5 cells were placed on 421 wells. All wells were screened by using hemin-BSA coated plates from ten days after the cell fusions. Only one well was surprisingly positive in all wells screened through twice cell fusions. The probability of hybridoma secreting antibody was extremely low compared with the normal cell fusion performed so far. The antibody was named to be 1D3. Competitive reaction using monoclonal antibody of 1D3(IgM,k) was subsequently carried out against hemin-PEG(free antigen) and hemin-BSA(fixed antigen). The result was shown in Fig. 1. In the figure, the following enzyme linked immunosorbent assay(ELISA) system was performed. Namely, hemin-BSA was first coated on 96 well immunoplate. Second, PEG or hemin-PEG which concentrations were varied from 0.015 to 5 mg/ml was competitively reacted with the monoclonal antibody 1D3 for one hour at room temperature. After washing the wells, POD labeled anti mouse IgM polyclonal antibody(rabbit) was third reacted to 1D3. Finally, the color development reaction based upon the labeled POD was performed using hydrogen peroxide as a substrate and o-phenylene diamine as a chromogen. The absorbance of 405 nm was measured to follow the extent of the reaction. As the inhibition by hemin-PEG clearly occured, the resulting monoclonal antibody should react against hemin. This is the first case to establish the anti hemin-antibody secreting cell.

Some protoporphyrin-PEG conjugates having other kinds of center metal of porphyrin ring were synthesized for the examination of immunological feature of the anti hemin monoclonal antibody. After the incorporation of metals, the modification with PEG[7] was carried out for obtaining proto-

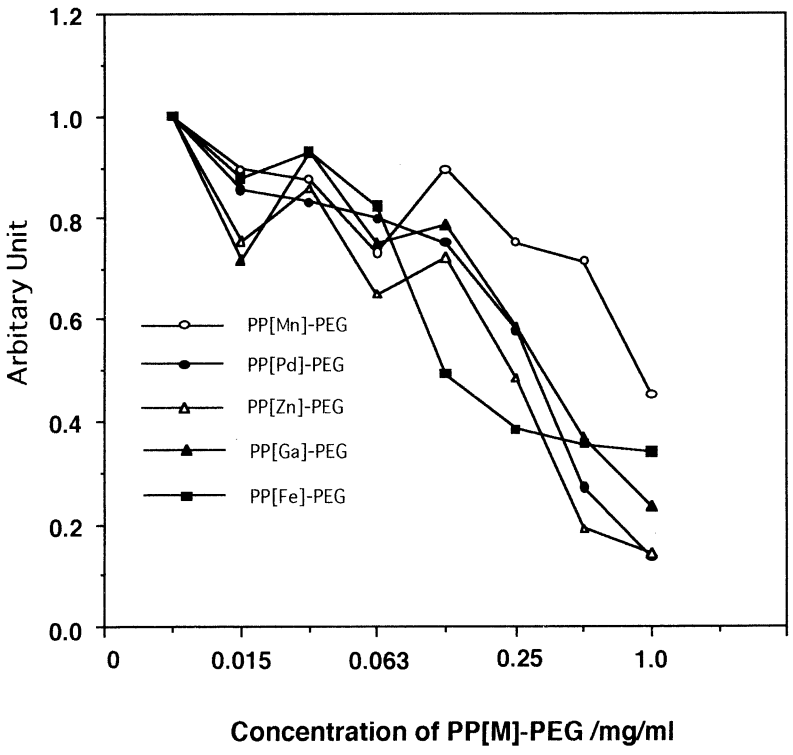

Fig.2. Immunological feature of the anti hemin antibody(1D3).

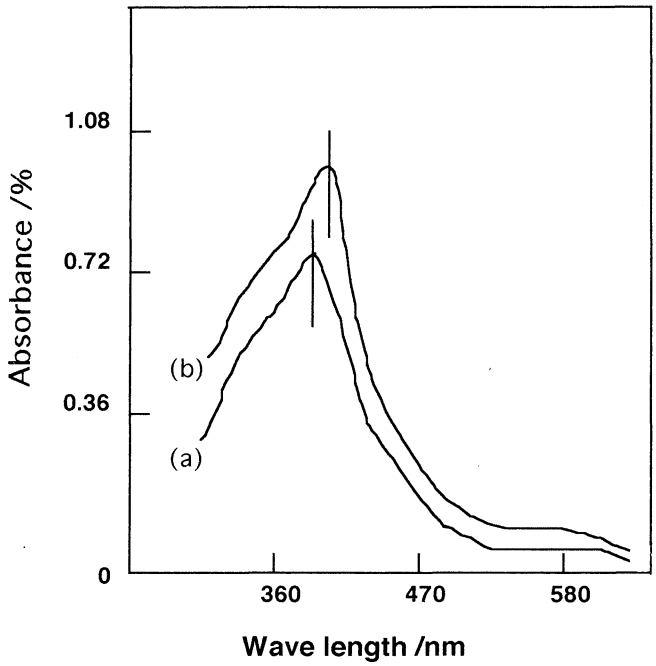

Fig.3. UV/VIS spectra for free hemin and the immunocomplex of the hemin and antibody. (a) represents the free hemin-PEG and (b) immunocomplex of antibody-hemin-PEG.

porphyrin-PEG conjugate (PP[M]-PEG; PP and M represent protoporphyrin and metal , respectively). Immunoreactivities for each PP[M]-PEG were shown in Fig. 2. In the results, all absorbance of 405 nm were taken to be unity at 0.015 mg/ml of PP[M]-PEG because the absorbance for each PP[M]-PEG was different and it is nesessary to calculate the value of 50% inhibition for each compound. As expected, PP[Fe]-PEG which is iron incorporated protoporphyrin, hemin, displayed the largest affinity to the antibody, 1D3, from the data of 50% inhibition. On the other hand, that of PP[Pd]-PEG, PP[Zn]-PEG and PP[Ga]-PEG was about one fifth in comparison with PP[Fe]-PEG. PP[Mn]-PEG had almost one tenth affinity against hemin-PEG. From these results, it is likely that the antibody loosely recognizes the metal substituted protoporphyrins.

From the substitution of metal ion in the center of porphyrin ring, some suggestions are derived with respect to the structure of the antigen recognition site of the monoclonal antibody. The recognition site might take in the major part of hemin molecule including the center metal of the porphyrin. Presumably, one or more amino acids in this antibody interacts to the center metal. In the case of hemoglobin, the iron of the hemin is coordinated with histidine and valin residue. Especially, the histidine residue strongly interacts with the iron so that the substitution by other metal containing porphyrin hardly occurs in hemoglobin. On the contrary, from Fig. 2, it is considered that the replacement of the different metal containing protoporphyrins can take place in the resulting antibody. Accordingly, the interaction of the center metal with the amino acid consisting the antigen recognition site might be weak in comparison with that of the hemin-globin protein.

The immunocomplex of the antibody with hemin was analyzed by UV/VIS spectroscopy. The spectra of free hemin-PEG and antibody-hemin complex are shown in Fig.3. Free hemin-PEG was first dissolved in 0.015 M PBS and then the anti hemin monoclonal antibody simply purified from the supernatant was added to react with hemin-PEG. Both of the band shift to long wave length by *ca.*10 nm and the increase of absorption of Soret band were observed. The band shift has been reported by Harada et al.[3] The immunocomplex of synthetic porphyrin, tetra carboxyphenyl protoporphyrin(TCPP), with the anti TCPP antibody had the band shift to longer wave length. In the case, the increase of the absorption band was not observed. On the other hand, Cochran et al.[4] produced the monoclonal antibody specific for N-methylmesoporphyrin on exploiting the catalytic antibody. The synthetic porphyrin was modified with methyl group at the position of one nitrogen

atom of porphyrin ring and thus iron atom is not involved in the porphyrin. From the report, a huge increase of Soret absorption band was observed when the antibody formed the complex with mesoporphyrin but the red shift did not occur. The increase of Soret absorption band is ascribed to the binding of mesoporphyrin with the hydrophobic pocket of the antibody. In our study, both of the red shift and the increasing of Soret absorption band were simultaneously observed when the anti hemin antibody reacted to hemin. The former might be caused by the electron transfer to hemin from the antibody. The increase of the Soret band is well understood by the explanation of Cochran et al., because the hemin is favourable to interact with the hydrophobic amino acids of the antibody.

In many heme proteins, histidine residue plays an important role with respect to the coordination of iron atom of the porphyrin. As the interaction is strong and specific, the metal substituted protoporphyrin is hardly incorporated into the hydrophobic pocket of those proteins. In contrast, the resulting anti hemin monoclonal antibody does not strictly recognize one special metal substituted porphyrin in the hemin recognition site of the antibody by considering the results in Fig. 2. Consequently, it is plausible that the interaction between center metal and amino acid residue is not strong unlike histidine of hemoglobin.

These points and the chemical behavior of oxygen with hemin-1D3 antibody complex will be clarified in near future.

(It is possible to provide a small aliquot of the monoclonal antibody,1D3, as the ascites fluid for the researcher who has interests to this kind of studies if one contacts to T. Uda.)

References

1) A. W. Schwabacher, M. I. Weinhouse, M. M. Auditor, and R. A. Lerner, *J. Am. Chem. Soc.*, **111**, 2344(1989).
2) A. Harada, K. Okamoto, M. Kamachi, T. Honda, and T. Miwatani, *Chem. Lett.*, **1990**, 917.
3) A. Harada, K. Okamnoto, and M. Kamachi, *Chem. Lett.*, **1991**, 953.
4) A. G. Cochran and P. G. Shultz, *J. Am. Chem. Soc.*, **112**, 9414(1990).
5) T. Usagawa, M. Nishimura, T. Uda, and Y. Nakahara, *J. Immunol. Methods*, **119**, 111(1989).
6) T. Usagawa, Y. Itoh, E. Hifumi, A. Takeyasu, Y. Nakahara, and T. Uda, *J. Immunol. Methods*, **157**, 143(1993).
7) K. Takahashi, A. Matsushima, Y. Sato, and Y. Inada, *Biochem. Biophys. Res. Commun.*, **138**, 283(1986).

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