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BINDING OF AUTOLOGOUS IgG TO HUMAN RED BLOOD CELLS BEFORE AND AFTER ATP-DEPLETION

SELECTIVE EXPOSURE OF BINDING SITES (AUTOANTIGENS) ON SPECTRIN-FREE VESICLES

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Binding of autologous IgG to fresh, ATP-depleted red blood cells as well as to spectrin-free vesicles was studied by a non-equilibrium binding assay using ^{125}I-iodinated protein A from *Staphylococcus aureus*. IgG binding was 14-times higher to spectrin-free vesicles than to ATP-maintaining red blood cells and 4-times higher than to ATP-depleted erythrocytes from which these vesicles were released. Protein A binding to vesicles that were released from washed and nutrient-deprived erythrocytes, was dependent on added autologous IgG. However, spectrin-free vesicles that were spontaneously released from erythrocytes conserved in whole blood, bound similar amounts of protein A with or without added autologous IgG (0.45–0.55 ng/μg band 3 protein). These findings demonstrate that opsonization of spectrin-free vesicles by autologous IgG occurs not only in the test tube, but also under blood blank conditions. The binding characteristics of IgG to spectrin-free vesicles are indicative of a natural autoantibody rather than an unspecific binding of autologous IgG. The preferential binding of IgG to spectrin-free vesicles implies a selective exposure of corresponding autoantigens in membrane regions that have lost cytoskeletal anchorage and bud off.

Introduction

ATP-depleted human erythrocytes are rapidly removed from the circulation as evident from transfusion studies using whole blood conserved for various times in acid/citrate/dextrose [1]. Survival of ATP-depleted erythrocytes in circulation is partially normalized by restoration of cellular ATP-levels [2,3]. The mechanism by which such cells are removed from the circulation remained elusive. In fact, it is not even known, whether such cells are phagocytized and whether this process requires opsonization by naturally occurring IgG-autoantibodies as is the case for senescent human red blood cells [4–6]. In order to eventually elucidate whether ATP-depletion correlates with exposure of autoantigens, we studied binding of autologous IgG to red blood cells before and after nutrient-deprivation which causes the cells to ATP-deplete. This question is difficult to attack, because ATP-depleting red blood cells undergo sequential shape changes from discocytes to echinocytes [7] and then to spheroechinocytes [7,8], which finally release spectrin-free vesicles [9,10] and thereby become spherocytes [11]. Since many interrelated biochemical changes follow a decrease

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Abbreviation: IgG, immunoglobulin G.

in ATP-content [12–18], it remains unknown at which state the cell becomes the target of macrophages. We chose to study binding of autologous IgG to spherocytes and to spectrin-free vesicles. The presence of two entities at this state of ATP-depletion allowed to analyze binding of autologous IgG to structurally different domains that originated from spheroechinocytes. While spectrin-free vesicles correspond to the tips of protrusions on spheroechinocytes [11,19,20], spherocytes represent the remainder. These structurally distinct entities were obtained by nutrient deprivation that causes ATP-depletion within 20–24 h and increasing vesicle release beyond 12 h [9]. In order to obtain sufficient quantities of spectrin-free vesicles, nutrient deprivation was extended to 36–40 h. Although the spectrin-free vesicles thus obtained are identical to those released up to 24 h [9], the remaining ATP-depleted erythrocytes have undergone some irreversible changes on the cytoplasmic side [16–18].

Binding of autologous IgG to intact erythrocytes has earlier been reported to reach a high extent, but only when determined at low ionic strength [21]. Some of this IgG binding was not Fab dependent [22], but had properties of an ionic binding to negatively charged groups [23], or was due to aggregated IgG [22]. In contrast to this, the few IgG molecules bound to senescent erythrocytes were firmly bound and resistant to washing [4,5]. Thus binding of autologous IgG was analyzed in this report in a non-equilibrium binding assay at high ionic strength, in order to probe for firmly bound autologous IgG of the type expected for an autoantibody.

Materials and Methods

Erythrocytes and spectrin-free vesicles from fresh blood

Erythrocytes were isolated from fresh whole blood collected in citrate/phosphate/dextrose from O Rh^+ donors. Erythrocytes were washed and freed from white cells as given elsewhere [24]. These erythrocytes were either used for IgG binding studies or were incubated under ATP-depleting or ATP-maintaining conditions. ATP-depletion was carried out according to Ref. 25 at 20% hematocrit and 310 mosM in a medium described elsewhere [9]. Spectrin-free vesicles were obtained from cells ATP-depleted for 36–40 h and were purified by a filtration technique described elsewhere [25]. The yield in vesicles was 11.4 ± 0.4 ($n = 3$) μg band 3 protein per ml of packed cells, if the supernates of ATP-depleted cells were processed alone, or 16.5 ± 1 ($n = 6$) μg band 3 protein, if the supernates were combined with the first wash of ATP-depleted cells. Control cells were incubated under ATP-maintaining conditions in a medium containing glucose, adenine, and inosine [9] under exactly the same conditions as ATP-depeleted cells.

Erythrocytes and spectrin-free vesicles from outdated blood

Blood from O Rh^+ donors was stored for 6–7 weeks at 4°C in citrate/phosphate/dextrose with occasional mixing of the content. The bulk of plasma and white cells was removed after erythrocytes sedimented by gravity. The erythrocyte-rich fraction was diluted and gently mixed with one volume of 150 mM NaCl, 5 mM $NaKHPO_4$ (pH 7.4). Cells were pelleted for 7 min at 2000 rpm in a Sorvall SS 34 rotor. The supernate containing spectrin-free vesicles and some contaminants was centrifuged for 50 min at 9000 rpm in a GSA rotor. The pelleted material was washed three times and recentrifuged for 15 min at 15 000 rpm in a SS 34 rotor. The final pellet was resuspended in 10 ml of buffer and was filtered through 0.8 μm Sartorius filters. The filtrate was centrifuged and the pelleted vesicles resuspended. Vesicles as well as the three times washed cells remaining from the first centrifugation were passed over columns containing α-cellulose and microcristalline cellulose [26] to remove white cell contaminants. Eluates were centrifuged. Vesicles or cells were washed three more times.

Density-separated young and old erythrocytes

Erythrocytes from fresh blood were separated according to their density on self forming gradients with Percoll (Pharmacia, Uppsala) following the protocol outlined in Ref. 24 with some modifications (Lutz, H.U. and Stringaro-Wipf, G., submitted). Washed cells were analyzed for their creatine contents as a cell-age parameter [27,28]. The creatine content of the light fraction over that

from the dense fraction was given as a ratio indicating the quality of separation into young and old cells. Young and old cells were then washed once in 50 mM glycylglycine, 5 mM $NaKHPO_4$, 90 mM NaCl, 250 mg/l penicillin G, 300 mg/l streptomycin, 0.6 mM adenine, 12 mM inosine, 10 mM D-glucose, 0.1 mM EDTA, 20 mM sucrose, 1 mM $MgCl_2$, and 50 μg/l phenylmethylsulfonylfluoride (pH 7.4, osmolarity 305–320 mosM). Cells were then passed at 50% hematocrit in the same buffer through a column containing α-cellulose and microcrystalline cellulose [26]. Eluted cells were washed three more times.

Autologous IgG and ^{125}I-iodinated protein A

Plasma was removed from blood collected in citrate/phosphate/dextrose and was supplemented with calcium to obtain sera. The calcium-free sera were treated with 0.4 mM diisopropylfluorophosphate and IgG was isolated, precentrifuged, and quantitated as given elsewhere [29]. IgG binding assays were carried out with IgG and erythrocytes from the same healthy blood donors, but autologous IgG and cells were not necessarily from the same blood collection. Protein A from *Staphylococcus aureus* (Pharmacia, Uppsala) was ^{125}I-iodinated with chloramine T according to Ref. 30. Labeled protein A was immediately separated from unreacted label by gelfiltration on Sephadex G-25 fine. The column was prerun with 1–5 mg gelatin to reduce adsorption of labeled protein A. The column was equilibrated and run with 150 mM NaCl, 5 mM $NaKHPO_4$, 1 mM EDTA, 1 mM NaI (pH 7.4). Eluted protein A was stored in small portions at −20°C in the column buffer supplemented with 1% gelatin. The specific activities ranged from $1 \cdot 10^7$–$4 \cdot 10^7$ cpm per μg. The specific activities are slightly overestimated, because they were based on a 100% recovery of protein A from gel filtrations.

SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out on slab gels according to Neville [31] as modified elsewhere [17]. Samples were denatured for 30 min at 37°C in electrophoresis sample buffer containing 1% SDS and 40 mM dithiothreitol. Gels were stained with Coomassie blue. The band 3 protein content in vesicles and cells was determined by densitometry of stained gels using an Integraph from Bender and Hobein, Zurich [25].

Binding of autologous IgG to erythrocytes and spectrin-free vesicles

Binding of autologous IgG was analyzed by a non-equilibrium binding assay which probes for IgG that remained cell- or vesicle-bound following several washes. Cells and vesicles, free from white cell contaminations, as verified by microscopic analysis, were washed once in a buffer that was varied depending on the type of sample analyzed: for spectrin-free vesicles and ATP-depleted cells: 150 mM NaCl, 5 mM $NaKHPO_4$ (pH 7.4); for young, old, fresh as well as ATP-maintaining cells: 60 mM glycylglycine, 5 mM $NaKHPO_4$, 103 mM NaCl, 10 mM D-glucose, 12 mM inosine, 0.6 mM adenine, 250 mg/l penicillin G, and 300 mg/l streptomycin (pH 7.4). 100-μl of a 5% cell suspension or 100 μl of resuspended vesicles were incubated in a total volume of 500 μl containing 5 mg/ml ovalbumin (Sigma, Grade V), 100 μg/ml phenylmethylsulfonylfluoride and with or without autologous IgG. The concentration of autologous IgG was in the range of 0–2 mg/ml. Since cells and vesicles were compared, added IgG was referred to a relative measure of the surface area, the content of band 3 protein in the incubation mixture containing either cells or vesicles [9]. Samples were rotated at 4°C for 90 min. Cells and vesicles were washed six times with the appropriate buffer. Cells were centrifuged for 0.5 min, vesicles for 15 min in an Eppendorf centrifuge. Washed cells or vesicles were supplemented with 48–95 ng ^{125}I-iodinated protein A. Samples were incubated at 4°C for 60 min. The suspensions were placed onto 10% dextran cushions (one ml in appropriate buffer) and centrifuged for 2.5 min and 60 min, respectively. The supernates and dextran cushions were removed and cells or vesicles resuspended, transferred to new tubes, washed three more times and analyzed for bound label in a gamma counter (Kontron, Zurich). Bound label was expressed in ng protein A per tube and was assayed in triplicate. To correct for loss of material during the entire procedure, the amount of a surface marker, band 3 protein, was determined for each pellet. Pellets were resuspended in electro-

phoresis sample buffer and analyzed on SDS-polyacrylamide gels. The relative amount of Coomassie blue bound to band 3 was determined and referred to that obtained from 15 μg of membrane protein applied to the same gel.

Results

Binding of autologous IgG to density-separated erythrocytes

The amount of ^{125}I-labeled protein A from *Staphylococcus aureus* which remained cell- or vesicle-bound in a non-equilibrium binding assay was determined as a relative measure of bound IgG. This number was referred to the content of band 3 as a reliable marker for surface-area for both red blood cells and vesicles [9,25]. This protocol was initially applied to determine cell-bound IgG on young and old red blood cells for which other techniques have previously been used [4,32]. We found a significant difference in protein A binding between density-separated old and young human red blood cells (Fig. 1). The difference amounts to 0.037 ng protein A per μg band 3, implying that old cells contain at least 80 protein A molecules more per cell than young cells (for details see legend to Fig. 1), if homogeneous populations are assumed. However, protein A binding to young cells was not nil [4], but rather 1.4-fold lower than to old cells. Fig. 1 further indicates that protein A binding to young or old cells did not increase when assayed after a preincubation with autologous IgG. Thus, cell separation and washing procedure did not result in exposure of new sites. These results illustrate that significant differences in protein A binding remained detectable after preincubation of red blood cells with up to 1000 μg of autologous IgG per μg band 3 protein.

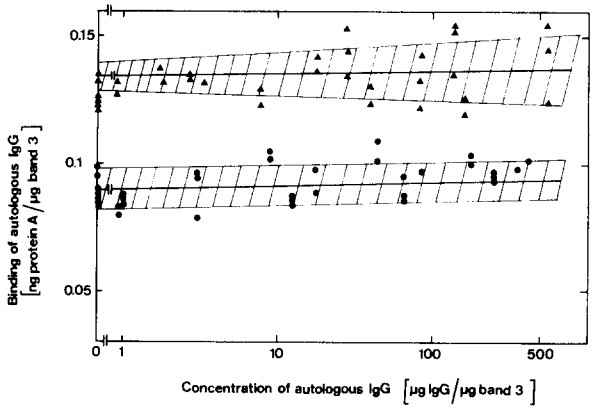

Fig. 1. IgG binding as measured by protein A to density-separated young and old human erythrocytes before and after preincubation with autologous IgG. Young and old erythrocytes were analyzed for cell-bound IgG by determining bound ^{125}I-iodinated protein A. The creatine content of young cells was 5.3 ± 1.0 ($n = 5$) times higher than in old cells. Binding is given in ng protein A per μg band 3 protein. The corresponding number of protein A molecules per cell (young, 193; old, 272) was estimated on the basis that young and old cells contain 10^6 copies of band 3 protein with a molecular weight of 100000 (for review, see Ref. 33). In reality the surface area of senescent cells is roughly 10% smaller [34] and with it the number of band 3 molecules. But since the specific activities of protein A were overestimated rather than underestimated (see Materials and Methods), these numbers in fact represent a minimum. Thus, the difference between young and old cells could range from 60–120 molecules per cell, if the specific activities were wrong by a factor of 2. IgG binding; ▲, old cells; ●, young cells. The hatched areas give ± 1 S.E. for all measurements originating from five independent experiments.

Binding of autologous IgG to ATP-maintaining and ATP-depleted red blood cells

Binding of protein A to red blood cells from whole cell populations reached 0.1 ng per μg band

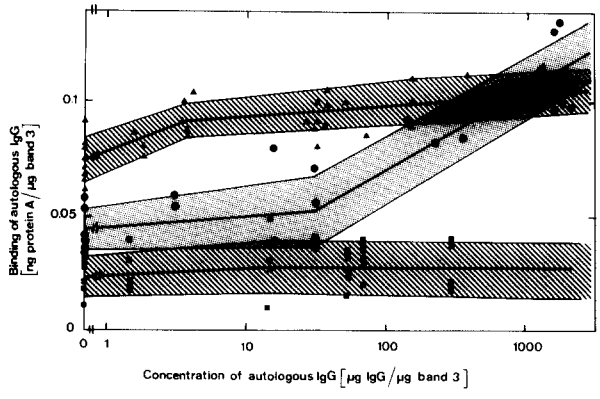

Fig. 2. IgG binding to freshly isolated, ATP-depleted, and ATP-maintaining erythrocytes before and after preincubation with autologous IgG. Protein A binding was either analyzed on cells from whole populations within a few hours after blood collection or following incubation for 36–40 h under ATP-maintaining or ATP-depleting conditions. For each assay system data from at least three independent experiments are shown. Averaged binding curves (heavy lines) were calculated from groups of four or more values at the same or similar IgG concentration and ± 1 S.E. is indicated by hatched or dotted areas. ▲, Freshly isolated cells; ●, ATP-depleted cells; ■, ATP-maintaining cells.

3 protein, when analyzed on freshly isolated cells after preincubation with autologous IgG (Fig. 2). While further washings of cells did not decrease bound protein A (not shown), incubation of these cells for 36–40 h at 37°C decreased the amount of protein A that could be bound in the absence of added IgG. The extended incubation in IgG-free medium apparently favored dissociation of bound IgG. This loss of protein A binding capacity was similar whether red blood cells maintained their ATP level or not (residual binding 0.02 and 0.035 ng, respectively). On the other hand, ATP-maintaining and ATP-depleted cells differed in protein A binding, when analyzed subsequently to a preincubation with autologous IgG (Fig. 2). While protein A binding to ATP-maintaining cells remained low, ATP-depleted cells showed an increased binding reaching similar amounts as observed on freshly isolated cells. It was not possible to verify whether protein A binding saturated at higher concentrations of autologous IgG than 1000 μg per μg band 3, because of increasing standard errors. Nevertheless, the comparison indicates that maintenance of normal ATP-levels is required for a low IgG binding. It remained, however, puzzling why IgG binding to ATP-depleted cells did not exceed binding to fresh cells. One explanation could be that IgG binding was primarily to the tips of spherechinocytic ATP-depleted cells which were released as spectrin-free vesicles from these cells. Evidence in favor of this conclusion was obtained by studying binding of autologous IgG to spectrin-free vesicles.

Binding of autologous IgG to spectrin-free vesicles

Protein A binding to spectrin-free vesicles was the same as to freshly isolated cells, when analyzed in the absence of autologous IgG (Fig. 3). Thus, the protein A binding capacity did not decrease on areas that budded off during ATP-depletion. Preincubation of spectrin-free vesicles with autologous IgG increased protein A binding. Binding reached saturation with 0.35 ng protein A per μg band 3 protein, when 500 μg of autologous IgG were added to vesicles containing 1 μg band 3 protein. This binding is 14-times higher than to ATP-maintaining and at least 4-times higher than to ATP-depleted red cells, from which these vesicles were released. These results demonstrate a prefer-

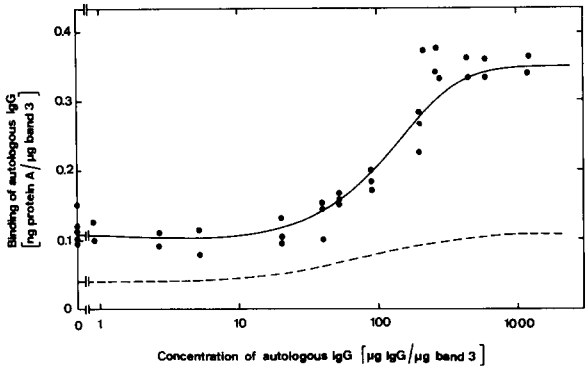

Fig. 3. IgG binding to spectrin-free vesicles released from erythrocytes that were ATP-depleted in serum-free medium (●). Data from four independent experiments. IgG binding to the corresponding ATP-depleted cells is shown by the dashed line (from Fig. 2).

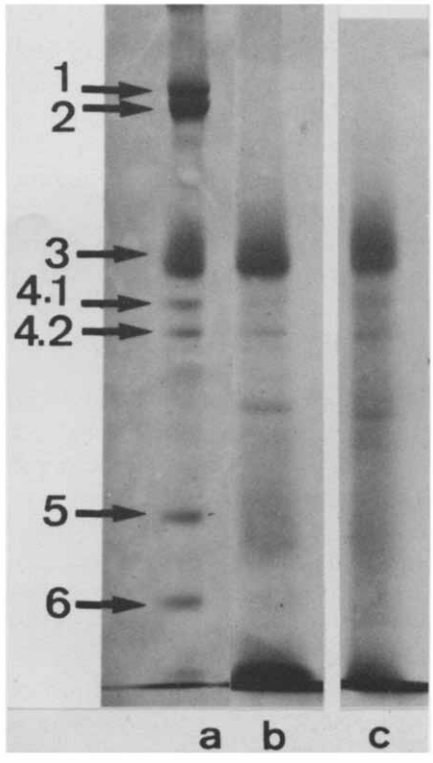

Fig. 4. Protein band patterns on SDS-polyacrylamide gels of spectrin-free vesicles obtained from outdated blood or from fresh blood by ATP-depletion. Spectrin-free vesicles were obtained either by ATP-depletion of fresh cells in serum-free medium or from whole blood stored for 6–7 weeks in citrate/phosphate/dextrose. Membranes were isolated from fresh cells as a control. (a) Membranes from freshly isolated cells (15 μg protein); (b) spectrin-free vesicles isolated from whole blood stored for 7 weeks in citrate/phosphate/dextrose; (c) spectrin-free vesicles from ATP-depleted cells in serum-free medium.

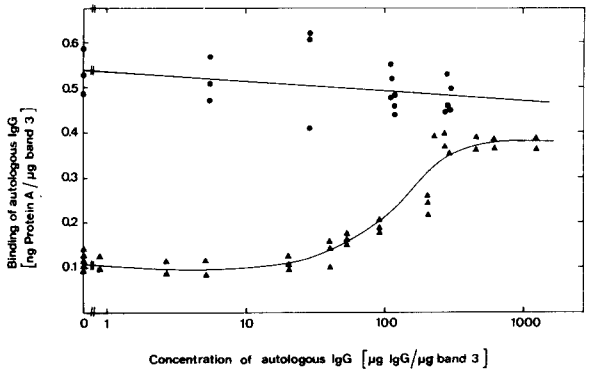

Fig. 5. IgG binding to spectrin-free vesicles from whole blood stored for 6–7 weeks in citrate/phosphate/dextrose (●), data from three independent experiments; ▲, IgG binding to spectrin-free vesicles from cells that were ATP-depleted in serum-free medium (from Fig. 3).

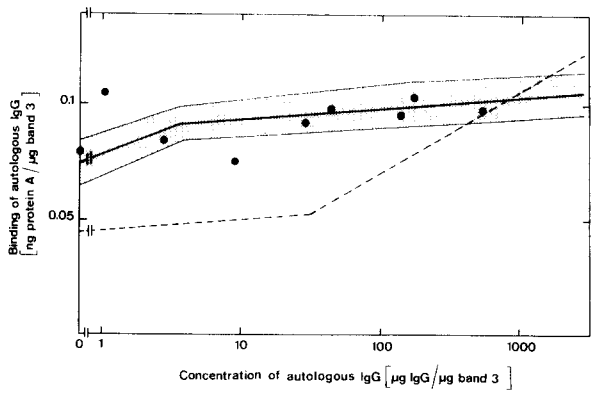

Fig. 6. IgG binding to erythrocytes from whole blood stored for 7 weeks in citrate/phosphate/dextrose (●), data from two independent experiments. The heavy line with the dotted area (±1 S.E.) is taken from Fig. 2 and shows IgG binding to freshly isolated cells; the dashed line shows the averaged IgG binding to ATP-depleted cells (from Fig. 2).

ential binding of autologous IgG to areas of red blood cells which bud off as spectrin-free vesicles. If this preferential binding to vesicles were physiologically relevant, it should occur during storage of whole blood, because red blood cells kept in autologous plasma also release spectrin-free vesicles [35,36]. Though vesicles were detected early during blood storage [17], amounts sufficient for binding studies were only obtained beyond the shelf life of banked blood, when red blood cells are partially ATP-depleted [35,36].

Spectrin-free vesicles released from red blood cells stored for 6–7 weeks under blood bank conditions show virtually the same protein patterns on SDS-polyacrylamide gel electrophoresis as those obtained from red blood cells that were ATP-depleted in serum-free medium (Fig. 4). Gel electrophoresis and protein A binding to these vesicles were carried out following complete removal of white cells (see Materials and Methods). As expected, these vesicles reveal at all concentrations of autologous IgG roughly the same, high amount of bound protein A (Fig. 5). Bound protein A is slightly higher than that determined for spectrin-free vesicles that were released in serum-free medium and preincubated with autologous IgG.

On the other hand, red blood cells stored for 6–7 weeks under blood bank conditions, which had released a small portion of their surface as spectrin-free vesicles, yet showed the same degree of protein A binding as freshly isolated erythrocytes (Fig. 6). Thus, binding of autologous IgG to spectrin-free vesicles from outdated blood further supports the conclusion that a preferential binding of autologous IgG occurs to areas that budded off.

Discussion

The data presented reveal a preferential binding of autologous IgG to regions of red blood cells which bud off and are released as spectrin-free vesicles during ATP-depletion. Binding of autologous IgG to vesicles is not simply a test tube phenomenon, but also occurs in conserved blood, in measurable amounts beyond the shelf life of banked blood.

IgG binding was assayed under conditions that probe for firmly bound IgG, e.g. IgG with a low dissociation constant. Hence, a sigmoidal, saturating IgG binding curve as found for vesicles may indicate bivalent binding of IgG to independent sites, because of the following reasons: in the presence of an excess of a bivalent ligand and only few sites per cell being occupied, the predominant form of binding is bivalent at equilibrium [37]. The non-equilibrium conditions chosen further select for this type of binding, because the rate of dissociation of bivalently bound IgG is considerably smaller than that of monovalently bound IgG [38,39]. Therefore, IgG binding to vesicles is indicative of an autoantibody binding rather than an unspecific binding of IgG which would not saturate

[22]. The nature of exposed autoantigens is not known. We can not exclude that exposure of autoantigens is due to chemical modification of surface components, although the surface constituents of spectrin-free vesicles are within the limits of accuracy the same as on intact cells: we have extensively characterized these vesicles and found that they are all right-side out and sealed [9]. Their band 3 protein content [9] and electrophoretic mobility remained unaltered [25]. Furthermore, chemical modification of 'self' is unlikely to generate autoantigens primarily on spectrin-free vesicles, because these vesicles orginated from ATP-depleting red blood cells and thus were subjected to the same conditions as the residual cells.

We think that exposure of autoantigens is intimately related to the loss of cytoskeletal restraints accompanying the formation of spectrin-free vesicles on the tips of spheroechinocytes. Evidence in favor of this explanation is given by a dramatic increase in binding of anti-blood group A antibodies to the tips of protrusions as compared to the remainder of spheroechinocytes [40]. A similar phenomenon might be responsible for a selective binding of an autoantibody-like substance from autologous IgG to spectrin-free vesicles. In fact, autologous IgG contains several autoantibodies to red blood cell membrane proteins, but only one of them appears to be directed to an integral membrane protein [29]. We have recently shown (Lutz, H.U. and Stringaro-Wipf, G., submitted) that this autoantibody is directed to exoplasmic portions of a subpopulation of band 3 protein. It is involved in recognizing senescent human red blood cells. Exposure of the corresponding autoantigen on senescent red blood cells is most likely due to the formation of a small number of oligomers of band 3 which may allow a bivalent and hence practically irreversible binding of autoantibody [41]. The same autoantibody might also bind to band 3 protein on spectrin-free vesicles, beause oligomerized band 3 protein is more likely to occur, when cytoskeletal restraints are missing. Schweizer et al. [42] probed for preexisting oligomers of band 3 protein on spectrin-free vesicles and intact red blood cells by studying the extent of rapidly cross-linked band 3 at low temperature, where lateral diffusion of proteins is practically inexistent [43]. They found a considerably higher cross-linkability for band 3 on vesicles than on intact red cells. A comparison of these data with the extent of IgG binding reported here, suggests a correlation between the cross-linkability of band 3 and the extent of IgG binding (Table I). The roughly 2-fold difference in extents is expected for a monomeric autoantigen and a bivalent autoantibody. Based on these results it is likely that IgG binding to spectrin-free vesicles is due to binding of a naturally occurring autoantibody to band 3 dimers or oligomers [41]. Whether this is in fact the case, will be answered by experiments in progress using purified autoantibodies.

TABLE I

CORRELATION BETWEEN CROSS-LINKABILITY OF BAND 3 PROTEIN AND BINDING OF AUTOLOGOUS IgG TO INTACT CELLS AND VESICLES

Type of sample	Cross-linkability [a]		Binding of autologous IgG	
	Percent of total label cross-linked	Ratio	ng protein A bound per μg band 3 protein	Ratio
Young erythrocytes	0.8 ± 0.3	1	0.090 ± 0.006	1
Old erythrocytes	0.9 ± 0.7	~1	0.127 ± 0.006	1.4
Spectrin-free vesicles	10	10	0.35 ± 0.01	3.9

[a] Data from Table II and Fig. 2C of Ref. 42. In brief: Erythrocytes or vesicles were treated with galactose oxidase and modified with [^{14}C]aniline in the presence of $NaCNBH_3$ such that the degree of modification reached 0.5 mole per mole band 3 protein in all cases. Cross-linking was subsequently carried out for 8 min at 4°C with dithiobis-(succinimidyl propionate) and was quantitated as outlined [42]. The numbers give the fraction of total label cross-linked to protein complexes exceeding a molecular weight of 140000.

IgG binding to intact young and old red blood cells was used initially to evaluate the non-equilibrium binding assay. The difference in IgG binding obtained for the two populations is comparable to that determined by Kay [4] using anti-human IgG coupled to SV 40. However, IgG binding to young cells was not zero [4] in our hands, but was 1.4-fold less than that to old cells. A similar ratio of 1.3 was reported by Szymanski et al. [32] using a sensitive agglutination technique. Since Kay applied microscopic techniques and analyzed binding to individual cells, whereas Szymanski and we studied cell populations, the discrepancy may be due to a methodological difference. On the other hand, we can not exclude that IgG binding to young and old red blood cells slightly differs in its K_D, such that an extended incubation in serum-free medium would result in dissociation of IgG primarily from young cells. This interpretation could explain why whole cell populations lose 2/3 of bound IgG during a 40-h incubation, but retain 1/3, an amount which corresponds to the difference in binding between young and old red blood cells. The data further suggest that the dissociable portion of IgG binds to a reversibly accessible site, because IgG rebinding is prevented by maintaining the cellular ATP content.

Non-equilibrium binding of autologous IgG to nutrient-deprived and thus ATP-depleted red blood cells was not significantly different from that determined for freshly isolated red blood cells. This was unexpected, but is probably due to the fact that these cells were analyzed for binding after a total of 12 washes which may have shed off virtually all preformed vesicles. Mechanical stress imposed on nutrient-deprived cells by one wash cycle increased the yield in vesicles by 44% over that determined after spontaneous release (see Material and Methods). Hence, non-equilibrium binding of autologous IgG to ATP-depleted red blood cells is representative for spherocytes rather than spheroechinocytes. Therefore, IgG binding to spectrin-free vesicles can be considered as IgG binding to the tips of protrusions on spheroechinocytes. The occurrence of IgG binding domains on these spheroechinocytes may eventually explain, why ATP-depleted red blood cells have a shortened survival, but may regain normal survival after shedding of vesicles and restoration of normal ATP levels as has been observed in experiments in which both sialic acid and ATP-contents were decreased [44].

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