

Detection of Monoclonal Antibodies Specific for Carbohydrate Epitopes Using Periodate Oxidation

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A method is described for determining whether particular monoclonal antibodies are specific for carbohydrate or non-carbohydrate antigenic determinants. In a model system consisting of the Lewis a human blood group determinant attached to either protein or lipid, mild periodate oxidation destroyed the carbohydrate determinant without altering protein or lipid epitopes. The technique was readily applied to antigens bound to plastic wells for ELISA, to nitrocellulose sheets for Western blots, and to thin layer chromatography (TLC) plates for TLC immunostaining. Mild periodate oxidation can prove useful during the early stages of hybridoma screening in order to select for or against anti-carbohydrate antibodies.

Key words: monoclonal antibody - carbohydrate - periodate - glycoprotein - glycolipids

Introduction

Cell surface glycoproteins and glycolipids play important roles in many aspects of development and immune function. With the advent of the hybridoma technology (Köhler and Milstein, 1975), it became possible to generate monoclonal antibodies specific for many different epitopes on these molecules. A first step in characterizing monoclonal antibodies directed against cell surface glycoconjugates is to determine if they are recognizing carbohydrate or protein epitopes.

Mild periodate oxidation at acid pH has been shown to cleave carbohydrate vicinal hydroxyl groups without altering the structure of polypeptide chains (Bobbitt, 1956). In this report we demonstrate that mild periodate oxidation at acid pH is a useful tool for detecting anti-carbohydrate monoclonal antibodies when used in conjunction with the following screening methods: (1) ELISA for glycoproteins and

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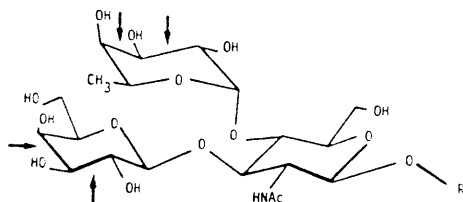


Fig. 1. Structure of the portion of the Lewis a human blood group determinant recognized by the CF4C4 monoclonal antibody. The arrows indicate the presumed periodate-sensitive sites.

glycolipids; (2) immunoblots for glycoproteins; and (3) thin layer chromatography (TLC) immunoblots for glycolipids. For these studies we utilized a model antigen, the Lewis a human blood group determinant attached to bovine serum albumin (Lewis a-BSA), as well as Lewis a active glycolipids. The terminal galactose and fucose moieties of the Lewis a determinant contain vicinal hydroxyl groups that are susceptible to mild periodate oxidation (Fig. 1). The use of the Lewis a-BSA synthetic glycoprotein allowed the simultaneous monitoring of the effect of periodate oxidation on the binding of anti-protein and anti-carbohydrate antibodies to the same molecule. The characterization of the periodate oxidation procedure provided in this report will allow this technique to be utilized with confidence to identify monoclonal antibodies recognizing carbohydrate epitopes on membrane glycoproteins and glycolipids.

Materials and Methods

Antibodies

The mouse-mouse hybridoma antibody CF4C4 (IgG₁, κ), which is specific for the Lewis a human blood group determinant, was characterized previously (Young et al., 1983). Antibodies specific for BSA were provided by Dr. David Benjamin and consisted of rabbit polyclonal antibodies raised against denatured BSA and a mixture of 22 murine monoclonal antibodies produced against native BSA and recognizing a variety of protein epitopes. Horseradish peroxidase (HRP) conjugates of goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Hyclone Laboratories, Logan, UT. The mouse hybridoma antibody HDP-1 (IgG₁, κ) which is specific for dinitrophenol (DNP) was provided by Dr. J. Fleischman.

Reagents

Periodic acid was obtained from Sigma Chemical Co., St. Louis, MO and MacAlaster Bicknell Co., Millville, NJ; sodium *m*-periodate was obtained from Sigma and Fisher Scientific, Pittsburgh, PA. Tween 20, bovine serum albumin (fraction V), *o*-phenylenediamine, Tris-base, sodium dodecyl sulfate, 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ABTS), sodium borohydride, Amido Black (Naphthol Blue Black) and glycine were obtained from Sigma. 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was obtained from Aldrich Chem-

Chemical Company, Milwaukee, WI. Protein A was obtained from Pharmacia Fine Chemicals, Piscataway, NJ, and iodinated by the Iodogen method (Fraker and Speck, 1978). Dinitrophenyl amino caproyl phosphatidylethanolamine (DNP-CapPE) was obtained from Avanti Polar-Lipids, Birmingham, AL.

Lewis a-BSA

The synthetic Lewis a hapten (Gal β 1 $\rightarrow$ 3(Fuc α 1 $\rightarrow$ 4)GlcNAc) covalently attached to bovine serum albumin (Lemieux et al., 1975) was provided by Dr. R.U. Lemieux. The degree of incorporation was 15 hapten residues/molecule BSA.

ELISA

(a) *Glycoproteins.* Lewis a-BSA or BSA, each at 1 μ g/ml in 150 mM NaCl, 10 mM NaPO₄, pH 7.4 (PBS), were permitted to attach to the wells of Immulon 1 plates (Dynatech, Alexandria, VA) for 1 h at room temperature. Plates were then flooded with PBS-Tween 20 (0.05%) and incubated an additional 30 min at room temperature after which they were washed 5 times with PBS-Tween 20. Plates were then rinsed with 50 mM sodium acetate buffer (pH 4.5) prior to exposure to periodate. Sets of wells were then exposed to varying concentrations of periodate (0–20 mM) in 50 mM sodium acetate buffer, pH 4.5, for 1 h at room temperature in the dark. Following a brief rinse with 50 mM sodium acetate the plates were then incubated with 50 mM sodium borohydride in PBS for 30 min at 23°C. This treatment reduces the aldehyde groups generated by periodate oxidation to alcohols and prevents non-specific cross-linking of antibody to antigen. Alternatively, the aldehyde groups can be blocked with 1% glycine. Both the periodate and borohydride solutions were prepared just prior to use. Following 5 washes with PBS-Tween 20 the plates were exposed to primary antibody for 1 h at room temperature, then washed 5 times with PBS-Tween 20, and subsequently incubated for 1 h with the appropriate secondary antibody (either HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG). Unbound secondary antibody was removed by 5 washes with PBS-Tween 20, and the extent of binding was monitored colorimetrically using 1 mM 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ABTS) or *o*-phenylenediamine (OPD) in the presence of 0.03% H₂O₂ as the peroxidase substrate. The reaction was allowed to proceed for 30 min after which the plates were read at a wave length of 405 nm for ABTS and 495 nm for OPD using a Titertek Multiscan (Flow Labs, McLean, VA). When using a non-BSA antigen, 0.1% BSA can be substituted for Tween 20 to block non-specific adsorption.

(b) *Glycolipids.* The ELISA procedure using Lewis a active glycolipids partially purified from human meconium was performed in a manner analogous to that used for the Lewis a-BSA with the following modifications. Plates were coated with DNP-CapPE or Lewis a active glycolipids (10 μ g/ml in water containing 0.02% sodium azide) by overnight incubation at 37°C (Young et al., 1979). Plates were blocked with 5% BSA in PBS and stored frozen. Thawed plates were washed 5 times with PBS-0.1% BSA prior to incubation with periodate. All incubations prior to substrate addition were performed in the presence of PBS containing 0.1% BSA.

Immunoblots

A 1:1 mixture of Lewis a-BSA and BSA were separated on a 7.5% polyacrylamide gel using the method of Laemmli (1970) without a stacking gel. The running buffer contained 0.1% SDS, and samples were denatured by heating at 100°C for 2 min in sample buffer (Laemmli, 1970). Following electrophoresis at constant current, the gel was placed in contact with a nitrocellulose sheet (Schleicher and Schuell, no. BA 83) and the proteins electroblotted (90 min at 150 mA in a Bio-Rad Trans-Blot apparatus) following the procedure of Towbin et al. (1979). The blotting buffer was 0.19 M glycine, 0.05 M Tris-base, pH 8.3, 10% methanol; in some cases, the methanol was deleted with little apparent effect. Following blotting the nitrocellulose sheet was stained with 0.1% Amido Black (Naphthol Blue Black) in 50% methanol-10% acetic acid and the acrylamide gel stained using the silver procedure of Merrill et al. (1979) in order to monitor the degree of transfer. Blots were destained in 30% isopropanol-10% acetic acid and then **blocked with PBS containing 0.05% Tween-20 for 30 min**. Blots were cut into numbered strips and adjacent pairs of strips were used for experimental and control conditions. All strips were rinsed briefly with 50 mM sodium acetate buffer (pH 4.5) after which control strips were incubated in the same buffer for 1 h. Experimental strips were exposed to varying concentrations of periodate (0.1–20 mM) in the pH 4.5 buffer for 1 h in the dark at 23°C. Control and experimental strips were then rinsed with sodium acetate buffer and exposed to 50 mM sodium borohydride in PBS for 30 min at 23°C.

Following 3 washes with PBS-Tween 20, the strips were exposed to primary antibody for 1 h at 23°C. Unbound primary antibody was removed by 3 washes with PBS-Tween 20 after which HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG in PBS was added for 1 h at 23°C. Following removal of unbound secondary antibody, 1 mM diaminobenzidine (DAB) in 10 mM Tris-HCl, pH 7.0, with 0.03% H₂O₂ was used as substrate for the HRP reaction. When using proteins other than BSA, unbound sites on the nitrocellulose can be blocked using 0.1% BSA in PBS; periodate oxidation can be performed before or after the blocking step.

Thin layer chromatography immunoblotting

Thin layer chromatography immunoblotting was performed using a modification of the technique of Brockhaus et al. (1981). All steps were performed at 23°C. Glycolipid samples were applied to an aluminum-backed high performance TLC sheet (E. Merck, F.R.G.) and developed with chloroform/methanol/water (55:40:11 v/v/v). After drying, the sheet was dipped in hexane containing 0.05% polyisobutylmethacrylate (Polysciences, Warrington, PA). The sheet was cut into lanes which were incubated with varying concentrations (0–20 mM) of sodium metaperiodate in 0.1 M sodium acetate (pH 6) for 1 h in the dark. In preliminary experiments sodium borohydride disrupted the silica gel on the sheet; therefore, to block the aldehyde groups formed by periodate treatment, the lanes were washed in PBS and then incubated in 20 mM glycine in 100 mM sodium phosphate buffer (pH 7.4) for 1 h. After washing, the lanes were blocked with 5% BSA in PBS for 2 h. The plate was then incubated for 1 h with antibody CF4C4 (1:1000 dilution of ascites

fluid in PBS) followed by washing and incubation with diluted ^{125}I -protein A for 1 h. After washing and drying, the sheet was exposed at -70°C for 1–2 h to Kodak XAR-5 film utilizing an intensifying screen.

Results

The effect of increasing concentrations of periodate on the binding of the anti-Lewis a antibody (CF4F4) to Lewis a-BSA attached to ELISA plates is shown in Fig. 2 (open bars). Complete loss of antibody binding occurred at a concentration of 5 mM periodate and greater than 90% inhibition was seen at 1.0 mM. In contrast, the binding of rabbit anti-BSA to Lewis a-BSA and to underivatized BSA was not significantly affected (hatched and solid bars, Fig. 2). In fact, the binding of rabbit anti-BSA to Lewis a derivatized BSA appeared to be enhanced by exposure to periodate (hatched bars, Fig. 2). The CF4F4 monoclonal antibody did not bind to untreated or periodate treated underivatized BSA.

Similar results were observed when Lewis a containing glycolipids were assayed by ELISA (Fig. 3). There was an 88% inhibition of binding of the anti-Lewis a monoclonal antibody after treatment of the antigen with 0.5 mM periodic acid and a 98% inhibition observed at 1 mM periodic acid. In order to determine if any of the loss of antibody binding after periodic acid treatment was due to release of the lipids from the Immulon 1 microtiter plate, a control experiment was performed on plates coated with DNP-CapPE. Table I shows that treatment of these plates for 1 h with concentrations of periodic acid up to 20 mM did not result in any significant loss of binding of an anti-DNP monoclonal antibody. This demonstrates that the loss of binding of anti-Lewis a monoclonal antibodies shown in Fig. 3 was due to oxidation

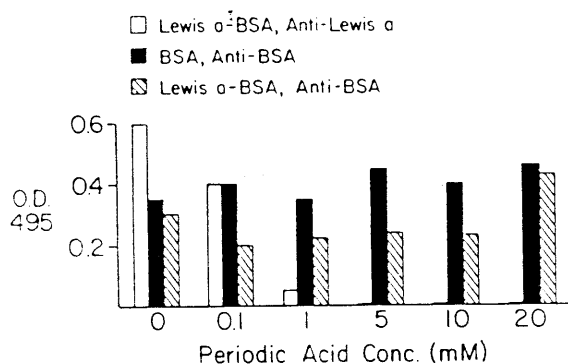


Fig. 2. Effect of periodate oxidation of Lewis a-BSA and BSA on the binding of anti-Lewis a or anti-BSA antibodies. The ELISA was performed as described in Materials and Methods using *O*-phenylenediamine as a substrate. Open bars represent binding of the CF4C4 monoclonal antibody to Lewis a antigen coupled to BSA, solid bars represent binding of rabbit anti-BSA antibodies to underivatized BSA, and hatched bars represent binding of rabbit anti-BSA antibodies to the Lewis a-BSA conjugate. No binding of CF4C4 antibody to underivatized BSA was observed.

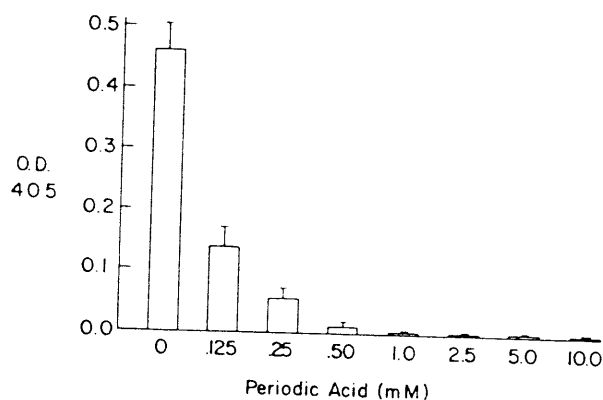


Fig. 3. Effect of periodate oxidation of Lewis x active glycolipids on the binding of an anti-Lewis x monoclonal antibody. The ELISA was performed as described in Materials and Methods using ABTS as the substrate. Error bars indicate the standard deviation for quadruplicate samples.

of the vicinal hydroxyl groups in the sugars and not due to release of the lipids from the surface of the microtiter plate.

Measurement of the binding of the anti-Lewis x monoclonal antibody to Lewis x containing glycolipids by ELISA was utilized to ask if the source of the periodate reagent had any significant effect on the dose dependence of inhibition of antibody binding. Periodic acid obtained from 2 different commercial sources and sodium *m*-periodate obtained from 2 different commercial sources were compared quantitatively on the same microtiter plate for their effect on antibody binding. In each case, a very similar dose-dependent inhibition of binding was observed (70–74% inhibition at 0.125 mM and 90–94% inhibition at 0.25 mM) suggesting that the source of the reagent is not a critical variable when using this technique.

In order to assess the effect of periodic acid oxidation on the binding of antibodies recognizing carbohydrate versus protein epitopes on the same glycopro-

TABLE I

EFFECT OF PERIODATE OXIDATION OF DNP-CapPE ON THE BINDING OF AN ANTI-DNP MONOCLONAL ANTIBODY

Treatment	OD ₄₀₅ ^a
Control ^b	0.357
0.5 mM	0.344
1.0 mM	0.339
5.0 mM	0.353
10.0 mM	0.373
20.0 mM	0.310

^a Average of 2 readings from each of 2 separate determinations.

^b No periodic acid but treated with the buffer (acetic acid-sodium acetate at pH 4.5) followed by sodium borohydride reduction.

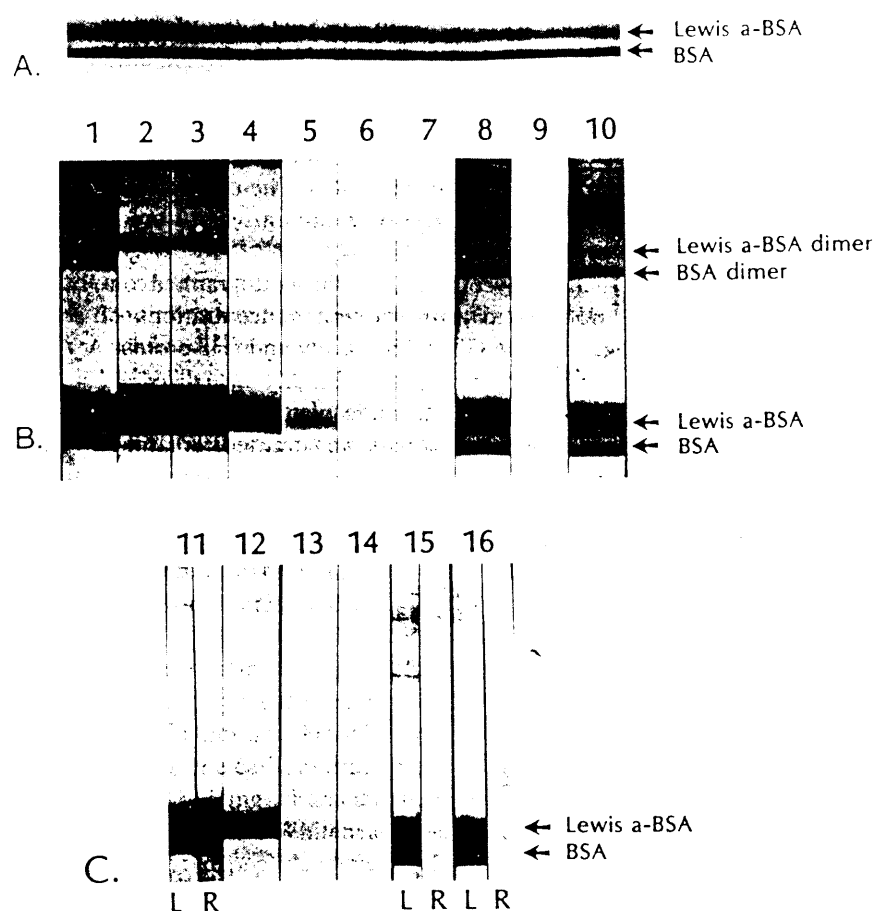


Fig. 4. Effects of increasing periodate concentration on the binding of anti-Lewis a and anti-BSA antibodies to electroblots of Lewis a-BSA and BSA. A: separation of Lewis a-BSA and BSA. The blot was prepared as described in Materials and Methods and stained with Amido Black. B: binding of CF4C4 anti-Lewis a monoclonal antibody or rabbit anti-BSA polyclonal antibody to the blot shown in A. Lanes 1, 8, and 10 were treated with rabbit anti-BSA antibodies (1:1000 dilution). Lanes 2, 3, 4, 5, 6, 7, and 9 were treated with anti-Lewis a antibody (12.5 μ g/ml). Lanes 1 and 2: control; lane 3: 0.1 mM periodic acid; lane 4: 0.5 mM periodic acid; lane 5: 1.0 mM periodic acid; lane 6: 5.0 mM periodic acid; lanes 7 and 8: 10.0 mM periodic acid; lanes 9 and 10: 20.0 mM periodic acid. C: binding of CF4C4 anti-Lewis a monoclonal antibody and mouse anti-BSA monoclonal antibodies to the blot shown in 4A. Lanes 11L, 15L, and 16L were treated with pooled supernatants from mouse anti-BSA monoclonal antibodies. Lanes 11R, 12, 13, 14, 15R, and 16R were treated with the anti-Lewis a monoclonal antibody (12.5 μ g/ml). Lane 11L and R: control; lanes 12: 0.5 mM periodic acid; lane 13: 1.0 mM periodic acid; lane 14: 5.0 mM periodic acid; lane 15L and R: 10.0 mM periodic acid; lane 16L and R: 20.0 mM periodic acid.

tein molecule by the Western (immuno-)blot procedure, the synthetic glycoprotein, Lewis a-BSA, was utilized. Equal amounts of BSA and Lewis a-BSA were electrophoresed and blotted as described in Materials and Methods. Two distinct bands were observed when the nitrocellulose blot was stained with Amido Black (Naphthol Blue Black) (Fig. 4A). Glycosylated BSA migrated more slowly than BSA and resulted in a somewhat broader band. In addition, the amount of Lewis a-BSA that transferred from the acrylamide gel to the nitrocellulose sheet was less than for the BSA alone (data not shown). Following treatment with 0.5 mM periodate a significant decrease in CF4F4 binding was observed (Fig. 4B). At concentrations greater than 5 mM periodate, binding of the anti-Lewis a monoclonal antibody was completely eliminated. By contrast, the binding of either rabbit anti-BSA (Fig. 4B) or a pooled group of mouse monoclonal anti-BSA antibodies (recognizing multiple epitopes) (Fig. 4C) was unaffected. Similar results were obtained for both the monomeric and dimeric forms of Lewis a-BSA (Fig. 4B). The dimeric species along with larger BSA aggregates were present in very low amounts and were not visible on the Amido Black-stained nitrocellulose blot (Fig. 4A).

Partially purified Lewis a-active glycolipid was chromatographed on multiple lanes of an HPTLC sheet and treated with varying concentrations of sodium *m*-periodate prior to incubation with the CF4F4 antibody and ^{125} I-protein A. In the absence of periodate, the Lewis a glycolipid was readily visualized as a band co-migrating with authentic Lewis a-active ceramide pentasaccharide (Fig. 5, lane A); no band was present when the specific antibody was omitted. Treatment with 10

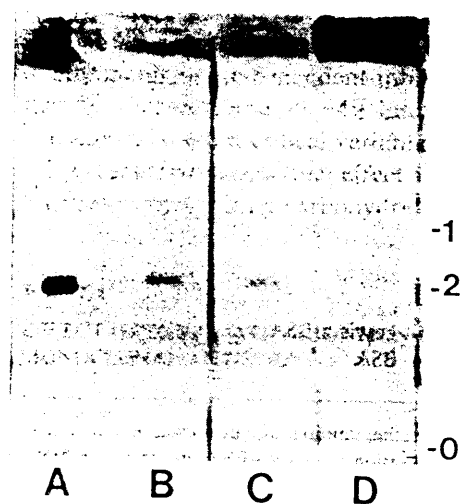


Fig. 5. Periodate sensitivity of Lewis a-active glycolipid as detected by autoradiography. Multiple lanes of an HPTLC sheet were chromatographed with Lewis a-active glycolipid followed by sequential treatment with periodate, glycine, anti-Lewis a antibody, and ^{125} I-protein A as described in Materials and Methods. Lane A: the periodate step consisted of pH 6.0 buffer in the absence of periodate; lanes B, C, D: 1 mM, 2.5 mM, and 10 mM periodate respectively. Glycolipid standards: 1, ceramide dihexoside; 2, Lewis a pentasaccharide from human small intestine (Smith et al., 1975). O, origin. The dark area at the front of the autoradiogram represents nonspecific staining.

mM periodate completely abolished the stained band (Fig. 5, lane D) while lower concentrations partially destroyed the reactive glycolipid (Fig. 5, lanes B and C).

Discussion

This paper reports a general method that permits the identification of monoclonal antibodies recognizing carbohydrate-containing epitopes associated with glycoproteins and glycolipids. This method involves periodate oxidation of vicinal hydroxyl groups on sugars to dialdehydes at acid pH and is applicable to Western blots and ELISA in the case of glycoproteins and ELISA and thin layer chromatograms in the case of glycolipids. We characterized this method utilizing the Lewis a blood group determinant attached both to lipid and to protein. Use of the Lewis a-BSA allowed us to demonstrate that periodate oxidation of this molecule abolished binding of a monoclonal antibody (CF4C4) specific for a Lewis a trisaccharide without altering the binding of mouse monoclonal or rabbit polyclonal antibodies recognizing a number of protein epitopes on the BSA molecule.

The conditions used for periodate oxidation determine the extent and types of cleavage achieved. Treatment at 0°C for 10 min with up to 5 mM periodate at acidic pH results in cleavage only between the exocyclic 7-, 8-, and 9-hydroxyl positions of terminal, unsubstituted sialic acid residues (Van Lenten and Ashwell, 1971). Such treatment has been utilized to distinguish between monoclonal antibodies specific for either *O*-acetylated or nonacetylated sialic acid residues in disialoganglioside GD3 (Cheresh et al., 1984). Oxidation for a slightly longer time at 25°C results in cleavage of terminal, non-reducing sugars as well as pyranosidically linked hexoses within the oligosaccharide chain in which carbon 3 is unsubstituted. Based on our present study, optimal conditions for this type of reaction consist of 10 mM periodate, pH 4.5, for 1 h at 25°C. Prolonged oxidation at higher oxidant concentrations destroyed amino acids, including those within polypeptides; the rate of oxidation varied for each amino acid but generally proceeded more rapidly at alkaline pH (Clamp and Hough, 1965). Periodate has been used to determine the participation of the carbohydrate moiety in the serologic reactivity of glycoproteins; the antibody binding characteristics of orosomucoid were unaffected by mild oxidation (Barker and Whitehead, 1963) but destroyed by harsh conditions which also degraded the peptide moiety (Krotoski and Weimer, 1966). In contrast, the serologic reactivity of the major egg glycoprotein of *Schistosoma mansoni* was reduced by both mild and drastic periodate oxidation, suggesting involvement of carbohydrate in at least some antigenic determinants (Hamburger et al., 1982).

The periodate oxidation techniques described here have been utilized to characterize a group of monoclonal antibodies recognizing a variety of *Chlamydomonas* flagellar membrane surface-exposed epitopes (Woodward et al., 1984). Periodic acid oxidation of flagellar glycoproteins abolishes the binding of a group of putative anti-carbohydrate monoclonal antibodies; a similar dose-dependent inhibition is observed using enzyme-linked immunoassays, Western blot analysis of SDS-acrylamide gels, and whole cell immunofluorescent localization procedures. The

binding of a group of IgG₃ monoclonal antibodies recognizing a negatively charged flagellar glycolipid is inhibited by periodate oxidation of the glycolipid, as assayed by enzyme-linked immunoassays on microtiter plates, by immuno-localization on thin layer chromatograms, and by whole cell immunofluorescent localization procedures.

Not all carbohydrate antigenic determinants are sensitive to periodate cleavage. Based on work with mouse anti-dextran, anti-carbohydrate antibodies can be divided roughly into 2 categories (Kabat, 1976): those having a groove type of binding site which bind at non-terminal locations along linear chains and those having a pocket or cavity type of binding site that react at the non-reducing termini of chains. Determinants consisting of linear sugar chains with linkages at carbon 3 should not be periodate sensitive and, in fact, the linear repeating structure of the i blood group antigen was only partially destroyed by periodate oxidation (Niemann et al., 1978). Determinants located at branch points, such as those recognized by some of the anti-I antibodies (Feizi et al., 1979), might also be periodate resistant. Interestingly, determinants containing partially *O*-acetylated sialic acids are also periodate resistant (Cheresh et al., 1984). Aside from the examples cited above, most anti-carbohydrate monoclonal antibodies are directed against non-reducing terminal carbohydrate structures (for a partial review see Hakomori and Kannagi, 1983). Since such structures are generally susceptible to periodate cleavage, mild periodate oxidation should alter these determinants enough to prevent antibody binding.

The method described in this paper is particularly useful with Western (immuno-)blots because modification of epitopes occurs after electrophoretic separation of proteins eliminating the problems associated with changes in electrophoretic mobility which result from enzymatic and chemical deglycosylation procedures. Electrophoretic separation of membrane components prior to periodate oxidation permits direct identification of glycoproteins possessing periodate sensitive epitopes.

Periodate oxidation is especially attractive as a tool to be used in screening for or against anti-carbohydrate monoclonal antibodies during the early stages following hybridoma fusion. The simplicity of the method makes it preferable to other deglycosylation techniques such as anhydrous hydrogen fluoride (Mort and Lampion, 1977) or alkaline borohydride treatment (Ogata and Lloyd, 1982). Such screening can be most easily accomplished by coating a pair of ELISA plates with antigen and then treating one of the plates with periodate. Antibodies binding only to the untreated plate would be classified as having anti-carbohydrate reactivity while those that bind to both plates would be considered to be directed against non-carbohydrate determinants, such as peptide epitopes, or against periodate-resistant carbohydrate moieties.

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