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Both Fcγ and complement receptors mediate transfer of immune complexes from erythrocytes to human macrophages under physiological flow conditions *in vitro*

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Summary

Abnormal clearance by the mononuclear phagocytic system of immune complexes (IC) is important in the pathogenesis of systemic lupus erythematosus (SLE). We have developed an *in vitro* model to investigate the cellular mechanisms involved in the transfer of soluble IC from erythrocytes to human macrophages under physiological flow conditions. In this assay, erythrocytes bearing fluorescently labelled IC are perfused over monolayers of human monocytes or monocyte-derived macrophages in a parallel-plate flow chamber, and transfer quantified using confocal microscopy and flow cytometry. Using aggregated human IgG as a model IC, we have been able to demonstrate transfer of IC from erythrocytes to macrophages. Blocking studies with specific neutralizing antibodies have shown that both complement and Fcγ receptors are required for IC transfer. Blockade of CR4 ($\alpha_x\beta_2$ integrin), FcγRIIa or FcγRIII reduced transfer, while anti-CR3 ($\alpha_m\beta_2$ integrin) had no effect. Blockade of CR3, FcγRIIa or FcγRIII also reduced the number of adhesive interactions between fluorescently labelled IC-bearing erythrocytes and macrophage monolayers. Taken together with the transfer data, this suggests differing roles for these receptors in the human IC transfer reaction that includes an adhesive function which facilitates IC processing by mononuclear phagocytes. Finally, a functional effect of the FcγRIIa R131/H131 polymorphism, important in susceptibility to SLE, has also been demonstrated using this model. Uptake of IgG_2 but not IgG_1-containing soluble IC was reduced by macrophages from individuals homozygous for the R131 allelic variant of the receptor.

Keywords: complement, Fcγ receptors, immune complexes, macrophages, systemic lupus erythematosus

Introduction

Immune complexes (IC) are cleared from the circulation in humans and non-human primates via the fixed mononuclear phagocytic system (MPS), located primarily in the liver and spleen [1,2]. The formation of IC in the circulation leads to activation of the classical pathway of complement, which in turn results in their opsonization by complement degradation products. These serve as ligands for complement receptor type 1 (CR1) on the surface of circulating erythrocytes, which transport IC to the MPS. Here, IC are transferred to fixed tissue macrophages in a reaction presumed to be mediated by Fcγ and complement receptors on the surface of the phagocyte [3,4]. The erythrocyte subsequently returns to the circulation with a reduced level of CR1 on its surface, likely to occur as a result of proteolytic cleavage of the receptor during the transfer reaction [4,5]. Additionally, the binding of IC to erythrocyte CR1 maintains IC within the circulation, preventing their interaction with the endothelium and/or extravasation into the tissues where potentially harmful effects can occur [6].

Defective processing of IC may allow persistence of IC within the circulation, with subsequent deposition in the tissues and tissue damage. This is best demonstrated by the prototypic IC-mediated disease, systemic lupus erythematosus (SLE). Several *in vivo* studies have demonstrated that the clearance of soluble IC is abnormal in patients with SLE. For example, when compared to normal subjects the mean half-time for initial clearance of radiolabelled heat-aggregated gamma globulin (HAGG) was shorter in patients with

SLE [7]. Experiments performed in our own laboratory using radiolabelled hepatitis B surface antigen (HBsAg)/anti-HBsAg IC demonstrated accelerated uptake of this probe by the liver, but subsequently defective retention of the IC within the organ, with return of the complexes to the circulation [8]. Uptake and retention of the IC by the spleen were also reduced. The hypothesis for this defective retention of IC was that only those complexes able to interact with both Fcγ and complement receptors are retained effectively within the organ. This hypothesis was tested later using smaller HBsAg/anti-HBsAg IC that activated complement poorly *in vitro* [9]. Again, defective retention of the IC was observed, supporting the notion that ligation of Fcγ is essential for the removal of IC by the liver in humans. Further evidence suggesting an important role for Fcγ receptors in IC processing in humans comes from several epidemiological studies investigating the frequency of genetically determined polymorphisms of Fcγ receptors in patients with SLE [10]. In some populations the R131 variant of FcγRIIa is more frequent in patients with SLE compared to normal controls, and in particular may increase susceptibility to the development of lupus nephritis [10–12]. This association is particularly strong in patients with anti-C1q antibodies, which are predominantly of the IgG_2 subclass [12]. Because the phagocytosis of IgG_2-coated erythrocytes by leucocytes from individuals homozygous for the H131 variant of FcγRIIa is more efficient *in vitro* than that by cells from R131 homozygotes [13], this raises the possibility that impaired clearance of IC containing anti-C1q antibodies in particular may be important in the pathogenesis of lupus nephritis.

The precise cellular mechanisms involved in the transfer of soluble IC from erythrocytes to macrophages remain to be delineated fully. In contrast to studies performed *in vivo*, few *in vitro* models have been developed to explore these mechanisms in detail. Emlen *et al.* investigated the transfer reaction between human erythrocytes and monocytes using HAGG as the model IC [3,14]. In their model, CR1 on the phagocyte was the most important receptor for the transfer of HAGG, while transfer from erythrocytes was a more efficient process than uptake from the fluid phase. More recent studies have used heteropoymer-based complexes, in which a monoclonal antibody (MoAb) to CR1 is chemically cross-linked to dsDNA, a potentially important antigen in SLE [4]. Using this approach, FcγRI was shown to be essential for the transfer of the complex from erythrocytes. Studies using heteropolymer complexes containing the bacteriophage ΦX174 and the murine macrophage $P388D_1$ cell line suggested that transfer from erythrocytes was dependent on Fcγ receptors on the phagocyte, with little contribution from complement receptors [15].

We have now developed a novel *in vitro* model to investigate the contribution of individual phagocytic receptors involved in the transfer of soluble IC from erythrocytes to human monocytes and monocyte-derived macrophages. In this model monolayers of these cells were exposed to IC delivered bound to human erythrocytes that were perfused through a parallel-plate flow chamber system, developed in our laboratory for the study of leucocyte–endothelial cell interactions [16,17]. We are able to demonstrate transfer of IC from erythrocytes to mononuclear cells using confocal microscopy. In recirculation experiments a progressive loss of IC from erythrocytes during perfusion over macrophage monolayers was observed, correlating with uptake by the macrophage monolayer. We have defined the complement and Fcγ receptors required for IC transfer under flow and those involved in the adhesive interaction between erythrocytes and macrophage monolayers. The data suggest differing roles for these receptors in the transfer reaction, including an adhesive function that facilitates IC processing by mononuclear phagocytes. Finally, we demonstrate a defect in the uptake of IgG_2 IC under flow by macrophages from individuals homozygous for the R131 variant of FcγRIIa.

Materials and methods

Antibodies

Mouse anti-human MoAb 10.1 (isotype IgG_1 kappa) against FcγRI (CD64) was obtained from Dako (Glostrup, Denmark). This antibody reacts with an epitope near the binding site for the Fc region of human IgG, blocking the binding of human IgG to this receptor [18]. Mouse anti-human MoAb 3G8 (IgG_1) against FcγRIII (CD16) was obtained from Immunotech (Marseille, France). This MoAb reacts with both FcγRIIIa and FcγRIIIb and blocks IC binding to the receptor. The mouse anti-human antibody IV.3 (IgG_{2b}) against FcγRII (CD32) was purified from mouse hybridoma supernatant (ATCC, Manassas, VA, USA). This MoAb binds preferentially to FcγRIIa rather than FcγRIIb [19], and blocks the binding of IgG to FcγRIIa [20]. The mouse anti-human CR1 MoAb To5 (IgG_1) was obtained from Dako. This antibody inhibits the binding of C3b to erythrocytes, neutrophils, B lymphocytes, monocytes and macrophages [21]. Mouse anti-human CR1 MoAb 3D9 was a gift from Professor J. Atkinson (University of Washington, St Louis, MO, USA). This blocks the binding of C3b to CR1 on erythrocytes [22]. The MoAb 3.9 against CR4 (CD11c) was a gift from Dr N. Hogg (Cancer Research UK, London) while the mouse anti-human CR3 MoAb 2LPM19c (IgG_1 kappa) was obtained from Dako [23]. FITC-and R-phycoerythrin-conjugated $F(ab')_2$ goat anti-human IgG (gamma-chain specific) antibodies were obtained from Sigma (St Louis, MD, USA). Alexa Fluor® 555-conjugated goat anti-mouse IgG_1 and Alexa Fluor® 488-conjugated rabbit anti-human IgG (whole molecule) were obtained from Molecular Probes (Eugene, OR, USA). The RGD tripeptide (Arg–Gly–Asp) was obtained from Sigma and was used at a concentration of 80 μM to inhibit C3b binding.

Immune complexes

HAGG was generated by heating a 10 mg/ml solution of human IgG (Sigma) in phosphate-buffered saline (PBS) at 63°C for 30 min. This was cooled immediately on ice and then microfuged at 1100 ***g*** for 15 min at 4°C to remove insoluble aggregates. HAGG was directly labelled with Alexa Fluor® 488 (Molecular Probes Inc.) according to the manufacturer's instructions to enable visualization of the model IC by fluorescent microscopy. The size of HAGG was estimated by sucrose density centrifugation and compared to monomeric human IgG. IC containing IgG_2 only were formed by heat aggregating human IgG_2 (Vital Products Inc., Boynton Beach, FL, USA). HBsAg was a gift from GlaxoSmithKline Biologicals (Rixensart, Belgium). The antigen was in the form of a large polymeric protein (MW of approximately 3000 kDa) and was stored in PBS at −70°C at a concentration of 0·5 mg/ml. Polyclonal anti-hepatitis B surface antigen (anti-HBsAg) was a gift from the Swiss Red Cross (Bern, Switzerland) and was prepared from the serum of patients hyperimmune against HBsAg. One vial of sterile lyophilized immunoglobulin (> 95% IgG_1) was dissolved in 10 ml sterile water (Antigen, Surrey, UK) to produce a final concentration of 50 U/ml. HBsAg/anti-HBsAg IC were formed as described previously [8,9] and were labelled fluorescently with Alexa Fluor® 488. Model IC were opsonized with 20% normal human serum as a complement source for 20 min at 37°C. Binding of C3 was optimal at this timepoint. IC formation and binding to erythrocyte CR1 was confirmed by precipitation in polyethylene glycol and radioimmunoassay, respectively, as described previously [9,24].

Cell isolation and culture

Human erythrocytes were purified from peripheral blood taken from healthy volunteers and stored in veronal buffered saline (VBS)/1% bovine serum albumin (BSA)/0·05% sodium azide at 4°C. Erythrocyte CR1 was enumerated by radioligand binding assay using MoAb E11 (a gift from Dr N. Hogg, Cancer Research UK, London), as described previously [25]. The mean CR1 number was 717 ± 106 molecules per cell. Peripheral blood mononuclear cells were isolated from healthy donors by density gradient centrifugation over Lymphoprep (Axis-Shield, Oslo, Norway). Monocytes were isolated by magnetic cell sorting with positive selection for $CD14^+$ cells [26]. Magnetic beads conjugated with mouse monoclonal anti-human CD14 were obtained from Miltenyi Biotec (Aubern, CA, USA). Separation of monocytes from lymphocytes was assessed by flow cytometry using appropriate primary and either fluorescein isothiocyanate (FITC)- or R-phycoerythrin secondary antibodies. Cell viability was > 95% as assessed by trypan blue exclusion. $CD14^+$ monocytes were differentiated into macrophages by culture in X VIVO 15 complete medium (BioWhittaker, Wokingham, UK) supplemented with 10% normal human serum (NHS) for 6–8 days. Cells were cultured in 9 cm^2 slide flaskettes (Nunc, Roskilde, Denmark) to form monolayers and were used in the flow chamber assay after either 1–2 (monocytes) or 6–8 (monocyte-derived macrophages) days in culture.

Parallel-plate flow chamber assay

Slides on which monocyte or macrophage monolayers had been formed were placed in the parallel-plate flow chamber (channel height 150 μm) and then placed under an inverted Diaphot 300 fluorescence microscope (Nikon, Melville, NY, USA) housed in a Perspex casing to maintain an ambient temperature of 37°C. In experiments where transfer of IC was assessed, either non-labelled or fluorescently labelled HAGG (10 μg/ml) was opsonized with 20% NHS then incubated with erythrocytes at 1×10^9/l (~10% haematocrit) in VBS for 10 min at 37°C. The cells were then washed twice in ice-cold VBS, resuspended in 5 ml VBS at 37°C and perfused over the monocyte or macrophage monolayer. Experiments were performed at 0·55 ml/min, which equates to a shear stress of 1 dyne/cm^2. Where specified, monolayers were preincubated for 30 min at 37°C with either 10 μg/ml neutralizing antibodies against individual Fcγ or complement receptors, or media containing appropriate subclass and isotype-matched control Ig at the same concentration, prior to exposure to IC.

In certain experiments, erythrocytes bearing IC were recirculated through the chamber over monocyte or macrophage monolayers by means of a peristaltic pump. Cells were recirculated for up to 30 min, with 200 μl aliquots removed at intervals and prepared for fluorescence activated cell sorter (FACS) analysis. This was performed on an Epics XL flow cytometer (Beckman Coulter, High Wycombe, UK). The percentage of cells staining positive for IC was determined using WinMDI software, version 2·5 (Scripps Research Institute, La Jolla, CA, USA). After exposure to fluorescently labelled IC, monocyte or macrophage monolayers were washed then fixed in 4% formaldehyde. Slides were then examined by a Zeiss LSM 5 Pascal confocal laser-scanning microscope (Zeiss Ltd, Welwyn Garden City, UK) equipped with Argon (excitation wavelength 488 nm) and HeNe (excitation wavelength 543 nm) lasers, using a ×40 water-dipping achroplan objective. Typically, multiple optical sections of samples, running through the whole depth of the cells, were captured and imaged using the software's automatic scanning mode. Fluorescent and differential interference contrast (DIC) images were obtained from 10 random areas per slide. Each image, which represents an area of 230 × 230 μm^2, typically contained between 10 and 25 cells. The number of cells that had taken up an IC, either bound to the cell surface or internalized, was counted and expressed as a percentage of the total number of cells seen in each area. As a secondary measure, the fluorescence of each individual cell that had taken up an IC was

measured using the LSM Pascal software (version 3·2). These values were averaged for each field of view to obtain a mean fluorescence value. For each experiment a control slide of monocytes or macrophages, which had not been exposed to fluorescently labelled IC, was used to obtain images that allowed adjustment of the level of background fluorescence.

Adhesion assay

To investigate further the nature of the interaction between IC-bearing erythrocytes and monocyte or macrophage monolayers, adhesion studies were performed with a modification of the protocol described above. Unlabelled HAGG was opsonized using NHS then incubated with 200 µl erythrocytes at 1×10^8/ml for 20 min at 37°C and the cells washed twice in ice-cold VBS. The erythrocytes were then resuspended in 1 ml VBS and were incubated with 5 µl of 1 M carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Inc.) for 30 min at 37°C. The cells were washed five times in VBS to ensure removal of excess CFSE and resuspended in 5 ml VBS at 37°C. CFSE-labelled IC-bearing erythrocytes or erythrocytes alone were perfused over monocyte or macrophage monolayers at 0·55 ml/min. Interactions between the flowing cells and the adhered monolayer were videotaped through the inverted microscope using a high sensitivity C2400 camera (Hamamatsu Photonics). Following an initial 2 min period to allow the flow chamber to equilibrate, 10 random fields were recorded per slide, each for 15 s. Analysis of the acquired images was performed off-line after the experiment using EML Cell Motion Analysis software (Ed Marcus Laboratories, Brighton, MA, USA). Ten-second images were acquired at 15 frames/s from each 15 s video recording and these were stored as a digital video file. The following indices were calculated automatically by the software: the total number of cells/field, the number of cells rolling/field, the mean velocity of the rolling cells in µm/s and the maximum and minimum velocity of individual cells. Arrested cells were defined as those moving < 5 µm in 10 s, while rolling cells were defined as those with a mean rolling velocity < 100 µm/s for at least 1 s [17,27].

Genotyping for the R131/H131 polymorphism of FcγRIIa

Genomic DNA was isolated from whole blood samples obtained from healthy donors. The polymerase chain reaction (PCR) for the R131/H131 polymorphism of FcγRIIa was adapted from Jiang *et al.* [28]. The allele-specific oligonucleotide primers used to amplify the FcγRIIa gene were obtained from Sigma-Genosys (Pampisford, Cambs, UK). DNA fragments were analysed on 3% w/v agarose gels with staining by ethidium bromide and visualization under ultraviolet light.

Statistics

Results are expressed as mean ± s.e.m. of at least three independent experiments, unless stated otherwise. Statistical analysis was performed using the non-parametric Mann–Whitney *U*-test, or one- or two-way analysis of variance (ANOVA) with Bonferroni's post-test corrections, where appropriate. These were performed using Prism software, version 3·0 (GraphPad Software Inc., San Diego, CA, USA). Results were considered statistically significant if $P < 0{\cdot}05$.

Results

Transfer of IC from erythrocytes to mononuclear cell monolayers

Erythrocytes bearing fluorescently labelled HAGG were perfused over monocyte or macrophage monolayers at 0·55 ml/min. Under these conditions uptake of HAGG could be demonstrated by confocal microscopy, confirming IC transfer (Fig. 1). The mean percentage of monocytes staining positive for HAGG was 28·60 ± 1·57%, compared to 43·20 ± 3·77% on monocyte-derived macrophages ($P = 0{\cdot}032$), corresponding to a mean fluorescence per field of view of 11·82 ± 2·41 and 20·91 ± 4·05, respectively. The

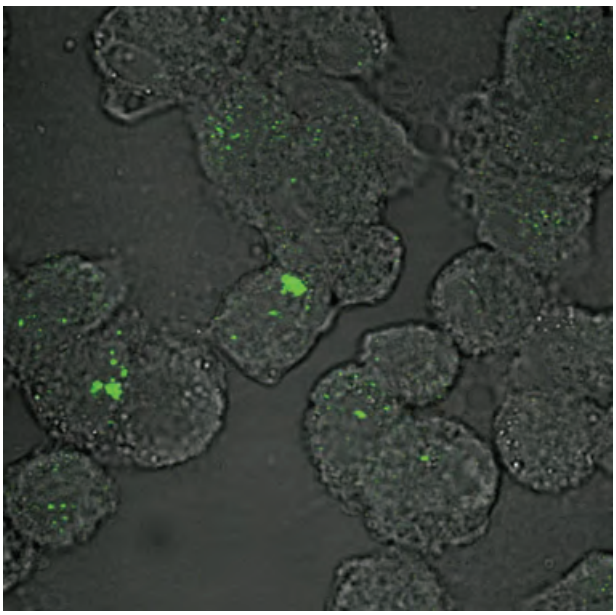

Fig. 1. Soluble immune complexes (IC) are transferred from flowing erythrocytes and internalized by monocytes. Heat-aggregated gamma globulin (HAGG) labelled with Alexa Fluor® 488 was opsonized using 20% human serum and incubated with erythrocytes for 10 min, prior to flow over monolayers of human monocytes at 0·55 ml/min. Cross-sectional confocal images are shown of the monocyte monolayer demonstrating IC that have been internalized by these cells after transfer from the flowing erythrocytes.

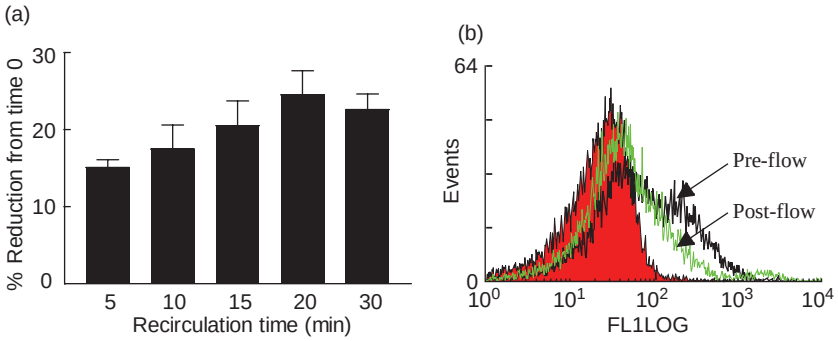

Fig. 2. Transfer of fluorescently labelled immune complexes (IC) from erythrocytes to mononuclear phagocytes flow conditions. The flow chamber system was adapted to allow continuous recirculation of erythrocytes bearing IC over monocyte monolayers. (a) Soluble IC were lost progressively from erythrocytes during recirculation: erythrocytes at a 1% haematocrit bearing opsonized heat-aggregated gamma globulin (HAGG) were recirculated over monocyte monolayers at 0·55 ml/min for 30 min. Aliquots were removed at intervals and stained using a fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody and analysed by flow cytometry. Results are expressed as the percentage reduction in the number of cells positive for HAGG relative to the number positive prior to flow (time 0). (b) A representative fluorescence activated cell sorter histogram showing a reduction in fluorescent signal on erythrocytes removed after 10 min of recirculation through the flow chamber.

addition of serum to the flow medium did not affect the transfer of opsonized HAGG from erythrocytes to monocyte or macrophage monolayers.

A small decrease in the number of flowing erythrocytes bearing IC was detected by flow cytometry after a single passage over monocyte or macrophage monolayers. This led to the hypothesis that with continued recirculation of erythrocytes, a progressive reduction in the number of cells bearing IC may reflect transfer of the IC to the phagocytes. In order to investigate this further, the flow chamber system was adapted to allow recirculation of the flowing cells by way of a peristaltic pump. Prior to recirculation, 50·93 ± 3·17% of erythrocytes bore HAGG. Results are expressed as the percentage reduction in the number of positive cells relative to the number positive prior to flow (time 0). There was a progressive reduction in the number of erythrocytes bearing HAGG with increasing recirculation times, a plateau being reached after 20–30 min (Fig. 2a,b). As illustrated, loss of HAGG from the flowing erythrocytes was rapid, with a 15·10 ± 1·01% reduction in the number of positive cells after 5 min of recirculation, consistent with transfer of the IC to the monocyte monolayer. A reduction in the number of erythrocytes bearing HAGG was not seen when cells were recirculated in the absence of monocytes or macrophages in the flow chamber.

We also explored fluid phase uptake of HAGG by monocyte and macrophage monolayers. Experiments were repeated using the same quantity of opsonized, labelled HAGG (2 μg), either bound to erythrocytes or in the fluid phase. The amount of HAGG bound to the erythrocytes was estimated using a radioligand binding assay, as previously described [9]. The percentage of monocyte-derived macrophages positive for HAGG was greater when HAGG was presented bound to erythrocytes, compared to presentation in the fluid phase (36·81 ± 5·73 *versus* 26·51 ± 6·35%; mean fluorescence intensity (MFI) 17·14 ± 3·10 *versus* 6·64 ± 1·42; $P = 0·013$). Similar results were obtained in parallel studies in which preblockade of erythrocyte CR1 was performed, which effectively also leads to delivery of IC in the fluid phase (data not shown).

Effect of Fcγ and complement receptor blockade on IC uptake and transfer

To investigate the role of individual phagocytic receptors in the uptake of IC transferred from erythrocytes, monocyte or macrophage monolayers were preincubated with specific neutralizing antibodies prior to exposure to HAGG. In addition to blockade of individual Fcγ and complement receptors, preincubation of macrophages with unlabelled HAGG at 100 μg/ml was used to occupy all Fcγ receptors prior to exposure to IC-bearing erythrocytes. Blockade of either FcγRIIa or FcγRIII on the macrophage monolayer significantly reduced the percentage of cells positive for fluorescently labelled HAGG after transfer from erythrocytes (Fig. 3). This was also observed with IC uptake quantified in terms of mean fluorescence per field of view (MF media 21·23 ± 3·89 *versus* FcγRIIa 10·77 ± 2·42 *versus* FcγRIII 11·44 ± 1·26; $P = 0·042$). Blockade of FcγRI had little effect on transfer, while blockade of either FcγRIIa or FcγRIII on the macrophage reduced transfer, each to a similar degree to that seen with unlabelled HAGG. Blockade of CR4 ($\alpha_x\beta_2$ integrin) led to a significant reduction in the transfer of the IC from erythrocytes to macrophages under flow conditions (Fig. 3). Blockade of the C3b binding site using the Arg–Gly–Asp tripeptide, or blockade of CR1 or CR3 ($\alpha_m\beta_2$ integrin) alone did not appear to reduce transfer of HAGG from erythrocytes. Studies were also performed with irrelevant subclass and isotype-matched control antibodies. These had minimal effects on the transfer process in this model, being

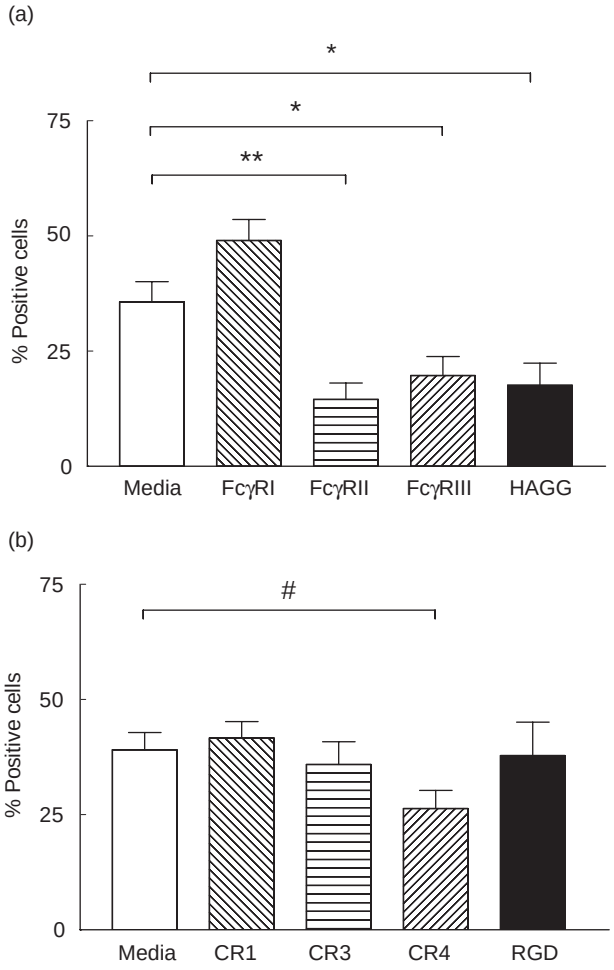

Fig. 3. Blockade of FcγRIIa, FcγRIII or CR4 reduces uptake by macrophages of immune complexes (IC) transferred from erythrocytes under physiological flow conditions *in vitro*. Heat-aggregated gamma globulin (HAGG) labelled with Alexa Fluor® 488 was opsonized and bound to erythrocytes. These were perfused at 0·55 ml/min over macrophage monolayers that had been pretreated specific neutralizing antibodies against (a) FcγRI, RIIa and RIII or with unlabelled HAGG, or (b) against CR1, CR3 or CR4 or with the RGD tripeptide. Uptake of labelled HAGG by the macrophages was quantified by confocal microscopy in terms of the percentage cells positive for HAGG. $^{*}P = 0{\cdot}015$ $^{**}P = 0{\cdot}003$ $\#P = 0{\cdot}033$.

comparable to the effect of preincubation with media alone (data not shown).

Similar experiments were performed to explore the uptake of HAGG from the fluid phase by both monocytes and monocyte-derived macrophages. Uptake of HAGG from the fluid phase by human monocytes was significantly reduced by blockade of FcγRIIa (MF media $22{\cdot}97 \pm 4{\cdot}67$ *versus* FcγRIIa $6{\cdot}88 \pm 0{\cdot}83$; $P = 0{\cdot}002$). Uptake of HAGG from the fluid phase by monocyte-derived macrophages was also reduced by blockade of FcγRIIa, but not by blockade of either FcγRI or RIII. Uptake of fluorescently labelled opsonized HAGG by monocytes was reduced by blockade of CR3 (MF $15{\cdot}26 \pm 3{\cdot}95$ *versus* $9{\cdot}69 \pm 1{\cdot}76$; $P = 0{\cdot}028$), but not by blockade of CR1 or CR4. Similarly, blockade of CR3 led to a reduction in uptake of HAGG by monocyte-derived macrophages (MF $12{\cdot}88 \pm 1{\cdot}97$ *versus* $8{\cdot}64 \pm 1{\cdot}12$; $P = 0{\cdot}028$).

Effect of IC size on the transfer reaction

Previous studies have shown that model IC formed at differing antigen to antibody ratios are handled differently *in vivo* in normal human subjects and in patients with SLE [8,9]. For example, small HBsAg/anti-HBsAg IC that fix complement poorly are cleared mainly by the liver in normal subjects and in patients with SLE by Fc-dependent mechanisms [9]. We therefore explored the effect of size, and hence complement-activating ability, of model IC on their transfer from erythrocytes to monocyte-derived macrophages under physiological flow conditions *in vitro*. HBsAg/anti-HBsAg IC were formed at the following antigen to antibody ratios: 1 : 4, 1 : 1 and 4 : 1. The ability of ^{125}I-labelled HBsAg/anti-HBsAg IC to activate complement and bind to erythrocyte CR1 was assessed using a radioimmunoassay, as described previously [9,25]. Their size was estimated using sucrose density gradient centrifugation. Erythrocytes bearing HBsAg/anti-HBsAg IC labelled with Alexa Fluor® 488 were perfused over macrophage monolayers with uptake of the IC quantified by confocal microscopy. Transfer of larger HBsAg/anti-HBsAg IC formed at an antigen to antibody ratio of 1 : 4 from erythrocytes to macrophage monolayers was greater than transfer from smaller complexes (Fig. 4a). This was also evident when transfer was quantified in terms of mean fluorescence per field of view (data not shown).

The effect of complement receptor blockade on the transfer reaction was also assessed using HBsAg/anti-HBsAg IC as the probe. We hypothesized that complement receptor blockade on the macrophage would have a greater effect on the uptake of larger HBsAg/anti-HBsAg IC (i.e. with a lower antigen: antibody ratio) than on the uptake of smaller complexes. Fluorescently labelled HBsAg/anti-HBsAg IC were opsonized using 20% human AB^{+} serum and bound to erythrocytes, before being perfused over macrophage monolayers preincubated with neutralizing antibodies against CR3 and CR4. CR1 on the macrophage had little effect on the transfer reaction using HAGG as the probe, and was therefore not studied in these experiments. Transfer of larger HBsAg/anti-HBsAg IC with an antigen : antibody ratio of 1 : 4 was greater than that of smaller complexes (Fig. 4b). As with the uptake of HAGG transferred from erythrocytes, transfer of HBsAg/anti-HBsAg IC was reduced by blockade of CR4 on the macrophage, although this was observed only with the larger complexes. This would be consistent with the presence of more C3b in the complex. In contrast to HAGG, blockade of CR3 also reduced transfer of these complexes, but only with those formed at an antigen: antibody ratio of 1 : 4.

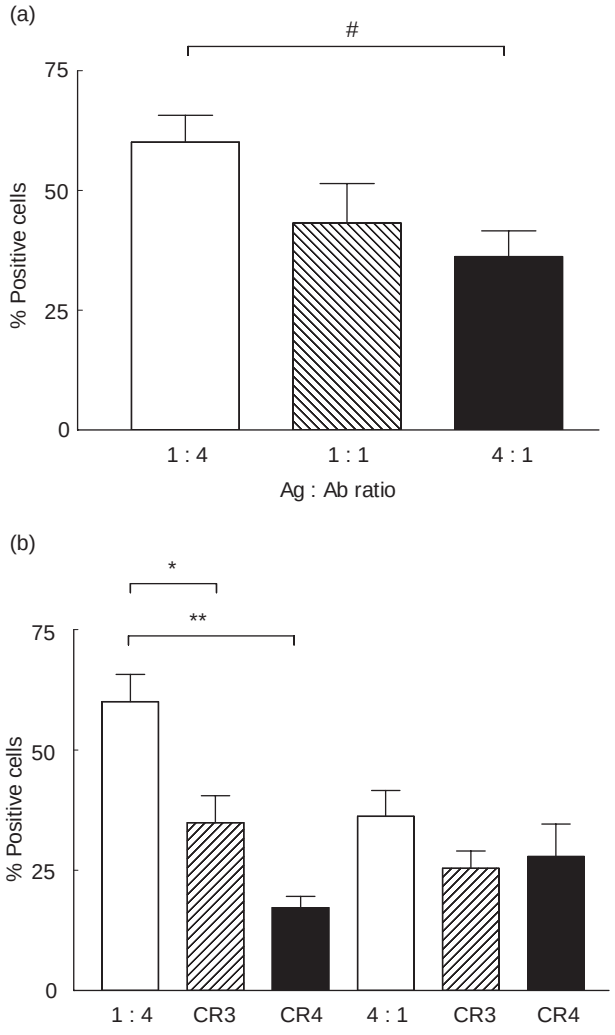

Fig. 4. Larger immune complexes (IC), formed at a lower antigen : antibody ratio, are transferred from erythrocytes to macrophages more efficiently than smaller complexes and their uptake is inhibited by blockade of CR4 on the macrophage. (a) Fluorescently labelled hepatitis B surface antigen (HBsAg)/anti-HBsAg IC were formed at varying antigen : antibody ratios, opsonized using human serum and bound to erythrocytes. These were perfused over monocyte-derived macrophages, with uptake by measured by confocal microscopy. (b) Erythrocytes bearing fluorescently labelled HBsAg/anti-HBsAg IC were perfused over macrophage monolayers that had been pretreated with neutralizing antibodies against complement receptor type 3 (CR3) or CR4. IC were formed at antigen : antibody ratios of either 1 : 4 or 4 : 1 and their uptake by the macrophages was quantified by confocal microscopy in terms of the percentage cells positive for the IC. $*P = 0{\cdot}028$ $**P = 0{\cdot}003$ $\#P = 0{\cdot}009$.

Adhesive interactions between IC-bearing erythrocytes and macrophage monolayers

The interaction between flowing erythrocytes and monocyte or macrophage monolayers was similar to that seen between leucocytes and endothelial cell monolayers [16,17]. The interaction *in vitro* between erythrocytes and cell monolayers has been studied previously in two situations. First, erythrocytes infected with *Plasmodium falciparum* have been shown to roll on and adhere to human umbilical vein endothelial cells (HUVEC), in an interaction mediated by CD36 and ICAM-1 on the endothelial cell [29] and by *P. falciparum* erythrocytes membrane protein I (PfEMPI) on the erythrocyte [30]. Secondly, a parallel-plate flow chamber assay has been used to demonstrate rolling of erythrocytes from patients with sickle cell disease on HUVEC and on microvascular endothelial cell monolayers [31]. It follows that erythrocytes bearing IC may interact directly with adhesion receptors on the monocyte or macrophage surface in the flow chamber assay. The data above suggest that CR4 has a role in the uptake of IC transferred from erythrocytes under flow conditions, while CR3 is involved in IC uptake from the fluid phase. The parallel-plate flow chamber assay was therefore adapted to investigate the adhesive interactions between IC-bearing erythrocytes and mononuclear phagocytes under physiological flow conditions.

Erythrocytes with or without HAGG were fluorescently labelled with CFSE and perfused over monocytes or macrophage monolayers at 0·55 ml/min. Significantly more rolling interactions were seen between erythrocytes bearing HAGG and monocyte monolayers than with erythrocytes alone (data not shown). Similar results were observed with macrophage monolayers. The number of arrested cells was also higher with erythrocytes bearing HAGG compared to erythrocytes alone. This was observed with monocyte monolayers (30 ± 8 *versus* 7 ± 3, $P = 0{\cdot}016$), although just failed to reach significance with macrophage monolayers (40 ± 14 *versus* 15 ± 1, $P = 0{\cdot}057$). Thus the number of adhesive interactions between erythrocytes and human mononuclear phagocyte monolayers was increased by the presence of opsonized IC on the surface of the erythrocyte, with similar numbers of interactions with macrophages compared to monocytes.

Effect of Fcγ and complement receptor blockade on the adhesive interaction between IC-bearing erythrocytes and monocyte or macrophage monolayers

To explore a role for complement receptors in mediating adhesion between IC-bearing erythrocytes and mononuclear phagocytes, the latter were preincubated with specific neutralizing MoAb against CR1, CR3 and CR4 prior to exposure to fluorescently labelled erythrocytes bearing HAGG opsonized using human serum. Blockade of CR3 led to a significant reduction in the number of IC-bearing erythrocytes rolling on macrophage monolayers (Fig. 5a). Blockade of CR3 also led to a reduction in the number of arrested cells (Fig. 5b). Blockade of CD36 or CD54 led to no detectable effect on any of the quantified interactions (monocytes, rolling cells: 24 ± 3 and 28 ± 5 *versus* 20 ± 3 cells/mm^2; arrested cells: 32 ± 3 and 29 ± 5 *versus* 29 ± 9 cells/mm^2, respectively). Blockade of either FcγRIIa or FcγRIII (using MoAb IV.3 and 3G8, respectively) led to a reduction in the number of

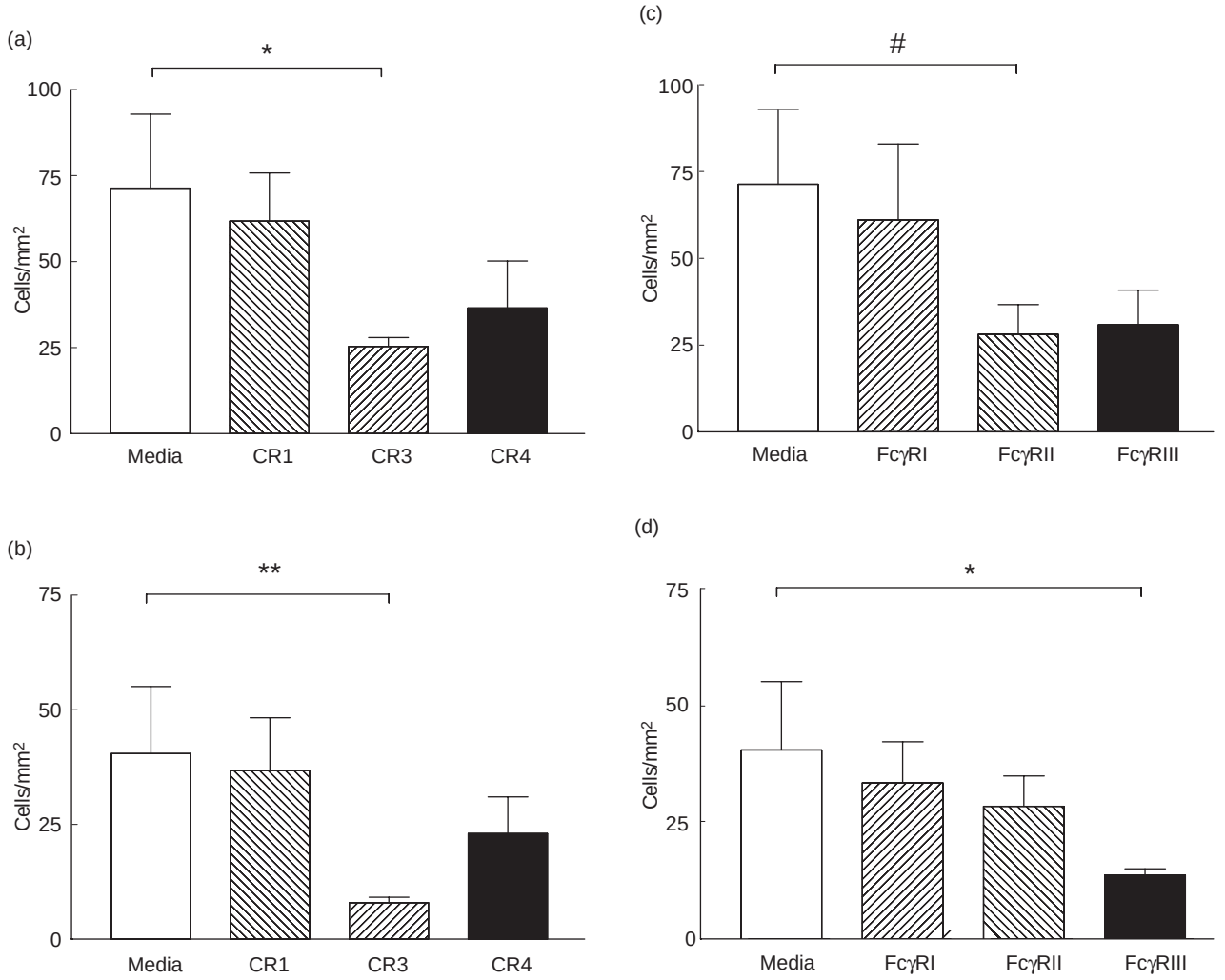

Fig. 5. Blockade of either CR3, FcγRIIa or FcγRIII reduces the number of adhesive interactions between erythrocytes bearing immune complexes (IC) and macrophage monolayers. Erythrocytes were incubated with opsonized heat-aggregated gamma globulin (HAGG), fluorescently labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and perfused over macrophage monolayers that had been preincubated with neutralizing antibodies against (a) and (b) complement receptor type 1 (CR1), CR3 and CR4 or (c, d) FcγRI, FcγRIIa and FcγRIII, or media containing control Ig. The number of (a) and (c) rolling or the number of (b, d) arrested cells in 10 random fields was quantified using Cell Motion Analysis software. Values are means ± s.e.m. ($n = 3$ independent experiments). $^*P = 0{\cdot}028$ $\#P = 0{\cdot}016$ $^{**}P = 0{\cdot}003$.

erythrocytes rolling on both monocyte and macrophage monolayers (Fig. 5c). However, only blockade of FcγRIII reduced the number of arrested cells (Fig. 5d). Blockade of FcγRI had no effect on either type of interaction.

Effect of the R131/H131 polymorphism of FcγRIIa on IC uptake by macrophages

Neutrophils from individuals homozygous for R131 phagocytose IgG_2-coated erythrocytes less efficiently than those from individuals homozygous for H131 [13], suggesting that the H131 variant of the receptor may be important in the clearance of IgG_2-containing IC. Further, the clearance of IgG-coated erythrocytes is prolonged in patients with SLE who are homozygous for the R131/H131 polymorphism of FcγRIIa [32]. We therefore investigated whether a functional effect of this polymorphism could be demonstrated using the *in vitro* flow chamber model. Genomic DNA was isolated from whole blood samples obtained from 24 normal donors and genotyping was performed using an allele-specific PCR [28]. Eight individuals were heterozygous for the polymorphism (33%), while 6/24 (25%) were homozygous for H131 and 10/24 (42%) homozygous for R131.

To investigate the effect of the R131/H131 polymorphism of FcγRIIa on the uptake of soluble IC by macrophages, peripheral blood monocytes were obtained from these donors and differentiated into macrophages. Macrophage monolayers were then exposed to the following soluble IC, fluorescently labelled with Alexa Fluor® 488: HBsAg/anti-HBsAg (IgG_1 isotype; antigen to antibody ratio of 1 : 4) and

heat-aggregated human IgG_2. These complexes were opsonized and perfused at 0·55 ml/min over the monolayers at a concentration of 10 μg/ml with uptake quantified by confocal microscopy. There was significantly less uptake of aggregated IgG_2 by monocyte-derived macrophages from individuals homozygous for R131 compared to those homozygous for H131 (Fig. 6a). Intermediate uptake was seen by macrophages from H131/R131 heterozygotes. However, no difference was seen in the uptake of HBsAg/anti-HBsAg IC (which contain mainly IgG_1 antibodies) by macrophages from individuals homozygous for R131 compared to those from H131 homozygotes. The same results were seen when uptake was quantified in terms of the mean fluorescence per field of view (Fig. 6b).

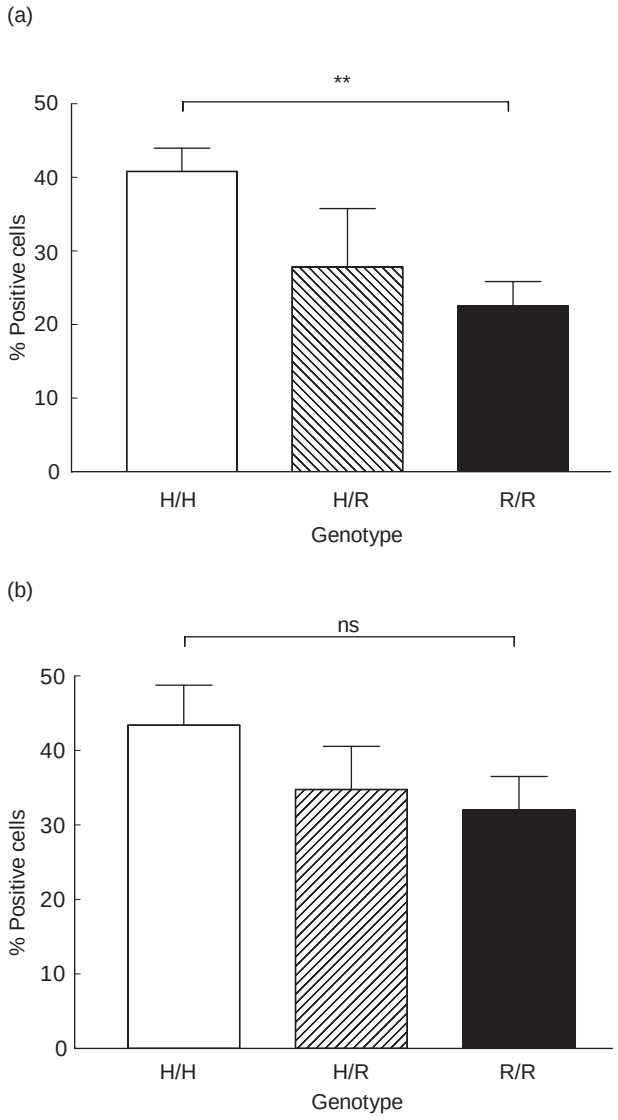

Fig. 6. Uptake of IgG_2 but not IgG_1-containing soluble immune complexes (IC) is reduced by macrophages from individuals homozygous for the R131 allelic variant of FcγRIIa. Monocytes were isolated from the peripheral blood of healthy individuals who had been genotyped for the R131/H131 polymorphism of FcγRIIa. These cells were differentiated into macrophages *in vitro* to form monolayers and then exposed to either fluorescently labelled (a) aggregated human IgG_2 or (b) hepatitis B surface antigen (HBsAg)/anti-HBsAg IC (which contain mainly IgG_1 antibodies) in the fluid phase under physiological flow conditions in the parallel-plate flow chamber. Uptake was quantified by confocal microscopy in terms of the number of the number of cells positive for IC per field of view. $^{**}P = 0{\cdot}001$.

Discussion

We have developed a novel *in vitro* model which allows investigation of the cellular mechanisms involved in the transfer of soluble IC from erythrocytes to macrophages under physiological flow conditions. Uptake by monocyte or macrophages of fluorescently labelled IC, presented bound to erythrocytes, has been demonstrated using confocal microscopy. This supports data from numerous *in vivo* studies in man and non-human primates which have demonstrated that erythrocytes are important in the clearance of IC, not only for their transport to the liver and spleen but also in the transfer reaction itself.

As an alternative method of detecting IC transfer, loss of HAGG from erythrocytes was measured using flow cytometry. This method, with or without the additional detection of uptake of the IC by phagocytes, has been used by others [4,33]. A progressive reduction in IC binding to erythrocytes was detected when these cells were recirculated over monocyte or macrophage monolayers. These experiments confirmed the rapid nature of the transfer process, with loss of HAGG detectable at 5 min and peaking at 20 min, and uniquely allowed *in vitro* modelling of the removal of IC from the erythrocyte surface by mononuclear phagocytes under flow conditions, akin to that seen *in vivo*. Because the transfer reaction could not be visualized directly during flow using the apparatus available, the rate of transfer and subsequent internalization of the IC could not be measured accurately. However, IC were clearly demonstrated within monocytes or macrophages when the cells were fixed, with the majority of the HAGG being present within the cell at this time-point. Fixing was carried out after a mean exposure time of the monolayer to the IC (either presented bound to erythrocytes or in the fluid phase) of 9 min, suggesting that transfer and internalization of the complex was rapid. Previous *in vitro* studies under static conditions have also suggested that transfer of model IC from erythrocytes to monocytes in rapid [3,4]. Subsequent time-lapse studies also confirm transfer times in the order of 5–10 min [33]. However, subsequent internalization was a slower process (30–60 min). This difference in internalization rate may have been due to the cell lines employed, the nature of the probe or the relative numbers of IC and cellular constituents in the model systems. However, it may also indicate that shear stresses induced by the flow conditions present in the current work affect internalization of IC by the phagocyte.

Recirculation experiments have also demonstrated that a proportion of IC clearly remain bound to erythrocytes at 9–10 min. This might suggest that those IC that are transferred to the adhered mononuclear cells may represent a more labile subfraction of the IC population. Complete transfer is unlikely to occur in the relatively short exposure time of 9 min, at the cell densities used in this model. Saturation of acceptor sites on the phagocyte monolayer is therefore unlikely to occur.

The studies described herein have also delineated the relative contribution of individual Fcγ and complement receptors in the uptake of soluble IC by mononuclear phagocytes under physiological flow. The phagocytic receptors involved in the uptake of soluble IC from the fluid phase have been investigated in one previous study [3]. Here, the uptake of opsonized HAGG from solution by monocytes under static conditions was reduced only by blockade of CR1, using the MoAb 3D9. Blockade of CR3 had no effect, while blockade of FcγRIIa had only a minor effect on uptake. The relative contribution of IC uptake from the fluid phase compared to that transferred from erythrocytes remains unclear, but is likely to be small and relevant only for small, poorly opsonized IC [9]. Therefore, in the present work we have chosen to focus on the phagocyte receptors involved in the IC transfer reaction rather than those required for the uptake of IC from the fluid phase.

Blockade of FcγRIIa on macrophages in our model reduced the uptake of HAGG that had been transferred from flowing erythrocytes. In addition, a role for FcγRIII was found. This supports evidence from *in vivo* studies in chimpanzees, in which blockade of this receptor using the same antibody (3G8) led to a reduction in the clearance of preformed dsDNA/anti-dsDNA IC [34]. However, blockade of FcγRI on the macrophage did not appear to affect transfer of HAGG in this flow model. This is in contrast to previous studies, in which blockade of FcγRI inhibited transfer of dsDNA-based heteropolymer complexes or dsDNA/anti-dsDNA IC from erythrocytes to U937 cells under static conditions [4]. Blockade of FcγRIIa or RIII had no effect on transfer to U937 cells in this study, although blockade of transfer was weakly inhibited by one of three MoAbs against CR1 (7G9). While their phenotype is broadly similar, U937 cells differ from mononuclear phagocytes with respect to the expression of Fcγ and complement receptors [35], making direct comparisons difficult. Their use in *in vitro* models of IC processing may therefore parallel less closely physiological IC clearance *in vivo*. The importance of FcγRI in the clearance of soluble IC is also questioned by the subsequent observation that only 20% of transferred dsDNA-based heteroplymer complexes are seen to co-localize with this receptor after transfer to THP-1 cells [33]. Comparison with these studies is, of course, limited further by their use of differing probes, which may subtly affect the route of uptake of the IC. This may be particularly true of heteropolymer complexes. These contain a mouse anti-human CR1 antibody rather than C3b, and may be less dependent on complement receptors on the phagocyte for their uptake.

With respect to the role of complement receptors on the macrophage in the transfer reaction, only blockade of CR4 led to a significant inhibition of transfer. This was a novel and somewhat surprising finding, as a role for this receptor in the clearance of soluble IC in humans has not been defined previously. Binding of opsonized particles to CR4 on macrophages is mediated via its ligand iC3b, so it follows that opsonization of soluble IC by complement may lead to their uptake via this receptor. However, blockade of CR1 or CR3, failed to significantly inhibit transfer of HAGG opsonized with complement in the flow chamber model, although blockade of CR3 in combination with blockade of FcγRIIa reduced transfer. Earlier studies also failed to demonstrate a role for CR3 in the transfer of HAGG from erythrocytes to monocytes, although CR1 appeared to be important [3]. It is clear that the relative importance of CR3 and CR4 in different model systems will be influenced by the incorporation of complement components into the IC during incubation with serum, and subsequently during binding to erythrocytes. The proportion of these ligands present in the complexes would then influence binding to these CR, as suggested by our data using HBsAg IC.

The role of FcγRIIa in the clearance of soluble IC by macrophages was investigated by exploring the effect of the R131/H131 polymorphism of the receptor. Reduced uptake from the fluid phase of aggregated human IgG_2 by macrophages from normal donors homozygous for the R131 variant of the receptor was observed, while no difference was seen in the uptake of HBsAg/anti-HBsAg IC, these being of the IgG_1 isotype. This difference was consistent with the observations of Salmon *et al.* [13], who demonstrated reduced phagocytosis of IgG_2 coated erythrocytes by neutrophils from individuals homozygous for R131. These *in vitro* observations support the hypothesis that there may be impaired clearance of potentially pathogenic IgG_2-containing IC in patients with SLE, in whom R131 homozygosity is, in certain populations, more frequent. No other *in vitro* models have addressed this question specifically. It should be noted, however, that the previous studies used neutrophils and a particulate rather than soluble IC, making direct comparison with the present work difficult [13]. A weakness of the present work is that two different probes were used, which may differ in more than just their respective IgG isotype. An alternative approach would be to use HAGG, which contains predominantly human IgG_1. However, HBsAg/anti-HBsAg IC contain a purely IgG_1 polyclonal antibody. Further, it is likely that these model soluble IC are cleared by broadly the same mechanisms compared to particulate IC, with Fcγ receptor polymorphisms influencing the degree of binding and internalization.

The parallel-plate flow chamber assay has uniquely allowed the investigation of the role of Fcγ and complement receptors in the adhesion of IC-bearing erythrocytes to

mononuclear phagocyte monolayers under flow conditions. Direct visualization of the transfer reaction has shown that the IC itself is likely to mediate this interaction via CR1 on the erythrocyte [33], although the receptor(s) involved on the phagocyte remain unclear. It is also unclear whether the receptors mediating this adhesive link are the same under static conditions to those mediating the interaction under flow. We hypothesized that blockade of specific complement receptors on the phagocyte would lead to a reduction in the adhesive interactions between IC-bearing erythrocytes and mononuclear phagocyte monolayers. In contrast to the data on the transfer of HAGG from erythrocytes to phagocytes under flow, these adhesion experiments demonstrated that CR3 on the latter rather than CR4 was more important with respect to the adhesion of erythrocytes bearing opsonized HAGG. Blockade of CR3 on human macrophages led to a significant reduction in both the interactions that were quantified. CR3 is likely to mediate, via C3b on the IC, adhesion of erythrocytes bearing HAGG to the macrophage, while CR4 is required for internalization of the complex with a less important adhesive function in the transfer reaction.

Blockade of FcγRIIa and RIII, but not FcγRI, on monocyte or macrophage monolayers also led to a reduction in the number of adhesive interactions with erythrocytes bearing HAGG. This is consistent with the hypothesis that adhesion may be mediated in part by ligation of these receptors via the Fc portion of IgG within the complex. Blockade of FcγRIIa had greatest effect on the number of rolling interactions, while blockade of FcγRIII significantly reduced the number of arrested cells. These observations may be explained by the co-operation that occurs between Fcγ and complement receptors in cell membranes [36,37]. In dual blockade experiments, the maximum reduction in the number of adhesive interactions was seen with blockade of CR3 and FcγRIII or CR3 and FcγRIIa. Co-operation between CR3 and Fc gamma RIIa during adhesion has been observed previously. Adhesion of CR3-transfected K562 cells (which endogenously express FcγRIIa but not CR3) to fibrinogen, was inhibited by cross-linking FcγRIIa using MoAb IV.3 [38]. Co-operation between CR3 and FcγRIIIb in neutrophil membranes has also been observed, but co-operation between CR3 and FcγRIIIa in an adhesive interaction in human monocytes has not been described. In contrast to interactions between sickle erythrocytes [31] or erythrocytes infected with *P. falciparum* and endothelial cell monolayers [29,30], adhesion of IC-bearing erythrocytes to mononuclear phagocyte monolayers was not influenced by blockade of either CD36 or ICAM-1. This was not unexpected, as the ligand mediating these interactions (i.e. the IC bearing C3b) would not be expected to interact with these receptors.

On the basis of these observations, we propose a series of reactions occurring under physiological flow between erythrocytes bearing opsonized soluble IC and monocyte or macrophage monolayers. In this model, IC-bearing erythrocytes roll on the monolayer, in a reaction mediated largely by FcγRIIa and RIIIa, with firm arrest prior to transfer being mediated by CR3 and FcγRIIIa. Uptake and internalization of the IC is then mediated by FcγRIIa, FcγRIIIa and CR4. Proteolytic cleavage of CR1 on the erythrocyte is a possible mechanism by which transfer is facilitated [4], leaving a 'stump' of CR1 on the erythrocyte surface. However, it is by no means proven that proteolytic cleavage is the only mechanism whereby IC transfer takes place, and there is a strong body of evidence that a mechanism involving membrane vesicles may be important [39,40]. Indeed, it has been postulated that whole patches of CR1-bearing membranes in the form of vesicles may be lost in the process. It is highly unlikely that IC simply dissociate from the erythrocyte during transfer, this being a very slow process [41]. Similarly, processing of the IC by Factor I is unlikely to play a significant part, as IC bearing C3dg are less likely to bind to the phagocyte. Our observation that the addition of serum, a potential source of Factor I, to the flow medium did not alter IC uptake supports this view.

Is the adhesion of erythrocytes via opsonized IC to mononuclear phagocytes likely to be important in the clearance of IC *in vivo*? It seems entirely plausible that some form of adhesive interaction occurs in the hepatic sinusoid or splenic sinus prior to transfer of the complex, as direct visualization of the transfer reaction has demonstrated strong linkage of erythrocytes and mononuclear cells via the IC without free IC being seen [33]. An IC coated with C3b and bound to erythrocyte CR1 is a ligand for several receptors on the phagocyte that are capable of mediating adhesion, either alone or by co-operating with other adhesive molecules in the cell membrane. Adhesion via the IC will bring the two cells within close proximity in order to maximally facilitate transfer. This hypothesis must, however, be considered in the context of the anatomy of the hepatic and splenic microvasculature. In contrast to the post-capillary venule, modelled extensively in relation to the inflammatory adhesion cascade, the hepatic sinusoid is short and has a variable diameter [42]. Further, its surface structure is probably less confluent, with interspersing Kupffer and endothelial cells and this may make flow somewhat turbulent. Thus, the rolling–arrest–transfer interaction may not truly occur *in vivo*, with simple trapping of the IC-bearing erythrocyte during its course through the sinusoid being a more common form of interaction. This is particularly likely to be the case in the spleen. However, studies of leucocyte migration to the liver have demonstrated a similar adhesion cascade in the sinusoid compared to that seen in the post-capillary venule [43,44]. The contribution of the hepatic sinusoidal endothelial cell in IC clearance must also be considered. These cells also express FcγRII and RIII, and are capable of ingesting IC *in vivo* [45,46]. However, the *in vivo* importance of these alternative mechanisms has not, to date, been demonstrated experimentally in humans.

Finally, the importance of the IC itself must also be considered when interpreting our results. Although broadly

similar mechanisms are likely to be involved in the uptake by macrophages of most types of soluble IC transferred from erythrocytes, differences between model IC and those found *in vivo* might influence the precise nature of the cellular interactions involved. For example, the ratio of Ig *versus* complement content, the resultant lattice structure or the nature of the antigen component itself may influence the receptors that are engaged on the phagocyte.

In conclusion, we have developed a novel human *in vitro* model to investigate the transfer of soluble IC from erythrocytes to mononuclear phagocytes. Our data support the notion that erythrocytes and their membrane receptor CR1 are likely to be important in IC transfer to macrophages, as suggested in previous *in vitro* and *in vivo* studies. More importantly, we have elucidated the role of individual receptors on the phagocyte in the uptake of IC transferred from the erythrocyte, and have demonstrated a functional effect of the R131/H131 polymorphism of FcγRIIa on the transfer reaction. This fully humanized model may now allow further elucidation of the abnormalities in this important process in patients with IC-mediated diseases such as SLE.

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