The International Journal of Transfusion Medicine
Vox Sanguinis

Vox Sanguinis (2009) **97**, 338–347

DOI: 10.1111/j.1423-0410.2009.001214.x

ORIGINAL PAPER

A pair of naturally occurring antibodies may dampen complement-dependent phagocytosis of red cells with a positive antiglobulin test in healthy blood donors

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Vox Sanguinis

Received: 4 April 2009,
revised 4 June 2009,
accepted 5 June 2009,
published online 30 June 2009

Background and Objective It is known that red blood cells (RBC) from healthy blood donors with a positive direct antiglobulin test (DAT) for IgG continue to circulate despite carrying elevated numbers of IgG molecules. To unravel the properties of these RBC-bound IgG, we studied them not only on whole RBC populations, but also on density-fractionated RBCs.

Materials and Methods The properties of acid-eluted RBC-bound IgG and plasma IgG were studied by ELISA for binding to RBC proteins and opsonins, and by blotting. *In vitro* phagocytosis was studied on density-separated RBCs.

Results IgG-DAT-positive blood donors carried most IgG molecules on dense RBCs and had more RBCs of high density than DAT-negative controls. Their densest RBCs were older than the oldest RBCs of DAT-negative controls, based on the band 4·1a/b ratio. *In vitro* phagocytosis of senescent RBCs from IgG-DAT-positive donors was 1·5 to 2 fold higher than that of senescent control cells, but the same or less in the presence of physiological IgG concentrations, implying that RBC-bound IgGs impaired complement-dependent uptake. The IgG molecules on these DAT-positive RBCs comprised anti-band 3 naturally occurring antibodies (NAbs) and were two- to fivefold enriched in anti-C3 and framework-specific anti-idiotypic NAbs as compared to controls. Correspondingly, anti-C3 and framework-specific anti-idiotypic NAbs were proportionally elevated in the plasma of two-thirds of DAT+ donors.

Conclusions Extra-binding of anti-C3 together with anti-idiotypic NAbs to senescent RBC-associated C3 fragments may suppress complement-dependent RBC phagocytosis and may prolong the *in vivo* life span of RBCs.

Key words: complement, direct antiglobulin test, naturally occurring autoantibodies, phagocytosis, red blood cells.

Introduction

One in 9000 to 14 000 healthy blood donors (HBD) [1] has red blood cells (RBC) that agglutinate in the presence of anti-immunoglobulins and thus show a positive direct antiglobulin test (IgG-DAT+). A varying number of IgG-DAT+ donors also has a positive DAT for complement C3 fragments [1,2]. IgG-DAT+ RBCs from HBD have been studied primarily by characterizing eluted IgG with regard to blood group specificity and IgG subclasses [2–4]. RBCs from these donors have two to 10 times as many IgG molecules (200 to 1000 IgG/RBC) on their surface as DAT– senescent RBCs [5]. IgG molecules eluted from RBCs of 18 of 20 IgG-DAT+ HBD inhibited binding of anti-Rhesus antibodies (anti-D, anti-E, anti-c) to homozygous antigen-positive RBCs largely irrespective of

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This work was supported by grants to HUL from the ETH (TH 22/01-1) and the SNF (31000-105831).

their antigen specificity [6]. Fifty-five per cent of these eluates agglutinated Rh_{null} RBCs, again suggesting that Rh antigens are at least not the only targets among RBC-bound IgG. Furthermore, all eluates also immunoprecipitated band 3 protein and in one case, eluted IgG reacted exclusively with band 3 [6]. It remained unclear whether immunoprecipitation of band 3 was due to the presence of anti-band 3 naturally occurring antibodies (anti-band 3 NAbs) [7] in these eluates or due to coprecipitation of Rh antigens and band 3 protein. In addition, a substantial portion of RBC-bound IgG had the properties of anti-idiotypic IgG [8].

To our knowledge this phenomenon has not been studied on young and old RBCs, despite that autologous IgG binds primarily to old RBC [9]. The majority of these IgG NAbs had anti-band 3 specificity and bound to oligomerized band 3 protein [10,11]. The acquisition of IgG NAbs on ageing RBC has since been studied in quite some detail [12,13]. It requires oxidative damage [14], haemichrome binding [15], band 3 oligomerization [14,16] and culminates in generation of $C3b_2$–IgG complexes [17]. Such complexes that carry dimeric C3b on one heavy chain [18] are preferentially formed on bound anti-band 3 NAbs [19], and act as potent precursors of the alternative C3 convertase [20]. Therefore we asked the question of whether the increased amounts of IgG molecules on IgG-DAT+ RBCs from HBD were bound to senescent cells rather than to all fractions of RBCs.

Materials and methods

DAT assay

We used the Micro-typing system (no. 50 830) by DiaMed SA, 1785 Cressier s/Morat, Switzerland.

RBC density separation

Filtered blood was centrifuged, and pelleted RBCs were density-separated as outlined [21]. Density-separated RBCs were fractionated into five or six portions numbered from top to bottom of the tube.

^{125}I-protein G binding for detection of RBC-bound IgG

Density-separated RBCs were washed three times with phosphate-buffered saline (PBS) (pH 7·4), resuspended to 2 to 3×10^9 cells/ml in PBS containing 1 g/l D-glucose, and incubated for 1 h at 0°C with an equal volume of ^{125}I-labelled protein G (8×10^6 cpm/μg) in PBS supplemented with 5 mg/ml ovalbumin. Three aliquots of this suspension were then centrifuged on a pre-cooled phthalate oil mixture (70% dibutyl phthalate and 30% dinonyl phthalate) [22] in a Beckman Microfuge (Beckman Coulter Inc., Fullerton, CA, USA). The tips of the frozen tubes, containing pelleted RBCs were cut off, cell-bound radioactivity was determined and referred to the number of RBCs in the pellet.

Elution and purification of IgG from fractionated RBCs

RBC from whole populations or density-separated RBCs were washed twice with PBS supplemented with 1 mM EDTA and 50 μg/ml phenylmethylsulfonyl fluoride (PMSF) (pH 7·4) and once with 155 mM NaCl. To elute IgG from RBCs of different cell age, 4×10^{11} washed RBCs were resuspended in 200 ml cold elution buffer (0·2 M glycine, 10 mM NaCl, osmolality 270 mOsm/kg, pH 2·5) and centrifuged for 5 min at 5800 *g*. The supernates were collected, and the pH was measured. If the pH value was higher than pH 2·7 the elution was repeated. Supernates from the first and the second elution were combined, neutralized with 1 M Tris (pH 9) containing 0·001% ovalbumin, and incubated for 1 h at room temperature with 5 M urea (f.c.) to dissociate IgG complexes [23]. This material was dialysed twice against PBS containing 0·08% azide, 50 μg/ml PMSF and 0·05% Tween 20 (pH 7·4) at 4 °C and passed through 1.5 ml Protein G Sepharose 4 fast flow (GE Healthcare GmbH, Otelfingen, Switzerland) at 4°C. Eluted IgG was neutralized, dialysed against PBS containing 0·08% azide and 0·02% Tween 20 (pH 7·4), and concentrated in Vivaspin 100 000 MWCO (Vivascience, Sigma-Aldrich, St Louis, MO, USA). An aliquot of the concentrated material was ^{125}I-iodinated. Where indicated, the iodinated samples were treated with 5 M urea for 1 h at room temperature and applied to a Sephacryl 300 column (1·5 × 30 cm) (Sigma-Aldrich). The peak corresponding to IgG was pooled and dialysed against PBS.

Membrane preparations and determination of the band 4·1a/b ratio

Density-separated RBCs were washed three times, membranes prepared, and membrane proteins run on SDS PAGE as described [24]. Using Coomassie blue staining, bands 4·1a and 4·1b were quantified with a GS-800 calibrated densitometer (Bio-Rad Laboratories, Hercules, CA, USA), and the ratio 4·1a/4·1b was calculated.

Isolation and immobilization of blood components

$F(ab')_2$ fragments of pooled, whole human IgG (Sandoglobulin, ZLB, Bern, Switzerland) were prepared as described [23]. Complement C3 was isolated from plasma as described elsewhere [25,26] and C3b and iC3b were prepared according to Sahu *et al.* [27]. Proteins were coupled to Affigel 10 or, where indicated, to Affigel 15 (Bio-Rad Laboratories), by overnight

incubation at 4°C at 2–3 mg/ml. Unreacted groups were blocked with 0·1 M ethanolamine in PBS (pH 7·4) for 1 h at 4°C.

Purification of anti-C3 NAbs

Anti-C3 NAbs were purified from IVIG [23], by first depleting of anti-IgG NAbs and then passing over C3 immobilized on Affigel 15.

Radioiodination of protein G and isolated antibodies

Recombinant protein G (Sigma-Aldrich), IgGs eluted from RBCs, anti-C3 and anti-F(ab′)$_2$ were labelled with ^{125}I-iodine (GE Healthcare) as described [28].

SDS-PAGE and Western blotting

Proteins were electrophoresed on SDS-PAGE as described [29]. Electrophoretically spread proteins were either stained or transferred onto polyvinylidene fluoride membranes (ImmobilonTM-P, Millipore Corporation, Billerica, MA, USA) and incubated overnight at room temperature with 5×10^5 to 1×10^6 cpm/ml ^{125}I-iodinated proteins in Replica buffer (20 mM Tris, 150 mM NaCl, pH 7·4) supplemented with 0·5% gelatin and 0·05% Tween 20. Membranes were washed three times with the given buffer, followed by three washes with Replica buffer only. Dried membranes were exposed to a phosphor screen and scanned with a Phosphoimager Storm 820 (Molecular Dynamics, GE Healthcare). Densitometry on blots was carried out by using a GS-800 calibrated densitometer (Bio-Rad).

ELISA

Chemobond plates (Dr E. Fischer, Dübendorf, Switzerland) were covalently coated with C3b, band 3 protein or F(ab′)$_2$ [30]. In ELISA assays for anti-C3/C3b and anti-F(ab′)$_2$ the accessible portion of IgG NAbs was determined by mixing equal volumes of plasma and PBS containing 10 mM EDTA. For the total concentrations of NAbs equal volumes of plasma and 10 M urea in PBS containing 10 mM EDTA were pre-incubated for 1 h at room temperature to dissociate IgG complexes. Then, samples were diluted with PBS containing 0·1% gelatin (GPBS pH 7·4) to 0·5% plasma and added to the plates (150 μl per well) in triplicates, and incubated for 90 min at 37°C. After washing alkaline-phosphatase-conjugated, heavy chain-specific anti-human IgG (1/9000) was applied and phosphatase-cleaved p-nitrophenylphosphate determined at 405 nm on a Dynatech MR5000 microplate reader (Bioconcept, Allschwil, Switzerland). Where indicated, anti-hinge IgG NAbs were depleted from plasma by incubating 1 ml of plasma for 30 min with 0·2 ml F(ab′)$_2$–Affigel at 4°C in a batch procedure and studying the supernate by ELISA.

Phagocytosis assay

Human monocytic leukaemia cells, THP-1, were maintained in suspension at a density of $2{\cdot}0 \times 10^5$/ml to 10^6/ml in RPMI 1640 medium containing 2 mM glutamine (Invitrogen, Carlsbad, CA, USA), 10% fetal bovine serum (Sigma-Aldrich), and 1% penicillin-streptavidin at 37°C in a humidified atmosphere containing 5% CO_2. For the phagocytosis assay $0{\cdot}5 \times 10^6$ cells/well were transferred into 24-well plates (Nunclon, Sigma Aldrich) containing sterile glass coverslips. THP-1 cells were differentiated into macrophage-like cells by incubating the cells with medium supplemented with 10 nM phorbol myristic acid for 48 h [31]. Differentiated THP-1 cells not only phagocytose via Fc receptors, but also reveal a similar set of complement receptors, reflecting monocyte-derived macrophages [32]. Density-separated, washed RBCs (0·5 ml/well, 1×10^7/ml) were incubated for 1 h at 37°C with differentiated THP-1 cells at approximately 10 RBC per macrophage. Non-ingested erythrocytes were eliminated by washing and lysing. The macrophages with ingested RBC were fixed with 3% paraformaldehyde and permeabilized by 0·1% Triton X-100 in PBS (pH 7·4). Ingested erythrocytes were stained with FITC-conjugated goat anti-human haemoglobin antibody (Bethyl, Montgomery, TX, USA) in PBS containing 3% BSA, and 0·05% Triton X-for 1 h. Coverslips were washed once with PBS containing 1% BSA and 0·01% Triton X-100, and twice with PBS containing 0·05% Triton X-100, and mounted on slides for examination with an Axiovert 100 M ZEISS fluorescence microscope (Carl Zeiss AG, Feldbach, ZH, Switzerland). The number of phagocytosed, fluorescently labelled RBCs was determined in four equally sized quadrants with 100–200 THP-1 cells on each coverslip. The numbers of phagocytosed RBCs per THP-1 cell were averaged +/–1SD and *P*-values were calculated using the Student's *t*-test.

Subjects studied

Whole blood drawings of 12 HBD with a positive DAT for IgG, but not for complement were obtained from the Regional Blood Transfusion Service, SRK, Zurich, Switzerland. As controls, drawings of matched IgG-DAT– HBD were used. Blood was collected in CPDA-1-containing regular blood donation kits and freed of white cells by inline filtration using Leucoflex LST2 filters (MacoPharma, Quebec, Canada). Blood was further processed at the day of collection or the next day. Table 1 lists the characteristics of IgG-DAT+ HBD. Informed consent was obtained from all HBD.

Results

Acid eluates from unseparated RBCs from donor 35 936 (DAT+2) contained IgG molecules that bound among RBC

Table 1 Characteristics of blood donors with positive DAT[a]

Donor ID	Sex	Age at first visit	DAT for IgG at first visit	DAT for IgG at last visit	DAT for C3d	Observ. time years	Ratio of IgG binding to C3bBb/C3b	EPO U/L	EPO year	Pathology
1 1831	Male	65	2+	2+	0	5	0·61	20	2005	None
2 9558	Female	62	1+	2+	0	6	ND	ND		Crohn's disease, plasmocytoma 2007
3 12 554	Male	43	3+	1+	0	8	0·69	ND		None
4 34 230	Female	47	2+	4+	1+	11	0·81	8	2004	None, died
5 35 936	Male	58	2+	2+	0	2	ND	ND		None
6 43 120	Male	52	3+	3+	0	9	ND	8	2005	Relapsing polychondritis
7 235 017	Female	53	4+	0	0	7	0·86	ND		None
8 253 343	Male	51	1+	3+	0	10	0·87	ND		Coxarthrosis, diabetes type 2
9 275 480	Male	57	2+	1+	0	2	0·86	ND		None
10 430 097	Male	32	2+	1+	0	4	ND	7	2006	None
11 400 953	Male	56	2+	1+	0	1	ND	16	2006	None
12 443 476	Female	25	1+	1+	0	2	ND	10	2006	None

[a]DAT assays were carried out for IgM, IgG and IgA. Data shown are for IgG. None of the donors had a positive DAT for IgM or IgA. DAT assays for C3d were performed repeatedly and were always negative except for one donor. The ratio of IgG binding to C3bBb/C3b was determined by ELISA according to ref. [33] and was determined where indicated to exclude that the donors had a C3 nephritic factor. Erythropoietin (EPO) concentrations were measured where indicated and illustrate that these blood donors did not have anemia (normal range: 8–22 U/l). All blood donors were healthy at first observation. Three of them developed a disease during the follow up.

proteins primarily to band 3 and somewhat to spectrin and actin (Fig. 1a). Surprisingly the strongest binding was to C3 and C3b as well as their polypeptides. IgG binding to intact IgG was visible, but was strongest to its light chain, suggesting the presence of anti-idiotypic NAbs. The binding to C3/C3b could only in part be due to anti-band 3 NAbs that have a framework-located affinity for C3, which is about 10 times lower than that to band 3 [28]. Correspondingly, eluted IgG bound five times better to immobilized C3 than purified anti-band 3 NAbs in a RIA (not shown). Hence, these eluates not only contain NAbs to RBC constituents, but substantial amounts of anti-idiotypic and anti-C3 NAbs. Anti-C3 NAbs are normally present in human plasma and IVIG, bind to C3 as well as to C3b [23], and even to the 65 kDa fragment of inactivated C3b (iC3b) (Fig. 1b). Since C3 fragments as well as anti-band 3 NAbs have earlier been found primarily on senescent RBCs [16,17], the majority of IgG molecules in

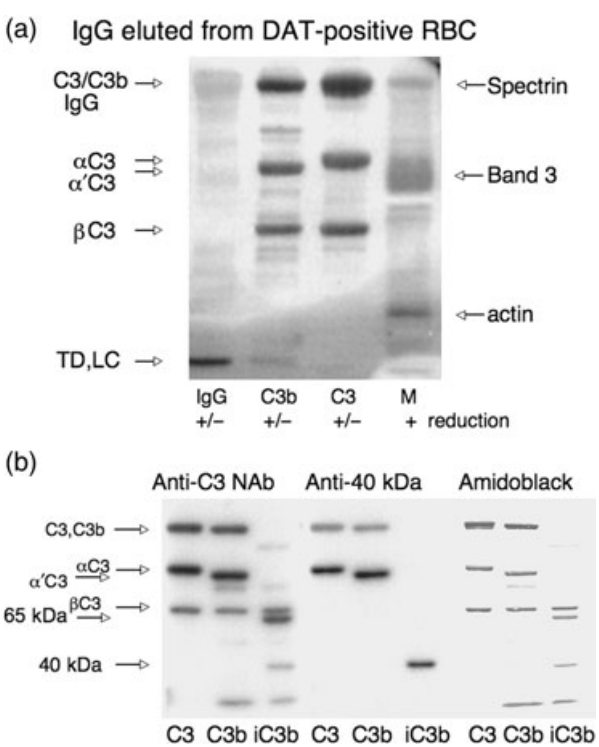

Fig. 1 (a) Binding specificity of IgG eluted from unseparated RBCs of a DAT+ HBD. (b) Comparison with the specificity of affinity purified anti-C3 NAbs. (a) IgG eluted from the whole population of RBCs from DAT+ donor 35936 was ^{125}I-iodinated and incubated with blots from SDS PAGE loaded with IgG, C3, C3b and RBC membrane proteins (M). The proteins were denatured with (+) or without reduction (–), alkylated and mixed prior to loading a total of 4 μg/lane for IgG, C3, C3b and 7 μg/lane for M. An autoradiograph is shown. TD stands for tracking dye, LC for IgG light chain. Similar results were found for eluates from two other DAT+ HBD. (b) Anti-C3 NAbs, affinity purified from IVIG and a commercially available monoclonal antibody against the C-terminal 40 kDa fragment of αC3 (Quidel, San Diego, CA, USA) were iodinated and incubated with blots obtained from SDS PAGE loaded as indicated with 1 μg/lane of reduced and unreduced and alkylated C3, C3b and 1 μg/lane of reduced and alkylated iC3b. The corresponding autoradiographs and an Amido black-stained blot are shown.

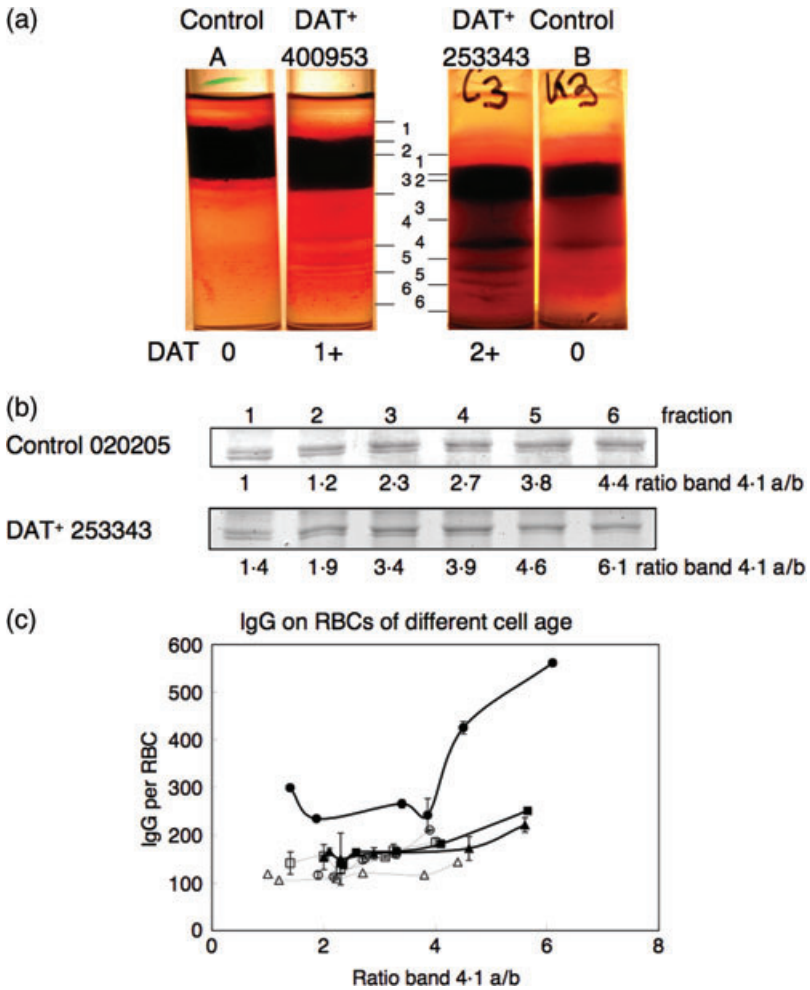

Fig. 2 RBCs from IgG-DAT+ HBD reach higher densities, are older and carry more IgG molecules than control RBCs. (a) RBCs from IgG-DAT+ HBD were density separated on Percoll gradients in two different experiments, along with RBCs from age- and sex-matched controls. The gradients were fractionated into 6 fractions. (b) Membranes were prepared from the fractionated RBCs and the band 4·1a/b ratios were determined from Coomassie blue-stained SDS PAGE, run with their reduced and alkylated membrane proteins. (c) RBC-bound IgG molecules were determined by ^{125}I-protein G binding to fractionated and washed RBCs. RBC-bound IgG (averaged data from triplicates +/– 1 standard deviation) is given as a function of the absolute cell age, represented by the band 4·1a/b ratio. The figure shows data from controls (empty symbols), and from DAT+ HBD (filled symbols): triangles, 430097, DAT+1; squares, 400953, DAT+1; circles, 253343, DAT+3.

eluates from RBCs of IgG-DAT+ HBD may originate from old rather than RBCs of any cell age. To test for this, we aimed at eluting RBCs of different cell age.

RBCs of different cell age were obtained by density centrifugation on Percoll gradients (Fig. 2a). RBCs of IgG-DAT+ HBD had more RBCs at high density than those from DAT– HBD (Fig. 2a, fractions 4–6). This phenomenon was not an effect of donor's age, because the control persons had the same age and gender as the blood donors with IgG-DAT+ RBCs, as shown for two pairs studied on different occasions. The most dense RBC fractions from IgG-DAT+ donors had a higher absolute cell age than those of controls, as judged from the band 4·1a/4·1b ratios. Oldest RBCs from IgG-DAT+1 donors contained at least as many IgG molecules as normal old RBCs and those from a IgG-DAT+3 donor significantly more (Fig. 2b,c). Thus, IgG-DAT+ RBC reached higher densities, higher absolute cell ages, and attracted more IgG NAbs than controls, but nevertheless remained in circulation.

Acid eluates were collected from density-fractionated RBCs, and IgG molecules were purified from them by adsorption to immobilized protein G. IgG from eluates of less dense RBCs of IgG-DAT+ HBD and controls bound similarly to intact IgG, less to C3, but not or very little to RBC membrane proteins and F(ab′)$_2$ (Fig. 3, younger RBCs). In contrast to this, IgG from eluates of most dense RBCs of both types of donors showed binding not only to IgG, but also to F(ab′)$_2$, C3, its polypeptides and to RBC membrane proteins, including band 3 (Fig. 2, old RBCs). Hence, the binding patterns of IgG eluted from both types of old RBCs did not differ qualitatively, suggesting that the difference between IgG from RBCs of IgG-DAT+ HBD and from DAT– HBD was quantitative or remained unrevealed because of IgG complex formation. To address this question, the same radioiodinated IgG preparations from senescent RBCs were pretreated with 5 M urea to dissociate pre-existing complexes, gel-filtered in 5 M urea to get rid of small molecular weight contaminants, and dialysed. Blots incubated with these dialysed preparations revealed a similar binding pattern for IgG-DAT+ HBDs and controls, but with significant quantitative differences (Fig. 3, after NAb dissociation). After NAb dissociation IgG binding to C3, its polypeptides, band 3, and F(ab′)$_2$ was at least twofold higher for IgG-DAT+ donors than for control RBCs (see legend to

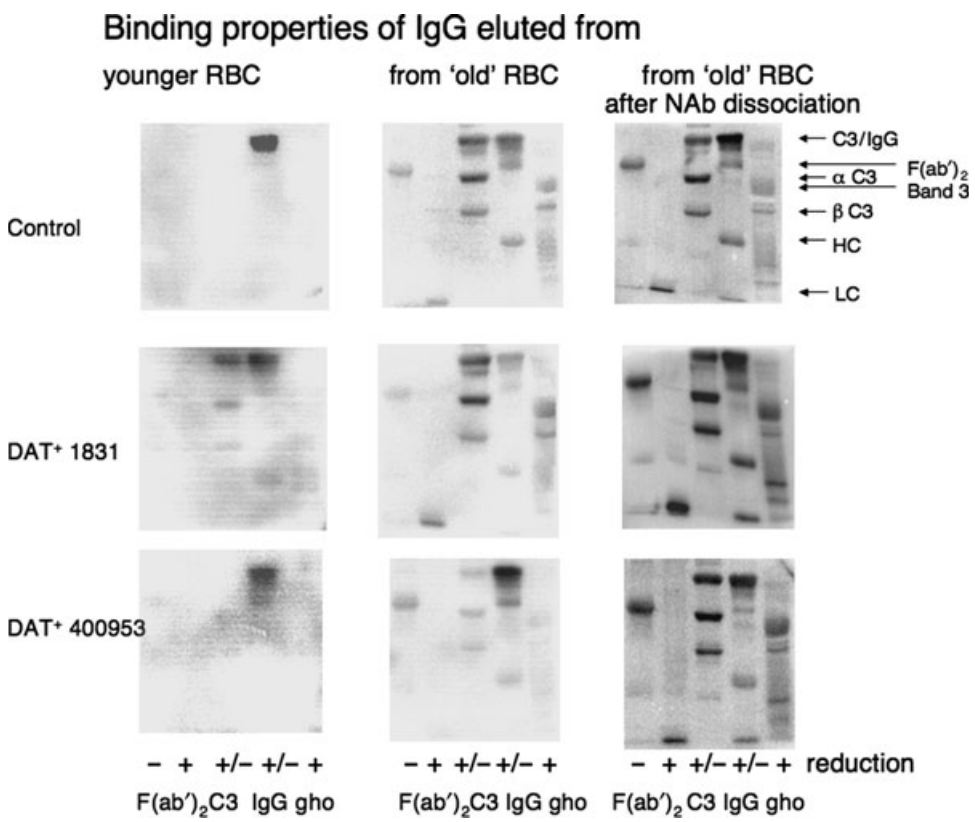

Fig. 3 Binding specificities of ^{125}I-IgG NAbs, eluted from younger and old RBCs before and after NAb complex dissociation. IgG NAbs eluted from RBC fractions 1 and 3 were pooled and referred to as 'IgG from younger cells'. A corresponding number of RBCs (4×10^{11}) from the densest fractions (the entire fraction 6 and a portion of fraction 5) was used to elute IgG from 'old RBCs'. IgG purified from eluates of RBCs was ^{125}I-iodinated and either directly applied to blots at $0{\cdot}5 \times 10^{6}$ cpm/ml (from younger RBCs, from 'old' RBCs) or was urea-pretreated to dissociate NAb complexes and dialysed. Blots were prepared from SDS PAGE containing: $F(ab')_2$ from human IgG, C3, whole human IgG, and membrane proteins of human RBCs (gho). Unreduced (–) and reduced (+) proteins were alkylated and loaded each at 2 μg/lane either separately ($F(ab')_2$ and ghosts) or after mixing (+/–) (C3, IgG). The blots illustrating binding properties of IgG from younger and old RBCs have been recorded within the same sensitivity range. The sensitivity range of the blots shown for old RBCs after NAb dissociation was the same, but differed from that used in the other rows. The urea treatment increased IgG binding for the two DAT+ samples (average) by 2·5-fold for unreduced $F(ab')_2$, by fivefold for reduced $F(ab')_2$, by 3·2-fold for C3, and by 2·8-fold for band 3, as determined by densitometry on blots recorded with the same sensitivity. The polypeptides are labelled and HC and LC stand for heavy and light chains from IgG.

Fig. 3). These findings suggest that the majority of eluted IgG molecules existed as complexes, enriched in anti-C3, anti-$F(ab')_2$ NAbs and also containing anti-band 3 NAbs. Thus, acid eluates from old RBCs of IgG-DAT+ HBD not only contained anti-band 3, but in addition elevated amounts of anti-C3 and anti-IgG NAbs. The majority of co-eluted anti-IgG NAbs were anti-idiotypic NAbs that bound to intact $F(ab')_2$ (Fig. 3), as well as to its light and shortened heavy chain as is obvious from the binding to polypeptides close to the tracking dye in lanes with reduced $F(ab')_2$ as seen after NAb dissociation (Fig. 3). This binding profile is reminiscent of that of anti-idiotypic NAbs rather than of anti-hinge NAbs, which bind primarily with high affinity to intact $F(ab')_2$ [34]. Hence, old RBCs from IgG-DAT+ HBD carry not only anti-band 3 NAbs, but also increased amounts of anti-C3 and anti-idiotypic NAbs.

Correspondingly, the plasma of most IgG-DAT+ HBD contained elevated concentrations of IgG anti-C3 and anti-$F(ab')_2$ NAbs, when studied by ELISA, as is best seen after dissociation of IgG complexes with urea (Fig. 4a). Since anti-$F(ab')_2$ NAbs comprise anti-hinge and anti-idiotypic NAbs, we studied a larger group of such donors (seven different donors, of which three were tested for two donations) repeatedly before and after depletion of the high affinity anti-hinge NAbs and plotted the total (+ urea) against the accessible concentrations (Fig. 4b). The total concentrations of anti-C3b NAbs exceeded the accessible concentrations by six- to 10-fold and only six of 10 plasma samples revealed higher concentrations than DAT-negative controls (panel A). As expected, depletion of anti-hinge NAbs did neither markedly alter anti-C3 NAb concentrations nor the number of samples differing from controls (panel B). In contrast to this, depletion of anti-hinge NAbs had a marked effect on anti-$F(ab')_2$ NAb readings (compare panel C with D). The accessible concentrations of anti-$F(ab')_2$ NAbs varied greatly prior to depletion of anti-hinge

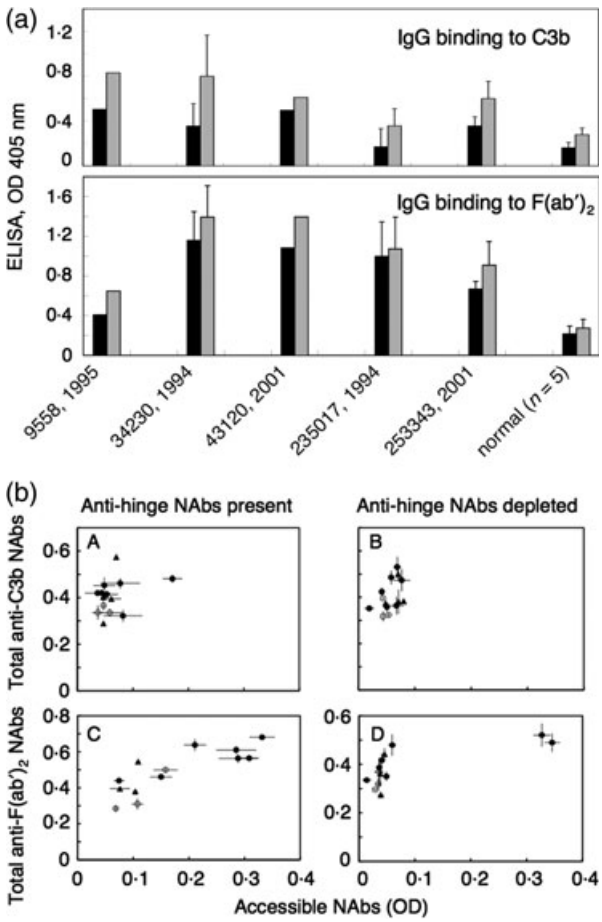

Fig. 4 Anti-C3b and anti-F(ab′)$_2$ NAbs in plasma of healthy DAT-positive donors and controls before and after a urea treatment (a) and before and after removal of anti-hinge NAbs (b). (a) ELISA assays were performed on plasma from the indicated IgG-DAT+ HBD and five DAT controls with different blood groups on immobilized C3b and F(ab′)$_2$ from IVIG. Plasma samples were either pretreated with 5 M urea (grey) or not (black) and diluted to 0·5%. Data were averaged from triplicates of 2 or three independent experiments and in the latter case + 1 standard deviation (SD) values are shown. (b) Binding of plasma IgG from controls (open circles) and IgG-DAT+ HBD (closed symbols) to C3b and F(ab′)$_2$ was analysed by ELISA before and after anti-hinge NAb depletion. Total anti-C3b and anti-F(ab′)$_2$ NAbs binding (urea-pretreated samples) is plotted vs. the accessible concentrations of the two types of NAbs (not urea-treated samples). Plasma of the following donors (year of donation) was used: 1831 (2001), 12 554 (1994), 34 230 (1994/2001), 43 230 (1996/2001), 235 017 (1994/2001), 253 343 (2001), 275 480 (1994). The results are from two to three determinations in triplicates with +/−1SD (circles) and from one triplicate (triangles). A pair of data points, originating from two blood donations of donor 43120, displayed an exceptionally high accessibility of anti-idiotypic NAbs (panel D). This donor had normal haematological parameters, but had acquired an autoimmune disease during the time of observation (relapsing polychondritis, Table 1).

NAbs (panel C). Following their depletion eight of 10 plasma samples showed a low accessibility of anti-idiotypes with the total concentrations being six to 10 times higher than the accessible portion (panel D, see legend for 2 data points), implying that anti-idiotypic NAbs (panel D) and anti-C3 NAbs (panel B) were largely complexed in plasma. Complexation was primarily between anti-idiotypes and anti-C3 NAbs, but also included anti-band 3 NAbs. In fact, plasma from one IgG-DAT+ HBD, but not control plasma dose-dependently induced a C3 binding to immobilized band 3 protein, which was lost when plasma was first absorbed on immobilized human IgG (not shown). Thus, anti-idiotypic NAbs bound to the framework of certain anti-C3 NAbs and to some extent to those of anti-band 3 NAbs, allowing all three NAbs to bind to their antigens. Binding of anti-C3 and anti-idiotypic NAbs to senescent cell-associated anti-band 3 NAbs can, however, not account for the majority of *in vivo* bound anti-C3 and anti-idiotypic NAbs, because anti-C3 and anti-idiotypic NAbs outnumbered anti-band 3 NAbs in eluates from old RBCs (see Figs 1 and 3). Therefore it is possible that anti-C3 and anti-idiotypic NAbs may also bind to C3 fragments on RBC and impair their phagocytosis.

In vitro phagocytosis of density-fractioned RBCs was studied by using THP-1 macrophage-like cells. The assays were performed either in the absence or the presence of 12 mg/ml of fluid phase, whole human IgG to simulate the inhibitory effect of physiological IgG concentrations on FcR-mediated phagocytosis [35]. In the absence of exogenous IgG old (fraction 5) and very old RBCs (fraction 6) from three IgG-DAT+ HBD were significantly better phagocytosed (1·5 to 2 times) than corresponding control cells with up to 10·5+/− 0·9 RBCs per 100 THP-1 cells for very old RBCs from donors 430 097 and 253 343 (Fig. 5). In the presence of exogenous IgG, however, old and very old control cells were as efficiently or even more efficiently phagocytosed than those from IgG-DAT+ HBD. In fact, in two cases (donor 400 953 and 430 097), phagocytosis of RBCs from IgG-DAT+ donors was lower under these conditions and significantly lower ($P < 0·03$) for donor 430 097. This suggests that senescent RBCs from IgG-DAT+ HBD were more efficiently phagocytosed than corresponding RBC fractions from DAT− cells, when both RBC-bound IgG and complement components exerted their stimulating effect. However, when FcR-mediated phagocytosis was suppressed, IgG-DAT+ RBCs were less efficiently phagocytosed as compared to IgG-DAT− control cells.

Discussion

RBCs of IgG-DAT+ HBD carry elevated numbers of IgG molecules primarily on senescent rather than on RBCs of any cell age. Correspondingly, *in vitro* phagocytosis of their senescent RBCs was 1·5- to twofold higher than that of senescent control cells. In contrast to this, under conditions

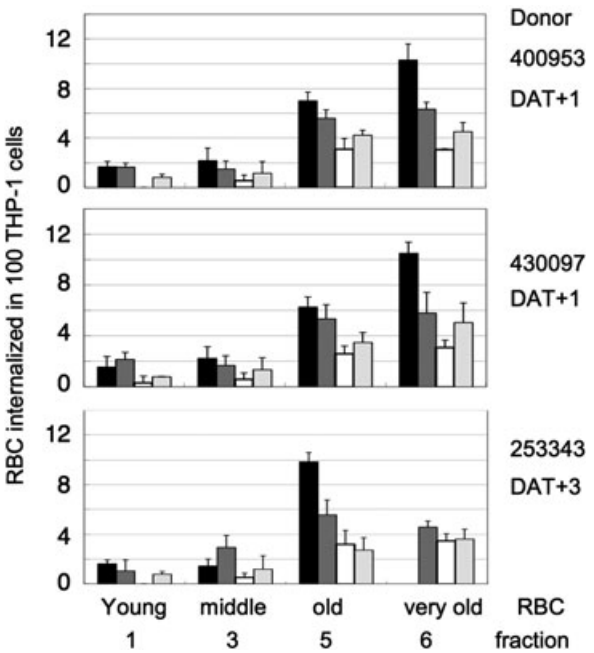

Fig. 5 Phagocytosis of RBCs from healthy IgG-DAT+ and DAT− HBD in the presence and the absence of fluid phase IgG. *In vitro* phagocytosis of density-fractionated RBCs from IgG-DAT+ HBD (black and empty bars) and DAT- HBD (grey and pointed bars). Results from three independent experiments + 1 standard deviation are shown for the given donors. Washed RBC were incubated with THP-1 cells in the presence (empty and pointed bars) or the absence (black and grey bars) of 12 mg/ml pooled whole human IgG. In the case of donor 253343 phagocytosis of the very old RBC could not be quantified in the absence of whole human IgG, because these RBC agglutinated, as was evident from the slides.

that suppress FcR-mediated phagocytosis and mimic the *in vivo* situation, senescent RBCs from IgG-DAT+ HBD were similarly or even less efficiently phagocytosed than senescent control cells. This suggests that complement-dependent phagocytosis of senescent RBC from these donors was impaired by the extra amounts of RBC-bound IgG, comprising anti-C3 and framework-specific anti-idiotpyic NAbs. Thus, senescent RBCs from IgG-DAT+ HBD may be protected from immediate *in vivo* and *in vitro* clearance in the presence of physiological IgG concentrations by additionally bound NAbs, which may have (i) impaired the interaction of RBC-bound C3 fragments with complement receptors and (ii) dampened C3b deposition onto RBC-associated anti-band 3 NAbs.

In IgG-DAT+ HBD anti-C3 and anti-idiotypic NAbs bound preferentially to senescent RBC that are known to carry anti-band 3 NAbs and C3b/iC3b molecules [11,14,16,17,36–39]. Since the amounts of RBC-bound anti-C3 and anti-idiotypic NAbs exceeded bound anti-band 3 NAbs, only a minor fraction of them could have interacted directly with RBC-associated anti-band 3 NAbs. Those that did bind to anti-band 3 NAbs could have impaired bound anti-band 3 NAbs from capturing C3b dimers, which these NAbs would otherwise have done preferentially [17,19]. A considerably larger portion of the two NAbs may have bound to C3 fragments on senescent RBCs, as illustrated in Fig. 6. Bound anti-C3 and anti-idiotypic NAbs together may prevent C3 fragments from eliciting phagocytosis via complement receptors *in vivo* and in the presence of physiological IgG concentrations *in vitro*. In the absence of IgG, *in vitro* phagocytosis of DAT+ RBC was indeed highest for RBC with the highest positive DAT, but the number of analysed cases was too small to correlate its extent with the strength of the DAT, as performed earlier [40].

The anti-C3 NAbs, which are increased in plasma of the majority of IgG-DAT+ HBD, are not pathological, because they do not preferentially bind to an assembled C3 convertase, as studied in some of the plasma samples (Table 1) and thus are different from nephritic factors [33]. Both anti-C3 and anti-idiotypic NAbs belong to the normal repertoire of NAbs of healthy individuals [23,41]. Hence, IgG-DAT+ HBD have transiently increased plasma concentrations of both NAbs at similar proportions as found in controls. The latter finding is relevant, because a significantly increased ratio of anti-C3/anti-idiotypes is expected to promote generation of amplifying C3 convertases, as shown by mixing the two purified NAbs at different ratios [23]. On the other hand, as long as the ratio of the two NAbs (anti-C3/anti-idiotypic NAbs) remains close to 1, increased concentrations of both NAbs may hinder RBC-bound anti-band 3 NAbs from capturing dimeric C3b [19], as well as RBC-bound C3b/iC3b from interacting with

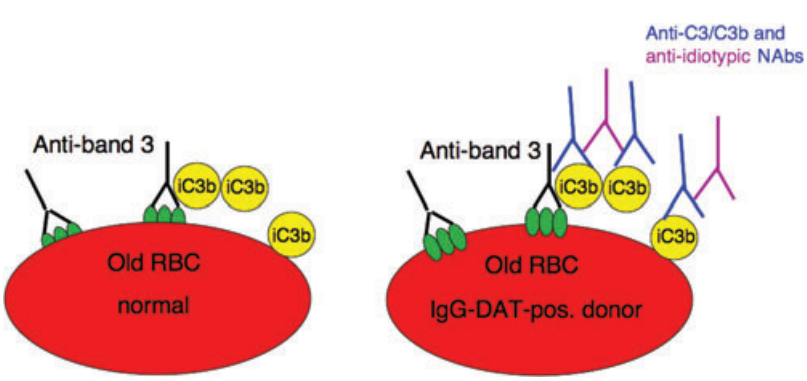

Fig. 6 Tentative explanation of the results. Old RBCs from IgG-DAT+ HBD carry in comparison to normal old RBCs not only anti-band 3 (black) and C3b/iC3b, but also anti-C3 (blue) and anti-idiotypic (violate) NAb complexes. Hence, overall they carry more IgG, but fewer C3b/iC3b molecules are accessible to complement receptors.

their receptors, as shown here. Increased concentrations of both NAbs at physiological ratio may exert beneficial effects by binding to C3 convertase precursors and thereby dampening C3 convertase generation (Jelezarova *et al.*, unpublished). The reason for the parallel up-regulation of both NAb concentrations in these blood donors is presently unknown and as such surprising, because most NAb concentrations are kept rather constant during adult life [42].

A comparison of our data with earlier characterizations of IgG molecules, eluted from RBC of DAT+ HBD, illustrates some parallels. In earlier studies a large portion of eluted IgG molecules did not bind to typed RBCs and was considered 'non-reactive' [43]. Indeed, the extent of IgG binding to RBC proteins rather than to opsonins was small in eluates from senescent RBCs. An even larger fraction was 'non-reactive' in the sense that it was complexed and could only be rendered reactive by a urea pretreatment, an approach that was not used at that time. The complexed material contained instead of paratope-specific [8] primarily framework-specific anti-idiotypic NAbs that did not prevent binding of anti-C3 NAbs to C3b/iC3b.

The fact that RBCs from IgG-DAT+ HBD not only reached a higher absolute cell age than cells of IgG-DAT– HBDs, but also a significantly higher density is unexpected in view of recent reports from Lew *et al.* [44,45]. These authors found that ageing human RBCs, after a steady increase in their density, undergo a density reversal at the very end of their lifespan by activation of a non-selective cation leak. In their experimental setting, the percentage of such light, but old RBC from healthy humans was, however, variable [44] and reached less than 0·03%, when taken from density gradients [46]. If the swelling occurred already *in vivo* to a significant extent, it would seem unlikely to find overaged RBCs at high densities as reported here. It is possible that such a swelling is facilitated not only by internal calcium ions, but in addition by mechanical/oxidative damage as is the case for sickle RBC [46]. In our experiments, mechanical and oxidative damage was minimal, because RBCs were pelleted from filtered blood, mixed with Percoll, and immediately separated on these self-forming gradients. RBCs processed in this manner from IgG-DAT+ HBD not only reached a higher absolute cell age, but also higher densities than those of normal senescent cells. The densest RBCs in their circulation had a higher absolute cell age than normal senescent RBC based on the band 4·1a/b ratio. Since this ratio reflects the extent of deamidation, which itself is exclusively time and temperature dependent [47], we propose that anti-C3 and anti-idiotypic NAbs prolonged the lifespan of IgG-DAT+ RBC. The suggested prolongation of the lifespan needs to be investigated by *in vivo* RBC survival studies and by determinations of erythropoietin or surrogate markers for erythropoietic activity (Table 1 shows preliminary data on erythropoietin for some of the donors).

Acknowledgements

We thank the blood donors with persistent positive DAT for participating in this study. We are grateful to Prof Virgilio Lew for helpful discussions.

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