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Enzymic oxidation of monoclonal antibodies by soluble and immobilized bifunctional enzyme complexes

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ABSTRACT

Site-specific modification of monoclonal antibodies was achieved by oxidation of the carbohydrate moieties of antibodies which are located remote from the antigen binding sites. Sialic acid and galactose are terminal sugars of these carbohydrate chains. Concomitant treatment of the antibodies with neuraminidase and galactose oxidase generated aldehyde groups in the oligosaccharide moieties of immunoglobulins which reacted selectively with amino or hydrazide groups of the matrix. Subsequent immobilization of neuraminidase and galactose oxidase on Eupergit C–adipic dihydrazide proved to be an efficient and selective system for the enzymic oxidation of the monoclonal antibodies without impairing their immunological activity. Oriented immobilization of enzymically oxidized monoclonal antibodies on hydrazide or amino Eupergit C derivatives thus leads to the formation of antibody matrix conjugates which possess high antigen-binding activities.

INTRODUCTION

Site-specific modification of antibodies and possible oriented immobilization may be achieved by chemical or enzymic oxidation of the carbohydrate moieties of the antibodies prior to carrier attachment[1–3]. As the carbohydrate residues on the Fc fragment of the antibody molecule are located remote from the binding site of the antibody, immobilization via this region usually does not impair the immunological activity of the antibody. Previous attempts have been described that utilize enzymic oxidation of various glycoproteins for labelling or attachment to liposomal membrane[1,4–6].

The contents and compositions of carbohydrates in immunoglobulin G (IgG) from rabbit, horse, human and bovine serum are very similar. Each molecule of IgG contains 0–1 residues of fucose, 3 of mannose, 1–2 of galactose, 3–4 of N-acetylglucosamine and 0–3 of sialic acid (N-acetylneuraminic acid; NANA)[7]. The sialic acid was shown to be terminal by its release with neuraminidase and by identification as the N-acetyl derivative by gas chromatography. Incubation with β-galac-

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tosidase subsequent to treatment with neuraminidase (NA) liberated galactose, indicating the presence of a sequence of NANA–GAL. In principle, each of these carbohydrate residues may be oxidized by specific enzymes to yield the corresponding aldehyde or keto derivatives.

In this work we used the oxidation of C-6 of galactose by galactose oxidase to form a corresponding C-6 aldehyde on galactose. Galactose oxidase, in combination with neuraminidase, may be used for the modification of galactosyl residues penultimate to sialic acid. The newly formed aldehyde groups may then react with hydrazide groups of the carrier [*e.g.*, Eupergit C–adipic dihydrazide (ADH)] to form stable covalent bonds of the antibody with the matrix. The data presented in this paper show that the co-immobilization of neuraminidase and galactose oxidase on Eupergit C–ADH beads provides an economical, efficient and selective system for the enzymic oxidation of monoclonal antibodies without impairing the immunological activity of the antibodies.

EXPERIMENTAL

Enzymes

Carboxypeptidase A (CPA) and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO, U.S.A.). Determination of the enzymatic activity of CPA and HRP were described previously[3].

Galactose oxidase (GO), Cat. No. G-3385, purchased from Sigma, was assayed according to the manufacturer's instructions. One unit of enzyme activity is defined the amount which produces an increase of 1.0 unit of absorbance at 425 nm in 1 min under the experimental conditions specified. Neuraminidase from *Clostridium perfringens*, Cat. No. 107590 (free from protease) was purchased from Boehringer (Mannheim F.R.G.). The enzymic activity of NA was determined according to Potier[8].

Preparation and purification of monoclonal antibodies

The monoclonal antibodies (mAbs) anti-CPA mAb100 and anti-HRP mAb2 were purified and characterized as described previously[3,9]

Enzyme-linked immunosorbent assay (ELISA)

ELISA plates were coated with intact or oxidized mAbs (10 μg/ml, 100 μl, 1 h at 37°C) and blocked by 1% fat milk for a further 1 h. HRP was added at various concentrations to the wells and incubated for a further 1 h at 37°C. The enzymic activity of antibody-bound HRP was determined as described previously[3].

Preparation of Eupergit C–ADH

Eupergit C beads (100 mg) were treated with ADH (Sigma) for 16 h at room temperature in 0.2 *M* sodium carbonate buffer (pH 9.0). The amount of ADH added to the carrier varied between 0.01 *M* (Eupergit C–$ADH_{0.01}$) and 0.1 *M* (Eupergit C–$ADH_{0.1}$). For immobilization of minute amounts of neuraminidase and galactose oxidase, 0.01 *M* ADH proved to be sufficient. The modified carrier was thoroughly washed with phosphate-buffered saline (pH 7.4) (PBS) and excess of oxirane groups of the Eupergit C were blocked with 0.2 *M* β-mercaptoethanol for 2 days at

room temperature. After thorough washing with 0.1 *M* acetate buffer (pH 5.5), the modified beads were stored at 4°C for up to 60 days for further use. With longer storage periods new oxirane groups are exposed to the solution and/or blocked β-mercaptoethanol groups are hydrolysed and additional blocking is required. The amount of ADH groups introduced onto the matrix was determined by reaction with trinitrobenzenesulphonic acid (TNBS)[10,11].

Chemical oxidation of the monoclonal antibodies

The chemical oxidation and oriented immobilization of anti-CPA mAb100 and anti-HRP mAb2 were carried out with sodium periodate in a one-step oxidation procedure as described previously[12]. A similar reaction in solution was carried out at 0°C in the dark using 10 m*M* sodium periodate. The reaction was stopped after 1 h by addition of 10 m*M* sodium thiosulphate.

Antigen-binding activity of native and oxidized antibodies

In order to determine the antigen-binding activity of native and oxidized antibodies, they were conjugated to intact Eupergit C or Eupergit C–ADH, respectively, as described previously[3,13]. The matrix–antibody conjugates (5 mg containing 10 μg of immobilized antibody) were incubated with a 5-fold molar excess of the respective antigen (HRP or CPA). After thorough washing of the beads with PBS, the respective enzymic activity was determined as described previously[3].

The antigen-binding activity of anti-HRP antibodies was also determined using an ELISA test: anti-HRP mAb2, chemically or enzymatically oxidized (100 μl, 10 μg/ml) was coated on a ELISA plate for 1 h at 37°C and blocked with milk for a further 1 h. HRP was added to the wells at various concentrations and incubated for a further 1 h at 37°C. In control experiments, unmodified antibody was assayed under the same conditions. The enzymic activity of antibody-bound HRP was determined as described previously[3].

Determination of aldehyde groups on the antibody molecule

Aldehyde groups on oxidized antibodies were determined using the avidin–biocytin–hydrazide system followed by a modified dot blotting method. A 3-μl volume of oxidized antibody was applied to nitrocellulose discs, dried and incubated with 10 μg/ml of biocytin–hydrazide for 1 h. After repeated washings with PBS and blocking with bovine serum albumin (BSA) for 1 h, avidin–alkaline phosphatase conjugate was added and the mixture incubated for 30 min at 37°C After repeated washings the amount of alkaline phosphatase bound to the blot was determined by hydrolysis of the soluble substrate *p*-nitrophenyl phosphate. The amount of alkaline phosphatase bound was directly proportional to the amounts of aldehyde groups on the oxidized antibodies. A calibration curve for lactoperoxidase–biotin containing a known number of biotin molecules per molecule of protein was used.

Enzymic oxidation of monoclonal antibodies by soluble neuraminidase and galactose oxidase

Anti-CPA mAb100 (10 μg in 0.1 ml of 0.1 *M* acetate buffer, pH 5.0) was incubated with neuraminidase containing 0.012 units (0.1 ml) at 37°C for 20 h with gentle shaking. After dialysis for 16 h against 0.1 *M* phosphate buffer (pH 6.0), the

mAb was treated with galactose oxidase containing 2.5 units (25 μl) for 17 h under the same conditions as for the neuraminidase treatment. Aliquots (25 μl) were withdrawn at various time intervals and newly formed aldehyde groups on the antibody molecule were determined as described above. Other aliquots were withdrawn and conjugated to Eupergit C–ADH in order to analyse for antigen-binding activity as described above.

Immobilization of neuraminidase and galactose oxidase on Eupergit C–ADH

Neuraminidase and galactose oxidase in 0.1 *M* acetate buffer (pH 5.5) were oxidized with 10 m*M* sodium periodate and immobilized onto Eupergit C–$ADH_{0.01}$ using a one-step oxidation procedure[12]. Three different preparations of co-immobilized neuraminidase and galactose oxidase were prepared by the reaction of 25 mg of the matrix with a mixture of enzymes containing total protein of 0.05, 0.1 and 0.5 mg in a 1:4 ratio (in units of enzymic activity) of galactose oxidase to neuraminidase. After coupling of the enzymes to the carrier, the preparations were treated with 0.2 *M* acetaldehyde in 0.1 *M* acetate buffer (pH 5.5) for 2 days to block the residual reactive hydrazide groups. The amount of enzymes immobilized on the carrier was determined by measuring the respective enzymic activities in the reaction mixture supernatants before and after the reaction.

In parallel experiments, galactose oxidase was immobilized separately by binding directly to Eupergit C or after oxidation to Eupergit C–$ADH_{0.01}$ as described above. Soluble neuraminidase was added to the reaction mixture. The enzymes were tested for their ability to oxidize the antibodies anti-CPA mAb100 and anti-HRP mAb2.

Enzymic oxidation of antibodies by co-immobilized enzymes

In order to determine the capability of the three Eupergit C–$ADH_{0.01}$-conjugated enzyme preparations to oxidize antibiodies, anti-CPA mAb100 (20 μg) was incubated with each of these preparations for various periods of time. The degree of oxidation of the antibodies was estimated by their immobilization by binding onto Eupergit C–$ADH_{0.1}$ beads as described above. In addition, a method for a concomitant determination of aldehyde groups formed on the antibody molecules and the immunological activity of these antibodies was developed: 25 mg of Eupergit C–$ADH_{0.01}$ containing 0.5 mg of immobilized enzymes were incubated with 20 μg of mAb100 and 20 μg of biocytin hydrazide. The supernatant containing the hydrazide derivative of mAb100 was bound to Eupergit C-conjugated streptavidin via the biocytin moicty. Thc amount of bound mAb directly proportional to number of aldehyde groups induced in the antibody molecule by enzymic oxidation. The enzymic activity of CPA immunologically bound to the immobilized mAb was determined as described previously[3].

Operational stability

The above three preparations were incubated repeatedly with new amounts of mAb for various periods of time. The operational stability was determined during 60 days.

RESULTS

Enzymic oxidation of monoclonal antibodies by a soluble bifunctional enzyme system

The applicability of soluble GO and NA to the enzymic oxidation of antibodies was investigated using soluble enzymes. The formation of aldehyde groups on the antibody molecules, the binding of oxidized antibodies onto the Eupergit C–ADH matrix and the antigen-binding activity of the immobilized antibodies were studied and optimized. For this purpose we developed an assay based on the reaction of biocytin–hydrazide with the aldehyde groups formed on the antibody molecules. The antibody-bound biocytin was then reacted with streptavidin-conjugated alkaline phosphatase. The activity of alkaline phosphatase was directly proportional to the number of aldehyde groups formed on the antibody molecules. By using of a calibration curve for a lactoperoxidase–biotin complex possessing a predetermined number of biotin residues per lactoperoxidase molecule, the number of aldehyde groups on the antibody molecule was determined. By this assay it was found that the oxidation by soluble enzymes produced a maximum of two aldehyde groups per mole of antibody, compared with 3.2–4 aldehyde groups obtained after periodate oxidation.

The antigen-binding activity of anti-HRP mAb2 was determined by an ELISA test. Enzymically and chemically oxidized antibodies and intact antibodies were adsorbed on a polystyrene plate and their binding activities for HRP were assayed. As show in Fig. 1, a substantial increase in immunological activity of mAb HRP2 occurred after enzymic oxidation, corresponding to 1.9 mol of HRP bound per mole of antibody, compared with 0.8 and 1.4 mol/mol obtained with the chemically oxidized and intact antibody, respectively.

As the enzymically oxidized antibody fully retained its antigen-binding capacity, we used the enzymic activity of the antigen (HRP) immunologically bound to the antibody to monitor the kinetics of the coupling of oxidized antibody to Eupergit C–$ADH_{0.1}$. As shown in Fig. 2, the enzymic activity of HRP bound to oxidized antibodies immobilized on Eupergit C–ADH is dependent on oxidation time. Treatment with NA increased the amount of antibody bound to the matrix, apparently owing to the release of sialic acid leading to exposure of galactose to oxidation by GO.

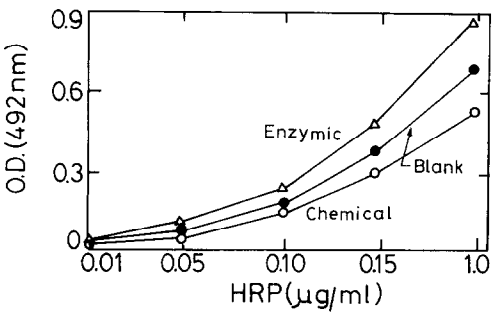

Fig. 1. Determination of immunological activity of anti-HRP mAb2 after (△) enzymic and (○) chemical oxidation and (●) of unmodified antibody measured by an ELISA assay.

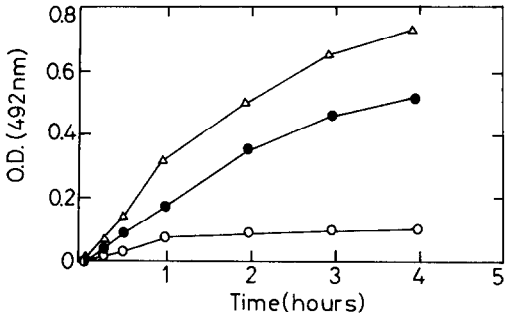

Fig. 2. Kinetics of enzymic oxidation of anti-HRP mAb2 with (△) neuraminidase–galactose oxidase or (●) galactose oxidase only. The oxidized antibodies and (○) unmodified antibody were immobilized on Eupergit C–$ADH_{0.1}$ and the enzymic of immunologically bound HRP was determined.

Enzymic oxidation of monoclonal antibodies by immobilized bifunctional enzyme complex

Neuraminidase and galactose oxidase (glycoproteins) were oxidized by reaction with sodium periodate using a one-step procedure[12]. Binding of the enzyme to Eupergit C–ADH was optimized by testing several preparations of Eupergit C–ADH, obtained by treatment of Eupergit C with various amounts of ADH and various concentrations of blocking reagent (β-mercaptoethanol). The enzymic activity of the co-immobilized NA and GO was measured by determination of newly formed aldehyde groups on the antibody molecule. As shown in Fig. 3, maximum activity of immobilized enzymes (2 mol of aldehyde per mole of anti-CPA mAb100) was obtained when 0.5 mg of the two enzymes (a ratio of 1:4 units of the enzymic activity of NA to GO) was co-immobilized onto Eupergit C–ADH prepared with 0.01 *M* ADH and blocked with 0.2 *M* β-mercaptoethanol for 16 h.

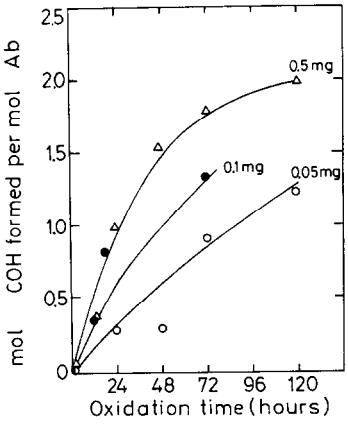

Fig. 3. Determination of aldehyde groups formed on anti-CPA mAb100 using the immobilized bifunctional enzymic system. Different amounts of immobilized enzymes (○, 0.05 mg; ●, 0.1 mg; △, 0.5 mg) were incubated with 10 μg of antibody. The enzymic activity of alkaline phosphatase–avidin measured as the increase in absorbance at 405 nm was proportional to the number of aldehyde groups formed.

TABLE I

ENZYMIC OXIDATION OF mAbs BY A HETEROGENEOUS COMPLEX OF NEURAMINIDASE AND GALACTOSE OXIDASE

Oxidation system	*Enzymic activity of AP–avidin*[a]	
	4 h[b]	*24 h*[b]
GO + NA	0.17	0.17
EC–GO + NA	0.02	0.07
EC–ADH–GO + NA	0.14	0.18
EC–ADH–GO–NA	ND[c]	0.15

[a] The values given indicate the absorbance at 405 nm as a measure of the enzymic activity of alkaline phosphatase (AP)

[b] Time of oxidation.

[c] Not determined.

The activity of a mixed preparation in which oxidized GO was immobilized onto Eupergit C–$ADH_{0.01}$ while native NA was added in solution was also determined. As shown in Table I, the mixed preparation was essentially as active as the two enzymes added in solution. In contrast, when native GO was immobilized directly onto Eupergit C its activity was lost. The co-immobilized enzyme preparation retained 80% of the original enzymic activity.

The antigen-binding capacity of oxidized anti-CPA mAb100 obtained using the co-immobilized bifunctional enzyme preparation was the same as the activity obtained with a mixture of the enzymes in solution. The amount of CPA bound to the immobilized antibody, as determined by the enzymic activity of the complex, corresponded to 6 μg of CPA per 20 μg of mAb or a molar ratio of 1.5 mol of antigen bound per mole of antibody.

DISCUSSION

Binding of antibodies on Eupergit C by reaction of their amino moieties with oxirane groups of the matrix may cause partial or complete loss of immunological activity. Site specific modification of antibodies by oxidation of their carbohydrate moieties and their oriented immobilization onto Eupergit C–ADH proved to be a highly efficient alternative procedure. As the sites of attachment of oligosaccharides to antibodies are specific and remote from the antibody-combining site, the selective coupling of newly formed aldehyde groups to hydrazide groups of the matrix often yields conjugates with unimpaired antigen-binding characteristics. Site-specific modification of antibodies by oxidation with sodium periodate as a means of their oriented immobilization onto insoluble matrices is widely used[1–3]. However, chemical modification by the periodate oxidation of vicinal hydroxyl groups of carbohydrate may also modify some amino acid residues, such as serine, threonine, proline and methionine, which, if located in the Fab region, may interfere with the immunological activity of the antibodies.

An enzymic procedure for the generation of aldehyde groups on the oligo-

saccharide moieties of antibodies utilizes the neuraminidase–galactose oxidase system[1,2]. In this procedure, terminal sialic acid residues are first removed by treatment with neuraminidase. Subsequent treatment with galactose oxidase results in the formation of aldehydes on the exposed galactose residues. This method has been used by Wilchek and Bayer[6] for the labelling of cell surface glycoproteins and by Chua *et al.*[5] to generate aldehyde on IgM immunoglobulins prior to coupling to liposomes. We have used soluble and immobilized bifunctional enzyme preparations of neuraminidase and galactose oxidase for the oxidation of affinity-purified anti-CPA mAb100 and anti-HRP mAb2. The course of oxidation was followed by determination of the aldehyde groups formed on the antibody molecules and by determination of the antigen-binding activities of the modified mAbs. Determination of the aldehyde groups in the oxidized antibodies by spectrophotometric methods, using TNBS[10,11] or 3-methyl-2-benzothiazolinone hydrazone hydrochloride[14], was not sensitive enough. Determination of aldehyde groups using the avidin–biocytin–hydrazide system[15,16] was adopted with a modification of the dot blotting method (see Experimental). This method proved to be highly sensitive, allowing the determination of 1–2 aldehyde groups formed on the antibodies after enzymic oxidation.

Enzymic oxidation of monoclonal antibodies is expected to be more specific than chemical oxidation. We indeed found the formation of a smaller number of aldehyde groups on the enzymically than on the chemically oxidized antibodies. Nevertheless, the 2 mol of aldehyde formed per mole of enzymically oxidized antibodies were sufficient to achieve efficient binding of the antibody to the matrix. These antibodies possessed a higher antigen-binding activity than the corresponding chemically oxidized antibodies immobilized on the same matrix. The amount of antigen bound to the corresponding antibody–matrix conjugate was close to the theoretical value of 2 mol of antigen (CPA or HRP) bound per mole of immobilized antibody. The co-immobilization of galactose oxidase and neuraminidase exhibits the well known advantages of immobilized enzymes such as repetitive use of the same enzymes, a good recovery of the antibodies and the possibility of continuous oxidation of mAb.

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REFERENCES

1 A. G. Morell, C. J. A. van den Hamer, J. H. Scheisberg and G. Ashwell, *J. Biol. Chem.*, 241 (1966) 3745.
2 D. J. O'Shaunessy and R. H. Quarles, *J. Immunol. Methods*, 99 (1987) 153.
3 G. Fleminger, E. Hadas, T. Wolf and B. Solomon, *Appl. Biochem. Biotechnol.*, 23 (1990) 123.
4 K. Y. Willan, B. Goldring, D. Givol and R. A. Dwek, *FEBS Lett.*, 80 (1977) 133.
5 M. M. Chua, M. M., S. T. Farr and F. Karush, *Biochim. Biophys. Acta*, 800 (1984) 291.
6 M. Wilchek and E. A. Bayer, *Immunol. Today*, 5 (1984) 39.
7 E. M. Press and R. R. Porter, In A. Gottschalk (editor), *Immunoglobulins in Glycoproteins*, Elsevier, Amsterdam, London, New York, 1966.
8 M. Potier, *Anal. Biochem.*, 94 (1979) 287.
9 B. Solomon, R. Koppel, D. Kenett and G. Fleminger, *Biochemistry*, 128 (1989) 1235.
10 J. F. McKelvy and Y. C. Lee, *Arch. Biochem. Biophys.*, 132 (1969) 99.
11 T. Miron and M. Wilchek, *J. Chromatogr.*, 215 (1981) 55.

12 G. Fleminger, B. Solomon, T. Wolf and E. Hadas, *Appl. Biochem. Biotechnol.*, in press.
13 B. Solomon, R. Kopper, G. Pines and E. Katchalski-Katzir, *Biotechnol. Bioeng.*, 28 (1986) 1213.
14 M. A. Paz, O. O. Blumenfeld, M. Rojkind, E. Henson, C. Furfine and P. M. Gallop, *Arch. Biochem. Biophys.*, 109 (1965) 548.
15 M. Wilchek and E. A. Bayer, *Anal. Biochem.*, 171 (1988) 1.
16 M. Wilchek and E. A. Bayer, *Methods Enzymol.*, 138 (1987) 429.