0273-2289/93/3803—0179$03.00

Recognition of Liposome-Bound Antigens by Antipeptide Antibody

YOSHINORI SOHMA, RYOJI FUJITA,
SHIGEO KATOH,* AND EIZO SADA

Chemical Engineering Department,
Faculty of Engineering, Kyoto University, Sakyo-Ku, Kyoto 606, Japan

Received September 18, 1992; Accepted November 23, 1992

ABSTRACT

In order to clarify the effects of the differences in physical states of antigens on recognition by antibodies in immunoassays, the binding characteristics of an antipeptide polyclonal antibody to the peptide and the corresponding protein were studied. The reactivity in the immunoliposome assay (ILA), as well as in the double-antibody sandwich ELISA, was identical to that in the solution. These results indicate that the conformation of liposome-bound antigen is changed little by coupling to liposomes and is almost the same as that of the native antigen in the liquid phase. It is desirable to assay by double-antibody sandwich ELISA or ILA to detect native proteins, and the latter is very easily performed.

Index Entries: Immunoliposome assay; antipeptide antibody; recognition of antigen; crossreactivity; ELISA.

INTRODUCTION

Immunoaffinity between antigen and antibody is so specific and strong that it is widely used in the biochemical and biomedical fields for the assays of biomaterials, especially for clinical assays. Among immunoassays the most popular method is enzyme linked immunosorbent assay (ELISA), but the method needs washing many times after each step for bound-free separation and long incubation. Therefore, fast and easily performed

*Author to whom all correspondence and reprint requests should be addressed.

methods, such as immunoliposome assays (ILA) and particle agglutination methods, have been developed. ILAs, which depend on the immune lysis of artificial phospholipid vesicles by complement, can be performed within 60 min in homogeneous systems. The physical states of antigens, however, are different among these methods. For example, antigens are physically adsorbed on the plastic surface of microtiter wells in the case of the conventional ELISA, whereas they are coupled covalently to phospholipids constituting liposomes in ILA. It is generally recognized that proteins become at least partly denatured when they are adsorbed to the surface of a plastic plate (*1*). Such differences may cause different reactivity of antibodies against the antigens.

Antipeptide antibodies, which are raised against parts of sequences of whole proteins and selected by the reactivity with the proteins in ELISA, might fail to bind to the proteins in solution because they tend to react extremely well with the denatured form of the corresponding proteins (*1*). Such antibodies can not be used for the purification of the native proteins from crude feeds. In clinical assays, the reactivity of antibodies against antigens in native states in vivo is an important indication of the physiological states of patients to be examined.

Thus, it is important to clarify the effects of the differences in physical states of antigens to the recognition by antibodies in immunoassays and also to develop a useful method for detection of proteins in native states. In the present work, the binding characteristics of an antipeptide polyclonal antibody that was raised against a part of the amino acid sequence of myoglobin to the peptide and the corresponding protein (myoglobin) were studied using ILA as well as the conventional and double-antibody sandwich ELISA. Since polyclonal antibodies should have many repertories of different binding characteristics, these results will be useful to known the applicability of these assays to obtain clones of monoclonal antibodies with suitable characteristics for antigen recognition.

MATERIALS AND METHODS

Materials

Myoglobin (from the sperm whale) was purchased from Sigma Chemical Co., St. Louis, MO. The peptide P121 (GNFGADAQGA), which represents residues 121–130 of myoglobin, was synthesized by the solid phase method and purified by a HPLC system (Shimadzu LC-6A) with a reverse-phase column. Keyhole limpet hemocyanin (KLH, Sigma Chemical Co.) and bovine serum albumin (BSA, Nacalai Tesque, Inc., Kyoto, Japan) were used as carrier proteins of the peptide for immunization and for ELISA, respectively. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphospha-

tidylethanolamine (DPPE) (Sigma Chemical Co.), cholesterol (Chol), and dicetylphosphate (DCP) (Nacalai Tesque, Inc.) were used for liposome preparation, and guinea pig whole complement (GPWC) was purchased from Diamedix Co. (Miami, FL). The chemicals used were of reagent grade.

Preparation of Antibodies

The peptide was coupled to KLH with glutaraldehyde (GA) according to the procedures of Mariani (*2*). A mixture of equal volume of an antigen solution and complete Freund's adjuvant (1 mL each) was injected into rabbits. Booster injections were repeated twice. Antisera were pooled, and specific antibodies were purified by affinity chromatography using antigen-Sepharose 4B (*3*). The equilibrium behaviors were determined by coupling these antibodies to CNBr-activated Sepharose 4B (Pharmacia LKB Biotech, Piscataway, NJ) and by measuring the adsorption capacity, as previously reported (*3*). According to Pauling et al. (*4*), the effective association constant K_0 for polyclonal antibody can be estimated by assuming that the free energy of antigen-antibody combination can be described by the normal distribution function.

$$y = 1\text{-}1 / \sqrt{\pi} \int_{-\infty}^{\infty} [e^{-\alpha^2} / (1 + K_0 C e^{\alpha\sigma})] \, d\alpha$$

$$\alpha = \ln(K / K_0) / \sigma$$

where C is the liquid phase concentration of free antigen; K is the association constant; σ is the heterogeneity of K; and y is the fractional saturation of antibody.

Preparation of Antigen-Coupled Liposomes

Antigen-coupled liposomes were prepared by the procedures previously reported (*5,6*). A lipid mixture containing DPPC (75 μmol), Chol (75 μmol), DCP (7.5 μmol), and *N*-[4-(*p*-maleimidophenyl)butyryl]DPPE (7.5 μmol) in chloroform was shaken vigorously for 10 min at 60°C after addition of 15 mL of 0.2*M* carboxyfluorescein (CF) as a marker, and then sonicated with a probe-type sonicator (Model US-300, Nissei) for 15 min under a nitrogen atmosphere. The antigens were modified with *N*-succinimidyl 3-(2-pyridyldithio) propionate, and pyridyldithio group introduced to the antigens was reduced with dithiothreitol as previously reported (*5*). The liposome suspension prepared (0.5–2.0 μmol-DPPC/mL), which was separated from untrapped CF by gel chromatography with Sephadex G-25, was mixed with an equal volume of the modified antigen solution (about 1 mg/mL) and reacted overnight at T_r under nitrogen. Uncoupled antigen was separated by gel chromatography with Sephacryl S-1000. The antigen-coupled liposomes were stored in a phosphate buffered saline (PBS) containing 0.1% gelatin at 4°C and were stable for 6 mo.

Conventional and Double-Antibody Sandwich ELISA

Microtiter plates (96 well, Falcon 3912) were coated with 0.1 mg/mL antigen (myoglobin and the P121 peptide conjugated BSA, which was a different carrier from KLH used in immunization) in PBS at 4°C overnight. After washing with PBS containing 0.05% Tween and blocking with 10 mg/mL BSA solution, the plates were incubated with antibody solution diluted in PBS at T_r for 2 h. After further washing, goat antirabbit IgG conjugated with horseradish peroxidase and the substrate 5-aminosalicylic acid with H_2O_2 were added. The absorbance at 405 nm was directly measured using a microplate reader (Bio-Rad Laboratories, Richmond, CA, Model 450).

In the case of double-antibody sandwich ELISA, wells were coated with 0.1 mg/mL mouse antiMb monoclonal antibody at first. The other procedures were the same as described above.

Measurement of Immune Lysis of Antigen-Coupled Liposomes

The antigen-coupled liposomes were diluted with PBS^{2+} (PBS plus 0.1% gelatin, 0.5 m*M* $MgCl_2$, and 0.15 m*M* $CaCl_2$, pH 7.4). To 0.8 mL of the appropriately diluted antigen-coupled liposomes (0.21 nmol-DPPC/mL), a specific amount of the antibody was added to obtain a desired final concentration (0.02–20 μg/mL) and preincubated at 37°C for 10 min. After addition of GPWC ($2CH_{50}$/mL), the mixture, final volume 1 mL,was incubated at 37°C. The fluorescent intensity of CF released from the liposomes was measured with a spectrofluorophotometer (Model RF-5000, Shimadzu, excitation: 490 nm, emission: 514 nm). The total amount of releasable CF in the liposomes was determined by lysing completely the liposomes with an addition of 4 mL of 1-propanol. We used the volume ratio 1:4 (solution: 1-propanol) because the total fluorescent intensity of releasable CF in the liposomes was little affected by the amount of 1-propanol added in the range of the ratio from 1:1 to 1:4.

The marker release was calculated by the following equation.

$$\text{Marker release (\%)} = (F_t - F_0) / (F_T - F_0) \times 100$$

where F_0 is the fluorescent intensity of the liposome suspension before addition of GPWC. F_t and F_T are the intensities of released CF at t min after addition of GPWC and of total releasable CF, respectively. The marker release reached a plateau at around 60 min, so an incubation time of 60 min was adopted.

Table 1
The Effective Association Constant and Heterogeneity of Polyclonal Antibodies

Antibody	Effective association constant K_0, M^{-1}	Heterogeneity σ
Antimyoglobin	2.6×10^6	4
AntiP121	5.6×10^5	3

Measurement of DPCC and Protein Concentrations

The concentration of phospholipids in the liposome suspension was measured by the cholineoxidase method using Phospholipid C-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (*7*). The calibration curve was obtained by use of phospholipid of the same concentration as the liposomes, and the concentration was expressed in nmol-DPPC/mL.

The concentration of antigens coupled to liposomes was measured by the procedure of Heath et al. (*8*), or by the amino acid analysis.

RESULTS AND DISCUSSION

Effective Association Constants of AntiP121 and AntiMb Antibodies

Table 1 summarizes the values of the effective association constant K_0 and the heterogeneity index σ between antigen and corresponding antibody, determined as described in the previous section. The K_0 value of the antiMb antibody was slightly larger than that of the antiP121 antibody. These values of the antiP121 antibody coupled to Sepharose 4B were equal to those measured by the dialysis method in liquid phase (data not shown). The antiP121 antibody coupled with Sepharose 4B failed to bind to native myoglobin in solution. The antiMb antibody coupled to Sepharose 4B also did not bind to P121 in solution.

Reactivity Between Antibodies and Antigens in ELISA

Fig. 1 shows the reactivities of antiMb antibody and antiP121 antibody in ELISA with myoglobin coated on microtiter plates. AntiP121 antibody crossreacted with the adsorbed antigen, although its reactivity was slightly lower than that of antiMb antibody. A nonspecific antibody did not bind to myoglobin adsorbed on the solid surface.

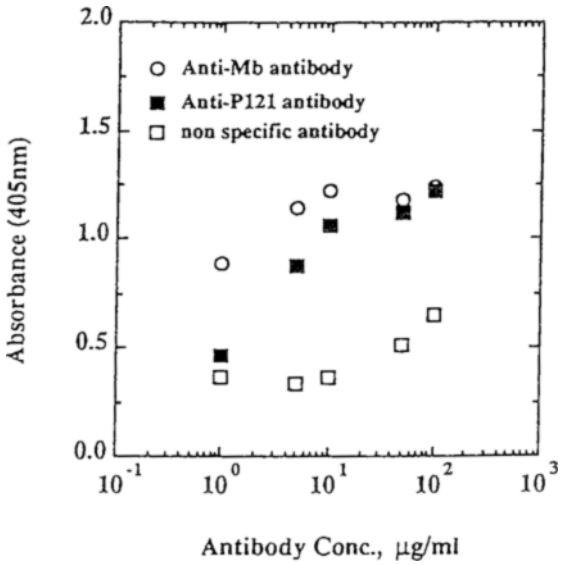

Fig. 1. Reactivity of the antiMb and antiP121 antibodies with myoglobin in ELISA.

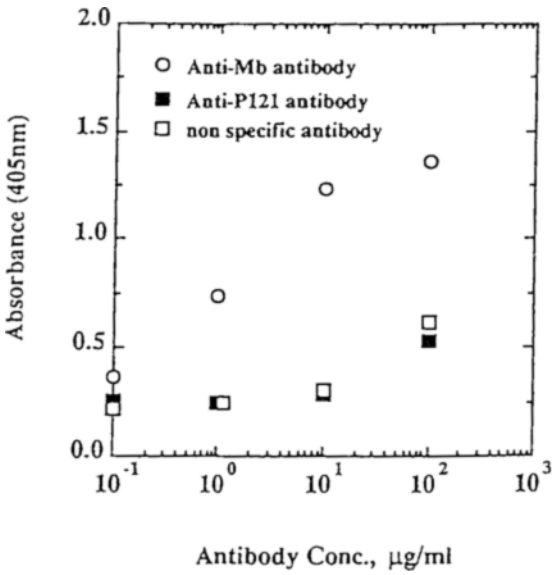

Fig. 2. Reactivity of the antiMb and antiP121 antibodies with myoglobin in double-antibody sandwich ELISA.

The reactivities of these antibodies measured by double-antibody sandwich ELISA, in which myoglobin was bound to a mouse antiMb monoclonal antibody coated on the surface of microtiter wells, are shown in Fig. 2. Naturally, the antiMb polyclonal antibody shows the positive result, but in this case the antiP121 antibody failed to crossreact with myoglobin in solution, which differs from the result with the conventional

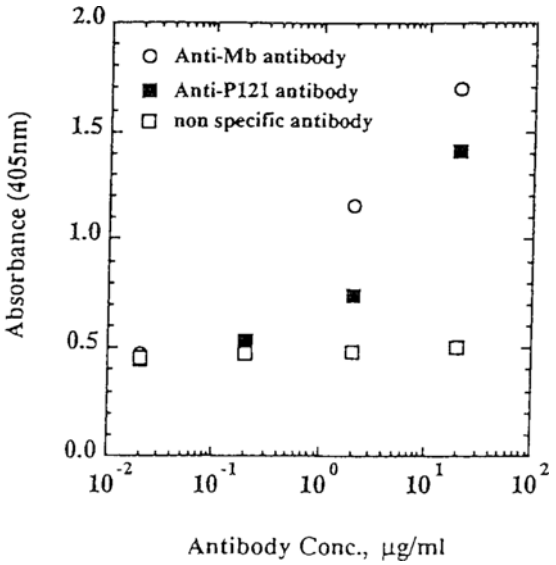

Fig. 3. Reactivity of the antiMb and antiP121 antibodies with P121-BSA conjugate in ELISA.

ELISA stated above. Since the mouse antiMb monoclonal antibody did not crossreact with the peptide P121 conjugate either in solution or by ELISA (data not shown), the corresponding region in myoglobin should be accessible to the antiP121 polyclonal antibody even when myoglobin binds to this monoclonal antibody. So, the failure of recognition of myoglobin bound to the monoclonal antibody by antiP121 polyclonal antibody indicates that some repertories of this antibody can recognize myoglobin when it is directly coated on the solid support, whose conformation might be changed by adsorption, but only little can crossreact with myoglobin, which has native conformation.

A peptide-carrier conjugate was prepared as an antigen for ELISA because the 10 residues' peptide did not bind well to the surface of microtiter plates. The peptide was coupled to BSA with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in order to couple the peptide to a different carrier by a different agent from those used in preparation of the immunogen (KLH and GA). Fig. 3 shows the reactivity of the antiMb and the antiP121 antibodies to the peptide-carrier conjugate coated on the microtiter plates. Both antibodies bound to the antigen. This was caused not by the conjugation of the peptide to carrier protein, but by adsorption of the conjugate antigen on the solid surface, because the antiMb antibody did not crossreact with the conjugate in liquid phase (data not shown). Adsorption of the conjugate may cause some change in the presentation of the peptide.

These results show that adsorption of antigens on the plastic surface of microtiter plates often cause changes in conformation of the antigens

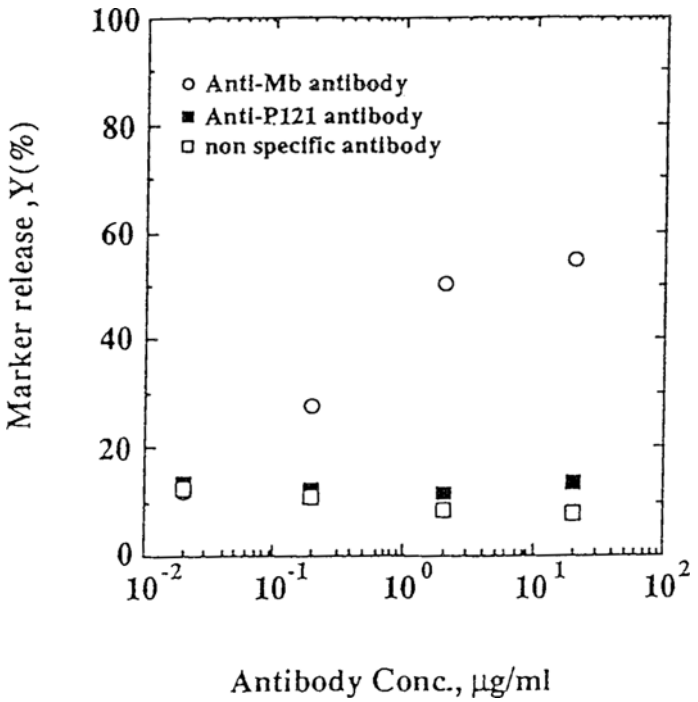

Fig. 4. Marker release from myoglobin-coupled liposomes with addition of antiMb and antiP121 antibodies (0.21 nmol-DPPC/mL, 2.80×10^{-3} mol-Mb/mol-DPPC).

and result in different crossreactivities between the antigens and antibodies. It is reported that this may be the cause of the different binding affinity of antipeptide monoclonal antibodies against native proteins (*1*), and that double-antibody sandwich ELISA must be applied to select clones reactive with native proteins.

Binding Characteristics Between Liposome-Bound Antigens and Antibodies

Fig. 4 shows the relationship between the marker release from myoglobin-coupled liposomes and the concentration of the antiMb and antiP121 polyclonal antibodies. The marker release increased with an increase in the concentration of the antiMb antibody. The immunoliposomes were lysed by the membrane attack complex, which was formed by the complement system activated by the antigen–antibody complex. In the case of the antiP121 antibody and nonspecific antibody, only a little marker release was observed, which was caused by the alternative pathway of complement activated without the formation of the antigen–antibody complex (*6*). Since there was no difference in the marker release between them, it is said that the antiP121 antibody could not bind to liposome-bound myoglobin.

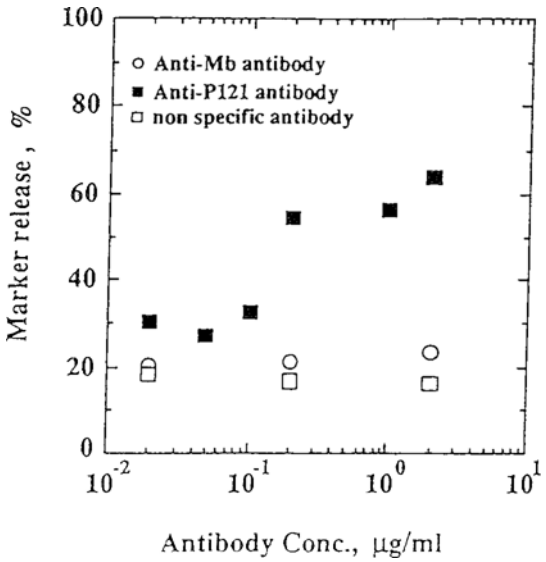

Fig. 5. Marker release from P121-coupled liposomes with addition of antiMb and antiP121 antibodies (0.21 nmol-DPPC/mL, 1.36×10^{-2} mol-P121/mol-DPPC).

A similar result was obtained in the case of liposome-bound peptide P121 (Fig. 5). Only the antiP121 antibody raised against the peptide bound to it. The addition of the antiMb antibody and nonspecific antibody did not cause the marker release by activation of the classical pathway of complement.

These binding characteristics are identical to those between antiP121 antibody and myoglobin, between antiMb antibody and P121 in solution, and also in the double-antibody sandwich ELISA. These results indicate that the conformation of liposome-bound antigen is changed little by coupling to liposomes and is almost the same as that of native antigen in liquid phase. Therefore, this method will be much more useful than double-antibody sandwich ELISA to select clones of monoclonal antibodies reactive with native proteins in a short time, i.e., 60 min, and without use of an extra antibody.

REFERENCES

1. Van Regenmortel, M. H. V., Braiand, J. P., Muller, S., and Plaué, S. (1988), *Synthetic Polypeptides As Antigens*, Elsevier, Amsterdam.
2. Mariani, M., Bracci, L., Presentini, R., Nucci, D., and Antoni, G. (1987), *Mol. Immunol.* **24,** 297.
3. Kondo, A., Takamatsu, H., Katoh, S., and Sada, E. (1990), *Biotech. and Bioeng.* **35,** 146.
4. Pauling, L., Pressman, D., and Grossberg, A. L. (1944), *J. Am. Chem. Soc.* **66,** 784.

5. Barbet, J., Patrick, M., and Leserman, L. D. (1981), *J. Supramol. Struct. Cell Biochem.* **16,** 243.
6. Kishimura, M., Yamaji, H., Fukuda, H., Katoh, S., and Sada, E. (1989), *J. Ferment. Bioeng.* **68,** 395.
7. Takayama, M., Itoh, S., Nagasaki, T., and Tanimizu, I. (1977), *Clin. Chem. Acta* **79,** 93.
8. Heath, T. D., Macher, B. A., and Papahadjopoulos, D. (1981), *Biochim. Biophys. Acta* **640,** 66.