Exchange Kinetics for a Heterogeneous Protein System on a Solid Surface

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The heterogeneous exchange process whereby IgG molecules adsorbed on a hydrophilic silica surface are replaced by fibrinogen molecules from the solution has been investigated by means of ^{125}I radiolabeling. Two characteristic times appear both in the desorption and replacement processes. At short times (on the order of 1 h), the process seems to correspond to an exchange mechanism which can be modeled by a first-order kinetic reaction with respect to the fibrinogen molecules. Furthermore, it also appears to be of first-order with respect to the adsorbed IgG molecules. The exchange rate constant is equal to $(3.84 \pm 0.05) \times 10^5$ L mol^{-1} h^{-1}. This generalizes previous conclusions drawn from homogeneous exchange studies both on polymer and protein systems. The long term behavior of the replacement process is characterized by a time scale which is identical to that of the desorption process. It thus seems to correspond to a desorption/readsorption process.

Introduction

Protein adsorption to solid surfaces plays a fundamental role in the field of biocompatible materials[1-3] such as blood compatible surfaces[4] or metallic surgical implants.[5] Under physiological conditions, biomaterial devices are in contact with various blood proteins such as kininogen, albumin, fibronectin, immunoglobulins, or fibrinogen.

These molecules can adsorb to such solid surfaces by two different mechanisms: either direct "adsorption" onto free available space or by a progressive exchange reaction which will be called "replacement". The first situation considers the direct contact of molecules with the bare sorbent surface, whereas in the second situation the proteins seem to come into contact with the surface through a progressive replacement of previously adsorbed molecules. These latter are then "released" from the surface. This term will be used to distinguish this process from pure "desorption" of adsorbed molecules from the surface, which is not influenced by the presence of proteins in solution. The exchange process can occur between two molecules of the same nature; this is a homogeneous exchange. However, biofluids are protein mixtures and exchange processes involving proteins of a different nature are also observed; these are called heterogeneous exchanges. Historically first observed with plasma proteins, this behavior is called the "Vroman effect".[6,7] Similar observations have also been reported for synthetic polymers.[8]

The structural properties of the adsorbed protein layer may be quite different for direct adsorption in comparison to replacement by an exchange mechanism. During the exchange process, a series of steps may occur, including interactions with already adsorbed molecules, diffusion within this initially formed layer, reptation of the incoming molecules, finally followed by annihilation of the links that the first fixed macromolecules had established with the surface. Consequently, the spatial orientation of the molecules may be quite different from that occurring with direct adsorption.

It was demonstrated experimentally that, for homopolymers of the same nature and same molecular weight, a first-order chemical reaction with respect to both the adsorbed polymers and the polymers in the solution describes the exchange reaction.[9] This result has been extended recently to the homogeneous exchange process whereby human polyclonal IgG molecules adsorbed onto latex particles were replaced by IgG molecules from the bulk solution; the value of 2.3×10^{-5} cm h^{-1} for the apparent exchange rate constant was the first given for proteins.[10] However, no results are available for heterogeneous exchange processes, and it is the aim of this paper to present experimental data devoted to this problem. We will, in particular, address the problem of the order of the exchange reaction to verify if the first-order law found previously, with respect to the molecules in the bulk, may constitute a general law or if it depends on the system.

The experiments were performed for a system in which human fibrinogen molecules (Fib) were brought in contact with human IgG molecules adsorbed onto a hydrophilic silica surface. These proteins have been selected mainly because of their predominant role in important biological reactions, such as clotting or antigen/antibody recognition, taking place at the interface between biomaterials and biofluids.

Materials and Methods

Lyophilized human polyclonal IgG (Mw ≈ 150 000), provided by the Centre Régional de Transfusion Sanguine

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(Strasbourg, France), isolated from blood given by about 1000 donors, was prepared as described in ref 11. Human fibrinogen (Fib, Mw ≈ 340 000), of grade L and coagulability >90%, was purchased from Kabi Vitrum (Stockholm, Sweden). Proteins were dissolved in a PBS buffer solution, prepared with 50 mM $Na_2HPO_4 \cdot 12H_2O$ and 0.15 M NaCl, the pH adjusted to 7.5 with 50 mM $NaH_2PO_4 \cdot 2H_2O$. In all experiments, the buffer was filtered with a Millex GV-0.22 μm filter and degassed before use. The same buffer was used for the rinsing steps throughout an experiment.

IgG and fibrinogen molecules were radiolabeled (^{125}I) using a technique adapted from the method of McFarlane[12] in which iodine monochloride is the iodinating agent. The amount of NaI was chosen so that only a small fraction of the molecules was labeled (this ratio was about 1:2300 for IgG and 1:700 for Fib molecules). The protein solutions were stored in a concentrated form (0.4−1% (w/w)) at −20 °C. Just before use, they were quickly thawed at 37 °C and diluted in PBS buffer to their final bulk concentrations. These concentrations were determined from the absorbance of the solutions at 280 nm (Beckman spectrophotometer, model 34) and by measuring the specific activities by γ counting (Minimaxi, United Technologies, Packard Instrument). All experiments were performed at room temperature (22 ± 2 °C).

The experimental apparatus used in the experiments has been described by Boumaza *et al.*[13] and consists of a silica tube of inner diameter 0.17 cm and length 21.5 cm. The tube is positioned so as to be centered in a circular NaI detector, jutting out a few centimeters from each side, the distance between the edges of the tube and the detector being regularly verified. A lead sheet surrounding the tube close to the entrance and exit of the detector allows the avoidance of edge effects in the signal shape, due to radioactivity coming from the portions of the tube or tubing situated on either side. A three-way valve enables alternate flows of solution and buffer, as well as diversion toward a waste vessel, to eliminate air bubbles and to stabilize the flux. No significative influence of iodine labeling was found for fibrinogen by adsorbing solutions of constant total concentration (45 ± 1 mg %) and by varying the relative labeled fractions (100, 75, and 51% of solution containing labeled molecules). We found a straight line which statistically intercepts the origin, indicating that the labeled species adsorb like the unlabeled ones. Similar observation was made previously for the same molecule adsorbing onto polyelectrolyte complexes.[14] We assume that labeled IgG molecules behave like fibrinogen during their adsorption onto the silica tube walls.

Protein or buffer solutions are injected at flow rates of about 2.5 or 5.0 mL h^{-1}. The corresponding interfacial shear rates (γ) are calculated using the Poiseuille equation (eq 1):

$$\gamma = \frac{4J}{\pi R^3} \qquad (1)$$

where J is the flow rate and R the radius of the tube. This gives γ values of 1.45 and 2.90 s^{-1}, respectively.

As discussed previously, the aim of our work was to study the kinetics of the exchange process between Fib and IgG, the IgG molecules being adsorbed first. An important parallel study is the determination of the ratio between the number of arriving Fib molecules and the number of leaving IgG molecules. Two sets of experiments were performed for this purpose, in which either Fib molecules were labeled, with IgG injected as an unlabeled solution, or the contrary. (Note: labeled molecules will be denoted with the superscript * throughout the text.) Before each experiment, the silica tube and the connected Teflon tubing were cleaned by leaving them filled with a 2% solution of a laboratory-use detergent (Hellmanex II, Helma, GmbH D-7840 Mullheim) overnight. After extensive rinsing with bidistilled water (MilliQ, Millipore), they were filled with a 10% sulfuric acid solution for about 5 min, followed by a rinse with water and PBS buffer solution.

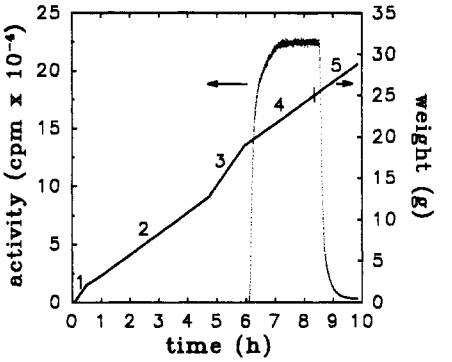

Figure 1. Dotted line: Evolution of the detected radioactivity (cpm × 10^{-4}) versus time in a typical IgG/Fib* exchange experiment. Solid line: Weight (g) of solutions injected versus time. The flow rates (fr) are calculated from the slope of these straight lines. Step 1, PBS buffer injection (fr: 5.0 mL h^{-1}); step 2, unlabeled IgG injection (fr: 2.5 mL h^{-1}, during 253 min, bulk concentration: 10.6 mg %); step 3, replacement of the solution by pure PBS buffer (fr: 5.0 mL h^{-1}, during 74 min); step 4, labeled Fib injection at bulk concentrations varying between 0 and 82 mg % (fr: 2.5 mL h^{-1}, during 143 min); step 5, PBS rinse of the Fib solution (fr: 2.5 mL h^{-1}, during 91 min).

An isotherm of IgG surface concentration as a function of bulk concentration was performed in order to determine a concentration range in which the IgG molecules cover the surface. Each series of experiments consisted of first adsorbing IgG molecules on the bare silica surface. A typical IgG/Fib* experiment starts (step 1, Figure 1) with injection of PBS buffer into the tube. The only signal is background noise (too low to appear on Figure 1), which must be taken into account in the further estimation of the adsorbed quantities. This step is followed (step 2, Figure 1) by injection of unlabeled IgG, at a flow rate of 2.5 mL h^{-1} during 253 min. The choice of the bulk concentration (≈10 mg %) will be discussed later. In step 3, the solution is replaced by buffer (rinse of 74 min at a flow rate of 5.0 mL h^{-1}). ^{125}I-Labeled Fib is then injected (step 4) at various concentrations (from 0 to 82 mg % (w/w)), during 143 min at a flow rate of 2.5 mL h^{-1}. The signal in Figure 1 was obtained with a Fib* solution of bulk concentration 41.1 mg %. We see that the radioactivity remains approximately constant at the end of step 4, indicating that the system has reached steady state. This was not the case for the lower Fib* solution concentrations, but since in the system IgG*/Fib we will compare the quantities of IgG* released in the same interval of time, it is here not of great importance to attain a stationary state (which would take a very long time at these low concentrations). The experiment is completed by a PBS rinse of the Fib* solution (step 5, Figure 1), at a flow rate of 2.5 mL h^{-1} during 91 min. The solutions injected are collected in a container placed on an electronic

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balance, allowing a precise continuous recording of the weight (and therefore volume) as a function of time. The flow rates are calculated from the slope of the resulting lines.

The increase in activity at the end of this step (which will be called a), in comparison with the background noise (given in steps 1–3 in the case of the initial injection of unlabeled IgG), is directly related to the amount of adsorbed Fib* molecules. The method we used to calculate the adsorbed labeled protein amounts was described earlier.[13] For each adsorption experiment, one measures the difference of the signal between step 4 (just before the rinsing step) and step 5 (at the end of the PBS rinse). This difference (ΔA) corresponds approximately to the radioactivity due to the amount of protein present in the bulk solution. The interfacial protein concentration (Γ) is then given by the relation:

$$\Gamma = 0.5RC_{bulk}\,(\Delta a/\Delta A) \qquad (2)$$

where R is the tube radius (R = 0.085 cm) and C_{bulk} (μg cm^{-3}) the protein solution concentration determined by absorbance at 280 nm and γ counting.

This method has the disadvantage that it could be affected by the desorption of any weakly bound protein layer during the rinsing step, leading to an underestimation of the value of the fixed protein amount which is proportional to Δa. On the other hand, the method presents the advantage that calibration is made for each individual experiment, allowing for intrinsic apparatus electronic variations. The precision on Γ can be estimated from the variation of Γ_{IgG^*} values found in our experiments. We obtained a mean value of Γ_{IgG^*} (at a solution concentration of 10.0 mg %) of (0.50 ± 0.06) μg cm^{-2}, with a relative error thus equal to about 10%.

In order to analyze the corresponding quantities of IgG molecules released during the exchange process with the Fib molecules, we performed a series of experiments in which IgG molecules were labeled, whereas Fib was injected as a cold solution (the concentrations varying from 25 to 62 mg %), with exactly the same time and flow rate conditions as in the IgG/Fib* experiments. The amount of departing IgG* was evaluated by taking the difference in the signal between the end of step 3 and the end of step 5 (as given in Figure 1, except that here the experiment is performed with labeled IgG molecules).

In another series of experiments, the quantitative (kinetic) analysis was performed on the IgG* leaving the surface by desorption or exchange with Fib molecules (over longer time periods than those used above). The kinetic analysis could not be done for experiments during which Fib* molecules replace adsorbed IgG molecules on the surface: the determination of kinetic constants in IgG/Fib* experiments would have required the knowledge of the precise law describing the filling of the tube with a labeled Fib solution, without any adsorption of the protein onto the surface, in order to subtract this contribution from the combined signal of Fib* adsorbed layer and bulk fluid. Moreover, since the signal due to the adsorbed Fib* is very small compared to the signal of the Fib* molecules in the bulk of the tube, it would be very difficult to extract the contribution of the surface radioactivity from the total signal.

Figure 2a represents the typical signal of such an IgG*/Fib desorption/replacement experiment. Again, the same sequence of injections as in an IgG/Fib* experiment was performed, except that, at the end of the experiment, there was no rinsing step with buffer (no step 5, Figure 1): the cold Fib solution injection was carried out during 237 min (at bulk concentrations varying between 0 and ≈100 mg %), during which the kinetics of the IgG* desorption/replacement process was studied (step 4, Figure 2a). For two experiments, Fib injection was pursued for about 3 days, in order to analyze the kinetics of either desorption or replacement over a long period. For these experiments one could attribute the decrease of the surface radioactivity either to a release of covalently bound iodine atoms from the adsorbed proteins, or to a release of iodine containing parts of the molecules due to an eventual hydrolysis. In order to quantify these effects, 20 mL of a labeled IgG solution (at a concentration of 14.8 mg %) were dialyzed against PBS buffer, using a dialysis membrane with molecular weight cutoff of 3500 g mol^{-1} (Spectrapore membrane tubing, Spectrum Medical Industries, Inc., Los Angeles, CA). By measuring the increase of radioactivity as a function of time in the outer medium, we determined that about 5% of the initial present radioactivity was lost after 72 h. (independent of the natural decrease of radioactivity), a value close to what was previously found for human albumin labeled via the same technique.[15] Thus, this could not affect the accuracy of our results.

For all experiments, we chose to integrate the signal of radioactivity over 10 s per channel on the multichannel analyzer. This time corresponded to a good compromise between an acceptable kinetic time resolution and a sufficient signal "height" (in comparison with the back-

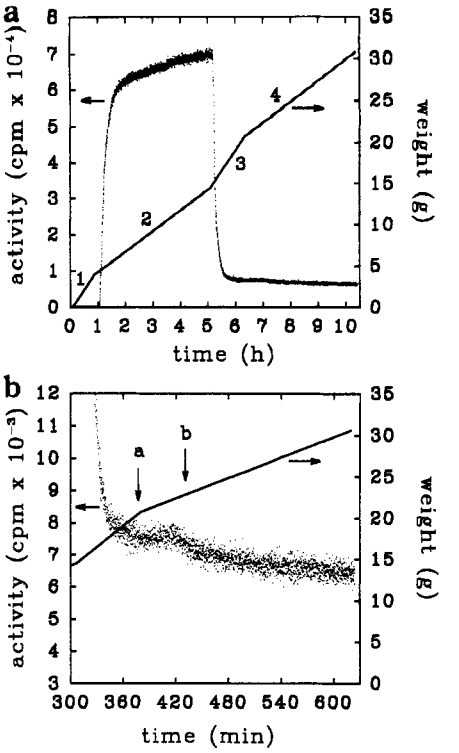

Figure 2. (a) Dotted Line: Evolution of the detected radioactivity (cpm × 10^{-4}) versus time in a typical IgG*/Fib exchange experiment (here, expt 4 (see Table 1), where C_{bulk}Fib = 26.4 mg %). Solid line: Weight (g) of solutions injected versus time. Step 1, PBS buffer injection (fr: 5.0 mL h^{-1}); step 2, labeled IgG injection (fr: 2.5 mL h^{-1}, during 253 min, bulk concentration: 10.0 mg %); step 3, replacement of the solution by pure PBS buffer (fr: 5.0 mL h^{-1}, during 74 min); step 4, unlabeled Fib injection at bulk concentrations varying between 0 and 100 mg % (fr: 2.5 mL h^{-1}, during 237 min or longer). The kinetics of the desorption/replacement process of the IgG* molecules is studied in step 4. (b) Enlargement of the final steps of the experiment of part a, in the time interval 300–625 min. The two arrows indicate (a) the start of step 4 (injection of the Fib solution, here at 26.4 mg %) and (b) the time (t_0) chosen to begin the kinetic analysis: 50 min after the beginning of Fib injection (see the text for explanations). Notice the slight increase of the signal between the two arrows.

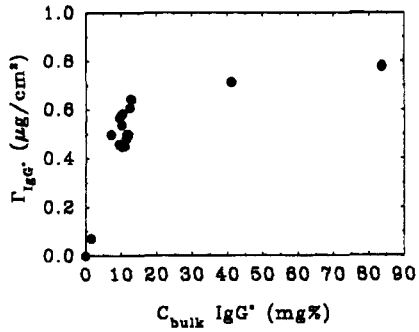

Figure 3. Adsorption isotherm of human IgG onto silica at a flow rate of 2.4 mL h^{-1} during 253 min (followed by a PBS rinse of 74 min at 4.8 mL h^{-1}). Each point represents an independent experiment.

ground noise), depending on the specific activity of the labeled protein.

Figure 2b shows an enlargement of the signal of Figure 2a after the addition of Fib. A complication was encountered linked to the phenomenon of interest. The alternate injection with two syringe pumps required the flow of the cold Fib solution through the Teflon tubing that was previously used for the injection of the labeled IgG molecules. Even after extensive rinsing of this part of the tubing with PBS buffer toward the waste vessel, IgG* molecules remain adsorbed on the walls. However, they desorb by exchange with the Fib molecules. The injected Fib solution thus carries these IgG* molecules along toward the silica tube, leading to an enhancement of the signal when starting step 4 (between arrows a and b, Figure 2b). This overshoot interferes with the beginning of the decrease in the signal due to the desorption/exchange mechanism. We could reduce this artifact by cleaning the tubing upstream from the three-way valve (during step 3, Figure 2a) with 2% Hellmanex and 10% H_2SO_4 solutions and by systematically letting the cold Fib solution flow toward the waste vessel during 20 min at 2.5 mL h^{-1} before injecting it into the tube. But this effect could not be avoided between the valve and the tube, this part being of course inaccessible to cleaning during injection of the buffer in step 3. For all desorption/exchange experiments, the kinetic analysis was therefore begun at time $t_0 = 50$ min (arrow b) after the beginning of Fib injection (arrow a, Figure 2b), in order to bypass this side effect to the investigated phenomenon. Note that the time necessary for the Fib molecules to attain the surface at the center of the tube by a diffusion/convection process[16] is about 30 min after the injection of the solution. This time $t_0 = 50$ min is thus sufficient for the replacement process to start.

Results and Discussion

IgG Isotherm. Figure 3 shows the isotherm of human IgG performed under the same conditions as the exchange experiments, i.e. adsorption at a flow rate of 2.4 mL h^{-1} during 252 min, followed by a PBS rinse of 74 min at 4.8 mL h^{-1}. The plateau was reached for a value of the surface concentration of 0.78 μg cm^{-2}. This corresponds to a coverage of 47% for an "end-on" and 250% for a "side-on" configuration, assuming ellipsoid dimensions of 23.5 × 4.4 × 4.4 nm^3 for the IgG molecule.[17] One can thus assume that the surface is totally covered by IgG molecules and that a large fraction of these molecules is adsorbed in an "end-on" configuration. The value of Γ corresponding to a surface covered by IgG molecules all adsorbed in a "side-on" configuration on the surface would be on the order of 0.32 μg cm^{-2}. The corresponding C_{bulk} value extrapolated from the isotherm is on the order of 3 mg %, indicating that a very low IgG concentration might be sufficient to attain total surface coverage under our experimental conditions, in the case of an optimization of the free space on the surface by molecules that would adopt a "side-on" orientation. However it cannot be excluded that the surface is not fully covered under such conditions.

We analyzed more precisely the kinetics of adsorption in an experiment in which the IgG* molecules were injected at 7.3 mg %: the signal increased linearly after the tube was filled. The slope (S_{exp}) of this line could thus be determined and the experimental rate constant of adsorption, k_{exp}, calculated by the relation (eq 3)[13]

$$k_{exp} = C_{bulk}^{-1}\left(\frac{d\Gamma}{dt}\right)_{exp} = \left(\frac{0.5R}{\Delta A}\right)S_{exp} \qquad (3)$$

We obtained for k_{exp} a value of 5.9×10^{-7} cm s^{-1}. This value may be compared to the Lévêque constant, k_{Lev},[18] which considers the extreme case of the kinetic process determined entirely by diffusion of the molecules to the interface:[13]

$$k_{Lev} = C_{bulk}^{-1}\left(\frac{d\Gamma}{dt}\right)_{Lev} = 0.54D^{2/3}\gamma^{1/3}z^{-1/3} \qquad (4)$$

D represents the diffusion coefficient, equal to 4×10^{-7} $cm^2 \cdot s^{-1}$ for the IgG molecule, γ is the shear rate at the wall of the tube, equal to 1.43 s^{-1} at the flow rate of this experiment (2.48 mL h^{-1}), and z is the distance to the tube entrance, equal to 10.75 cm at the center of the detector. We found $k_{Lev} = 1.5 \times 10^{-5}$ cm s^{-1}, which is about 25 times higher than the k_{exp} value. We can thus conclude that, at this shear rate, the process is not controlled by transport to the interface, but is surface controlled.

These bulk concentrations (≈10 mg %) therefore probably correspond to complete coverage of the surface, perhaps in a mixture of "side-on" and "end-on" configurations, even though the maximum coverage is not reached. Thus, direct adsorption of the Fib molecules should not occur at the very beginning of the kinetic analysis in desorption/replacement experiments. Hence, we chose to adsorb the IgG molecules at a bulk concentration of about 10 mg % (Figure 3), i.e. at 0.5 μg cm^{-2}.

For comparison, a similar kinetic approach allowed us, by evaluating the slope of the signal during the injection of labeled Fib, to calculate an approximate value of k_{exp} for adsorption of Fib* molecules on an IgG layer (IgG/Fib* experiments). With the help of eqs 3 and 4, we found $k_{Lev} = 9.4 \times 10^{-6}$ cm s^{-1} (the diffusion coefficient of fibrinogen being equal to 2×10^{-7} $cm^2 \cdot s^{-1}$). This value was between 30 and 200 times higher than the k_{exp} values, for Fib* solutions of concentrations varying between 10 and 60 mg %, respectively. Again, the process is controlled by the reaction taking place at the interface.

Desorption and Release of Adsorbed Molecules from the Surface. We first investigated the dynamical behavior of the system under study in the long time range. Thus, the kinetics of desorption of IgG* molecules was analyzed over a period of 3 days as PBS buffer flowed through the silica tube (experiment 1, Table 1). One can

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Table 1. Experimental Data Obtained from IgG*/Fib Desorption/Release Experiments[a]

expt	Γ_{IgG}* (pmol cm^{-2})	C_{bulk}Fib (mg %)	K_1 (min^{-1})	K_2 (min^{-1})	A (pmol cm^{-2})	B (pmol cm^{-2})	Γ_∞* (pmol cm^{-2})
1	3.1	0	0.006 585	0.000 375	0.19	0.92	1.9
2	3.2	0	0.006 702	*b*	0.16	0.90	2.1
3	3.0	10.0	0.011 447	*b*	0.30	0.64	2.1
4	3.0	26.4	0.012 710	*b*	0.31	0.74	1.9
5	3.0	28	0.010 040	0.000 377	0.43	0.72	1.9
6	3.0	51.0	0.017 658	*b*	0.28	0.83	1.9
7	3.3	76.3	0.019 611	*b*	0.38	1.2	1.7
8	3.3	101.5	0.027 338	*b*	0.37	1.9	1.1

[a] K_1 and K_2: desorption/release rate constants obtained by adjusting the signal with the function $A\exp(-K_1t) + B\exp(-K_2t) + \Gamma_\infty$* (eq 5). Γ_{IgG}* is the adsorbed IgG* amount at t_0. The parameter values A, B, and $\Gamma\infty$* were obtained in cpm and then converted using eq 2. *b* Assumed to be 0.000376 min^{-1}.

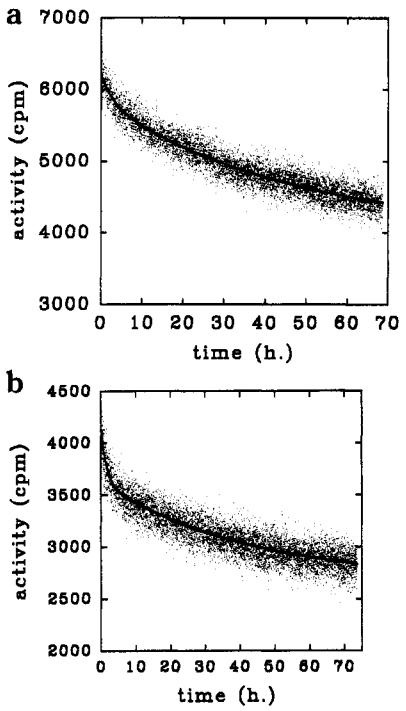

Figure 4. (a) Experiment 1 (Table 1): kinetics of desorption of IgG* molecules. Pure PBS buffer flowed through the silica tube over a period of 3 days (step 4, Figure 2a). The experimental data were fitted with the help of eq 5. (b) Experiment 5 (Table 1): kinetics of desorption/replacement of IgG* molecules. The unlabeled Fib solution (C_{bulk} 28 mg %) flowed through the silica tube over a period of 3 days. At $t = t_0 + 65.5$ h (end of continuous line), injection was followed by pure PBS buffer (same flow rate) during 8 h, without apparently affecting the process evolution. Solid line: data were fitted with eq 5 in the interval of time $t_0 + 65.5$ h. For each part, values for K_1 and K_2 parameters are given in Table 2. Only one in 10 points is represented, but all points are used for the fits. The represented activity is corrected by taking into account the natural radioactive decrease of ^{125}I.

observe (Figure 4a) that the process is rapid at the beginning of the signal, becoming much slower after a few hours.

As a first trial, we fit the experimental data by a simple decreasing exponential function: $\Gamma^* = A\exp(-kt) + \Gamma_\infty^*$ (Γ^* being the crude signal), but this function fit the data very poorly at short times; one characteristic time is not sufficient to describe the process. On the other hand, the whole signal could be correctly fit (Figure 4a, continuous line) with a double exponential function of the form (eq 5)

$$\Gamma^* = A\exp(-K_1t) + B\exp(-K_2t) + \Gamma_\infty^* \qquad (5)$$

where A and B are two constants to which we can give the dimension of a surface concentration (μg or pmol/cm^2), K_1 and K_2 are two characteristic rate constants (which correspond to the inverse of two characteristic times), and Γ_∞* is the signal which would be observed at infinite time (t_∞). Parameter values are collected in Table 2. We see that K_1 is about 18 times greater than K_2, revealing again two distinct regimes.

Table 2. Values of the Parameters Corresponding to the Adjustment of the Data of Experiments 1 and 5 with Equation 6

parameter	expt 1	expt 5
A (cpm)	371	534
B (cpm)	1824	960
K_1 (min^{-1})	0.006585	0.010040
K_2 (min^{-1})	0.000375	0.000377
Γ_∞* (cpm)	4035	2653

The same effect may be seen in the kinetics of the departure of IgG* molecules in the presence of Fib molecules over long times (expt 5, Table 1): instead of PBS buffer, the unlabeled Fib solution at a mean C_{bulk} of 28 ± 2 mg % flowed through the silica tube. Every 16 h, corresponding to a volume of 40 mL injected, a Fib solution was freshly prepared and injected without interruption. At $t = t_0 + 65.5$ h, the tube was rinsed with pure PBS buffer during 8 h (at the same flow rate as for Fib). This caused no apparent change in the behavior of the signal. Again, a fast and a slow release mechanism of IgG* molecules could be distinguished (Figure 4b). As in expt 1, the data were fitted with eq 5 (Figure 4b, continuous line); parameter values are given in Table 2. K_1 is here about 27 times larger than K_2. Hence, we can conclude that in the presence of Fib molecules, there are also at least two desorption processes with distinct characteristic times. Moreover, as appears in Table 2, the long time rate constant K_2 is independent (mean value of 0.000 376 min^{-1}, which corresponds to a characteristic time τ_2 on the order of 44 h) of the presence or absence of Fib molecules. This reveals that at long times, the release of IgG molecules is independent of the presence of Fib molecules in solution and thus corresponds to a pure desorption process. On the other hand, the process related to the short characteristic time is influenced by the presence of fibrinogen molecules in the solution. In particular, this process becomes more rapid in the presence of these molecules.

These results seem to indicate that there are three population types for adsorbed IgG molecules on the surface: (i) a population that is irreversibly fixed on the surface and that neither desorbs nor exchanges; (ii) a population that is strongly adsorbed on the surface but which can be desorbed or released from it through a slow desorption process the kinetics of which seems uninfluenced by the presence of proteins in the solution, denoted as population type 2; and (iii) a population that is loosely attached to the surface, and thus easily desorbable or exchangeable, called type 1. The desorption or release

process of these proteins from the surface is charaterized by a rate constant, $K_1(C_{Fib})$, which is a function of the bulk protein concentration. It is this process that we will now examine. In addition, the fact that the whole release process can be described by eq 5 seems to indicate that these three kinds of populations are each independent of each other. Furthermore, the release/desorption process can be modeled by a first-order kinetic reaction with respect to the IgG* molecules on the surface, since the form of the integrated rate equation is a double exponential: this means that the process is governed by two regimes, each one being of order one with respect to the adsorbed molecules. Consequently, the mechanism of exchange here may imply the formation of a bimolecular transition Fib/IgG complex on the surface.

This model is certainly an oversimplified situation and it is expected that the release process is not governed by only two time constants but by a continuous spectrum of time constants. However, we do not have access to this spectrum. The two time constant equation (eq 5), the simplest found to be satisfactory within the precision of our data, will thus be used to analyze all of our experiments.

To investigate the processes governed by the rapid time scale K_1^{-1}, we concentrated our attention on the behavior of the desorption/release process over a period of about 190 min starting from t_0. The data were fitted using eq 5, imposing for K_2 the value 0.000376 min^{-1} found previously. The values of the rate constant K_1 determined at various bulk concentrations of fibrinogen are reported in Table 1 and plotted in Figure 5. The corresponding relaxation times $\tau_1 = K_1^{-1}$ lies within the interval 30−150 min for the range of Fib concentrations investigated. The straight line corresponds to a least-squares fit; the 95% confidence interval is given by the two dotted lines: this demonstrates that K_1 varies linearly with the Fib solution concentration.

The linear dependence of K_1 with C_{Fib} and the finite value of K_1 for $C_{Fib} = 0$ indicate that at short times the type 1 release from the surface of IgG molecules in the presence of Fib molecules in the bulk takes place by two different mechanisms: (i) a diminution of the adsorbed amount of IgG molecules on the surface independent of the presence of Fib molecules in the bulk (This process can thus be considered to be desorption. The rate constant of this process is found to be on the order of 0.007 min^{-1}.) and (ii) a release of the molecules from the surface influenced by the presence of Fib molecules in the bulk. In particular, the release flux is directly proportional to the concentration C_{Fib}. It is thus expected that this process corresponds to an exchange mechanism which would follow a first-order kinetic reaction with respect to the fibrinogen molecules in the bulk. Such a conclusion has already been obtained for the homogeneous exchange processes of proteins and polymers. The exchange rate constant k_e can be estimated from the slope of the line in Figure 5 and is on the order of $(384 \pm 5) \times 10^3$ L mol^{-1} h^{-1} (0.000 188 (mg %)$^{-1}$ min^{-1}) for an initial Γ_{IgG^*} value of 0.50 μg cm^{-2}. This value should be compared to $(5.8 \pm 1.0) \times 10^3$ L $mol^{-1}h^{-1}$ found for the homogeneous exchange process between IgG molecules adsorbed on latex particles and IgG molecules from the bulk solution.

The parameters A, B, and Γ_∞* could only be estimated approximately in the moderate-time experiments. Hence, in order to refine the values of these parameters, we refitted the signals of experiments 1−8 by imposing not only K_2 but also the values of K_1 as determined from the line of Figure 5. The results are given in Table 1 but the precision of these preexponential parameters is still limited. We will thus not discuss their evolution with

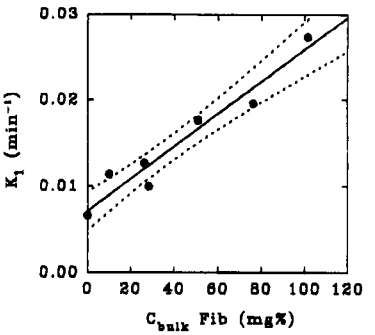

Figure 5. Variation of K_1 versus C_{bulk}Fib. Solid curve: least-squares fit to a straight line. Dashed curves: confidence interval of 95%.

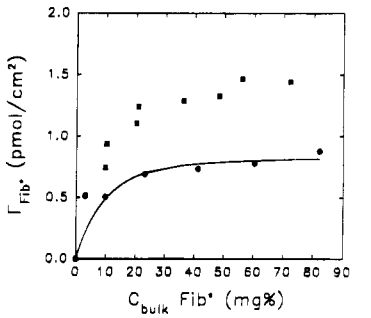

Figure 6. Adsorption isotherm of human Fib* onto silica at a flow rate of 2.4 mL h^{-1} during 207 min (followed by a PBS rinse of 78 min at 4.8 mL h^{-1}). Amounts of Fib* molecules adsorbed on a cold IgG layer. The experimental procedure is that described in Figure 1. Data were adjusted by eq 6. Parameter values are $A' = 0.445$ pmol cm^{-2}, $k_F = 0.000\,935$ (mg %)$^{-1}$ min^{-1}. In both curves, each point results from an independent experiment.

C_{Fib}. However, it must be pointed out that even if these preexponential parameters are not defined precisely, the value of K_1 is reliable because it depends on the slope and curvature of the signal with time, and these are well-defined (see Figures 2b and 4a,b).

In order to verify that the release process of IgG molecules of type 1 influenced by the presence of Fib molecules in the bulk is indeed due to an exchange process, it is necessary to verify that while the IgG molecules leave the surface, Fib molecules adsorb on the surface.

Adsorption of Fib and Replacement of IgG Molecules. As has already been pointed out, our experimental setup did not allow us to follow the adsorption/replacement kinetics of fibrinogen, due to the small contribution of the surface compared to the bulk activity. The Fib* adsorption experiments were thus conducted by keeping the contact time between the solution and the surface constant and equal to 143 min, Fib* injection being followed by a rinse of the tube by pure buffer. The quantity of fibrinogen remaining on the surface was then determined. Figure 6 represents the quantity of Fib* adsorbed on the surface previously in contact with an IgG solution (lower curve), as a function of the bulk concentration. The adsorption isotherm of Fib* onto the bare silica surface, obtained under similar experimental conditions, is also represented for comparison (upper curve). We thus deduce that, indeed, Fib molecules adsorb or replace IgG molecules on the surface. In order to determine the ratio between arriving Fib molecules and departing IgG molecules (F/I), we performed a series of experiments in which we determined the amount of IgG* molecules released from the surface by exchange or desorption, under exactly the same conditions as in the experiments realized with labeled Fib. Data are given in Table 3. The determination

Table 3. Ratio of Arriving Fib* on Leaving IgG* Molecules (F/I)

C_{bulk}Fib[a] (mg %)	Γ_{Fib}* (pmol cm^{-2})	$\Delta\Gamma_{IgG}$* (pmol cm^{-2})	F/I
25.0	0.66	0.60	1.1
47.9	0.76	0.57	1.3
53.3	0.78	0.85	0.92
61.7	0.81	0.58	1.4

[a] Fib molecules are labeled or not (see the text for experimental procedure). F/I = $\Gamma_{Fib*}/\Delta\Gamma_{IgG*}$.

of this F/I ratio requires the knowledge of the amount of Fib* adsorbed for each C_{Fib} reported in Table 3. This was obtained approximately by fitting the lower curve of Figure 6 by the help of a fitting function (eq 6) obtained by several assumptions developed in the Appendix. Γ_{Fib}, the number of protein molecules fixed per unit area, was thus described by:

$$\Gamma_{Fib} = \frac{(k_{d1}/(1 - K_1/K) + k_e C_{Fib})A'}{K_1}(1 - e^{-K_1 t}) + \frac{B'}{1 - K_2/K}(1 - e^{-K_2 t}) - \left(\frac{k_{d1}A'}{K - K_1} + \frac{K_2 B'}{K - K_2}\right)(1 - e^{-Kt}) \quad (6)$$

where $K = k_F C_{Fib}$. The fitting parameters are A' and k_F, the ratio A'/B' being held constant and equal to $^1/_3$, as was observed in IgG*/Fib experiments (Table 1). All other parameters retain their previous meaning. A' and B' correspond to the number (A') of Fib molecules that are fixed to the surface by replacement of an IgG molecule by exchange or the number (B') adsorbing on a surface left free by desorption of an IgG molecule, through processes involving IgG molecules of respectively types 1 and 2. k_F represents the adsorption rate constant on the free available surface. The proposed model is far from being unique in fitting the experimental observations. The proposed function (eq 6) must thus essentially be considered as a simple fitting function, based on a model that is compatible with our findings. The best fit was obtained with the following values: $A' = 0.445$ pmol cm^{-2} and $k_F = 0.000935$ (mg %)$^{-1}$ min^{-1}. The constant k_F value is thus found to be about 5 times k_e, which seems plausible. Indeed, Fib molecules should adsorb more rapidly on sufficiently large regions of free available surface than by exchange with already anchored IgG molecules. By comparison with the mean value of A (see Table 1), one finds from the value of A' (and thus also B') that the ratio between the number of Fib molecules that adsorb on the surface and the number of IgG molecules leaving the surface is of about 1.3.

If one calculates this ratio by comparing directly the number of Fib molecules that reach the surface, estimated from expression (6), and the number of IgG molecules that have left the surface during the same time, one finds a ratio on the order of 1.2 (Table 3). These results make the hypothesis of an exchange process on the surface highly probable, even if it could not be strictly proven with our experimental approach.

Conclusion

In the present work, we have focused our attention on the study of the protein exchange mechanism for the heterogeneous IgG/fibrinogen system, under flow and on a hydrophilic silica surface. According to the presented model, three important conclusions could be drawn: (i) Exchange occurs rapidly in the first few hours of the process. At long times, this is followed by a slow desorption mechanism independent of the presence of the molecules in solution, which seems thus to correspond to a desorption/adsorption mechanism. A rapid and a slow regime are also observed in the pure desorption signal. (ii) The order of the exchange reaction with respect to the Fib molecules in solution is one. (iii) The order of the exchange reaction with respect to the IgG adsorbed molecules also seems to be one, implying the formation of a bimolecular transition complex on the surface. The value of k_e, the exchange rate constant, could be estimated at $(3.84 \pm 0.05) \times 10^5$ L mol^{-1} h^{-1}. This value is much higher than for the IgG/IgG homogeneous exchange.[10] This result was completed by the analysis at a given time of the ratio F/I between arriving Fib and leaving IgG molecules, which was found to be on the order of 1.

Appendix

The evolution of the amount of fibrinogen molecules that adsorb on the surface or exchange IgG adsorbed molecules can be described by the following model: as we have seen, three kinds of adsorbed IgG molecules seem to be present on the surface. The molecules of type 1 can either desorb from the surface with a rate constant k_{d1} (on the order of 0.007 min^{-1}) or be released from it through an exchange process which is first-order with respect to the Fib molecules in solution and to the adsorbed IgG molecules. The exchange rate constant is k_e (0.000188 (mg %)$^{-1}$ min^{-1}). The evolution of the amount of IgG molecules of type 1 on the surface is thus given by

$$\frac{d\Gamma_{IgG}^{(1)}}{dt} = -k_e C_{Fib}\Gamma_{IgG}^{(1)} - k_{d1}\Gamma_{IgG}^{(1)} = -K_1\Gamma_{IgG}^{(1)} \quad (A1)$$

The molecules of type 2 can only desorb from the surface, with a rate constant equal to K_2, so that the amount of molecules of type 2, $\Gamma_{IgG}^{(1)}$ follows the equation

$$\frac{d\Gamma_{IgG}^{(2)}}{dt} = -K_2\Gamma_{IgG}^{(2)} \quad (A2)$$

We assume that each time an IgG molecule desorbs from the surface, whatever the type of IgG molecule it is, it leaves a "hole" on the surface. These holes can then be filled with fibrinogen molecules through a readsorption process. This adsorption rate for fibrinogen is taken equal to k_F. Let Γ_{Fib} and Γ_h represent the number of fibrinogen molecules bound to the surface and the number of holes per unit area, respectively. These quantities follow the equations

$$\frac{d\Gamma_h}{dt} = k_{d1}\Gamma_{IgG}^{(1)} + K_2\Gamma_{IgG}^{(2)} - k_F C_{Fib}\Gamma_h \quad (A3)$$

and

$$\frac{d\Gamma_{Fib}}{dt} = k_F C_{Fib}\Gamma_h + k_e C_{Fib}\Gamma_{IgG}^{(1)} \quad (A4)$$

This model assumes that the IgG/Fib replacement process does not affect the size of the holes. To determine the boundary conditions we assume that at time $t = 0$, Γ_{Fib} and Γ_h are both equal to zero. One then finds for Γ_{Fib} the following relation with C_{Fib}:

$$\Gamma_{Fib} = \frac{k_F C_{Fib}\alpha + k_e C_{Fib}A'}{K_1}(1 - e^{-K_1 t}) + \frac{k_F C_{Fib}\beta}{K_2}(1 - e^{-K_2 t}) - (\alpha + \beta)(1 - e^{-k_F C_{Fib} t}) \quad (A5)$$

with α and β given by

$$\alpha = \frac{k_{d1}A'}{(k_F - k_e)C_{Fib} - k_{d1}} \tag{A6}$$

$$\beta = \frac{K_2B'}{k_FC_{Fib} - K_2} \tag{A7}$$

Relation (A5) was used as the fitting function to account for the lower curve of Figure 6. A' and B' correspond to the initial amounts of IgG molecules of types 1 and 2.

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