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Enhanced activity of immobilized dimethylmaleic anhydride-protected poly- and monoclonal antibodies

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ABSTRACT

The effect of reversible protection of the free amino groups of poly- and monoclonal antibodies by dimethylmaleic anhydride on their binding activity following immobilization onto various carriers was studied. The treatment with dimethylmaleic anhydride resulted in a 1.6–1.8-fold increase in the activity of immobilized goat anti-mouse immunoglobulin antibody immobilized onto different epoxy containing carriers and a 3–10.7-fold increase in the activity of immobilized monoclonal antibodies specific for carboxypeptidase A. The increase in activity was most pronounced at low antigen to carrier loads and over a wide range of modifier to protein ratios. The application of reversible protection of antibodies may permit the development of highly active immobilized antibody preparations for use in immunoaffinity purification.

INTRODUCTION

Immobilization of antibodies (or other biologically active proteins) onto solid matrices often leads to partial or complete loss of their antigen-binding activity, as a result of either (a) chemical modification of amino residues essential for activity, (b) steric hindrance caused by the attachment of the antibody to the matrix at a region close to its active site or (c) conformational changes and/or restriction of the intramolecular mobility imposed by multi-point interaction with the matrix.

The susceptibility of individual antibody molecules to loss of antigen-binding activity by mechanism (a) or (b) would depend on their chemical composition. These effects may therefore vary between different antibody molecules. On the other hand, as the number and distribution of free amines are roughly similar in most antibody molecules, it is logical to expect that most if not all of them would suffer a loss of activity due to multi-point attachment of the amines to the carrier. This problem may be more severe with carriers that have a high density of reactive groups, such as Eupergit C.

Eupergit C is a polymethylmethacrylamide-based polymer bearing oxirane

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groups which may bind proteins mainly via reaction with their amino groups. Thiol moieties, which may also react with oxirane groups of Eupergit C, are less recurrent in the molecules of most proteins.

Recently, the use of dimethylmaleic anhydride (DMA) for enhancement of the activity of antibodies conjugated with methotrexate has been described[1]. It was suggested that DMA treatment served to protect amino acid residues essential for antibody activity.

We hypothesized that reversible protection of some free amines of the antibody molecule by DMA prior to its immobilization onto Eupergit C might serve on the one hand to protect essential amino acid residues in the antigen-binding site of the antibody, and on the other to reduce the number of attachment points between antibody and carrier. Thus, the activity of the immobilized antibody may be enhanced. The results of experiments described in this paper tend to support this hypothesis.

EXPERIMENTAL

Materials

Immunoaffinity-purified goat anti-mouse Fc antibodies were obtained from Biomakor (Nes-Ziona, Israel). Two products based on Eupergit C were used (1) CB6200, which are 6 mm in diameter poly(methyl methacrylate) beads, coated with non-porous 1-μm diameter, Eupergit C beads; and (2) standard Eupergit C, which is 150-μm diameter porous beads. Both products were obtained from Rohm (Darmstadt, F.R.G.). Epoxy-activated Sepharose 6B was obtained from Pharmacia (Uppsala, Sweden). Poly(vinyl alcohol)-epoxy (PVA-epoxy) was obtained from Riedel-de Haën (Seelze, F.R.G.). All other materials were purchased from Sigma (St. Louis, MO, U.S.A.) unless specified otherwise.

Monoclonal antibodies

CB6F1 mice were immunized with two injections, ten days apart, of 10 μg of carboxypeptidase A (CPA) in 0.2 ml of phosphate-buffered saline (PBS) emulsified in an equal volume of Freund's complete adjuvant. After a further 20 days, their spleen cells were fused with NSO myeloma cells[2] as described previously[3]. The resulting hybridoma cultures were first tested by enzyme-linked immunosorbent assay (ELISA) for secretion of antibodies recognizing CPA. In a second screening, the cultures were tested by reverse ELISA (as described below) for their ability to immobilize CPA without affecting its enzymic activity.

A similar approach was utilized for the production of another antibody specific for the enzyme horseradish peroxidase (HRP), which was named mAb HRP2, except that the reverse ELISA was used as the only screening procedure.

Ascites fluid was produced in CB6F1 mice following priming with Freund's incomplete adjuvant as described previously[4].

All the mAbs used in this study were purified by the use of protein A–Sepharose (Bio-Rad Labs.) as described previously[5].

ELISA

The wells of microtitre ELISA plates were coated with the protein being tested.

They were exposed for 16 h to a solution containing 1 μg of protein per well in 0.1 ml of 0.1 *M* carbonate buffer (pH 9.6) at 4°C. After washing several times with tap water, mAb (hybridoma culture media or ascites fluids diluted in skim milk containing 1% fat) was added (0.1 ml per well). After 1 h at 37°C, the wells were washed five times with tap water. To each well, 0.1 ml of goat anti-mouse immunoglobulin G (IgG–HRP complex (Bio-Rad Labs.), diluted 1:3000 with skim milk (1% fat), was added and the plates were incubated at 37°C for 1 h. After washing several times with tap water, 0.1 ml of *o*-phenylenediamine reagent was added to each well and the colour allowed to develop. The *o*-phenylenediamine reagent contained 2 mg of 1,2-phenylenediamine in 1 ml of 50 m*M* sodium citrate buffer (pH 5.0) containing 0.08% hydrogen peroxide. The activity was determined by measuring of the rate of increase in absorbance at 492 nm compared with a non-specific absorbance at 405 nm in a EAR400 ELISA Reader (SLT, Grodig, Austria).

Reverse ELISA

Microtitre ELISA plates were coated with rabbit anti-mouse Ig antibodies (Sigma) (10 μg per ml of PBS, 100 μl per well, for 16 h at 4°C) and washed several times with tap water. Hybridoma culture fluids were added to the wells (100 μl per well) the plates were incubated for 1 h at 37°C and washed several times with tap water. CPA (100 μl, 10 μg per ml of PBS) was added to the wells and the plates were incubated for 1 h at 37°C and washed several times with PBS. CPA substrate (100 μl per well of PBS) was added to the wells and the plates were incubated for 1h at 37°C. CPA activity was measured by the ninhydrin method on the ELISA plates as follows: to 0.1 ml of fresh ninhydrin reagent (3% ninhydrin in methyl Cellosolve) were added 0.05 ml of $2 \cdot 10^{-4}$ *M* sodium cyanide in 3.8 *M* sodium acetate (pH 5.3), sample was then added and the mixture was heated at 95°C for 20 min, cooled and read in ELISA reader at 550 nm.

A similar reverse ELISA procedure was used for the determination of the activity of the mAb HRP2 except that the substrate used was the *o*-phenylenediamine substrate solution (above) as described for the regular ELISA.

Immobilization of antibodies on Eupergit C, PVA-epoxy and epoxy-activated Sepharose 6B

DMA method. DMA [dissolved in dimethylformamide (DMF)] was added to antibodies [5–10 μg per sample, concentration 0.3–1.3 mg/ml, 5–30 μl, in 25 m*M* sodium borate buffer (pH 9.5) + 1 *M* NaCl] at a 400 molar excess, keeping the final DMF concentration in the antibody solution below 5%. The solution was incubated for 30–60 min at 0°C. The antibodies were then added to carrier beads [0.5 mg in 5 μl of 1 *M* potassium phosphate (pH 7.5)] in a Beckman tube and incubated for 4 h at room temperature and then 16 h at 4°C. Excess oxirane groups were blocked by incubating the carrier beads–antibody conjugate with 2-mercaptoethanol (2ME) solution (final concentration 0.2 *M*) for 4 h at room temperature. The beads were then washed with PBS and the DMA was hydrolysed by incubation at acidic pH [50 m*M* citrate buffer (pH 5.5) for 1 h at room temperature], followed by washing with PBS.

Standard method. The method is identical with the above DMA method except that the antibodies were not reacted with the DMA prior to their immobilization and the DMA was not hydrolysed by the acid treatment after blocking with 2ME solution.

Immobilization of antibodies onto CB6200 beads

Standard method. A 10-μg amount of anti-Fc antibodies was bound to the bead in 0.3 ml of 1 *M* potassium phosphate buffer (pH 7.5). The beads were incubated with the antibody for 16 h at 4°C and washed with PBS. The beads were then blocked with 2ME (0.2 *M* for 4 h at room temperature) and finally washed with PBS.

DMA method. The pretreatment of antibodies with DMA, binding of the antibodies to the bead, blocking with 2ME and hydrolysis of DMA were as described above.

Determination of the activity of anti-CPA antibodies

CPA was added to the Eupergit C–antibody conjugate (at more than 2 mol per mole of input antibody) in PBS + 0.5 *M* NaCl and incubated for 1 h at room temperature with shaking. The non-bound CPA was washed with PBS. Hippuryl-L-phenylalanine (10 m*M* in PBS + 0.5 *M* NaCl, 50 μl per sample) was added to the sample and incubated for 10 min with shaking. A sample was removed for determination of the amount of product by the ninhydrin method[6].

Determination of the activity of anti-mouse Fc antibodies immobilized on CB6200 beads

MAb HRP2 was applied to the beads (10 μg of antibody per bead in 0.3 ml of low-fat milk) and incubated for 1 h at room temperature. Following washing with water, HRP was added to the beads (5 μg per bead in 0.3 ml of low-fat milk) the beads were incubated for 1 h at room temperature and washed with PBS. The beads were then transferred into the wells of a 24-well plate (Costar) and to each well was added 1 ml of *o*-phenylenediamine substrate solution. The plates were placed on a horizontal rotating table, mixed at 200 rpm for 5 min and then the reaction was stopped by the addition to each well of 0.5 ml of 4 *M* HCl. The colour developed was analysed by the ELISA reader as described above.

Determination of the activity of the anti-mouse Fc antibodies immobilized on Eupergit C standard, PVA-epoxy and epoxy-activated Sepharose 6B

Anti-mouse Fc antibodies were immobilized on 5 mg of carrier. MAb HRP2 (50 μg in 25 μl of milk) was then added, the tubes were incubated for 1 h at room temperature and then the carrier beads were washed with PBS. To each tube was added HRP (50 μg of enzyme in 25 μl of milk) and the tubes were incubated for 1 h at ambient temperature, with shaking, and the carrier beads were then washed with PBS. The beads were dispersed in 1 ml of PBS and a 50-μl sample was transferred into a fresh tube and mixed with 1 ml of *o*-phenylenediamine substrate solution. Colour development was stopped after 1 min by the addition of 0.5 ml of 4 *M* HCl. The colour was analysed by the ELISA reader as described above.

RESULTS

The immobilization of goat anti-mouse Fc antibodies was selected as a model system for the study of the effect of DMA on the activity of immobilized polyclonal antibodies. The immobilized anti-mouse Fc antibodies were used for the binding of mAb HRP2 and the amount of the HRP enzyme which was bound by the immobilized mAb served for the determination of the activity of the anti-mouse Fc antibodies. The

TABLE I

EFFECT OF DMA ON THE ACTIVITY OF POLYCLONAL ANTI-MOUSE Fc ANTIBODIES IMMOBILIZED ON DIFFERENT CARRIERS

Carrier	*Antibody activity*[a]		*Improvement factor*
	Standard method	*DMA method*	
CB6200	1.060	1.949	1.84
	–	0.341[b]	0.32
Eupergit C standard	0.740	1.214	1.64
Epoxy-activated Sepharose 6B	0.023	0.040	1.74
PVA-epoxy	0.086	0.156	1.81

[a] Antibody activity is expressed as A_{492} resulting from the activity of horseradish peroxidase which is bound to mAb HRP2 which is bound by the immobilized anti-mouse Fc antibodies. Assay was performed as described under Experimental. Background resulting from the direct immobilization of mAb HRP2 or horseradish peroxidase on the carrier was subtracted.

[b] Blocking with 2-mercaptoethanol was performed after hydrolysis of the bound DMA.

results of these experiments are summarized in Table I. As can be seen, reversible protection of the antibodies with DMA allowed the formation of more active immobilized anti-mouse Fc antibodies. Similar activity improvement factors were obtained for the different carriers used.

When the DMA was removed from the immobilized antibodies prior to elimination of unreacted oxirane groups, a decrease in activity of antibodies immobilized on Eupergit C was observed (Table I). A similar result was obtained when excess of DMA was removed from the system (without hydrolysing the bound DMA) before the unreacted oxirane groups were eliminated (data not shown).

Immobilization of mAbs specific for the enzyme CPA which do not inhibit enzymic activity was selected as a model system for the study of the effect of DMA treatment on the activity of immobilized mAbs. The activity of the immobilized

TABLE II

ACTIVITY OF ANTI-CPA MONOCLONAL ANTIBODIES IMMOBILIZED ON EUPERGIT C BY THE STANDARD AND DMA METHODS

Monoclonal antibody	*Antibody activity*[a]		*Improvement factor*
	Standard method	*DMA method*	
CPA1	0.040	0.362	9.0
CPA8	0.060	0.216	3.6
CPA9	0.082	0.242	3.0
CPA14	0.102	0.300	3.0
CPA18	0.030	0.322	10.7

[a] Antibody activity is expressed as A_{550} resulting from the activity of carboxypeptidase A bound by the immobilized antibody. CPA activity was measured by the ninhydrin method as described under Experimental. Background resulting from the activity of carboxypeptidase A directly immobilized on Eupergit C was subtracted.

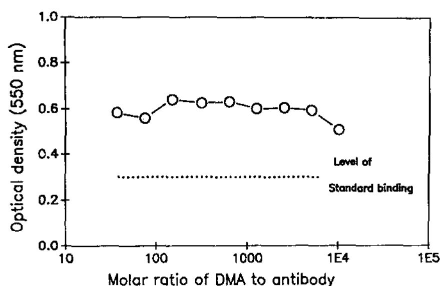

Fig. 1. Effect of varying the ratio of DMA per antibody on the activity of immobilized monoclonal antibody CPA8. The graph depicts the binding activity of the immobilized antibody as manifested by the activity of the CPA bound to the antibody.

antibodies was evaluated through measurements of the enzymic activity of CPA bound by the antibody (for details see Experimental). Five different mAbs were tested in this assay. As can be seen in Table II, antibodies immobilized after protection with DMA possessed a higher CPA binding activity compared with the standard immobilization method.

The optimum ratio of DMA to protein necessary for achieving the above response was examined in another experiment. MAb CPA8 was immobilized on Eupergit C following pretreatment with different doses of DMA. As can be seen in Fig. 1, a similar effect of the DMA treatment on the activity of the immobilized antibody was observed over a wide range of DMA to protein ratios.

The effect of the DMA treatment on the activity of antibodies immobilized at different loads on Eupergit C was also studied. As can be seen in Fig. 2, maximum improvement in the activity of immobilized mAb CPA8 was observed at low antibody loads and was less pronounced at high antibody loads.

DISCUSSION

Several possible mechanisms may explain the increase in activity of antibodies immobilized on Eupergit C, epoxy-activated Sepharose 6B and PVA-epoxy achieved by employing reversible protection by DMA.

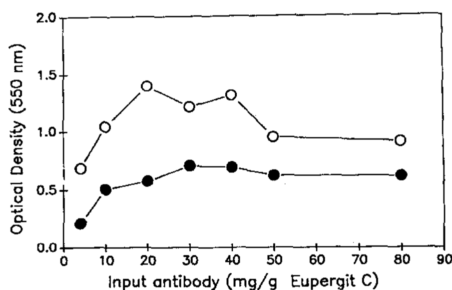

Fig. 2. Immobilization of different doses of (○) DMA-treated and (●) untreated monoclonal antibody CPA8 on Eupergit C. The graph depicts the binding activity of the immobilized antibody as manifested by the activity of the enzyme CPA bound by the antibody.

Blocking of most of the free amines on treatment of the protein with DMA would result in the formation of fewer bonds between the protein and matrix, down to a single bond. This would result in immobilization of antibodies having as native a conformation and intramolecular mobility as possible and therefore having antigen-binding properties close to those of free antibodies.

Failure to eliminate the residual oxirane groups prior to hydrolysis of the bound DMA or even prior to removal of the excess of DMA from the solution resulted in a decrease in the activity of the antibodies immobilized on Eupergit C in comparison with untreated antibodies. This result indicates that additional bonds between the unblocked carrier and the newly exposed amines may take place. Thus, formation of multiple bonds between the native antibody molecule and the carrier, which are inhibited by pretreatment of the antibody with DMA, are at least partially responsible for the loss of the activity of the immobilized antibodies.

Treatment of the antibody with DMA would block mainly the most reactive amines of the antibody molecule. Hence the DMA-protected antibody would be immobilized via the less reactive amines, those which in the native protein would have less chance of interacting with the matrix. This reorientation of the protein–matrix site of interaction may cause an increase in the activity of the immobilized antibody if the most reactive amines are located near the active site while the less reactive amines are remote from it. On the other hand, it may happen that the protein–matrix interaction site would be relocated to an area close to the active site, in which case a decrease in the activity of the immobilized antibody should be observed.

As the number and distribution of amines in different immunoglobulin molecules is similar, the DMA treatment is expected to lower the number of antibody–matrix interactions, and consequently increase the activity, of most immobilized antibody molecules. On the other hand, the effect of reorientation of the antibody–matrix binding site is likely to be variable between different mAbs, as discussed above. Our experiments demonstrated a marked improvement in the activity of immobilized polyclonal antibodies and all five mAbs tested. Hence one might expect that this novel procedure could be suitable for preserving the activity of the majority of immobilized mAbs.

To the best of our knowledge there have been no previous reports on the use of reversible amino group blocking reagents for protecting the activity of immobilized proteins. Hence the DMA method for immobilization of antibodies, and probably also other proteins, on Eupergit C or other carriers should be a valuable adjunct to currently available immobilization methods.

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