Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 9890–9894, October 1992
Biochemistry

Competitive adsorption of human immunoglobulin G and albumin: Consequences for structure and reactivity of the adsorbed layer

(antigen–antibody reactivity/exchange reaction/protein denaturation)

E. LUTANIE*†, J. C. VOEGEL*‡, P. SCHAAF†§, M. FREUND¶, J. P. CAZENAVE¶, AND A. SCHMITT†

*Centre de Recherches Odontologiques, Institut National de la Santé et de la Recherche Médicale, CJF 92-04, Université Louis Pasteur 1, Place de l'Hôpital, 67000 Strasbourg, France; †Institut Charles Sadron, Centre National de la Recherche Scientifique–Université Louis Pasteur, 6, rue Boussingault, 67083 Strasbourg, France; §Ecole Européenne des Hautes Etudes des Industries Chimiques de Strasbourg 1, rue Blaise Pascal, BP296F 67008 Strasbourg Cédex, France; and ¶Centre Régional de Transfusion Sanguine, Institut National de la Santé et de la Recherche Médicale, Unité 311, 10, rue Spielmann, 67085 Strasbourg Cédex, France

Communicated by Howard Reiss, July 6, 1992 (received for review January 6, 1992)

ABSTRACT **The affinity of polyclonal anti-IgG for human IgG adsorbed on silica surfaces was investigated by two complementary techniques, scanning angle reflectometry and ^{125}I radiotracing. Special attention was paid to compare the reactivity of IgG adsorbed directly or by exchange with already adsorbed albumin. In particular it was shown that (*i*) in the first case (direct adsorption) the reaction between anti-IgG and adsorbed IgG was in the ratio 1:1 and (*ii*) in the second case (adsorption by exchange) there was no reaction.**

Most natural systems, essentially biofluids, contain different kinds of proteins mutually competing for adsorption at any exposed surface. Understanding the mechanism of competitive protein adsorption is therefore of great interest for the development of biocompatible materials. Extensive work, including adsorption experiments in which single protein solutions and solutions of protein mixtures were used (1, 2), has been carried out to obtain more insight into this phenomenon. Most investigations have been performed with three plasma proteins: (*i*) fibrinogen, due to its physiological role in hemostasis, (*ii*) albumin, due to its abundance in plasma, and (*iii*) IgG, since it is the most important among the globulins.

From competitive adsorption experiments it is usually found that the sorbent surface is initially populated by the smaller and more abundantly occurring molecules, which exhibit a faster rate of diffusion. At later stages, these adsorbed molecules must be displaced by other molecules of higher molecular weight having a stronger tendency to adsorb but with a smaller diffusion coefficient. This phenomenon, which is representative of what has been termed the "Vroman effect," has been demonstrated by Vroman and Adams (3) for fibrinogen. In studies involving fibrinogen, IgG, and albumin at long adsorption times, it is observed that the preference for the adsorbent surface decreases in this order (4, 5). Nevertheless, when comparing the adsorption affinity of proteins of different sizes, the differences in their electrical charge and conformational stability and in their hydrophobicity or that of the surface must also be taken into account. Indeed, Brash and Uniyal (6) have shown that the exchange sequence (albumin, IgG, fibrinogen, fibronectin, factor XII, high molecular weight kininogen) commonly reported on hydrophilic surfaces like glass (7) does not apply to hydrophobic polystyrene or polyethylene.

To our knowledge, no results have been reported on the relationship between competitive adsorption and antigen–antibody reactions. Competitive adsorption may, however, have implications for the use of antigen–antibody assays involving the adsorption of protein antigens from blood plasma containing other proteins. In this regard, we studied the reactivity of adsorbed IgG toward anti-IgG (IgY) molecules in three situations: IgG molecules were immobilized either alone or from a binary IgG/albumin mixture on a bare silica surface or were adsorbed from an IgG solution onto an albumin-precoated surface. Two experimental techniques were used, scanning angle reflectometry (SAR) (8), which is sensitive to the optical parameters of the adsorbed layer, and radiolabeling, which gives information on the quantities of adsorbed proteins.

MATERIALS AND METHODS

Adsorbents. Hydrophilic silica was employed in two different forms. (*i*) The hemicylindrical silica cell (Herasil from Hereaus) used for SAR measurements was cleaned overnight by treatment with sulfochromic acid for about 15 hr to remove all organic material before each experiment. Twenty rinses with deionized filtered water (Super Q, Millipore) were then performed. Without drying of the surface, the cell was filled with buffer solution and the arms of the reflectometer were positioned adequately to select an angle of incidence close to the Brewster angle.

(*ii*) Silica microbeads (Verre et Technique, Arcueil, France) had a mean diameter of 36 μm and a specific surface area of 760 cm^2/g as determined with a Coulter Counter (model TA2, Coultronics). Before use, the beads were treated with concentrated HCl and thoroughly washed in double-distilled water. They were then immersed in a concentrated sulfochromic acid solution overnight at room temperature and finally rinsed extensively with deionized filtered water (Super Q, Millipore). This treatment was repeated twice before the beads were lyophilized.

Proteins. Polyclonal IgY from hen eggs ($M_r \approx 170{,}000$), human albumin ($M_r \approx 69{,}000$), and human IgG ($M_r \approx 150{,}000$) were provided by the Centre Régional de Transfusion Sanguine (Strasbourg, France). IgY was isolated from the egg yolk of hens immunized against human IgG (9, 10) and was purified by three successive precipitations in poly(ethylene glycol) 6000 at 3.5%, 12%, and 12% (wt/vol) followed by 2% (vol/vol) chloroform extraction (11). The product was used without further purification and stored at −70°C as a 2% (wt/vol) solution. Human albumin and polyclonal human IgG were prepared from plasma by successive ethanol precipitations (12). Purified human albumin was stored at −70°C as a 17% (wt/vol) solution. IgG for intravenous injection was isolated from blood given by ≈1000 donors. The lyophilized product was reconstituted in phosphate-buffered saline (PBS) as a 5% (wt/vol) solution and stored frozen at −70°C

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Abbreviations: IgY, anti-IgG; SAR, scanning angle reflectometry.
‡To whom reprint requests should be addressed.

until needed. Proteins were further characterized by SDS/PAGE, stained with Coomassie blue, and judged to be at least 95% pure.

PBS was prepared by dissolving 10 mmol of $NaH_2PO_4 \cdot 2H_2O$ (Merck, Microselect), 100 mmol of NaCl (Prolabo, Paris), and 1 mmol of NaN_3 (Merck) per liter of deionized filtered water (Super Q, Millipore) and pH was adjusted to 7.8 with concentrated NaOH. The isoelectric pH values of the proteins were measured by isoelectric focusing. They were 4.7 for albumin, between 5.2 and 6.55 for IgY, and between 5.95 and 6.85 for IgG (13, 14).

Iodination of Proteins. The method of protein iodination (^{125}I) was adapted from the technique of McFarlane (15) in which iodine monochloride is the iodinating agent. Solution concentrations were measured by absorbance at 280 nm, using extinction coefficients of 5.3 [1% (wt/vol) solution, 1-cm path], 13.8, and 14.1 for albumin, IgG, and IgY, respectively (14, 16), and specific activities were determined by γ counting. Corrections taking into account γ-ray absorption by the glass beads were applied for the determination of protein adsorption (17).

Adsorption Procedures. *SAR.* The method has been described (8, 18). It is based on the variation of the reflection coefficient of a light wave polarized in the plane of incidence (p-wave) around the Brewster angle after adsorption. A schematic representation of the reflectometer is shown in Fig. 1. The light source of a 5-mW He–Ne laser (λ = 632.8 nm) producing almost linearly polarized light, and polarization parallel to the plane of incidence is selected by two polarizers (P1 and P2), each having an extinction coefficient of about 10^{-5}. The experimental cell consists of a hemicylindrical silica block, optically polished at the plane interface (S). A microscopic pinhole (PH) of diameter 100 μm is fixed in front of the photomultiplier (PM) and selects a well-defined reflected angle with angular precision of $\pm 0.01°$. Small rotations around the Brewster angle θ_B are obtained by means of microcontrolled rotation and translation devices (M rot. and M trans. in Fig. 1).

Once the silica surface had been equilibrated with buffer solution, the reflected intensity was recorded at various angles near θ_B. These data provided the reference signal, corresponding to an interface of the Fresnel type. A protein solution of known concentration was then injected into the cell to replace the buffer, and as adsorption proceeded at the interface the reflectivity around θ_B increased (Fig. 2). Once quasiequilibrium had been attained, the protein solution was replaced by solvent. After a constant signal was obtained, the reflected intensity was again measured as a function of

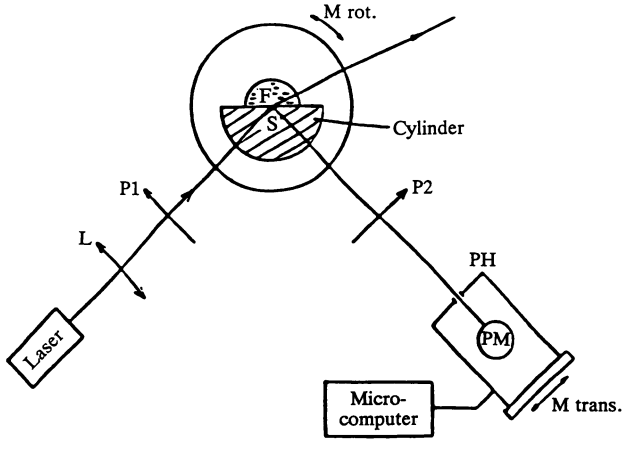

FIG. 1. Schematic experimental system. The lower hemicylinder is made of silica, with refractive index n = 1.457; F represents the thermostated liquid phase and L is a focusing lens. Other parts are described in the text.

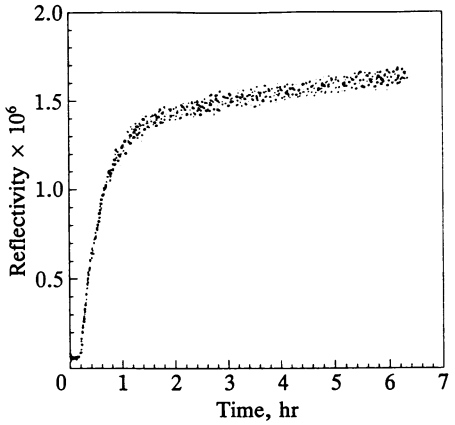

FIG. 2. Variation of the reflectivity coefficient of a p-wave at the Brewster angle as a function of IgG adsorption time for an IgG bulk solution concentration of 0.05% (wt/wt).

incidence angle to determine the mean parameters characterizing the adsorbed layer.

To enable calculation of structural information, we adopted for the interface the commonly used model of a homogeneous isotropic layer defined by a thickness (L_0) and a mean refractive index (n). Previous studies of the adsorption of fibrinogen and albumin on the same substrate have shown that the reproducibility of SAR is 15–20% for determination of L_0 and n of the adsorbed layer. The product $\Delta n \cdot L_0$ (where Δn is the refractive index difference between layer and solvent) is measured with the same precision. In addition, most of the experiments reported in this paper were repeated at least twice.

Radiotracing. The technique used in radiotracer experiments has been described (19). Briefly, protein or buffer solutions were directly added or injected with a variable-speed syringe pump (Razel, Stanford, CT) into a 24.7-cm^3 cell containing the adsorbent. To minimize extraneous protein adsorption, the inner surface of the cell was passivated prior to experiments (20). Adsorption of labeled proteins was estimated by measuring the activity of the adsorbent in a γ counter (Minimaxi γ, United Technologies, Packard Instrument).

A single or mixed protein solution of known concentration was added to the cell filled with a given quality of nonporous glass beads. Adsorption and exchange experiments were carried out with stirring at 27°C using 1–1.4 g of precisely weighted glass beads. After a given time period, stirring was stopped to allow the beads to settle (10–15 min). Three samples of 100–200 μl of supernatant were then withdrawn, the activity was determined in a γ counter, and the amount of protein adsorbed or exchanged was estimated by difference. At the final stage 400–500 mg of beads were withdrawn, immersed in PBS, rinsed three times, and dried at 50°C for 48 hr before triplicate radioactive counting (three samples of 100 mg). Measurement of the radioactivity of the supernatant solution after each rinse allowed us to verify that no significant desorption occurred during the rinsing procedure (17). This was not the case in the experiments performed by Beissinger and Leonard (21), who observed important differences between amounts of proteins taken up with or without rinsing.

In some experiments, the adsorbed layer was allowed to react with a solution of IgY. Once the previous solution had been removed from the cell, it was rinsed four times with PBS and then filled with the IgY solution. During these rinses, care was taken that the adsorption surfaces never came into contact with air but remained at all times immersed in buffer. Adsorption was carried out for a given time period, after

which the beads were withdrawn and their activity was determined as described. Some experiments were repeated, and the general precision of the radiotracer measurements in the present system (17) was about 15%.

RESULTS AND DISCUSSION

SAR Experiments. IgG adsorption from a simple IgG protein solution and from a solution of an IgG/albumin mixture was investigated using SAR by varying the contact time (t_c) between surface and solution from 1 min to a few hours. After a time t_c, when the solution was replaced by buffer, it was observed that the temporal variation of the reflectivity at the interface showed similar behavior for the 0.05% IgG (Fig. 2) and 0.05% IgG/0.05% albumin (data not shown) systems. Moreover, the corresponding reflected intensities measured around the Brewster angle at long adsorption times led to almost identical values for the two layer thicknesses ($L_{0_1} = 20$ nm and $L_{0_2} = 21$ nm) and refractive index differences between layer and solvent ($\Delta n_1 = 22 \times 10^{-3}$ and $\Delta n_2 = 24 \times 10^{-3}$). These data are to be compared to an optical thickness L_0 of 3.6 nm and a Δn value of 30×10^{-3} for a pure albumin layer on the same substrate. The corresponding amounts of adsorbed protein (Γ) were calculated by assuming the refractive index increment dn/dc to be the same as for fibrinogen (0.18 cm^3/g). We found $\Gamma_1 = 0.25$ μg/cm^2 and $\Gamma_2 = 0.28$ μg/cm^2 for the two layers, compared with about 0.06 μg/cm^2 for adsorption of albumin on the same surface (8). These results indicate that in the second case, at long adsorption times, albumin is largely replaced by γ-globulin at the interface, in agreement with the "Vroman effect" (3) reported by many authors for hydrophilic sorbent surfaces and for systems that are at least partly diffusion-controlled. This is probably the case in SAR experiments, where adsorption requires transport of protein molecules to the interface, followed by physical and/or chemical reaction at the surface (8). This point was further investigated by radiotracer experiments.

We also examined by SAR the reactivity of IgY proteins against adsorbed IgG molecules, and a very striking observation was made concerning antigen–antibody reactivity (Fig. 3). Indeed, a significant amount of IgY from a 0.01% solution bound to IgG adsorbed from a 0.05% protein solution, whereas no reaction occurred for IgG adsorbed from a 0.05% IgG/0.05% albumin mixture. In the first situation, the reflectivity measurements for the total adsorbed protein layer led to a value of 0.48 μg/cm^2. Considering the Γ_1 value relative to a simple IgG layer, we calculate that IgY molecules are adsorbed at a surface concentration of $\Gamma_{IgY} = \Gamma_{tot} - \Gamma_1 = 0.23$ μg/cm^2, which suggests that IgY and IgG molecules react quantitatively in the ratio 1:1.

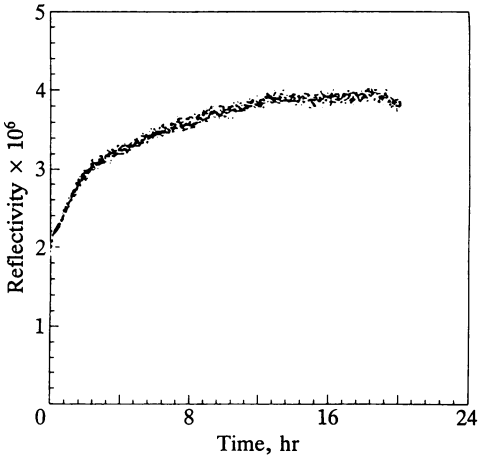

FIG. 3. Variation of the reflectivity coefficient of a p-wave at the Brewster angle as a function of IgY adsorption time for a silica/preadsorbed IgG interface (Fig. 2) in the presence of an IgY bulk solution concentration of 0.01%.

The apparently contradictory result obtained for the second adsorption situation (IgG adsorbed from an IgG/albumin mixture) indicates that the interaction between albumin and IgG molecules led to an alteration of the latter with respect to their recognition by IgY. Clearly, the main difference between adsorption from single and mixed solutions is that in the first case IgG molecules are directly adsorbed, whereas in the second case they are bound mostly by way of an exchange process. Since antibodies are known to be directed toward structural domains of macromolecules, it may be assumed that the exchange mechanism alters IgG so that the resulting molecular conformation does not allow reaction with IgY. This lack of reactivity might be explained by (*a*) some steric effect induced by structural changes during the exchange process, which could prevent the interaction of IgY with surface adsorbed IgG, or (*b*) alteration of the IgG antigenic binding sites due to conformational changes during exchange with albumin. Nevertheless, these hypotheses seem to be more applicable to monoclonal antibodies specific for only one antigenic domain than to polyclonal IgY molecules possessing multiple binding sites for antigen–antibody recognition. Monoclonal antibodies specific for a single antigenic domain would be useful to define the exact domain(s) involved in such changes (22).

Table 1. Quantities of radiolabeled albumin and IgG adsorbed for various adsorption conditions

Exp.	Protein, mg in 100 ml of bulk solution		IgG adsorption time, min	Adsorbed ^{125}I-protein, μg/cm^2	
	Albumin	IgG		Albumin	IgG
1a	0	50	180		0.110
2a	50*	50	0	0.210	
2b†	50	50*	180	0.173	0.056
2c	50	50*	360	0.167	
2d	50	50*	1170	0.121	
3a†	50	50	180	0.013	0.120
3b†	50	50	360	0.014	0.114
3c	50	50	450		0.119
3d	10	10	180		0.086
3e	50	50	60	0.013	
3f	50	50	1350	0.015	

In each experiment only one protein was radiolabeled. Experiments started always with 3 hr of albumin adsorption followed, after albumin withdrawal, by addition of IgG.
*IgG was added after adsorption of albumin onto the bare silica surface for 3 hr.
†Adsorption data obtained from two different experiments.

Radiotracer Experiments. To gain a deeper insight into the adsorption processes under study and their correlation with antigen–antibody reactions, some additional investigations were carried out using the technique of protein radiolabeling. A series of experiments was first performed to check the results obtained by SAR, which had led us to suspect the inability of exchanged IgG molecules to specifically react with their corresponding IgY antibodies. In these studies, the fact that adsorption of radiolabeled proteins is generally not limited by protein diffusion to the surface (23) had to be taken into account and appropriate adsorption conditions were therefore designed to model a diffusion-controlled adsorption process, as in the SAR investigations. Therefore, the following procedure was used for competitive adsorption experiments. The surface of bare silica microbeads was first saturated with a 0.05% albumin solution over a period of 3 hr, and then albumin was withdrawn and γ-globulin was added to the bulk solution and adsorption was continued for an additional IgG adsorption time. Finally, after withdrawal of the IgG, the reactivity of the adsorbed layer was tested by addition of a 0.01% solution of IgY.

Quantities of adsorbed IgG and albumin are reported in Table 1 for different adsorption situations, each situation being defined by unique values of protein bulk solution composition and adsorption time. Measurements of IgY bound to adsorbed IgG for the different experiments of Table 1 are presented in Table 2. Considering the molar ratio of the reacting proteins in the bulk solution (IgG/IgY ratio = 1.13) and assuming a one-to-one reaction between IgG and IgY macromolecules, the calculated value of the IgY surface concentration ($\Gamma_{IgY} = \Gamma_{IgG} \times 1.13 = 0.11\ \mu g/cm^2 \times 1.13 = 0.124\ \mu g/cm^2$) obtained for the situation 1a is very close to the experimental result (0.129 $\mu g/cm^2$). These findings are thus in total agreement with the former SAR measurements.

On the other hand, concerning antigen–antibody reactions, some apparently contradictory results were obtained in the case of IgG adsorbed on an albumin-precoated surface. Whereas no reaction was observed by reflectometry, IgY binding was radioactively detected at significant surface concentrations of 0.028 and 0.038 $\mu g/cm^2$ (Table 2, situation 2b). In view of these observations and assuming that no reaction can occur between IgY and IgG adsorbed by IgG–albumin exchange, we suggest that IgY may be immobilized on the surface by exchange with IgG or, preferably, albumin molecules within the adsorbed layer. To test this hypothesis, further experiments were carried out with radiolabeled proteins. The data obtained for albumin and IgG are represented in Figs. 4 and 5, respectively, and show that both proteins are effectively replaced by IgY, with albumin being exchanged to a larger extent.

Nevertheless, quantitative analysis of these results was required to establish whether a protein exchange process could explain the measured quantities of bound IgY. Considering the experimental values (Table 1, experiments 2a and 2b) for the amounts of displaced albumin (0.210 − 0.173 = 0.037 $\mu g/cm^2$) and bound IgG (0.056 $\mu g/cm^2$), we first estimate the number of moles of initially adsorbed albumin exchanged for one mole of IgG before the addition of IgY, which is ≈1.5. Since the molecular weights of IgG and IgY are very close, we suppose that albumin is exchanged for IgY in a similar quantitative manner. On the basis of this hypothesis, the experimental results for the quantities of labeled albumin and IgG remaining on the surface after addition of a solution of nonlabeled IgY (Figs. 4 and 5) may be used to calculate the theoretical amounts of IgY, $\Gamma_{IgY/alb}$, and $\Gamma_{IgY/IgG}$ adsorbed by exchange with albumin and γ-globulin. Taking the IgY/IgG and IgY/albumin molecular weight ratios to be 1.13 and 2.17, respectively, we calculate that at 100 min, $\Gamma_{IgY} = \Gamma_{IgY/alb} + \Gamma_{IgY/IgG} = 0.0213\ \mu g/cm^2 + 6.8 \times 10^{-3}\ \mu g/cm^2 = 0.028\ \mu g/cm^2$ with $\Gamma_{IgY/alb} = 0.01472\ \mu g/cm^2 \times 2.17/1.5$ (0.01472 $\mu g/cm^2$ being the amount of albumin displaced by IgY molecules) and $\Gamma_{IgY/IgG} = 6 \times 10^{-3}\ \mu g/cm^2 \times 1.13 = 6.8 \times 10^{-3}\ \mu g/cm^2$ (6×10^{-3} $\mu g/cm^2$ being the amount of albumin displaced by IgG molecules). Similarly, after 4 hr of reaction with IgY, the quantity of IgY adsorbed becomes

Table 2. Quantities of IgY adsorbed for various adsorption times and IgG adsorption conditions

Exp.	Adsorption time for 0.01% IgY, min	Adsorbed IgY, $\mu g/cm^2$	Technique
1a	100	0.230	SAR
	100	0.129	Rad
3a	100	≈0	SAR
	100	0.050	Rad
3e	60	0.051	Rad
2b	100	0.028	Rad
	240	0.038	Rad

Experimental albumin and IgG adsorption conditions are those defined in Table 1. Rad, radiotracer.

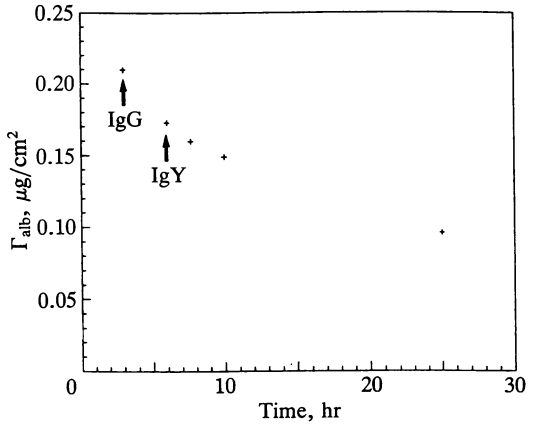

FIG. 4. Quantities of adsorbed albumin remaining on the surface as a function of time after (*a*) initial adsorption of a 0.05% solution of radiolabeled albumin from time 0 to 3 hr; (*b*) injection of a 0.05% solution of unlabeled IgG at 3 hr; and (*c*) rinsing and injection of a 0.01% solution of unlabeled IgY at 6 hr.

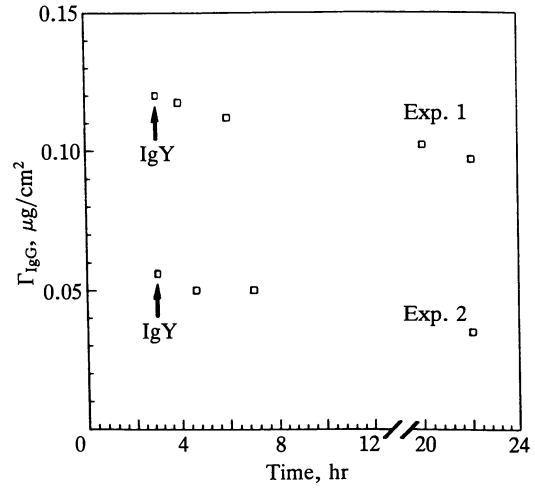

FIG. 5. Quantities of adsorbed IgG remaining on the surface as a function of time after rinsing and addition of a 0.01% solution of unlabeled IgY for two experiments: Exp. 1, IgG was mixed into a 0.05% solution of albumin and adsorbed from time 0 to 3 hr; Exp. 2, IgG was added to the albumin solution after albumin adsorption for 3 hr onto the bare silica surface and then adsorbed from time 0 to 3 hr.

0.046 $\mu g/cm^2$ (value estimated from the amount of albumin and IgG displaced, 0.027 $\mu g/cm^2$ and 6×10^{-3} $\mu g/cm^2$, respectively, by IgY after 4 hr of reaction time). As seen in Table 2 (situation 2b), good agreement is obtained between measured and predicted values. This concordance does not prove that exchanged IgG does not specifically react with IgY, but such a conclusion seems to be compatible with the overall considerations of our study.

In this perspective, it was interesting to examine the reactivity of an adsorbed layer containing some IgG molecules directly adsorbed and others fixed by exchange with albumin molecules. Additional experiments were therefore performed using an IgG/albumin mixture, where at early adsorption times both proteins interact with the surface. Results are summarized in Tables 1 (experiments 3a–3f) and 2 (experiment 3a). As expected, it is found that IgY molecules are adsorbed at a surface concentration (0.05 $\mu g/cm^2$) intermediate between those observed for an IgG solution adsorbed on bare silica (direct adsorption, 0.129 $\mu g/cm^2$) and on albumin-precoated silica (adsorption by exchange, 0.028 $\mu g/cm^2$).

Conclusions. The main conclusion emerging from the present study is that the phenomenon of protein exchange occurring during adsorption from a solution containing a protein mixture might have a substantial impact on the immunogenic properties of the adsorbed molecules, leading in the case of IgG to alteration of their antigen–antibody reactivity (24). To our knowledge such an effect has not to date been reported. Several authors have observed that adsorption of antigen or antibody molecules onto a solid surface could induce an increase or decrease of the immunoreactivity of their specific sites, but the role played by the adsorption mechanism was not considered. If adsorption of antigen molecules by exchange processes can effectively render them unreactive toward their corresponding antibodies, it is clear that the data obtained from solid-phase immunoassays should be interpreted with caution. However, a fuller understanding of the correlations between protein exchange and immunospecific reactions will require further studies.

Technical assistance of G. Maennel and S. Behr was greatly appreciated. We thank H. Zins for typing the manuscript and J. Mulvihill for critical reading and improvement of the English text. P.S. and A.S. thank the Société Biomérieux (Lyon) and Institut National de la Santé et de la Recherche Médicale for financial support (Contrat de Recherche Externe nos. 899016 and CJF 92-04).

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