Binding of the Brucella abortus Lipopolysaccharide O-chain Fragment to a Monoclonal Antibody

QUANTITATIVE ANALYSIS BY FLUORESCENCE QUENCHING AND POLARIZATION*

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An antigenic O-chain polysaccharide fragment derived from Brucella abortus lipopolysaccharide was labeled with 14.8 ± 1.8 (n = 5) and 52.3 ± 2.4 (n = 3) μmol of fluorescein/g of polysaccharide (designated FL₁ and FL₂, respectively) for use in investigating the binding of O-chain to a specific murine antibody YsT9 under equilibrium conditions. Upon binding to YsT9, the fluorescence of FL₁ and FL₂ was quenched 45–57% with no shift in the excitation and emission spectra, and polarization of fluorescence increased by 300–335%. With fluorescence quenching and polarization as sensitive signals for antibody-bound labeled O-chains, the equilibrium constants for binding of FL₁, FL₂, and unlabeled O-chain to YsT9 were determined to be within a similar order (1.5 x 10⁷ to 2.0 x 10⁷ M⁻¹) using a nonlinear curve fitting approach rather than Scatchard analysis. These results indicated that covalent attachment of fluorescein groups to the O-chain did not influence the recognition of the YsT9-defined epitope by the antibody. The reversibility of the O-chain-antibody reaction was also demonstrated by showing a rapid depolarization of the labeled O-chain-antibody complex in the presence of unlabeled O-chain, suggesting that this displacement experiment could be exploited to quantify the Brucella polysaccharide antigen. The study described here provides a useful model for characterization of the complex formation between a carbohydrate-binding protein and a carbohydrate ligand and also for the design of a homogeneous assay system to quantitate antigens or antibodies of clinical interest.

Brucella abortus, a Gram-negative bacterium of the genus Brucella, is pathogenic for animals, causing abortions in cattle and a debilitating fever (undulant fever) in humans. The smooth-type lipopolysaccharide (S-LPS)¹ of B. abortus is recognized as an immunodominant cell surface molecule involved in the antibody response to the infection (1, 2). Hydrolysis of the B. abortus S-LPS with mild acid yields a specific polysaccharide fragment constituted by an O-chain linear homopolymer of 1,2-linked 4,6-dideoxy-4-formamido-α-D-mannopyranosyl units (2–5). The O-chain polysaccharide (designated the O-chain) is linked via a core oligosaccharide to lipid A, which anchors the LPS molecule to the outer membrane (2). Structural studies revealed that B. abortus and Yersinia enterocolitica serotype O:9 share a common O-chain polysaccharide (3, 6). It has been demonstrated with murine monoclonal antibodies that antibodies specific for the O-chain or S-LPS are protective in passive immunity to B. abortus infection (7, 8). The humoral immune response to LPS in B. abortus-infected cattle was previously monitored for antibody titer and content of immunoglobulin subclass (9, 10), but characterization of an anti-B. abortus antiserum in terms of antibody affinity had not been described. Antibody affinity has been recognized as an important factor in the immune response in terms of clearance of pathogens, response to vaccination, or pathogenesis of infectious diseases (11–13). It has become clear that the antibody affinity is very likely to influence the efficiency of the assay employed for its quantification (11, 14).

Because of the importance of B. abortus in animal and human infections and the great value of the O-chain in the diagnosis of brucellosis (2, 15), it was considered that characterization of the complex formation between the O-chain and a specific monoclonal antibody in terms of affinity would provide a useful analysis model that could be exploited to assess the antibody response to infection or vaccination and could assist in the design of a more efficient assay system where the O-chain and a monoclonal antibody are employed for the diagnosis of brucellosis. This paper reports purification of B. abortus O-chain and modification of it with fluorescein isothiocyanate for use in measurement of the equilibrium (affinity) constant for binding of the O-chain to a specific murine antibody YsT9 by fluorescence quenching and polarization. The antibody YsT9 was characterized as binding to a linear α-1,2-linked tetrasaccharide determinant that was common to the Brucella A and M, and Y. enterocolitica O:9 polysaccharide antigens (16, 17). The reversibility of the O-chain-antibody reaction was also examined by displacement experiments using the labeled O-chain probe. This is the first application of such techniques to the investigation of binding of a bacterial polysaccharide fragment to the corresponding monoclonal antibody on a quantitative basis. The study described here demonstrated that the chemical linkage of fluorescein groups to a bacterial carbohydrate antigen provides a probe suitable for quantitation of the complex formation between a carbohydrate-binding antibody and a carbohydrate.

MATERIALS AND METHODS

Reagents—Fluorescein isothiocyanate (FITC) isomer I was purchased from Sigma; Affi-Prep Polymyxin gel, from Bio-Rad; HPLC columns of TSK G2000 SWG (21.5 x 600 mm) and TSK G2000 SW (7.5 x 600 mm), from Phenomenex (Torrance, CA). Monoclonal antibody (mAb) YsT9 (IgG₃) specific for the B. abortus O-chain, originally developed by Bundle et al. (18), was purified from ascitic fluid through a column of Protein G-Sepharose (1 x 6.4 cm). A mAb, M178, which

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¹ The abbreviations used are: S-LPS, smooth-type lipopolysaccharide; LPS, lipopolysaccharide; FITC, fluorescein isothiocyanate; HPLC, high pressure liquid chromatography; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
reacts with *Salmonella typhimurium* and isolates of most *Salmonella* O serogroups, was obtained from the Animal Diseases Research Institute (Nepean, Ontario, Canada).

**Purification of the O-chain Polysaccharide Fragment from B. abortus**—Freeze-dried B. abortus S1119–3 cells (10 g) were suspended in 400 ml of 2% (v/v) acetic acid and autoclaved at 121°C. After the suspension was cooled on ice and neutralized to pH 7.0 with NaOH, trichloroacetic acid (20 g) was added with stirring for 40 min. The suspension was then centrifuged at 6,000 × g for 1 h. The supernatant (designated the crude extract) was collected, dialyzed against H$_2$O overnight, and lyophilized.

The freeze crude extract was divided into three aliquots. Each aliquot was dissolved in 15 ml of 10 mM phosphate buffer (pH 6.8) containing 100 mM NaCl (Buffer A) and centrifuged at 10,000 × g for 15 min at 4°C to remove the insoluble material. The supernatant was then applied, at 0.5 ml/min, to a column of Affi-Prep Polymyxin gel (25 × 110 mm) pre-equilibrated with Buffer A. Fractions of 5 ml were collected and monitored for absorption at 200 nm. Fractions of unbound material (designated the crude polysaccharide fraction) were collected, dialyzed against H$_2$O, and lyophilized.

The crude polysaccharide fraction (250 µl, 4 mg/ml) in 0.1 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ (Buffer B, pH 7.0) was applied, at 1 ml/min, to a column of TSK G2000 SWG (7.5 × 600 mm), which had been connected to a LKB HFLC system and pre-equilibrated with Buffer B. Fractions of 1 ml were collected and monitored for absorption at 200 nm or assayed for total sugar according to the colorimetric method (19). For large scale preparation of the O-chain, the crude polysaccharide fraction (1.3 ml, 20 mg/ml) was loaded, at 1 ml/min, onto a column of TSK G2000 SWG (21.5 × 600 mm). The elution profile on TSK G2000 SW (7.5 × 600 mm) was shown in Fig. 1. Fractions of peak 1 and peak 2 were pooled separately, dialyzed against H$_2$O, and lyophilized.

**Fluorescein Labebling of the O-chain—SDS-PAGE of the O-chain preparation (prior to labeling)** with both Coomassie Blue and silver staining procedures showed that the preparation did not contain any protein components. Lyophilized O-chain polysaccharide (3 mg) was dissolved in 0.5 ml of 0.1 N NaOH and incubated for 1 h at 37°C. FITC was reacted with the O-chain as described for Western blotting (22) with the exception that samples were mixed with an equal volume of the sample buffer and loaded onto a gel resolving gels using a Bio-Rad mini-gel apparatus. Sodium dodecyl sulfate (SDS)-PAGE was performed according to the method of Laemmli (21) with 4% stacking gels and 15% resolving gels using a Bio-Rad mini-gel apparatus. Sodium dodecyl sulfate was omitted in all the buffers. The polysaccharide samples were mixed with an equal volume of the sample buffer and loaded onto a gel without heat treatment. Dot immunoblots were carried out basically as described for Western blotting (22) with the exception that samples were applied directly to a nitrocellulose membrane.

**Optical Methods**—A Pharmacia LKB Ultrospec Plus spectrophotometer (Pharmacia Biotech, Baie D'Urfe, Quebec, Canada) was used to measure the absorbance. A SLM-Amino model 8000C spectrfluorometer equipped with an IBM microcomputer and a circulating water bath was used for determination of fluorescence excitation and emission spectra. Excitation and emission bandwidths were 8 and 16 nm, respectively. Excitation and emission wavelengths were 490 nm and 520 nm, respectively. Spectra were smoothed using the SLM data manipulation software. Fluorescence intensity of a given sample was determined at optimal excitation (485 nm) and emission (517 nm) wavelengths. Fluorescence polarization was measured using a FFM-1 fluorescence polarization analyzer (Jolley Consulting and Research, Inc., Round Lake, IL).

**Titrination Experiments**—A solution of fluorescein-labeled O-chain at 9 × 10$^{-5}$ g/liter for FL _1_ or 3 × 10$^{-5}$ g/liter for FL _2_ was prepared in 2 ml of PBS and titrated with 5-µl aliquots of a specific or control antibody solution. The steady-state fluorescence intensity or polarization was measured after incubation for 10 min. The values of fluorescence intensity or polarization were corrected for background readings. The cumulative titrating volume was kept less than 4% of the initial volume of the antigen solution. In these titration experiments, the labeled O-chain was used at a considerably low concentration (at least 100-fold lower than that of total antibody), so that the difference between the concentrations of free and total antibody was negligible at reaction equilibrium.

**Displacement Experiments**—Displacement of the fluorescein-labeled O-chain from the antibody with the unlabeled O-chain was performed in PBS (2 ml) containing FL _1_ (9 × 10$^{-5}$ g/liter) and antibody (5 × 10$^{-5}$ x or FL _2_ (3 × 10$^{-5}$ g/liter) and antibody (3.3 × 10$^{-5}$ m). After reaching the steady-state polarization, the labeled antigen/antibody mixture was titrated with 5-µl aliquots of an unlabeled B. abortus O-chain or *S. typhimurium* LPS stock solution. Steady-state fluorescence polarization was measured after incubation for 10 min. The accumulative titrating volume was kept below 4% of the initial volume of the labeled antigen/antibody solution. All titration experiments were performed at 25°C.

**Analysis of Fluorescence Quenching Data**—The fluorescence of fluorescein-labeled O-chain was effectively quenched, upon binding to the antibody YsT9. The quenching value, Q, as defined in Equation 1, was used to estimate the equilibrium (affinity) constant for the antigen-antibody reaction.

$$ Q = \frac{F - F}{F} \quad \text{(Eq. 1)} $$

$F$ and $F$ are the fluorescence intensities of the labeled antigen in the absence and presence of antibody, respectively. The labeled and unlabeled O-chain were designated Ag$^*$ and Ag, respectively. According to the law of mass action, the equilibrium constant, K, for binding of the labeled O-chain to antibody is described by Equation 2.

$$ K = \frac{[Ag^*][Ab]}{[Ag][Ab^*]} \quad \text{(Eq. 2)} $$

[Ag$^*$][Ab], [Ag], [Ag$^*$], and [Ab] are the concentrations of antigen-antibody complex, free antigen binding site, free antigen, bound antigen, and total antigen, respectively.

Equation 2 can be rewritten as shown below.

$$ Q = \frac{[Ag^*][Ab]}{[Ag][Ab^*]} \quad \text{(Eq. 3)} $$

[Ag$^*$]/[Ag$^*$] is the mole fraction ($f_b$) of bound labeled antigen, which can be replaced by $Q/Q_m$, $Q_m$ is the maximal fluorescence quenching. Thus, we obtain Equation 4.

$$ Q = \frac{K[Ab]}{1 + K[Ab]} \quad \text{(Eq. 4)} $$

[Ab] is approximately equal to the total concentration of antigen binding sites, [Ab], under the conditions employed. Nonlinear curve fitting through the data points in a plot of $Q$ versus [Ab], according to Equation 4 gives the values of $K$ and $Q_m$.

**Analysis of Fluorescence Polarization Data**—Fluorescence polarization ($P$) and anisotropy ($A$) are two interrelated parameters that describe the same phenomenon and are related by the equation shown below.

$$ A = \frac{2P}{3 - P} \quad \text{(Eq. 5)} $$

However, only fluorescence anisotropy was used in the data analysis because, unlike polarization, the anisotropy of a fluorescent solution is the arithmetic average of component fluorescence anisotropies in the solution (23). The fraction of fluorescence intensity due to bound labeled antigen, $F_b$, is determined from the fluorescence anisotropy as outlined below.

$$ F_b = \frac{A - A_0}{A_0 - A} \quad \text{(Eq. 6)} $$

$A_0$ is the fluorescence anisotropy of the labeled antigen alone, and $A_0$ is the limiting anisotropy of bound labeled antigen. The values of $F_b$ are related to fluorescence quenching ($Q$) by the formula (23) shown in Equation 7.
By substituting Equation 7 into Equation 4, $V_t$ and $V_o$ show fluorescein fluorescence.

Equation 5 can be derived by fitting a nonlinear curve fitting through the data points in a plot of $F_b$ versus $[Ab]$ according to Equation 8.

$K_1 = \frac{[Ag^*]+[Ab]}{[Ag^{\text{Ag}}]+[Ag^*]-[Ag^*][Ab]}$  

(Eq. 13)

The present experimental conditions employed the labeled O-chain concentration considerably lower than that of antibody and the unlabeled O-chain concentration considerably higher than that of antibody, so that $[Ab] = [Ab]_0$. Thus, we obtain Equation 15.

$$f_b = \frac{K_1[Ab]}{1 + K_1[Ag] + K_2[Ab]}$$  

(Eq. 15)

The values of $f_b$ can be calculated by the ratio of $Q$ to $Q_n$ according to Equation 7. $K_1$ and $K_2$ can be derived by fitting a nonlinear curve through the data points in a plot of $f_b$ versus $[Ag]$ according to Equation 15.

RESULTS

Purification and Fluorescein Labeling of the B. abortus O-chain—As shown in Fig. 1, three $A_{200}$ peaks were resolved on a HPLC size-exclusion column with distribution coefficient $K_{av}$ values of 0.32 (peak 1), 0.69 (peak 2), and 1.0 (peak 3), respectively (each number represents the mean of duplicate determinations). The peak 1 and peak 2 materials, after modification with fluorescein and prior to being purified on a Sephadex G-25 column, were analyzed by the anti-O-chain mAb YsT9 on dot nitrocellulose membrane-immobilized fluorescein-labeled peak 1 and peak 2 taken under UV illumination, showing an equal fluorescence intensity for both labeled preparations (5 and 10 µl of each preparation were used); inset b, dot immunoblot analysis of the membrane-bound samples from inset a using the antibody YsT9 as a probe; inset c, photograph of PAGE analysis of fluorescein-labeled peak 1 (10 µl) and peak 2 (10 µl) taken under UV illumination, showing fluorescein fluorescence.
Panel a

Excitation and emission bandpasses were 8 and 16 nm, respectively.

Panel b

1. The emission maximum (Fig. 2). When either FL1 or FL2 was mixed with antibody YsT9, fluorescein-labeled O-chain preparations (2 g/liter) with antibody M178 (5 g/liter) or mixed with antibody (5 × 10⁻⁸ M) in 2 ml of PBS was incubated for 10 min in the dark prior to collecting the spectrum data. The excitation spectra (curves 1, 3, and 5) were obtained by measuring fluorescence at 520 nm; the emission spectra (curves 2, 4, and 6) were obtained with excitation at 490 nm. Excitation and emission bandpasses were 8 and 16 nm, respectively.

Panel a, labeled O-chain FL1, alone (curves 1 and 2), FL1 plus the antibody YsT9 (curves 5 and 6), and FL1 plus the control antibody M178 (curves 3 and 4). Panel b, labeled O-chain FL2 alone (curves 1 and 2), FL2 plus the antibody YsT9 (curves 5 and 6), and FL2 plus the control antibody M178 (curves 3 and 4).

Fluorescence excitation and emission spectra of fluorescein-labeled B. abortus O-chain. The labeled O-chain alone (FL1 = 9 × 10⁻⁵ g/liter; FL2 = 3 × 10⁻⁵ g/liter) or mixed with antibody (5 × 10⁻⁸ M) in 2 ml of PBS was incubated for 10 min in the dark prior to collecting the spectrum data. The excitation spectra (curves 1, 3, and 5) were obtained by measuring fluorescence at 520 nm; the emission spectra (curves 2, 4, and 6) were obtained with excitation at 490 nm. Excitation and emission bandpasses were 8 and 16 nm, respectively.

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Fluorescence quenching of the fluorescein-labeled O-chain as a function of antibody concentration. Three separate titrations of both FL1 and FL2 with the antibody YsT9 are shown in a and b, respectively. The antibody concentration used here and also in Fig. 4 is expressed as that of total antigen binding sites.

The binding of FL1 to the antibody YsT9 was also studied by a second approach: fluorescence polarization, which would increase as a consequence of the effective increase in molecular volume when the labeled O-chain binds to the large antibody molecule. The fluorescence anisotropy, calculated from the polarization data according to Equation 5, was applied to the data analysis. Fig. 4a showed that the fluorescence anisotropy of FL1 increased as the concentration of YsT9 was increased, approaching the limiting anisotropy. The value of limiting anisotropy was determined to be 0.216 by linear extrapolation through the data points of a double reciprocal plot of 1/A versus [Ab]o (Fig. 3a, inset c) according to Equation 4.

The effect of antibody concentration on the fluorescence anisotropy of FL1 was studied by a second approach: fluorescence polarization, which would increase as a consequence of the effective increase in molecular volume when the labeled O-chain binds to the large antibody molecule. The fluorescence anisotropy, calculated from the polarization data according to Equation 5, was applied to the data analysis. Fig. 4a showed that the fluorescence anisotropy of FL1 increased as the concentration of YsT9 was increased, approaching the limiting anisotropy. The value of limiting anisotropy was determined to be 0.216 by linear extrapolation through the data points of a double reciprocal plot of 1/A versus [Ab]o (Fig. 3a, inset c) according to Equation 4.

Effect of Antibody on Fluorescence Characteristics of Labeled O-chain—The excitation and emission spectra of both fluorescein-labeled O-chain preparations (i.e. FL1 and FL2) were determined in the absence and presence of the antibody YsT9. Reaction of either FL1 (9 × 10⁻⁵ g/liter) or FL2 (3 × 10⁻⁵ g/liter) with YsT9 (5 × 10⁻⁸ M) caused no shift in their excitation and emission spectra but a strong decline in the fluorescence maximum (Fig. 2). When either FL1 or FL2 was mixed with the control antibody M178, no change in fluorescence anisotropy was observed (Fig. 4a), indicating that the increase in the anisotropy of FL1 was specific to the interaction with the anti-O-chain antibody. This quenching signal can be employed to measure the antibody-bound labeled O-chain in the binding equilibrium study.

Binding of the O-chain to Antibody—Binding of FL1 to the antibody YsT9 was investigated by measuring the fluorescence quenching of the labeled O-chain when titrated with the antibody. Fig. 3a showed that quenching of fluorescence of FL1 increased as the concentration of antibody was increased, approaching a plateau value. The equilibrium constant (K) and the maximal fluorescence quenching (Qm) were calculated to be 1.79 × 10⁷ M⁻¹ and 0.45, respectively, by fitting a nonlinear curve through the data points of a plot of Q versus [Ab]o (Fig. 3a) according to Equation 4.

Effect of Fluorescein Labeling on Binding of the O-chain to Antibody—To determine whether the extent of fluorescein labeling affects the binding of O-chain to antibody, a second labeled O-chain preparation (FL2) with a degree of modification 3.5-fold higher than that of FL1 was employed in the binding study. Fluorescence quenching and anisotropy of FL2 as a function of antibody concentration were shown in Figs. 3b and 4c, respectively. As found with FL1, both fluorescence quenching and anisotropy of FL2 increased as the concentration of antibody was increased, approaching a plateau value. The equilibrium constant (K) and the maximal fluorescence quenching (Qm) were calculated to be 1.79 × 10⁷ M⁻¹ and 0.45, respectively, by fitting a nonlinear curve through the data points of a plot of Q versus [Ab]o (Fig. 3a) according to Equation 4.

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ing and anisotropy of FL2 approached a limiting value as the concentration of YsT9 was increased (Figs. 3b and 4c). By performing a nonlinear curve-fitting according to Equation 4, a \( K \) of 2.04 \( \times 10^7 \) M\(^{-1} \) and a \( Q_m \) of 0.57 were derived from the FL2 fluorescence quenching data (Fig. 3b). Analysis of the FL2 polarization data yields a limiting anisotropy of 0.219 by linear extrapolation (Fig. 4c, inset) and a \( K \) of 1.80 \( \times 10^7 \) M\(^{-1} \) by nonlinear curve-fitting (Fig. 4d) according to Equation 8 with a \( Q_m \) of 0.57.

Reversibility of the O-chain-Antibody Reaction—The reversibility of binding of the O-chain to antibody was tested by displacing the fluorescein-labeled antigen from the antibody with the unlabeled O-chain. A mixture of FL1 (9 \( \times 10^{-5} \) g/liter) and YsT9 (5 \( \times 10^{-8} \) m) or FL2 (3 \( \times 10^{-5} \) g/liter) and YsT9 (3.3 \( \times 10^{-8} \) m) was prepared to give the steady-state anisotropy values of 0.159 \( \pm \) 0.002 (n = 6) and 0.162 \( \pm \) 0.001 (n = 6), respectively. This corresponded to approximately 80% bound labeled O-chain in each case. Displacement experiments were performed by adding aliquots of the unlabeled O-chain to the labeled O-chain/antibody solution. As shown in Fig. 5, the fluorescence anisotropy decreased for both FL1 and FL2 as the unlabeled O-chain concentration was increased, approaching that of the labeled O-chain alone. When \( S. typhimurium \) LPS was added in a separate titration, no depolarization was observed (Fig. 5), indicating that displacement was specific to the O-chain. The relationship of \( f_b \) with the unlabeled O-chain concentration was shown in the insets of Fig. 5. Taking an average value of \( K_1 \) for FL1 to be 1.65 \( \times 10^5 \) M\(^{-1} \) (Table I), a \( K_2 \) of 1.49 \( \times 10^3 \) liter/g for the unlabeled O-chain was obtained by nonlinear curve fitting through the data points in a plot of \( f_b \) versus [Ag], (Fig. 5a, inset) according to Equation 15. Using an average value of \( K_1 \) for FL2 (1.90 \( \times 10^7 \) M\(^{-1} \), Table I), a \( K_2 \) of 1.48 \( \times 10^3 \) liter/g for the unlabeled O-chain was derived from the data in Fig. 5b. Taking the molecular mass of O-chain to be 13 kDa (24), the equilibrium constant (\( K \)) in terms of M\(^{-1} \) for the unlabeled O-chain was calculated to be 1.93 \( \times 10^5 \) M\(^{-1} \) from the data in Fig. 5a and 1.82 \( \times 10^5 \) M\(^{-1} \) from the data in Fig. 5b.

**DISCUSSION**

The \( B. abortus \) O-chain polysaccharide fragment (O-chain) has been successfully labeled with fluorescein for use in a homogeneous assay strategy to characterize the binding of a carbohydrate antigen to its antibody on a quantitative basis. Covalent attachment of fluorescein groups to the O-chain, presumably via reacting with hydroxyl groups on the polysaccharide antigen (25), has provided a sensitive and reliable signal.
for bound antigen. Surprisingly, there have been few reports on labeling of carbohydrate molecules with a fluorescent probe available in the literature (25). To the best of our knowledge, this study represents the first use of a fluorescently labeled O-chain derived from the outer membrane LPS of a pathogenic bacterium for the determination of carbohydrate-binding antibody affinity by fluorescence quenching and polarization techniques. These techniques have been a powerful tool in the study of molecular interactions in a variety of biological systems including the antigen-antibody reaction (23, 26–29). The previous study of antigen-antibody reaction using the fluorescence quenching and polarization techniques mainly focused on a system with an antigen either being a fluorescent hapten or a fluorescently labeled protein. The present study has extended the application of such techniques to characterization of the complex formation between a carbohydrate antigen and its antibody. Carbohydrate antigens labeled with a radioactive isotope (30, 31) or conjugated with enzyme (32, 33) have been employed for the measurement of antibody specificity and affinity. This is time-consuming due to separation of free antigen from bound antigen and/or is potentially hazardous due to the radioactive material. Moreover, covalent attachment of enzyme to polysaccharides resulted in a significant loss of enzyme activity (33). As demonstrated here, labeling of a carbohydrate antigen with fluorescein groups is an alternative approach to determine the affinity constant for a carbohydrate-binding antibody and avoids some difficulties associated with other immunoassays.

Analysis of molecular interactions could become complicated due to the multivalency of interacting molecules. In the present study, two antigen binding sites per antibody molecule were taken into consideration for the data analysis. As a linear homopolymer, the O-chain possibly has multiple YsT9-defined epitopes. This multivalency should not affect the results of the data analysis, because it does not alter the molar ratio of bound antigen to total antigen (i.e. the mole fraction of bound O-chain) in the binding assays, which is a variable of the binding equations derived in this study. The B. abortus O-chain has a molecular mass of 13 kDa (24), which is considerably lower than that of an antibody IgG molecule (150 kDa). Based on this fact, an assumption has been made to facilitate the data analysis; the O-chain was small enough so that the binding of the O-chain molecule to each antigen binding site is independent. The fact that plots of 1/Ab versus [Ab], (inset of Fig. 4a) or 1/Q versus [Ab], (data not shown) are linear suggests that the O-chain-antibody interaction has the characteristics of a hapten-antibody system (34). One possible explanation for the O-chain exhibiting such characteristics is that binding of a large antibody molecule to an epitope on a small O-chain fragment may prevent more antibodies from binding to other possible epitopes on the same O-chain molecule due to steric hindrances. The present study has applied the nonlinear curve fitting approach rather than the Scatchard plot for the data analysis because, as addressed by Wei and Herron (34), (i) the latter approach was thought to violate the assumptions of the least-squares method and (ii) the linearized variables used in Scatchard analysis tend to have higher uncertainties due to error propagation than the directly measured variables. It may be noted covalent linkage of fluorescein to the O-chain provided an extremely sensitive signal for bound antigen and the labeled O-chain was used at a concentration considerably lower than the total antibody concentration in the assay. Under such conditions, the latter may be regarded as approximately constant and equal to the equilibrium concentration. This approximation treatment has simplified the data analysis and appears to be valid as judged by the fact that three independent analysis procedures derived a similar order of equilibrium constants for the labeled O-chain preparations either with a low or a high degree of modification, and the unlabeled O-chain (Table I).

Modification of the O-chain with fluorescein did not affect the antibody affinity constant, indicating that the intrinsic affinity had been measured. Therefore, the affinity of various antisera for B. abortus O-chain as measured by the fluorescein derivative of O-chain can be compared. Although used here to characterize the binding of B. abortus O-chain to antibody, the carbohydrate-fluorescein conjugate approach has the potential application in any bacterial LPS-binding antibody or carbohydrate-binding receptor systems.

The practical implication of this study is that the fluorescence quenching or the increase in polarization of the labeled O-chain due to the formation of antigen-antibody complex may be exploited to yield a new diagnostic tool for brucellosis. In fact, a fluorescence polarization assay for detection of antibody to B. abortus has been developed in our laboratory (35). Another interesting result of this study is that addition of the unlabeled antigen resulted in fluorescence depolarization of the labeled O-chain-antibody complex. This result indicates that the labeled O-chain is released from the antibody in the presence of the unlabeled O-chain and that the O-chain-antibody reaction is in dynamic equilibrium. The specific displacement of this type can be used to quantitate the bacterial polysaccharide antigen. One of the advantages with the displacement assay system is that the polysaccharide antigen to be quantitated is not necessarily in a purified form because the displacement was highly specific (Fig. 5).

In summary, fluorescein labeling of the B. abortus O-chain has provided a probe suitable for characterization of the binding of O-chain to antibody and a useful model system for the study of the complex formation between a carbohydrate-binding protein and a carbohydrate ligand.

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### Table I

| Antigen preparation | Fluorescence quenching | Fluorescence polarization | Displacement experiment |
|---------------------|------------------------|---------------------------|-------------------------|
| FL₁                 | 1.79 × 10⁻⁷            | 1.50 × 10⁻⁷               | 1.93 × 10⁻⁷             |
| FL₂                 | 2.04 × 10⁻⁷            | 1.90 × 10⁻⁷               | 1.82 × 10⁻⁷             |
| Unlabeled O-chain   |                        |                           |                         |

a FL₁ and FL₂ are the labeled O-chains with substitutions of 1.8 ± 1.8 (n = 5) and 52.3 ± 2.4 (n = 3) μmol of fluorescein/g of polysaccharide, respectively.

### Analysis of Molecular Interaction

Comparison of equilibrium binding parameters determined by three procedures for the binding of B. abortus O-chain to a monoclonal antibody YsT9

For B. abortus O-polysaccharide to a mAb

| Antigen preparation | Fluorescence quenching | Fluorescence polarization | Displacement experiment |
|---------------------|------------------------|---------------------------|-------------------------|
| FL₁                 | 1.79 × 10⁻⁷            | 1.50 × 10⁻⁷               | 1.93 × 10⁻⁷             |
| FL₂                 | 2.04 × 10⁻⁷            | 1.90 × 10⁻⁷               | 1.82 × 10⁻⁷             |
| Unlabeled O-chain   |                        |                           |                         |

a FL₁ and FL₂ are the labeled O-chains with substitutions of 1.8 ± 1.8 (n = 5) and 52.3 ± 2.4 (n = 3) μmol of fluorescein/g of polysaccharide, respectively.
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