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Assessment Of Heterogeneity in Antibody−Antigen Displacement Reactions

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The intrinsic binding characteristics of monoclonal antibodies are modified upon immobilization onto a solid-phase matrix. Factors such as the distribution in affinity must therefore be taken into consideration in order to predict the kinetics of antibody binding at solid−liquid interfaces. A mathematical analysis is presented herein that allows the assessment of heterogeneity in the affinity of monoclonal antibodies immobilized onto a solid support. This model is based on a modified version of the Sips distribution function adapted to the conditions of a solid-phase displacement assay in flow. An assay for trinitrotoluene (TNT) provides the data to evaluate the extent of heterogeneity introduced by immobilization of antibodies in a flow immunoassay. We determined the index of antibody heterogeneity on two solid supports, controlled-pore glass beads and agarose beads, coated with a monoclonal anti-TNT antibody at varying densities. The data confirm that the threshold for crossover from homogeneous to heterogeneous forms of the reaction isotherm is different in displacement reactions than in association−dissociation reactions. Our analysis shows that the measured displacement isotherm is consistent with a homogeneous or only moderately heterogenous distribution of relative affinities.

Analytical methods using immobilized antibodies conducted under flow conditions are gaining increasing popularity for clinical and environmental testing (for a review, see refs 1 and 2). These solid-phase methods include chromatographic assays,[3,4] surface plasmon resonance assays,[5−7] flow injection assays (FIA),[8] and a continuous-flow immunoassay.[9] The flow immunoassay has become exceptionally attractive for fast analyses since it is performed under nonequilibrium conditions and thus does not require incubation periods. However, several obstacles have been encountered in the assessment of such systems. In particular, classical concepts for describing equilibrium kinetics for antibody−antigen interactions in fluid phase systems do not adequately explain nonequilibrium kinetics of antibody−antigen binding at solid−liquid surfaces.[10] In a previous study, we developed a preliminary theoretical framework utilizing mass law to describe the kinetics of antigen binding to immobilized antibodies in flow under nonequilibrium conditions.[11] Using this theoretical framework, the behavior of antigen displacement events in a flow immunoassay at changing flow rates[12] and at different antibody densities on solid support[13] could be successfully predicted.

One potential problem with the methods using immobilized antibodies is the fact that monoclonal antibodies that are identical in structure and homogeneous in binding kinetics *in solution* may be converted to a population that has a heterogeneous capacity to bind to the antigen when the antibody is immobilized *on the solid support*. The functional activity of each immobilized antibody is likely to be affected to a different extent depending on the geometry of the attachment site on the solid support and the amino acid residue(s) of the antibody molecule used for immobilization. Thus, the purpose of this study is to assess the heterogeneity in affinity of immobilized monoclonal antibodies.

To predict the kinetics of antibody binding at solid−liquid interfaces, therefore, a fundamental understanding of the degree of heterogeneity in affinity is required. The standard technique for assessing antibody heterogeneity is to measure the isotherm for an association−dissociation reaction between antibodies and antigens. The measured association−dissociation isotherm is related to the distribution of antibody heterogeneity using the Sips equation.[14] In a displacement assay using immobilized antibodies, it is not possible to measure the association/dissociation isotherm; only the displacement isotherm can be measured. For that reason, the standard technique must be generalized.

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(1) Mattiasson, B.; Borrebaeck, C. Novel approaches to enzyme-immunoassay. In *Enzyme-immunoassay*; Maggio, E. T., Ed.; CRC Press: Cleveland, OH, 1980; pp 213−248.
(2) Eddowes, M. J. *Biosensors* **1987**/**88**, *3*, 1−15.
(3) Hage, D. S.; Kao, P. C. *Anal. Chem.* **1991**, *63*, 586−595.
(4) Cassidy, S. C.; Janis, L. J.; Regnier, F. E. *Anal. Chem.* **1992**, *64*, 1973−1977.
(5) Attridge, J. W.; Daniels, P. B.; Deacon, J. K.; Robinson, G. A.; Davidson, G. P. *Biosens. Bioelectron.* **1991**, *6*, 201−214.
(6) Karlsson, R.; Michaelson, A.; Mattson, L. *J. Immunol. Methods* **1991**, *145*, 229−240.
(7) Pellequer, J. L.; Van Regenmortel, M. H. *J. Immunol. Methods* **1993**, *166*, 133−143.
(8) Puchades, R.; Maquieira, A.; Atienza, J.; Montoya, A. *Crit. Rev. Anal. Chem.* **1992**, *23*, 301−321.
(9) Kusterbeck, A. W.; Wemhoff, G. A.; Charles, P.; Bredehorst, R.; Ligler, F. S. *J. Immunol. Methods* **1990**, *135*, 191−197.
(10) Ligler, F. S.; Rabbany, S. Y. In *Synthetic Microstructures in Biological Research*; Schnur, J. M., Ed.; Plenum Press: New York, 1992; pp 67−75.
(11) Rabbany, S. Y.; Wemhoff, G.; Kusterbeck, A. W.; Bredehorst, R.; Ligler, F. S. *FASEB J.* **1992**, *6*, 6242.
(12) Wemhoff, G. A.; Rabbany, S. Y.; Kusterbeck, A. W.; Ogert, R. A.; Bredehorst, R.; Ligler, F. S. *J. Immunol. Methods* **1992**, *156*, 223−230.
(13) Rabbany, S. Y.; Kusterbeck, A. W.; Bredehorst, R.; Ligler, F. S. *J. Immunol. Methods* **1994**, *168*, 227−234.
(14) Sips, R. *J. Chem. Phys.* **1948**, *16*, 490−495.

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In the accompanying theoretical paper,[15] the Sips equation is generalized to describe displacement reactions. Hence, in this study, the displacement isotherms are measured in a flow immunosensor under a variety of conditions. The measured displacement isotherms are then related to the distribution of antibody affinity using the theory of Selinger and Rabbany.[15]

In this study, the theoretical model of Selinger and Rabbany is used to assess the heterogeneity in affinity of monoclonal antibodies, immobilized via their primary amino groups onto a solid support, in a solid-phase displacement immunoassay. For an ideal homogeneous antibody population in solution undergoing association and dissociation, the binding curve (bound vs free) is expected to be in the form of a natural exponential as described by the Michaelis−Menten approach, in analogy to the reciprocal Langmuir plot. The introduction of heterogeneity in a monoclonal antibody population will cause nonlinear behavior of the Langmuir and Scatchard plots. Such heterogeneous antibody populations have been modeled using a function similar to a normal distribution function.[14,16] The approach presented herein is based on a modified version of the Sips distribution function adapted to the conditions of a solid-phase displacement immunoassay. Similar to association−dissociation reactions, the low-concentration limit of the displacement isotherm is dominated by the high-affinity tail of distribution of relative affinity. However, the modified Langmuir isotherm for a displacement reaction predicts the square root relationship in contrast to the linear Langmuir isotherm observed for an association−dissociation reaction. The threshold for the index of heterogeneity in a displacement assay is $a = {}^1/_2$, making such reactions less sensitive to heterogeneity by shifting it to a lower detection limit. To evaluate the degree of heterogeneity generated by antibody immobilization, we analyzed the displacement reaction and the index of antibody heterogeneity under varying conditions using a displacement flow immunoassay for trinitrotoluene (TNT). The theoretical results of the Rabbany−Selinger paper are derived for an equilibrium system. In this paper, we apply this theory to a nonequilibrium flow immunoassay. Although this is an approximation, in practice it is justified by the experimental result that changing the flow rate does not change the measured isotherms and hence the heterogeneity index.

MATERIALS AND METHODS

Purification of Monoclonal Antibody (MoAb)11B3. Murine MoAb 11B3 (IgG_1) developed against trinitrobenzenesulfonate (TNBS)-derivatized ovalbumin[17] was purified from ascites fluid by protein G affinity chromatography as described.[18] Homogeneity of purified MoAb 11B3 was confirmed by SDS−polyacrylamide gelelectrophoresis.[19]

Synthesis of *N*α-[^{3}H]Acetyl-*N*ϵ-TNP-L-lysine. *N*ϵ-TNP-L-lysine (5.7 mg, 16 μmol; Research Organics Inc., Cleveland, OH) in 1.0 mL of ice-cold dimethylformamidc (DMF) was reacted with [^{3}H]acetic anhydride (16 μmol, 1.85 GBq/mmol; DuPont NEN, Bad Homburg, Germany), and 60 μL of NaOH for 30 min at room temperature. After the addition of nonlabeled acetic anhydride (5 μmol), DMF was removed by distillation, *N*α-[^{3}H]acetyl-*N*ϵ-TNP-L-lysine (3.4×10^4 cpm/nmol) was obtained in 68% yield as determined by absorption at 345 nm.

Equilibrium Dialysis. The affinity of purified MoAb 11B3 for *N*α-[^{3}H]acetyl-*N*ϵ-TNP-L-lysine under fluid-phase conditions was determined by equilibrium dialysis using an equilibrium microvolume (100 μL) dialyzer with 12−14 kDa cutoff adhering dialysis membrane (Hoefer-Serva, Heidelberg, Germany). Prior to use, the membranes were boiled for 5 min in 5% (w/v) Na_2CO_3 containing 50 mM EDTA, followed by three washing steps in boiling H_2O. Purified MoAb 11B3 (30 μg) in 100 μL of 0.15 M phosphate-buffered saline (PBS), pH 7.2, was placed on one side of the dialysis membrane and varying amounts of *N*α-[^{3}H]acetyl-*N*ϵ-TNP-L-lysine (10^{-6}−10^{-8} M) in 100 μL of PBS, pH 7.2, on the other side. After 15 h off equilibration at 4 °C in the dark, aliquots were removed from each side of the dialysis chambers and counted in a liquid scintillation counter. Calculation of the affinity constant was performed as described.[20]

Antibody Immobilization. Monoclonal antibody 11B3 with specificity for TNT was immobilized at two different densities onto tresyl chloride-activated agarose and controlled-pore glass (CPG; CPG, Inc., Fairfield NJ) beads. Tresyl chloride-activated agarose (1 g; Pierce Chemical, Rockville, IL) was washed in cold distilled water for 1 h, with rotation, followed by four washes with coupling buffer (0.1 M $NaHCO_3$, pH 8.6, containing 0.5 M NaCl) on a Büchner-type filtering funnel (Fisher Scientific). Purified antibody (1.3 mg; concentration 0.3 mg/mL) was immobilized onto the agarose, by a 2-h incubation at room temperature with end-over-end rotation in coupling buffer. The unbound antibody was removed by washing four times with 1 mL of coupling buffer. Remaining coupling sites were blocked with 0.1 M Tris-HCl, pH 8.0, for 4 h. The final concentration of anti-TNT antibody immobilized was determined indirectly by a standard bicinchoninic acid (BCA) protein assay (Pierce Chemical) to be 2 nmol of antibody/mL of gel. The matrix was stored in PBS, pH 7.4, at 4 °C.

Prior to antibody immobilization onto the CPG, 10 g of CPG beads, 120−200 mesh, ∼500-Å pores (CPG, Inc.) were washed with several volumes of concentrated nitric acid (100 mL) in a 90 °C shaking water bath for 16−24 h. The contents were transferred to a 30-mL filtering funnel and washed with deionized water until a neutral pH was reached (at least 500 mL). Two aliquots, containing 2 g of beads each, were silanized with 10% (glycidoxypropyl)trimethoxysilane (GPTMS; Fluka Chemical, Buchs, Switzerland) in water at pH 3.5 for 2 h at 90 °C with shaking. After being washed sequentially with 100 mL each distilled water, acetone, and diethyl ether, the beads were again washed three times with 20 mL of acetone and then activated in a solution of 10 mL of acetone containing 130 μL of 2,2,2-trifluoroethanesulfonyl chloride (tresyl chloride; Sigma Chemical Co., Saint Louis, MO) and 200 μL of pyridine (Fluka Chemical) for 30 min at 4 °C, with a second addition of tresyl chloride and pyridine after the first 15 min. The beads were placed in a filtering funnel and washed with 30 mL each of acetone, acetone−5 mM HCl (70:30 v/v), acetone−5 mM HCl (50:50 v/v), acetone−5 mM HCl (30:70 v/v), 5 mM HCl, 1 mM HCl, and coupling buffer. The anti-TNT antibody was immobilized onto CPG, at concentrations of 0.3 or 0.8 mg/mL, by overnight incubation in coupling buffer, and any available

(15) Selinger, J. V.; Rabbany, S. Y. *Anal. Chem.* **1997**, *69*, 170−174.
(16) Berzofsky, J. A.; Epstein, S. L.; Berkower, I. J. In *Fundamentals of Immunology*; Paul, W. E., Ed.; Raven Press: Hagerstown, MD, 1989; pp 315−356.
(17) Whelan, J. P.; Kusterbeck, A. W.; Wemhoff, G. A.; Bredehorst, R.; Ligler, F. S. *Anal. Chem.* **1993**, *65*, 3561−3565.
(18) Åkerström, B.; Brodin, T.; Reis, K.; Björck, L. *J. Immunol.* **1985**, *135*, 2589−2592.
(19) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.
(20) Nisonoff, A.; Pressman, D. *J. Immunol. Methods* **1958**, *80*, 417−428.

binding sites not filled with antibody were again blocked with 0.1 M Tris-HCl, pH 8.0, for 4 h with rotation. The matrix was stored in PBS at 4 °C.

Preparation of Texas Red-Labeled TNB. To prepare a labeled analog of TNT, 2 mg of 2,4,6-trinitrobenzenesulfonic acid (TNBSA) (Pierce Chemicals) was dissolved in 1 mL of borate-buffered saline (BBS), pH 8.5−50% ethanol. This solution was added to 5 mL of BBS−50% ethanol containing 5.2 mg of Texas Red cadaverine (TR-CV; Molecular Probes, Eugene, OR). Under these conditions, the final molar ratio of TNBS to TR-CV was 0.9:1 (1.13 mM TNBS−1.26 mM TR-CV). The reaction mixture was vortexed and incubated overnight with rotation at 4 °C. The solution was dried and redissolved in 1 mL of methanol. The fluorophore-labeled product was purified by preparative thin-layer chromatography on silica gel, using type PLK5F silica plates (Whatman, Maidstone, KY). The preparative plate was developed with methanol−chloroform (1:3), and a single band with $R_f = 0.85$ was extracted with methanol. The final product was lyophilized, resuspended in distilled water containing 20% EtOH, and stored at 4 °C. The concentration of Texas Red cadaverine conjugate (TR-CV−TNB) was calculated based on the absorbance at 596 nm using Texas Red cadaverine, with an extinction coefficient of 90 000, as a standard.

Saturation of Immobilized Antibody with Labeled TNB. A 100-μL bed volume of agarose gel or CPG beads coated with the anti-TNB monoclonal antibody 11B3 was placed in a disposable microcolumn (Isolabs, Inc., Akron, OH), and excess buffer was removed by vacuum suctioning (note: precise volumes of solid matrix, at such low quantities, are difficult to transfer to the columns). Antibody binding sites were saturated with 100 μL of 25 μM TR-CV−TNB in PBS, by overnight incubation at room temperature.

Flow Immunoassay. The apparatus for measuring the antigen response in the flow immunoassay included a Jasco 821-FP fluorometer (Jasco International Co., Tokyo, Japan), equipped with a 12-μL flow cell, a Hewlett-Packard integrator, a Rabbit-Plus peristaltic pump (Rainin Instruments, Emeryville, CA), and a Rheodyne five-way valve (Rainin Instruments, Emeryville, CA) employed as a low-pressure sample injector. Connecting tubing was 0.6-mm-i.d. Teflon (Upchurch, Oak Harbor, WA). A Model HP-9114B disk drive (Hewlett-Packard, Inc., Rockville, MD) was utilized to store analog output from the fluorometer for subsequent analysis. The microcolumn containing the saturated matrix was connected to the system and washed with standard flow buffer of PBS, pH 7.4, containing 0.1% reduced Triton X-100 (Aldrich Chemical, Milwaukee, WI) and 12.5% EtOH until the background fluorescence was stable. Fluorescence was monitored at an excitation of 583 nm and an emission of 607 nm.

For sample testing, a continuously flowing buffer stream was established through the column at flow rates of 0.1, 0.5, 1.0, and 2.0 mL/min. Column effluent was directed through a spectrofluorometer. All TNT samples and negative controls were diluted in flow buffer and 100-μL aliquots were introduced into the flow stream using the low-pressure sample injector. A stock solution of TNT (414 μg/mL) was prepared in 100% ethanol by slow rotation at room temperature in a glass vial and used to prepare a standard curve with concentrations ranging from 9.4 to 1200 ng/mL. A mixture of phenylalanine (8 μg/mL) and lysine (8 μg/mL) was used as a negative control.

Determination of Bound Labeled Antigen. For calculations of the total amount of labeled antigen bound to the immobilized antibody, a repetitive displacement experiment was performed. A molar concentration of TNT 100 times greater than that of antibody present on the matrix was repeatedly applied at a flow rate of 0.1 mL/min. The resulting displacement was monitored until the signal approached the original baseline, indicating depletion of the labeled antigen from the matrix. The total displaceable labeled antigen bound by the antibody at the beginning of the experiment was calculated by adding the amount collected for all samples.

MATHEMATICAL ANALYSIS

The displacement kinetics of the fluorophore-labeled antigen from the immobilized antibody in the flow immunoassay are represented by the following reaction utilizing the law of mass action:

$$\mathrm{AbAg^*} + \mathrm{Ag} \rightleftharpoons \mathrm{AbAg} + \mathrm{Ag^*} \qquad (1)$$

where AbAg* is the complex of immobilized antibody and labeled antigen, Ag is the unlabeled antigen added during each sample injection, AbAg is the complex of immobilized antibody and unlabeled antigen, and Ag* is the labeled antigen displaced from the antibody. Ongoing reactions undetectable in the system are (i) the displacement of an unlabeled antigen by another unlabeled antigen, (ii) the displacement of labeled antigen by another already displaced labeled antigen, and (iii) the displacement of unlabeled antigen by already displaced labeled antigen.

To describe and predict the kinetics of immobilized antibody-antigen interaction in the flow immunoassay, expressions previously formulated for systems at equilibrium are utilized. For an association−dissociation reaction, the Langmuir isotherm predicts

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK\,[\mathrm{Ag}]} \qquad (2)$$

where r represents the number of reactants bound per antibody molecule *in solution* at equilibrium, n is the maximum number of reactants that can be bound per antibody molecule (antibody valence), K is the antibody affinity constant, and [Ag] is the amount of free antigen *in solution*.

In the flow immunoassay, we can only detect those reactions at the surface that will displace a detectable labeled antigen from an immobilized antibody. The parameter r is therefore replaced by a new parameter for immobilized systems in flow, r_{im}:

$$r_{im} = \text{displaced Ag*/displaceable Ag*} \qquad (3)$$

where displaced Ag* is the amount of labeled antigen displaced per sample injection and displaceable Ag* is the amount of bound immobilized labeled antigen before each injection. Furthermore, since total bound Ag* has been used to calculate active antibody binding sites, the valence parameter n, in practice, is 1. For a displacement reaction rather than an association−dissociation

reaction, the modified Langmuir isotherm[15] becomes

$$\frac{1}{r_{im}} = \frac{1 + (K_{disp}[Ag_{loaded}]/[Ag^*Ab])^{1/2}}{(K_{disp}[Ag_{loaded}]/[Ag^*Ab])^{1/2}} = 1 + \left(\frac{[Ag^*Ab]}{K_{disp}[Ag_{loaded}]}\right)^{1/2} \quad (4)$$

where K is replaced by K_{disp}, which is defined as the apparent displacement constant:

$$K_{disp} = [Ag^*][AbAg]/[Ag][AbAg^*] \quad (5)$$

Correspondingly, the amount of free antigen in solution, [Ag], is herein replaced by the amount of loaded antigen, $[Ag_{loaded}]$. In solution kinetics, antibody and antigen concentrations are expressed in moles per liter, which leads to an affinity constant in liters per mole. It should be noted that, in this analysis, the apparent displacement constant (K_{disp}) is dimensionless.

Experimental binding data often deviate from the predictions of the Langmuir equation. This deviation has been ascribed to heterogeneity of affinities within an antibody population. The standard approach to determine the level of antibody heterogeneity in fluid phase assays is the Sips relationship.[20] The Sips equation relates the measured binding isotherm to the heterogeneity of the antibody population. Selinger and Rabbany[15] have generalized the Sips equation to describe both association−dissociation and displacement reactions. In this generalization, it is assumed that the distribution function $N(K_{disp})$ of the apparent displacement constant K_{disp} decays as a power law:

$$N(K_{disp}) \propto K_{disp}^{-a-1} \quad (6)$$

for large K_{disp}. The exponent a is the index of heterogeneity of the antibody population. As *a decreases* the population becomes *more heterogeneous.* A Sips plot, where $\log r/(n - r)$ is plotted against log [Ag], produces a straight line of slope a (heterogeneity index), allowing the determination of the K (affinity). For an association−dissociation reaction, the reaction isotherm for a heterogeneous system of antibodies

$$\log r = \log [Ag] + \log K \quad \text{for} \quad a > 1 \quad (7)$$

$$\log r = a \log [Ag] + \log I[\pi\alpha/\sin \pi a] \quad \text{for} \quad 0 < a < 1 \quad (8)$$

allows determination of the index of heterogeneity (a) in the range from 0 to 1. As it decreases from 1 (no heterogeneity) toward 0, the heterogeneity increases. By contrast, for a displacement reaction, the reaction isotherm becomes

$$\log r = {}^1/_2 \log [Ag] - 1/_2 \log [AbAg^*] + \log K_{disp}^{1/2} \quad \text{for} \quad a > {}^1/_2 \quad (9)$$

$$\log r = a \log [Ag] - a \log [AbAg^*] + \log [2\pi\alpha/\sin 2\pi a] \quad \text{for} \quad 0 < a < {}^1/_2 \quad (10)$$

The constant K_{disp} is an empirically determined function of not only the intrinsic dissociation constant (k_d) of the AbAg* complex but also factors such as rebinding of dissociated labeled antigen and the effect of immobilization on antibody activity. Application of this modified Sips equation to the flow system allows experimental determination of an apparent displacement constant (K_{disp}) and the index of heterogeneity (a). The above equations imply that an association−dissociation reaction is sensitive to heterogeneity in the range of $0 < a < 1$, while a displacement reaction is sensitive to heterogeneity in the narrower range $0 < a < 0.5$. Thus, a displacement reaction is less susceptible to heterogeneity than an association−dissociation reaction, but the value of index of heterogeneity (a) can still indicate when an antibody population becomes very heterogeneous in a displacement reaction.

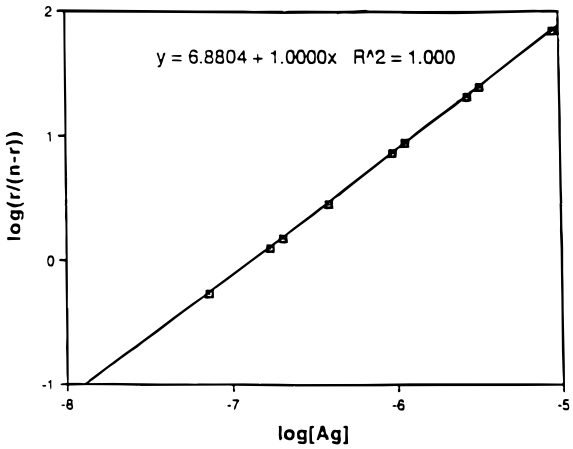

Figure 1. Sips plot of binding data for equilibrium dialysis studies with tritium-labeled L-lysine, undergoing association−dissociation reactions. Equation illustrating the linear fit of the data is shown where the index of heterogeneity (a) is determined from the slope.

RESULTS

Assessment of Heterogeneity. The data for the equilibrium dialysis with tritium-labeled L-lysine, undergoing association−dissociation reactions, was analyzed to ascertain the degree of heterogeneity in the affinity of antibody binding in solution. It is hypothesized that monoclonal antibodies have a homogeneous affinity at equilibrium (i.e., the heterogeneity index $a = 1$). Figure 1 shows the Sips plot, whereby the slope of the line reflects the index of heterogeneity (a). The data for the equilibrium dialysis with tritium-labeled L-lysine, undergoing association−dissociation reactions, was analyzed to ascertain the degree of heterogeneity in the affinity of antibody binding in solution. It is hypothesized that monoclonal antibodies have a homogeneous affinity at equilibrium (i.e., the heterogeneity index $a = 1$). Figure 1 shows the Sips plot, whereby the slope of the line reflects the index of heterogeneity (a). As expected $a = 1$, confirming that this monoclonal antibody is homogeneous in solution.

Figure 2A illustrates the displacement of Texas Red cadaverine-labeled TNB from the binding sites of monoclonal antibody 11B3 immobilized onto CPG at a density of 2 nmol/mL matrix by various concentrations of TNT loaded at a flow rate of 0.1 mL/min. Shown as the dose−response curves obtained from two different experiments. For increasing concentrations of loaded Ag (TNT), corresponding increases were observed in the total displaced Ag*. The dose−response curves are nonlinear. Since the data for each dose−response curve were obtained from repetitive displacement experiments using a single column,

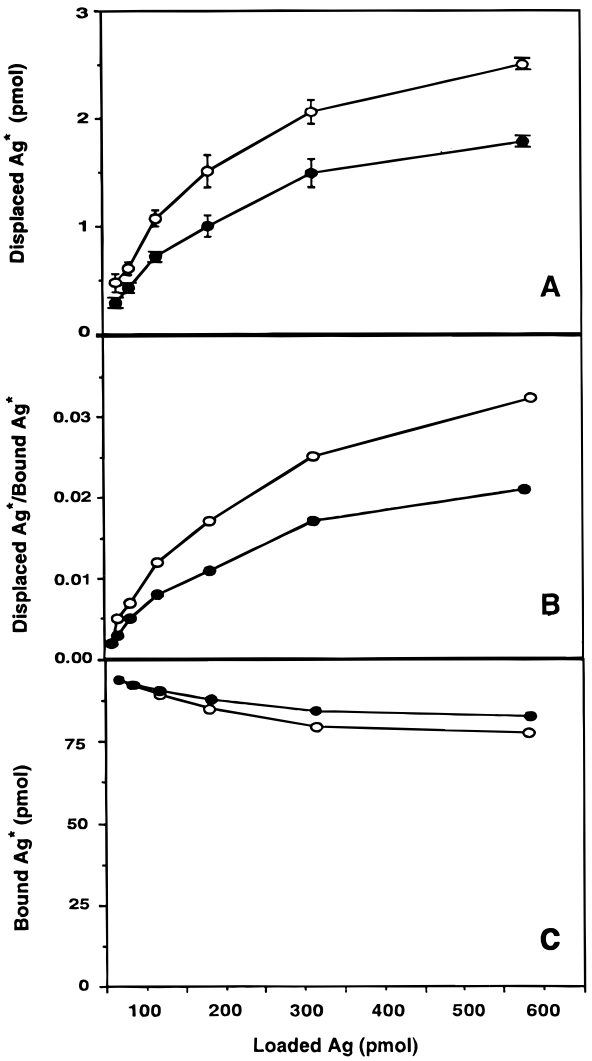

Figure 2. Displacement of Texas Red cadaverine-labeled TNB from antibody immobilized on CPG beads. (A) Displaced labeled antigen (Ag*) as a function of loaded TNT concentration for two different experiments. The mean values and standard errors for triplicate runs are shown for two separate experiments at a flow rate of 0.1 mL/min. (B) Displaced labeled antigen normalized by bound antigen as a function of loaded TNT. (C) Bound labeled antigen remaining on the column as a function of loaded TNT concentration. Each experiment is conducted on a different column, with triplicate samples at each loaded TNT concentration.

depletion of labeled antigen had to be eliminated as reason for the nonlinearity. When displaced Ag* was normalized against bound labeled Ag* and plotted as a function of loaded TNT, the nonlinear nature of the dose−response curve remained (Figure 2B). Therefore, we determined the amount of Ag* bound to the column. The data in Figure 2C demonstrate that less than 25% of total displaceable antigen (bound Ag*) had been displaced after completion of all displacement experiments and that depletion of Ag* is not responsible for the nonlinear nature of the dose−response curves in Figure 2A.

Figure 3 depicts a double−reciprocal plot of the same data. To compare the data with the predictions of Selinger−Rabbany theory for displacement reactions, the Sips plot of log [r_{im}] vs log [Ag] was drawn. As shown in Figure 4 this plot is linear, with a slope of $\sim^1/_2$. This slope is consistent with eq 9 rather than eq 10. This result shows that the immobilized antibodies are either homogeneous or only moderately heterogeneous, with $a > {}^1/_2$.

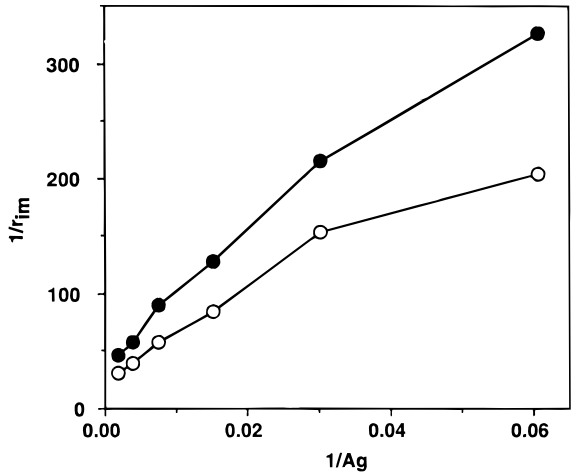

Figure 3. Double-reciprocal plot for the binding data presented in Figure 2. The term r_{im} represents the amount of displaced labeled antigen as a function of total bound.

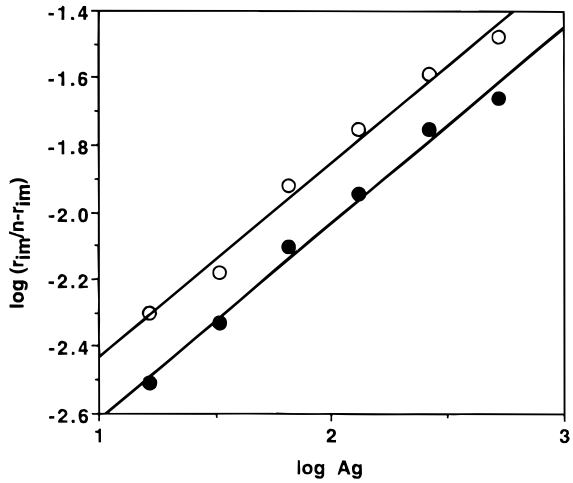

Figure 4. Sips plot of binding data adapted to the solid phase immunoassay. The index of heterogeneity (a) is determined from the slope, and the apparent displacement constant (K_{disp}) is calculated from the x-intercept. Equations illustrating the linear fit of the data are $y = 3.004 + 0.575x$, $R^2 = 0.987$ and $y = -3.199 + 0.583x$, $R^2 = 0.990$.

Effect of Flow Rate. To examine the effect of flow rate on the index of heterogeneity, identical experiments were also conducted at flow rates of 0.5 and 2.0 mL/min. Two experiments were performed at each flow rate, with triplicate samples at each concentration of loaded TNT. Figure 5 depicts the Sips plots for the displacement data obtained from flow rates of 0.5 (A) and 2 mL/min (B). For both flow rates, the slope of the Sips plots was $\sim^1/_2$, indicating that the immobilized antibodies are either homogeneous or only moderately heterogeneous, with $a > {}^1/_2$.

Effect of Antibody Density. To examine the role of antibody density on both the displacement constant and the index of heterogeneity, analogous studies were carried out with antibody densities of 2 and 5 nmol of antibody/mL of CPG beads at a flow rate of 1.0 mL/min. Figure 6 illustrates the displaced Ag* as a function of loaded TNT under identical experimental conditions. The amount of displaced Ag* per loaded antigen is ~2-fold higher using the matrix with the 5 nmol/mL antibody concentration. The insets in Figure 6 illustrate the double-reciprocal plots at these two densities. Comparison of the indexes of the heterogeneity for the two antibody densities does not show a significant difference (Figure 7).

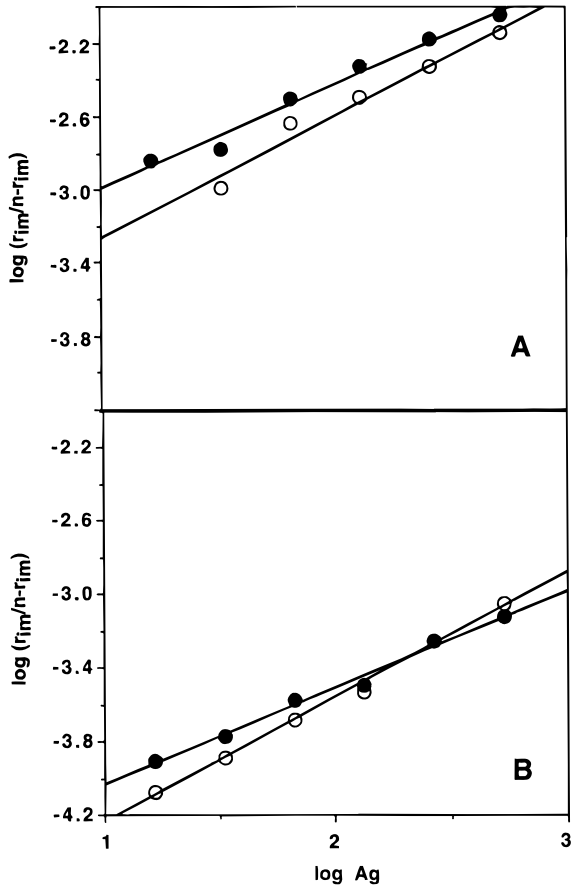

Figure 5. Sips plot for the binding data. Variations in flow rate of 0.5 (A) and 2.0 mL/min (B) at an antibody density of 2 nmol/mL using CPG as the solid support are shown. Equations illustrating the linear fit of the data are $y = -3.924 + 0.663x$, $R^2 = 0.968$ and $y = -3.558 + 0.565x$, $R^2 = 0.980$ for 0.5 mL/min and $y = -4.918 + 0.680x$, $R^2 = 0.995$ and $y = -4.555 + 0.525x$, $R^2 = 0.989$ for 2.0 mL/min, respectively.

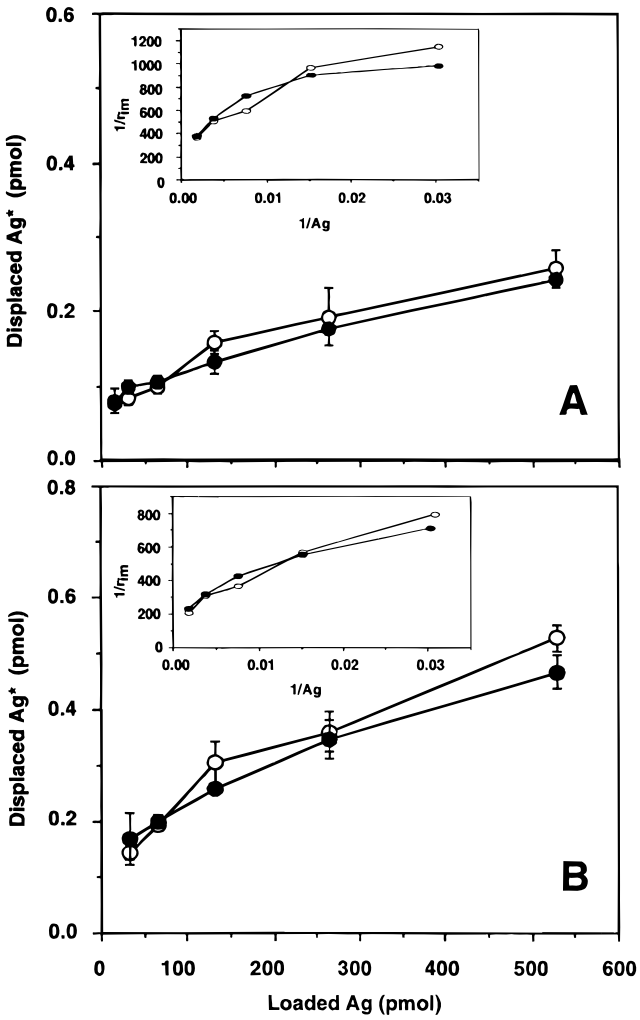

Figure 6. Displaced labeled antigen (Ag*) as a function of loaded TNT concentration at a flow rate of 1 mL/min and two antibody densities: (A) 2 nmol/mL; (B) 5 nmol/mL. Double-reciprocal plots of the binding data, illustrating nonlinear characteristics, are shown in the insets. Shown are the data for two different experiments.

Effect of the Solid Support Properties. To evaluate the role of solid support properties on the index of heterogeneity, CPG (~500 Å) was compared to agarose beads, a more hydrophilic solid support with heterogeneous pore size. Both supports were coated with virtually identical amounts of monoclonal antibody (11B3) per milliliter of matrix using a tresyl chloride linkage to terminal amine groups on the antibody. Figure 8 illustrates the displaced Ag* as a function of TNT loaded on to the column for both the CPG and agarose matrices at a flow rate of 0.1 mL/min. The Sips plot demonstrates that the index of the heterogeneity is lower (i.e., more heterogeneous) when agarose is used as a matrix (Figure 9).

DISCUSSION

This study addresses the possibility that monoclonal antibodies may not maintain a uniform affinity after immobilization. Homogeneous monoclonal anti-TNT antibodies were immobilized onto porous matrices via their primary amino groups. We examined the possibility that the homogeneous antibody population may have been transformed into a population with heterogeneous binding capacities. Since an antibody molecule contains multiple primary amino groups, the specific amino group(s) used for immobilization cannot be controlled. Consequently, antigen binding is likely to be affected to a certain extent by immobilization. For example, an attachment site in close proximity to the antigen binding region might cause a decrease in affinity due to stearic hindrance effects. Since impaired antigen binding affects the prediction of antibody behavior at solid−liquid interfaces in flow, heterogeneity in affinity must be taken into consideration for the design of optimized flow immunoassays. Other sources of heterogeneity could include surface chemistry, local geometry of solid support, and density of antibody immobilized onto the solid support.

To assess the extent of heterogeneity that may be introduced in the affinity of immobilized monoclonal antibodies, a modified Sips relationship for displacement assays was developed.[15] Similar to the original Sips relation, the modified equation assumes that antibody affinity exhibits a distribution function. However, in a solid-phase assay, we are only concerned with interactions at the surface of the matrix that will displace a detectable labeled antigen from immobilized antibody molecules. Hence, the parameter r was replaced by r_{im} and the intrinsic affinity constant K by the apparent displacement constant K_{disp}. The constant K_{disp} is a function of not only the intrinsic dissociation constant k_d of immobilized AbAg* complexes but also of other factors introduced by immobilization. Such factors include nonspecific interaction of the solid support with labeled antigen and, depending on the location of the immobilization site on the solid support and the

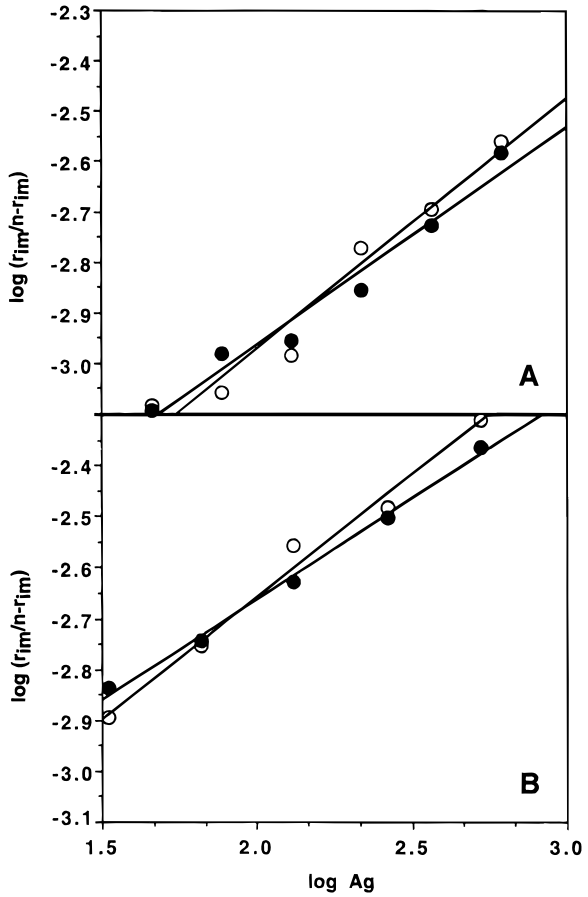

Figure 7. Sips plot of data presented in Figure 5 for two different antibody densities at the flow rate of 1.0 mL/min. Equations illustrating the linear fit of the data are $y = -3.592 + 0.372x$, $R^2 = 0.957$ and $y = -3.507 + 0.325x$, $R^2 = 0.966$ for 2 nmol/mL (A) and $y = -3.613 + 0.478x$, $R^2 = 0.987$ and $y = -3.452 + 0.395x$, $R^2 = 0.995$ for 5 nmol/mL (B).

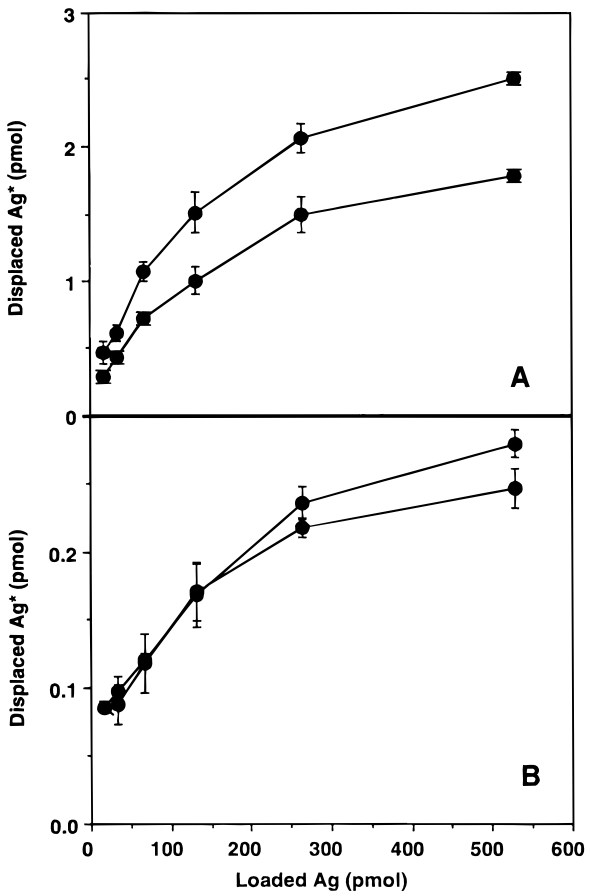

Figure 8. Displaced labeled antigen (Ag*) as a function of loaded TNT concentration at a flow rate of 0.1 mL/min, using two different support matrices for antibody immobilization: (A) CPG; (B) agarose.

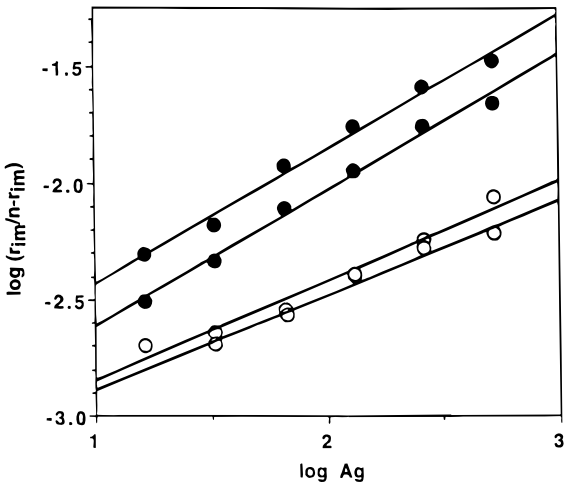

Figure 9. Sips plot of data presented in Figure 8 for two different support matrices. Equations illustrating the linear fit of the data are $y = -3.004 + 0.575x$, $R^2 = 0.987$ and $y = -3.199 + 0.583x$, $R^2 = 0.990$ for CPG and $y = -3.281 + 0.432x$, $R^2 = 0.971$ and $y = -3.294 + 0.409x$, $R^2 = 0.977$ for agarose, respectively.

attachement site(s) on the antibody molecule, a limited accessibility of the antigen binding sites to injected antigen.

Utilizing the modified Sips distribution function, several parameters known to influence the performance of flow immunoassays were evaluated for their effect on the extent of heterogeneity in affinity. Since solid support properties may have an impact on the index of heterogeneity, two very different solid supports were explored. Controlled-pore glass beads and agarose vary in their hydrophilicity, the method of covalent antibody immobilization, and their pore size. Agarose beads are more hydrophilic than CPG beads, although the glass beads were coated with (glycidoxypropyl)trimethoxysilane to create a hydrophilic surface. Also, the pores of CPG beads are more homogeneous than the agarose beads, which have pores of widely varying diameters. As a result, the sites for antibody immobilization on both matrices are likely to exhibit different local geometries, which may affect the exposure of the immobilized antibodies to injected antigen as well as the diffusion and transport of dissociated antigen away from the antigen binding region.

The effect of antibody density on heterogeneity was also examined since it is well-known that solid supports with high local antibody density display higher apparent affinities than supports with low antibody densities.[21,22] In regions of higher antibody density, rapid reassociation of dissociated antigen is favored instead of diffusion and transport away from the surface.[13] Furthermore, it has been observed that antibodies may adsorb to surfaces in clusters and these clusters exhibit increased affinity for antigen.[22] Such antibody clusters display an increased apparent affinity,[21,23] and the antibodies within the clusters are likely to be

(21) Schramm, W.; Paek, S.-H. *Anal. Biochem.* **1992**, *205*, 47−56.
(22) Nygren, H. *J. Immunol. Methods* **1988**, *114*, 107.
(23) Werthen, M.; Nygren, H. *J. Immunol. Methods* **1988**, *115*, 71−78.

organized in a homogeneous fashion due to their lateral intermolecular interactions. While the data confirm that the threshold for crossover from homogeneous to heterogeneous is lower in displacement assays, the antibody density studies demonstrate that extent of heterogeneity is not affected by different densities since a homogeneous or only moderately heterogenous distribution of relative affinities is observed.

While variation of flow rate would not be expected to introduce heterogeneity in a displacement assay, higher flow rates limit the time for displacement of the labeled antigen by antigen in the sample. Hence if immobilization has introduced heterogeneity, altering the flow rate may alter antigen transport and shear characteristics, thus selecting a subpopulation of antibodies which can participate in the displacement reaction. A 20-fold increase in the flow rate from 0.1 to 2.0 mL/min decreased the apparent displacement constant K_{disp} of AbAg* complexes immobilized onto CPG beads by ~2 orders of magnitude but seemed to have a negligible effect on the index of heterogeneity.

In the flow displacement assay, the experimental conditions have been carefully monitored to minimize the effect of unoccupied antibody binding sites Ab. In applying the equations described here to other displacement systems, it must first be verified that the spontaneous dissociation of Ag* from the binding sites, creating unoccupied sites, does not increase the measured [Ag*] and that the binding of Ag to unoccupied antibody binding sites does not decrease the measured [Ag*]. Such analyses of the flow displacement assay were completed prior to the experiments described here.[12] Alternatively, the theory must be generalized to include the effect of unoccupied antibody sites Ab.

The data of this study demonstrate that the modified Sips distribution function, developed for displacement assays, is useful for evaluating the impact of different assay conditions on the antigen binding function of immobilized antibodies. Using the modified Sips distribution, other assays can be individually tailored in order to optimize the reproducibility and sensitivity. Our analysis provides an analytical tool with which to optimize the selection of the solid support, the geometry of the immunoassay, and the method of immobilization. This study shows that immobilization does not substantially increase the heterogeneity of monoclonal antibodies in a displacement assay.

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