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Research Paper

Immunoadsorption of Alloantibodies onto Erythroid Membrane Antigens Encapsulated into Polymeric Microparticles

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Purpose. Classical immunoadsorbents used for the removal of deleterious molecules in blood such as auto-antibodies are prepared by covalent coupling of antigens onto previously chemically activated supports. Such a chemical treatment may induce a potential toxicity which can be reduced if new immunoadsorbents are prepared by encapsulating erythrocytes-ghosts carrying antigens inside polymeric and porous microparticles.
Materials and Methods. Erythrocyte-ghosts obtained by hemolysis in hypotonic buffer were encapsulated into ethylcellulose microparticles by w/o/w emulsification. The porosity of microparticles was evaluated by mercury porosimetry. The adsorption ability of encapsulated antigens was evaluated by hemagglutination after contact in tube or elution in column with polyclonal antibody solutions or human blood-plasma.
Results. The encapsulation process did not significantly alter the evaluated antigens since a significant decrease in anti-A (from 256 to 4) as well as anti-Kell (from 64 to 2) antibody titer has been observed in column after eight chromatographic runs (2 h). The higher the ghost concentration (total protein content of 6 mg/ml), the higher the adsorption capacity.
Conclusion. Encapsulation, currently used for drug delivery purposes, may consequently also be applied to the design of new immunoadsorbents as biomaterials.

KEY WORDS: alloantibodies; biomaterials; immunoadsorption; microparticles; red blood cell antigens.

INTRODUCTION

Extracorporeal immunoadsorption has been shown to be a powerful process in reducing circulating antigens (1) or immunoglobulins and immune complexes responsible for many diseases (2–5) as well as organ rejection after transplantation (6,7). In addition, it is the rescue treatment in patients refractory to conventional therapy. Moreover, owing to the shortage of human donor organs or tissues, xenotransplantation might be considered: however, in order to prevent a severe antibody-mediated reaction, it would be necessary to remove the xenoreactive antibodies which cause hyperacute organ rejection. The limitations of classical treatments such as plasmapheresis or plasma exchange (i.e. non-specific removal of plasma components) are well known. On the other hand, immunoadsorption could provide a specific elimination of pathogenic molecules and avoid plasma replacement. It is consequently considered superior to conventional plasmapheresis (8) and is generally used in patients when less expensive treatments have failed. More specific sorbents allowing antibody removal has been clinically tested with an acetylcholine receptor peptide for patients suffering myasthenia gravis, complement component C1q or protein A in systemic lupus erythematosus patients with threatened renal function and linear peptide ligands mimicking the β_1-adrenoreceptor in idiopathic dilated cardiomyopathy of autoimmune origin (9). However, the current immunoadsorbents are prepared by covalently binding ligands (protein A, antibodies or antigens) onto inert supports previously activated by rather toxic chemical reagents (i.e. cyanogen bromide). In addition, protein A immunoadsorption, currently widely used, allows the removal of all IgG (except IgG3) and may induce immunomodulation (10). Moreover, in order to prevent microbial growth during storage, reusable protein A-Sepharose gel column are primed with ethyl mercury thiosalicylate which can induce toxicity to patients (11). In addition, the potential leakage of the ligand from the support (due to the breakage of the covalent bond (12)) may contaminate the reinfused plasma and lead to side effects in patients, especially when the ligand is of animal origin (13). Based on these limitations, we developed

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previously an original approach consisting in encapsulating ABO antigens in porous non-biodegradable polymer microparticles (14). Since it is difficult to obtain purified ABO antigens, erythrocyte ghosts carrying antigens (thus preserved in their natural environment) were prepared and encapsulated into microparticles. Consequently, no coupling chemistry was required. The process efficiency, tested in a tube set-up with A and B antibodies, was found to dramatically decrease the titer of the antibody solutions. A similar system was developed after encapsulation of goat anti-apolipoprotein B polyclonal antibodies in order to deplete apolipoproteins B from plasma of patients suffering familial hypercholesterolemia (15).

We here extend the demonstration that erythroid membrane antigens can be non-covalently immobilized by entrapment into porous, non-biodegradable polymeric microparticles and used in columns in order to efficiently and selectively remove the specific antibodies of the encapsulated antigens. Such a removal can be useful in unusual clinical settings such as ABO-incompatible allogeneic haematopoietic stem cell transplantation (16). The A antigens of the ABO blood group system and the Kell antigens were used as model, both anchored in the RBC membrane as ghosts, but differing from their chemical nature (carbohydrate or protein, respectively) and membrane density.

MATERIALS AND METHODS

Materials

Ethylcellulose (MW 146,300 Da) was supplied by Sigma (Saint-Louis, MO, USA). Polyvinylalcohol (PVA, MW 30,000 Da, 88% hydrolyzed) and Tween® 80, also purchased from Sigma, were chosen as non-ionic surface active agents in order to stabilize the second emulsion and to decrease the hydrophobicity of microparticles as well. Red blood cells and ACD-A blood-plasma from healthy donors as well as human polyclonal anti-A antibodies were obtained from the "Etablissement Français du Sang, site de Nancy" (France). Human polyclonal anti-Kell antibodies were supplied by Sanofi Diagnostics Pasteur (Marnes-la-Coquette, France). Annexin V-fluorescein isothiocyanate (FITC) and anti-glycophorin A -phycoerythrin (PE) monoclonal antibodies (mAb) were provided from Bender Med Systems (Alexis Corporation, Switzerland) and Becton Dickinson (San Jose, CA, USA), respectively. All other chemical reagents were of analytical grade and used as supplied.

Methods

Preparation of Red Blood Cell Ghosts

Ghosts from red blood cells of blood groups A1 or B carrying also K antigens were prepared by hemolysis in hypotonic buffer at pH 7.4 according to Dodge et al. (17) with minor modifications : erythrocytes (10 mL) were mixed under stirring with isotonic buffer (20 mL) pH 7.4 (50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 150 mM NaCl) containing 0.037% ethylenediaminetetraacetic acid (EDTA) (w/v) and 0.017% phenylmethylsulfonyl fluoride (PMSF) (w/v) and washed by two successive centrifugations (1,700 g) for 5 min. Erythrocytes were subsequently added dropwise under magnetic stirring to the hypotonic solution (40 mL) pH 7.4 (100 mM NaH_2PO_4, 100 mM Na_2HPO_4) containing 0.037% EDTA (w/v) and 0.012% aminoethylbenzenesulfonyl fluoride (AEBSF). After the mixture was gently shaken for 15 min (100 rpm), ghosts were washed by successive centrifugations at 20,000 g for 15 min until all hemoglobin, as detected by spectroscopy at 560 nm, was removed and stored at 4°C in isotonic buffer. The total protein content in the ghosts suspension was determined by a colorimetric method (18).

Flow Cytometry

Analysis of both intact red blood cell (RBC) membrane and ghost suspension was performed using glycophorin A as specific erythroid marker and annexin V as probe of phosphatidylserine. Non- and stimulated red blood cells and ghost suspensions adjusted at 1×10^8 cells/ml were first incubated with 5 μg/ml of FITC-labelled annexin V for 10 min at room temperature (20–22°C) in the presence of 0.5 mM/l $CaCl_2$ in the dark. Then, they were labelled with a specific erythroid marker (PE-labelled anti-glycophorin A antibody) under previously determined saturating conditions: 100 μl of cell suspension were incubated with 10 μl of mAb during 30 min at room temperature in the dark. Intact RBC and ghost suspensions were then washed with Cell Wash solution (Becton Dickinson). Samples were analyzed with a FACS Calibur® flow cytometer (Becton Dickinson, San Jose, CA) equipped with Cell Quest® software. The instrument's optical alignment, laser output, and photomultiplier settings were checked each day using appropriate calibration beads (Calibrite®, Becton Dickinson). The analysis required the acquisition of 10,000 events, for each sample. The FITC and PE fluorescence were displayed (x axis) on a dot plot (y axis: cell size) in a logarithmic scale.

Preparation of Polymeric Microparticles

Ethylcellulose (MW 146,300) microparticles were prepared by the emulsification and solvent evaporation technique (14,15) modified as followed: 1 ml of ghosts (2, 4 and 6 mg/ml of total protein content) suspended in the isotonic buffer was emulsified by vigorous magnetic stirring (3 min) in methylene chloride (10 ml) containing ethylcellulose (500 mg). This first emulsion (water-in-oil) was then poured into water (1,400 ml) containing 0.6 g of polyvinylalcohol and 1.5 ml of Tween 80. Microparticles were then spontaneously obtained after solvent evaporation followed by filtration.

Unloaded microparticles as well as particles encapsulating ghosts labelled with fluorescein isothiocyanate (FITC) were prepared in the same way.

Ghost and Microparticle Size

The mean diameter of microparticles was evaluated by laser light diffraction using a Mastersizer (Malvern Instruments, UK) whereas the size of ghosts was measured by laser light diffusion with a Zetasizer (Malvern Instruments). Each sample was measured in triplicate and all normalized with respect to glass beads standard or a polystyrene standard suspension (Malvern Instruments).

Mercury Porosimetry

The size distribution of microparticle pores larger than 6 nm was measured by mercury porosimetry (19) (Autopore III—Micromeritics Co., Atlanta, USA). The pressure ranged between 11.6 10^{-1} and 16204.4 10^{-1} mPa, and the contact angle and surface tension were considered to be 130° and 485 dynes/cm, respectively at 20°C. The calculation of pore size was determined according to Washburn equation (20).

FITC Labelling of Membrane Proteins Bound Onto Ghosts

Fluorescein isothiocyanate was bound to membrane proteins according to the method previously described by Clausen and Bernkop-Schnürch (21). Briefly, 2 mg of FITC dissolved in dimethylsulfoxide (1 ml) was slowly added in 0.1 M sodium carbonate (20 ml) containing 1 ml of erythrocyte ghosts suspended in isotonic buffer (6 g/l). After 8 h of incubation at 4°C under gentle magnetic stirring (150 rpm), the coupling reaction was stopped by adding an ammonium chloride solution at a final concentration of 50 mM. The resulting ghost-FITC conjugate was incubated for 2 h at 4°C, and isolated after two dialysis in PBS at pH 7.4 before being concentrated by centrifugation at 25,000 g for 15 min.

Confocal Laser Scanning Microscopy (CLSM)

A Biorad MRC 1024 Laser Scanning Confocal Imaging System (Hemel Hempstead, UK) equipped with an argon ion laser (American Laser Corp., Salt Lake City, UT, USA) and a Zeiss Axiovert 100 microscope (Carl Zeiss, Oberkochen, Germany) was used to qualitatively detect the fluorescent proteins into polymeric particles dispersed in a neutral oil (miglyol 812). The laser was adjusted in the green fluorescent mode which yielded an excitation wavelength at 488 nm. All confocal fluorescent pictures were taken with a ×40 objective (oil immersion, numeric aperture 1.30). The software used for the CLSM imaging was Laser Sharp MRC-1024 Version 3.1 (Bio-Rad, Deisenhofen, Germany).

Adsorption Capacity

Experiments were first performed in glass hemolysis tubes (75×12 mm) with blood group A ghost-loaded microparticles (25 mg) suspended in 0.5 ml of anti-A antibody solution, under gentle mixing by rotation at room temperature.

Then, in order to demonstrate the feasibility of the concept in column, as it would be performed for clinical treatments, ethylcellulose microparticles (750 mg) were packed in a glass column (10.0×1.1 cm) and washed with PBS pH 7.4 (130 mM NaCl, 5mM Na_2HPO_4, 1mM KH_2PO_4 containing 0.05% Tween® 80). Fifteen milliliters of human blood plasma supplemented with anti-A or anti-Kell antibodies were loaded on the column (1 ml/min). Flushing with PBS was then carried out until 15 ml of blood plasma were recovered: the same 15 ml were passed eight consecutive times (2 h) in the same experiment under the same conditions. The adsorption capacity was evaluated by determining the antibody titers before and after each adsorption experiments by hemagglutination in saline in tubes (anti-A) or in Liss-Coombs in gel (Sepharose, DiaMed AG, Swizerland) (anti-Kell).

In the same way, similar experiments performed with blood plasma supplemented with anti-A antibodies were tested with both blank microparticles without ghost and microparticles containing ghosts from blood group B, in order to evaluate the specificity of microparticles.

RESULTS

Flow Cytometry

In order to check the ghost membrane organization after red blood cell hemolysis, the phospholipid organization of both red blood cells and ghosts was compared by flow cytometry. As shown in Fig. 1, after labelling with annexin-FITC and anti-glycophorin A-PE, rested and stimulated red cells and ghosts expressed 98, 99 and 95% of glycophorin A, respectively (Fig. 1a). On the contrary, no expression of annexin was detected for red cells and ghosts, whereas as expected, annexin was expressed on stimulated red cells (positive control) (Fig. 1b).

Characterization of Microparticles

In order to both confirm the encapsulation of erythrocyte ghosts inside ethylcellulose microparticles and evaluate their distribution within the polymeric matrix, erythrocyte ghosts were labelled with FITC. As observed by laser scanning confocal microscopy (Fig. 2), ghosts were successfully entrapped into microparticles since fluorescence was detected all over the particles. That also clearly demonstrated the homogeneous distribution of erythrocyte ghosts inside the polymeric matrix of particles. Moreover, erythrocyte ghosts loaded microparticles appeared spherical and exhibited a mean diameter of 400 μm. The polymeric wall was porous (Fig. 3) and characterized by pores whose size ranged from 6 nm to 23 μm, with a major population (75%) ranging from 10 nm to 1 μm.

Adsorption Capacity

Experiments were performed in tubes and in a chromatographic column in order to test the adsorption capacity of both encapsulated A antigens of carbohydrate nature (ABO system) (Fig. 4) and Kell antigens of protein nature (Fig. 5) after contact with each antibody solution (anti-A and anti-Kell) and blood-plasma containing antibodies. Similar results in antibody titer decrease were also obtained after adsorption of anti-B antibodies onto ghosts carrying B antigens entrapped into microparticles (data not shown). Whether it was in tubes or in column, the ratio between the mass of microparticles and the volume of the medium containing antibodies was similar. The results obtained in tubes confirmed those obtained previously (14). However, for both antibodies tested, a more significant decrease in the antibody titer occurred when the immunoadsorption was performed in column. In addition, the adsorption capacity of ghost-loaded microparticles depends on the total entrapped protein content (2, 4 and 6 mg/ml). The more the protein content the more important the adsorption capacity and

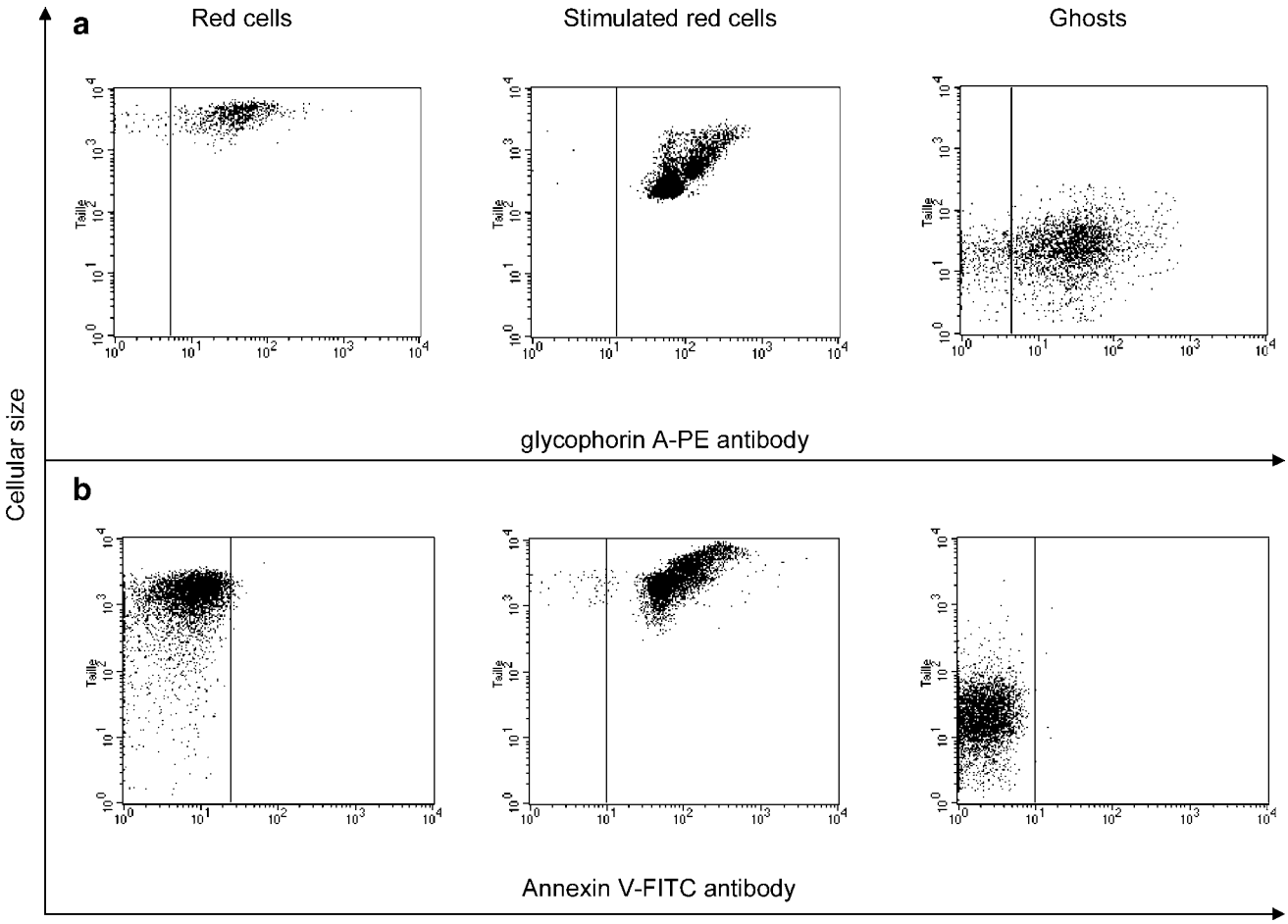

Fig. 1. Flow cytometric analysis of glycophorin A expression **a** and annexin V binding **b** on intact red cells, stimulated red cells and ghosts. All samples were positive for glycophorin A, as a specific erythroid marker. Annexin V binding was negative for both resting red cells and ghosts, conversely stimulated red cells bound annexin V as positive control.

consequently the lower the final antibody titer. Indeed, when 6 mg/mL of total proteins were encapsulated, the antibody titer reached 4 and 2 in column for anti-A and anti-Kell antibodies, respectively, whereas it decreased only down to 32 and 16 in tubes. Moreover, the depletion rate is much faster in column than in tubes. Indeed, for instance after 1 h of contact in tube (corresponding to four runs of 15 min in column), no adsorption was observed whereas the antibody titer was already reduced to 8 in column in the case of 6 mg/ml of protein concentration. It can also be observed that the decrease in antibody titer is faster and greater for the Kell system compared to the ABO system. Indeed, after 1 h of contact in tube, a four-fold fall in anti-Kell antibody titer was observed whereas no adsorption was noticed with the ABO system regardless the protein concentration.

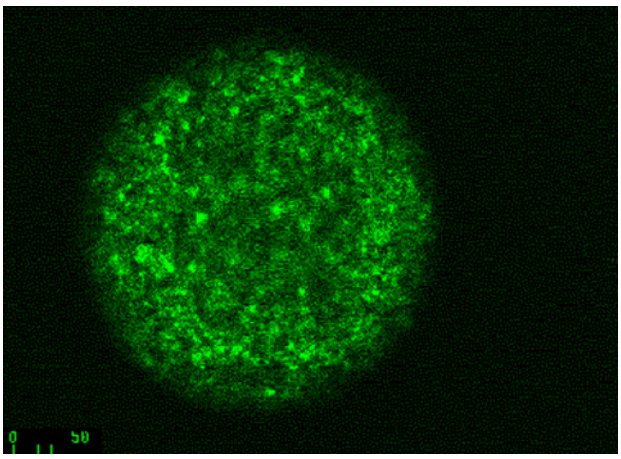

Fig. 2. Distribution of FITC-labelled ghosts encapsulated into ethylcellulose microparticles observed by confocal laser scanning microscopy. Fluorescence excitation was performed at 488 nm and emission at 520 nm.

DISCUSSION

Immunoadsorption represents an efficient process in order to eliminate specific pathogenic molecules from blood-plasma of patients and to save replacement solutions as it is performed in plasma exchange treatment. In addition, during the immunoadsorption process, no dilution of normal plasma proteins occurs, allowing the treatment of larger plasma volumes, leading to a dramatic reduction of the pathogenic molecules in a single session. Many patients suffering disorders such as hypercholesterolemia and autoimmune diseases and refractory to conventional therapy were successfully treated by immunoadsorption (9). However,

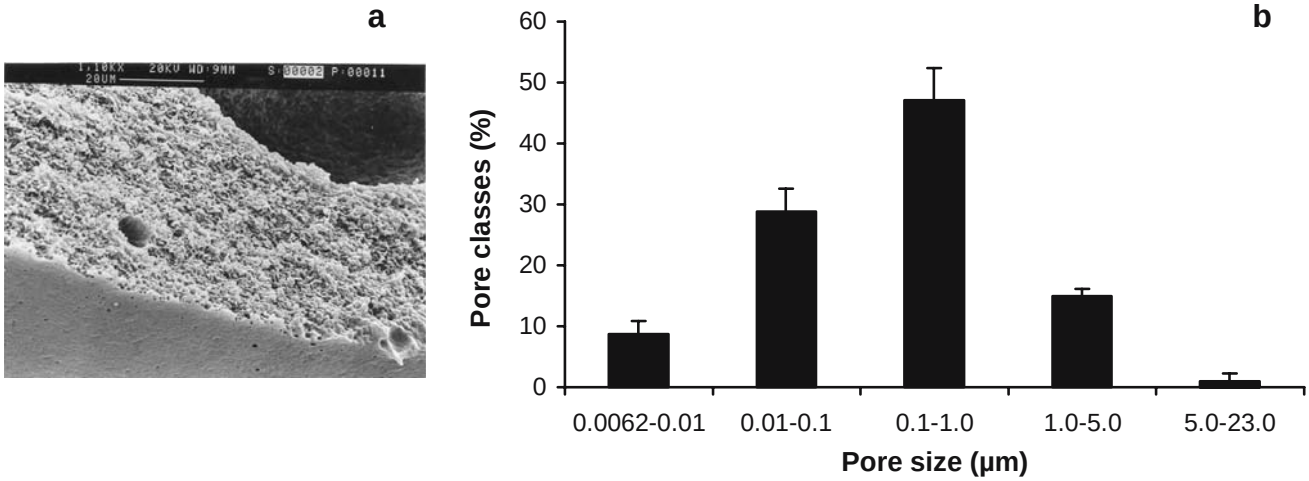

Fig. 3. **a** Morphology of the outer surface of ethylcellulose microparticles showing the porosity of the polymeric wall observed with a scanning electron microscope (Cambridge model S240, Leica Cambridge Ltd., Cambridge, UK). **b** Percentage of pore classes of the polymeric wall of microparticles evaluated by mercury porosimetry (n=3).

immunoadsorption columns have one major limitation that is the chemical coupling of both protein A or a ligand specific to the pathogenic molecule to be removed. Thus, our approach, based on the entrapment of antigens into porous polymeric microparticles, eliminates the chemical coupling and allows specific removal of antibodies. Moreover, the purification step currently necessary for proteins before their binding on the support becomes redundant since erythrocyte ghosts carrying antigens were immobilized into microparticles by encapsulation, thus preserving antigens in their natural environment. However, there are also some limitations with our new approach. The most critical points are the harvesting of erythrocytes in a sufficient volume and their shelf-life, which has a direct influence on the time between sampling from volunteers and the ghost transformation. Transformation of erythrocytes into ghosts is a well established process with no major critical point. Erythrocyte ghosts often seal after their preparation, exposing antigens either inside or outside, thus favoring or not the interaction with their corresponding antibody. Since the specific erythroid marker glycophorin A was still expressed on ghosts it can be postulated that glycophorin A remained accessible after ghost preparation thus preserving the erythroid lineage characteristics. On the other hand, a main feature of the plasma membrane bilayer is the asymmetrical distribution of lipids and charges between the internal and external surfaces. It is well-known that ghosts may tend to seal as closed vesicles exposing the anionic phospholipids either inside or outside, depending mainly on the experimental conditions (22). It was consequently important to check if the natural

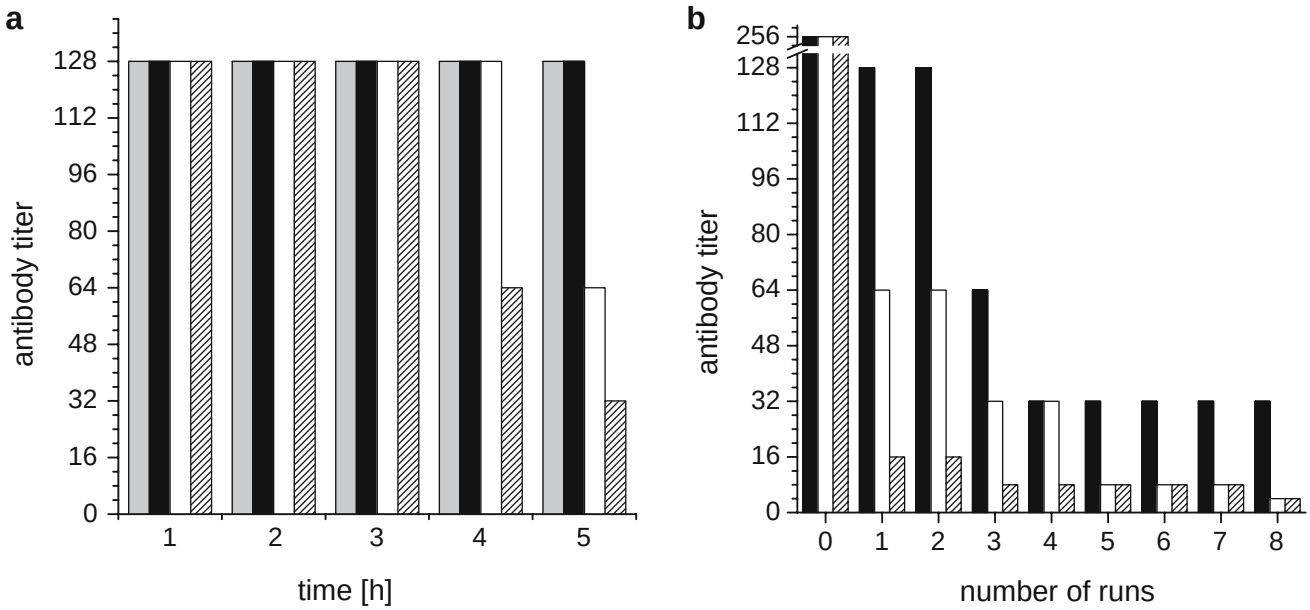

Fig. 4. Adsorption ability, expressed as a decrease in the antibody titer, of unloaded microparticles (*grey*) and blood-group A ghosts (two (*black*), four (*white*) and six (*hatched*) mg/ml of total protein content) encapsulated into ethylcellulose microparticles and tested **a** in tube (25 mg of microparticles in 500 µl of polyclonal anti-A antibody solution) or **b** packed in a column (750 mg) loaded 8 consecutive times with 15 ml of human blood-plasma supplemented with polyclonal anti-A antibodies, at a flow rate of 1 ml/min.

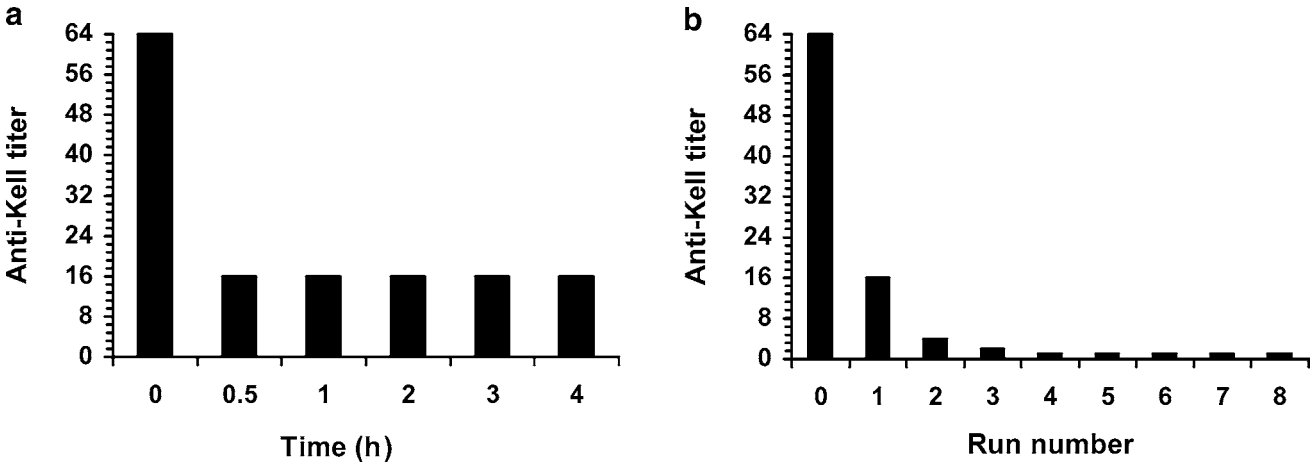

Fig. 5. Adsorption ability, expressed as a decrease in the antibody titer, of blood-group B ghosts carrying Kel antigens (six (*black*) mg/ml of total protein content) encapsulated into ethylcellulose microparticles and tested **a** in tube (25 mg of microparticles in 500 μl of anti-Kell antibody solution) or **b** packed in a column (750 mg) loaded eight consecutive times with 15 ml of human blood-plasma supplemented with anti-Kell antibodies, at a flow rate of 1 ml/min.

membrane organization of red cells was preserved after the ghost preparation. Indeed, on intact red blood cells, anionic phospholipids are exposed inside, thus conferring the right-side-out structure. Annexin V, which binds to the anionic phospholipid phosphatidylserine, did not bind on intact red cells and ghosts indicating that membrane phospholipids were not disorganized after ghost preparation and/or that vesiculation of unsealed ghosts occurred as initially, thus preserving the right-side-out structure. On the contrary, annexin V was bound onto ionophore-treated red cells which are known to expose phosphatidylserine on their outer layer (23). Indeed, after activation of red cells, anionic phospholipids are exposed outside by the flip-flop mechanism conferring the inside-out membrane structure and allowing the binding of annexin V as observed on Fig. 1b. It can be concluded that the initial membrane organization was preserved after ghost preparation and that the "resealing" of erythrocyte membrane ghosts occurred as initially observed with intact red cells.

As previously shown, the integrity of antigens was preserved after the ghost preparation (24). Suspensions of blood-group A ghosts (2, 4 and 6 mg/ml) were encapsulated within ethylcellulose microparticles which exhibited an average diameter of 400±36 μm. The microparticles manufacturing process (ratio polymer mass/organic solvent volume, volume of the second emulsion and speed in agitation during the emulsification step) allowed the design of discrete and porous particles (Fig. 3a). Such a porous structure is in favour of an easy permeation of antibodies inside microparticles. Indeed, antibodies have to be able to cross the polymeric wall in order to adsorb onto antigens. On the contrary, antigens have to remain inside microparticles in order to prevent contamination of the eluted solutions or blood plasma. 83% of pores ranged from 6 nm to 1 μm (Fig. 3b) both allowing the entrance of the antibodies of ABO and Kell systems. In addition, since (1) antigens are strongly anchored into the ghost membrane, (2) ghosts are entrapped into the core of the polymeric matrix of ethylcellulose (Fig. 2) and (3) antibodies have a strong affinity and avidity for their corresponding antigens, both antigens and antibody/antigen complexes are not likely to be released even for the highest population of pores (5–23 μm). Nevertheless, the pore size of the microparticles has to be compared to the red cells or ghosts diameter. Blood red cells have an average diameter around 7 μm. Only 1% of the microparticles pores are larger than 5 μm (Fig. 3b). Therefore the possibility of ghost release into the plasma is unlikely due to the very dense structure of the polymer matrix (Fig. 3a). Indeed, it has to be kept in mind that the entity to be released would be the ghost membrane (carrying the antigens/antibodies complex) and not the release of a small molecule. In addition, it should also be possible to adapt the manufacturing process in order to have all pores smaller than 7 μm (25,26).

The encapsulation process seems efficient since the ghost distribution appears very homogeneous in the polymeric matrix (Fig. 2). The encapsulation process, including contact with organic solvent and shear stress, had no major effect on the biological activity of antigens since a suitable adsorption capacity was maintained. Indeed, when microparticles were suspended in hemolysis tubes in a polyclonal anti-A antibody solution, the initial titer (128) decreased slowly down to 64 after 4 h (Fig. 4a). In the same way, the initial titer of anti-Kell antibodies (64) showed a decrease to 16 after 4 h of contact (Fig. 5a). However, for both antibodies, the adsorption mechanism was lower and slower than that observed with unencapsulated ghosts (14). Indeed, the polymeric wall definitely acts as a barrier which slows down adsorption but also prevents the release of antigen/antibody complexes once they have occurred.

Ghosts-encapsulated microparticles packed into a glass chromatographic column loaded with the corresponding antibody solutions (ratio microparticle mass/antibody solution volume being kept similar than in tubes) displayed a better adsorption capacity than in hemolysis tubes (Figs. 4b and 5b), probably owing to a closer and physical forced contact between fluids and microparticles in column and consequently a better wettability.

Figure 4 displays the influence of the initial ghost concentration (based on the total protein content) on the adsorption capacity of antigens. The higher the protein concentration, the higher the adsorption capacity. Indeed, particles prepared with the highest ghost content (6 mg/ml)

allowed the most efficient depletion since after only one chromatographic run (15 min) the antibody titer decreased from 256 to 16. Although the trend is the same for microparticles in tubes, it has to be noted that it took 5 h to reduce the antibody titer from 128 to 32 for a 6 mg/ml ghost concentration. That corresponds to a two-fold decrease in anti-A antibodies. Similar results in antibody titer fall were obtained after one run (15 min) for the 4 mg/ml concentration and after three runs (45 min) for the 2 mg/ml concentration. In the case of 6 mg/ml concentration, there is a four-fold reduction in antibody titer after only onr run (15 min). At the end of the experimental time in column (2 h) a three-fold antibody titer reduction was observed with 2 mg of total proteins/mL, whereas a six-fold fall occurred with microparticles containing ghosts at 4 and 6 mg of total protein content/ml. This demonstrates the higher efficiency of the column system into which microparticles were packed. Indeed, in tubes, microparticles tend to float at the water/air interface thus not favouring an intimate contact between the fluid and the solid microparticles. An initial ghost concentration of 6 mg/ml was therefore chosen as the optimal concentration. Besides the antigens of carbohydrate nature (ABO system), ghosts also contain protein antigens. Based on the above mentioned results regarding the ABO system, it was important to verify if the proposed microparticles were also able to deplete antibodies with encapsulated protein antigens. Thus, the Kell system was chosen as model. Figure 5 shows that it took only one chromatographic run to decrease the anti-Kell titer from 64 to 16. No difference between the experiments in tube or in column was observed after 1 h since in both cases the anti-Kell titer was reduced from 64 to 16 (a two-fold reduction). However, in tube there was no further decrease in the anti-Kell titer since the same value was obtained after 4 h of contact. On the other hand the anti-Kell titer was reduced from 4 after the second chromatographic run (30 min) to 1 after the fourth run (1 h). No further reduction was observed between the fourth and the eighth runs. Most of anti-Kell antibodies were adsorbed and consequently depleted from plasma as soon as after 1 h. The faster and more important decrease in antibody titer observed with the Kell system compared to the ABO system can be explained by the difference in both antigen density and antibody molecular weight. Indeed, 8 to 12×10^5 A antigens are anchored into A RBC membrane whereas the Kell antigen density ranges from 2,300 to 6,000 (27). It can be considered that an overcrowding occurs with the ABO system, hampering an easy uptake of antibodies onto membrane antigens. Indeed, since anti-A antibodies are actually a blend of IgM, IgG and IgA, and especially IgM, this hypothesis is strengthened by the fact that IgM with their large size, may also contribute to overcrowding compared to anti-Kell antibodies which are essentially IgG characterized by a smaller size.

Since no decrease in antibody titer and consequently no adsorption was observed with both unloaded microparticles and non specific antibodies of the encapsulated antigen (i.e. antigens A with anti-B antibodies, data not shown), that demonstrates the specificity of the immunoadsorbent. In addition, no released antigens were detected by hemagglutination into the eluted medium, which confirms that ghosts did not escape from the polymeric microparticles. Furthermore, when immunoadsorption was performed in column with blood-plasma, no overt clotting was observed, probably owing to the blood anticoagulation treatment by citrate. This could consequently be expected during a clinical immunoadsorption trial since blood of patients is heparinized in order to prevent coagulation. Nevertheless, further investigations concerning nonspecific adsorptions are still needed in order to prevent immunological disorders owing to incompatibility (28,29) although the surfactants (polyvinylalcohol and Tween 80) used for microparticle manufacturing are well-known to decrease the hydrophobicity of polymeric particles and might minimize nonspecific adsorptions onto the polymeric surface. In addition, ethylcellulose is widely used in the pharmaceutical industry and is recognized as an inert and biocompatible polymer. Furthermore, in order to demonstrate the lack of thrombogenic activity of the ethylcellulose polymer, plasma was eluted through a column packed with encapsulated ghosts carrying AB antigens. Clotting times such as prothrombin time and APTT (activated partial thromboplastin time) were determined before and after plasma elution under the same conditions as described in "Methods." The two clotting times explore extrinsic and intrinsic pathways of coagulation, respectively. Blank microparticles (with no ghosts inside) were also tested as controls. In addition, fibrinogen, Immunoglobulins G and albumin were also quantified before and after immunoadsorption. No changes in the tested parameters have been observed. Prothrombin time was stable and APTT was 36.5 and 37.1 s, whereas albumin was 28.8 and 28.1 g/l and immunoglobulins G 7.76 and 7.61 g/l before and after immunoadsorption, respectively. Such results allow us to conclude that there is no depletion in the coagulation factors. Therefore, this preliminary experiment shows that, upon plasma contact (there would be no contact with blood in our system since blood cells and plasma would be separated before elution as it exists with the currently marketed systems), ethylcellulose microparticles do not seem to have a thrombogenic activity. Furthermore no gross clotting was noticed by naked eye inspection.

Overall the same results are found for either ABO or Kell antigens in both tubes and column. Indeed, the maximum antibody adsorption was 75% and more than 95% for both types of antigens in tubes and column, respectively. That may open up new opportunities in the treatment of immune diseases or increase the success rate in organ transplantation by treating patients by extracorporeal immunoadsorption in order to deplete blood-plasma from autoantibodies or anti-HLA antibodies, respectively.

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