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Role of Physiologic Autoantibody in the Removal of Senescent Human Red Cells

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The mechanism by which mononuclear phagocytes distinguish mature "self" from senescent "self" was investigated. Evidence is presented indicating that human mononuclear phagocytes distinguish senescent RBC from mature RBC on the basis of selective Ig attachment to the membranes of senescent cells. This Ig, eluted from senescent human RBC, was shown to be IgG and free of other Igs by immunodiffusion, immunoelectrophoresis, and polyacrylamide gel electrophoresis. The IgG was polyclonal with respect to light chains. The eluted IgG reattaches to homologous stored RBC, but not to mature autologous or allogeneic RBC, via the Fab region. It then initiates phagocytosis of these stored RBC by mononuclear phagocytes. Evidence suggests that the IgG is directed against altered membrane receptors. Thus, this IgG may be a "physiologic" autoantibody and contribute to the maintenance of homeostasis by performing regulatory functions.

Key words: scanning electron microscopy, cellular aging, autoantibodies, red blood cells

Mononuclear phagocytes* can distinguish mature "self" from senescent "self" cells. This is reflected by their ability to phagocytize cells that have reached the end of their functional life-span, while sparing the mature cells. For example, mononuclear phagocytes

*A select WHO committee [1] has recommended that actively phagocytic mononuclear cells of any system, including those of the reticuloendothelial system, be reclassified as the mononuclear phagocyte system (MPS) on the basis of common functional properties (ie, phagocytosis) and of their common origin from the blood monocyte which is itself derived from a bone marrow precursor cell. In accordance with this recommedation, the terminology, "mononuclear phagocyte," will be used throughout this manuscript.

Abbreviations: ID, immunodiffusion; IEP, Immunoelectrophoresis; Ig, Immunoglobulin; PAGE, polyacrylamide gel electrophoresis; RBC, red blood cells.

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of the liver and spleen remove syngeneic lymphocytes as well as antibody-coated red blood cells (RBC) [2–4]. Studies on the fate of aged RBC indicate that they are eliminated intracellularly by mononuclear phagocytes rather than by osmotic lysis both in vitro and in situ [2–9]. In this way, mononuclear phagocytes may perform an essential homeostatic role by permitting the more efficient mature cells to carry out their vital functions without hindrance from the less efficient senescent cells, or by pathological reactions that could arise as a consequence of senescent cells dying and decaying within the organism.

Previous experiments performed in this laboratory suggest that mononuclear phagocytes can distinguish senescent from mature self RBC on the basis of selective attachment of autologous immunoglobulin (Ig)G to the membrane of senescent RBC [10]. The presence of IgG on senescent cells is presumptive evidence that an immunological receptor-IgG binding has occurred. However, such evidence by itself does not establish that the Ig is a homeostatic autoantibody directed against normal constituents of the cell membrane. Definitive evidence for the role autoantibodies play in the selective removal of senescent cells can be obtained by first dissociating the antibodies from senescent cells and by then demonstrating their specific immunologic reattachment to homologous senescent but not to mature cells, leading to destruction of only the senescent cells by mononuclear phagocytes. This report presents evidence that IgG binds to the surface of senescent cells via the Fab region and initiates their phagocytosis by macrophages. The evidence suggests that the IgG may be an autoantibody directed against altered cell membrane molecules.

Human RBC were utilized as a model system because mononuclear phagocytes routinely phagocytize RBC at the end of their 120 day life-span, and they are an ideal experimental system in many respects. Large numbers of RBC are readily available, and senescent cells can be easily be separated from mature cells. RBC membranes have been extensively characterized biochemically, and they have a smooth regular surface that does not "cap" or ingest labels. Two biological assays are available: a phagocytosis assay, which assesses recognition of senescent cells by mononuclear phagocytes [2, 10], and a scanning immunoelectron microscopy labeling technique, which enables detection of as few as 2 IgG molecules per cell and assesses density and distribution of receptor sites [10–13].

MATERIALS AND METHODS

Isolation of Ig From Old RBC

Blood was obtained from 18 healthy individuals 19 to 34 years of age and one who was 50 years old. Young RBC were removed, and the remaining RBC were washed 3 times with 50 volumes of phosphate buffered saline (PBS), pH 7.4. Ig was eluted from 50–250 ml of packed RBC by the method of Kochwa and Rosenfield [14], because methods of elution which did not result in dissociation of antigen-antibody bonds (eg, temperature and glycine-NaOH buffer, pH 8), did not result in Ig elution in our hands. Preliminary experiments indicated that Ig could be eluted from old, but not young, RBC. The RBC eluate from nine individuals was assessed without further purification. Antibody from the RBC eluates of each of 10 individuals was isolated with an anti-Fab immuno-absorbent column. Fab was obtained from normal human IgG by papain digestion and column chromatography [15, 16]. Purity was determined by immunodiffusion (ID), immunoelectrophoresis (IEP), and polyacrylamide gel electrophoresis (PAGE). Antisera to Fab was obtained by immunizing a goat with 8 weekly injections of 1 mg of Fab that was pure as

determined by ID and IEP. After 10 weeks, the goat was bled and the immune serum was incubated with an immunoabsorbent that was made by binding Fab covalently to Sepharose 4B through a 13 atom spacer (aminohexyl derivative of Sepharose 4B, [17]). After thorough washing, the beads were poured into a 7.5 ml glass column (Bio Rad), and the antibodies specific for Fab were eluted with 0.1 M glycine-HCl, pH 2.3. The eluted antibody, determined with a recording spectrophotometer, was neutralized with 1 N NaOH, concentrated, and dialysed against PBS, pH 7.4. The anti-Fab antibodies obtained by this procedure were specific for Fab and reacted with pure IgA and IgM as well as IgG, as determined by ID and IEP. Conversely, specific anti-IgA, IgG, and IgM reagents reacted only with the specific Ig class. Anti-Fab specific antibodies were coupled to aminohexyl Sepharose 4B and used to isolate Ig from RBC eluates. The Ig was eluted from the anti-Fab column in the same manner as described for anti-Fab antibodies, then concentrated with an Amicon Diaflow with PM 10 filter. The quantity of IgG eluted per 100 ml of packed RBC was 10.27 ± 3.03 μg (mean ± SE), as determined by radial immunodiffusion with Ultra Low Level Diffu-gen plates, lot #55822 (Oxford Laboratories, Foster City, CA).

Mononuclear Phagocytes

Mononuclear cells from human peripheral blood were isolated on "Lymphoprep" and washed three times with Medium 199. The percentage of mononuclear phagocytes was determined by spreading an aliquot of mononuclear cells on a slide, or by making a cytocentrifuge preparation of mononuclear cells, staining with May-Gruenwald-Giemsa or with peroxidase [18], and performing differential cell counts with a 100X oil-immersion objective (Zeiss). The number of mononuclear phagocytes was calculated by multiplying the total number of mononuclear cells times the percentage of mononuclear phagocytes. Polymorphonuclear leukocyte contamination was less than 1%. Mononuclear cells were diluted with bicarbonate buffered Medium 199 with Methicillin/Gentamycin (100 μg/ml) and glutamine so that the concentration of mononuclear phagocytes was $6–10 \times 10^5$/ml, and 1 ml was pipetted into each tube. After a 1 h incubation at 37° in an atmosphere of humidified air containing 5% CO_2, nonadherent cells were removed by vigorous washing.

Phagocytosis Assay

The phagocytosis assay utilized for these studies measured phagocytosis rather than attachment, osmotic lysis, or adhesion of RBC to glass tubes. Light microscopy and transmission electron microscopy of cultures after 30 min and 3 h demonstrated that RBC in various stages of degradation were present inside mononuclear phagocytes, with some phagocytes having as many as 20 RBC inside them [2, 19]. Scanning electron microscopy of the cultures after 3 h demonstrated that only RBC that were partially engulfed were visible on the surface [2]. When young RBC were added to 20 tubes containing mononuclear phagocytes, 100% of the RBC were recovered 3 h later. When RBC stored without Ig were added to 16 tubes containing mononuclear phagocytes, 98–100% of the RBC were recovered 3 h later. Negative controls were utilized throughout the study. These included young RBC, RBC aged in vitro and incubated in medium without serum or in Ig-depleted serum, neuraminidase-treated RBC incubated in culture medium, and young RBC incubated in allogeneic Ig eluted from senescent cells. These cells were not phagocytized, as indicated by the recovery rates noted above. Mononuclear phagocyte viability at the end of culture was 98–100%, as determined by trypan blue dye exclusion.

Young and Senescent RBC

RBC from freshly drawn blood were depleted of white cells and reticulocytes, then separated into young, middle-aged, and old (senescent) populations by their difference in density [20, 21]. At the end of the density separation, young RBC are at the top of the gradient, and old RBC are at the bottom, as determined by the distribution of recently synthesized ^{59}Fe-labeled RBC [20, 21].

Stored RBC

Freshly isolated young RBC were washed 3 times with 50 volumes of Medium 199, resuspended at a concentration of 10% in Medium 199 without serum to avoid IgG binding, and transferred to tissue culture flasks. RBC were stored for 2–4 wk at 4°.

Not all of the RBC populations were needed for the purpose of these experiments. Those in excess of the required numbers remained in storage for a total of five months. When these cells were subsequently examined, it was found that all old RBC and approximately half of the middle-aged RBC had lysed. The young RBC populations, however, were essentially free of lysed cells. This reaffirms that the method of cell separation employed in the present experiment is successful and that different cell populations have different membrane properties. It suggests that storage of RBC in vitro may be a reasonable parallel model for aging in vivo because young RBC survive at least 150 days, and that old RBC have the potential for lysis if stored, without macrophages, for an extended period of time. It is possible that the mechanism by which membrane lesions are generated during storage in vitro may not be the same as that by which lesions are generated in vivo as cells age. It is possible, however, that the lesions themselves are identical.

Incubation of RBC With Ig Eluted From Senescent Cells

Stored RBC were washed and resuspended to a concentration of $1.5–2.0 \times 10^9$ RBC per ml. Twenty micrograms of Ig were added to 1 ml of the cell suspension which was incubated at 37° for 30–60 min in a shaking water bath. The RBC were then washed 3 times with medium and incubated with mononuclear phagocytes. Approximately 15–20 RBC were added per phagocyte, and the volume was adjusted to 0.24–0.40 ml per tube. Following incubation for 3 h at 37°, the percentage of phagocytized RBC was calculated as described previously [10]. This culture method supports a maximum of $\approx$ 50% phagocytosis.

The maximum percentage of phagocytosis obtainable appears to be limited by depletion of nutrients in the media. Addition of new medium at the end of 3 h results in additional phagocytosis of RBC: 46 ± 11% of the RBC were phagocytized during 6 h when new medium was not added, whereas 61 ± 2% were phagocytized when new medium was added after the first 3 h incubation. If old RBC are added to cultures that have been incubated without RBC for the first 3 h, without changing medium, there is no significant phagocytosis (only 8 ± 7% of the RBC were phagocytized). Finally, an additional 40 ± 3% of the old RBC, harvested from cultures after a 3 h incubation, are phagocytized when added to new cultures with fresh medium.

Neuraminidase-Treated RBC

Freshly isolated young RBC were washed 3 times with 50 volumes of Medium 199; incubated with 3.35 units of Vibrio Cholerae neuraminidase (Behring Diagnostics, which was tested and found to be lipase and protease free) per 10^{10} RBC at 37° in PBS, pH 6.8,

TABLE I. Characterization of Antibody Eluted From Senescent RBC*

Individuals	IgA			IgG			IgM			Albumin			Kappa		Lambda	
	ID	IEP	PAGE	ID	IEP	PAGE	ID	IEP	PAGE	ID	IEP	PAGE	ID	IEP	ID	IEP
1	–	–	–	+	+	+	–	–	–	–	+	+	+	–	+	+
2[a]	–	–	–	+	+	+	–	–	–	–	–	–	+	+	+	+
3[b]	–	–	–	+	+	+	–	–	–	–	–	–	+	+	+	+
4[b]	–	–	–	+	+	+	–	–	–	–	+	+	+	–	+	+
5	–	–	–	+	+	+	–	–	–	–	–	–	ND	ND	ND	ND
6	–	–	–	+	+	+	–	–	–	–	–	+	ND	ND	ND	ND
7	–	–	–	+	+	+	–	–	–	–	–	–	ND	ND	ND	ND
8	–	–	–	+	+	+	–	–	–	–	–	–	ND	ND	ND	ND
9[b]	–	–	–	+	+	+	–	–	–	–	–	–	+	–	+	+

*The results of the first 9 out of 15 individuals are presented here, for the latter 6 individuals gave similar results; ND, not done. Eluted antibody was identified by ID, IEP, and PAGE. For PAGE, 50 and 100 μg of protein were loaded on the gels. A "plus" indicates that the component was present; a "minus" indicates that it was not detected. ID was performed with Hyland ID plates (pattern C). IEPs were performed with barbital-barbital sodium buffer, pH 8.2 ($\Gamma = 0.05$), at a constant current of 34 mamp (approximately 51 volts) for 3 h at room temperature using IEP plates from Meloy. PAGE was performed by the method of Laemmli (22). The following anti-sera were used anti-human: whole serum and anti-human albumin (Meloy, Springfield, VA), anti-kappa, anti-lambda, anti-IgA, anti-IgG, anti-IgM, and anti-F(ab)$_2$ (Tago Immunodiagnostics, Burlingame, CA), and anti-Fc (Miles Labs., Kankakee, IL).
[a]Sodium dodecyl sulfate polyacrylamide gel electrophoresis was also performed and the results revealed only one Ig.
[b]Antibody eluted but not purified with anti-Fab immunoabsorbent.

TABLE II. IgG Binding and Specificity

	RBC group	Macrophage source	IgG source[a]	% Phagocytosis ± SEM[b]
Control groups				
Positive	Senescent, freshly isolated	Autologous	none added	50 ± 4
Negative	Young, freshly isolated	Autologous	none added	0
	Young, freshly isolated	Autologous	Allogeneic	5 ± 3
	Young, stored	Autologous	none added	0
	Young, stored	Allogeneic	none added	0
Experimental groups				
	Young, stored	Autologous	Autologous	46 ± 0
	Young, stored	Autologous	Allogeneic	27 ± 0
	Young, stored	Allogeneic	Autologous	56 ± 4

[a]Twenty μg of IgG eluted from senescent cells was added to 1 ml of RBC suspension containing $1.5–2.0 \times 10^9$ cells. In contrast, when stored RBC were incubated in either 0.6 mg or 6 mg of pooled, normal human IgG, the percentage of phagocytosis was 13 ± 2 and 35 ± 9, respectively. This confirmed the specificity of the binding of IgG eluted from senescent cells, since even a 300-fold greater concentration of pooled, normal human IgG was not as efficient as the eluted IgG in initiating phagocytosis.
[b]SEM, standard error of the mean from three triplicate cultures from each of three experiments.

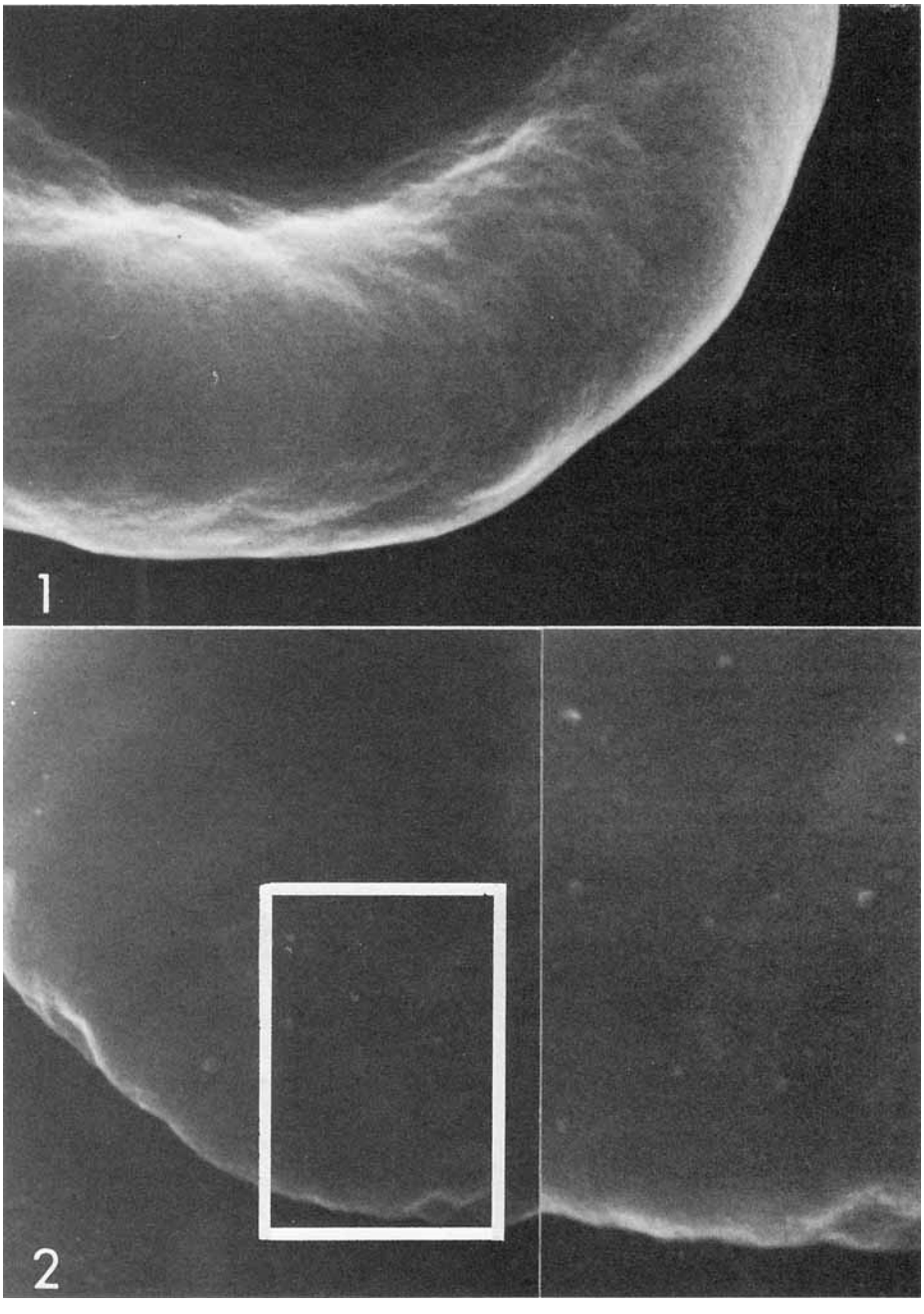

Fig. 1. Scanning electron micrograph of a typical stored RBC, incubated with goat antihuman IgG, and then rabbit antigoat conjugated to ferritin as a control for the antibody binding and specificity experiments. No ferritin is visible (× 31,250).

Fig. 2. Scanning electron micrograph of stored RBC, incubated with autologous IgG eluted from senescent cells, and then goat antihuman IgG followed by rabbit antigoat conjugated to ferritin. Ferritin is visible on the cell surface, even at low magnification. All RBC in these preparations were labeled with the ferritin. × 22,857. The inset on the right shows a higher magnification of the area within the white square (× 56,500).

for 30–60 min; and washed again. They were incubated in Medium 199 containing 20 μg of either autologous or allogeneic Ig eluted from senescent cells, washed 3 times, and incubated with autologous mononuclear phagocytes.

Scanning Immunoelectron Microscopy

Aliquots of the RBC used in phagocytosis experiments were incubated in the IgG fraction of goat antihuman IgG for 30 min at 37°, washed, incubated in rabbit antigoat IgG conjugated to ferritin for 30 min at 4°, washed again, and prepared for scanning electron microscopy [10–13]. Preparations were viewed with an Hitachi HFS-2 field emission, scanning electron microscope with 3 nm resolution. Between 220 and 300 cells were viewed in each preparation at magnifications from 20,000 to 100,000. Scanning time per preparation was 18–36 h.

RESULTS

Identification and Characterization of Old RBC Ig

The Ig eluted from RBC aged in situ was shown to be an IgG containing kappa and lambda light chains by immunodiffusion and immunoelectrophoresis (Table I). Other Igs were not detected by immunodiffusion, immunoelectrophoresis, or polyacrylamide gel electrophoresis.

IgG Binding and Specificity Experiments

In order to determine whether the IgG eluted from old RBC aged in situ would reattach to homologous cells, the IgG was incubated with autologous or allogeneic young stored RBC. These RBC were then washed and incubated with autologous mononuclear phagocytes. The results of these experiments are summarized in Table II. The percentage of phagocytosis of stored RBC incubated with autologous IgG and then with autologous mononuclear phagocytes is essentially the same as that of RBC aged in situ. Mononuclear phagocytes phagocytized autologous stored RBC incubated with autologous IgG, as well as allogeneic stored RBC incubated with autologous IgG. However, they did not phagocytize allogeneic cells that had not been incubated with IgG, nor did they phagocytize young allogeneic cells that were incubated with allogeneic IgG. IgG was demonstrated on the surface of stored RBC incubated with IgG eluted from autologous or allogeneic cells with scanning immunoelectron microscopy (Figs. 1 and 2).

TABLE III. Phagocytosis of Stored RBC ("0" RBC) Incubated With IgG Eluted From Senescent Cells Before and After Absorption With Stored RBC or Freshly Isolated Young RBC (YRBC)

Experiment	Quantity[a] (μg)	Phagocytosis (%) ± SEM[b]		
		Before absorption	After absorption with YRBC	After absorption with "0" RBC
1	3	49 ± 2	43 ± 9	0
2	3	35 ± 1	34 ± 7	0
3	3	43 ± 11	46 ± 16	0

[a]Quantity (μg) of IgG added to 1.5×10^8 RBC in 1 ml of Medium 199.
[b]SEM, standard error of the mean of triplicate or quadruplicate cultures.

TABLE IV. Phagocytosis of Stored RBC Incubated With Pooled Human IgG Before or After Absorption of the IgG With Stored RBC*

Experiment	Quantity of IgG (μg)[a]	Phagocytosis (%) Before absorption (Mean ± SEM)[b]	After absorption
1	30	42 ± 17	0
2	30	32 ± 4	0
3	300	52 ± 2	0
4	300	28 ± 6	0

*IgG was absorbed overnight at 4° with RBC aged in vitro.
[a]The quantity of IgG (μg) was added to 2×10^8 RBC which were incubated for 30 min at 24° and then for 30 min at 4°, washed, and incubated with autologous mononuclear phagocytes. The quantity of IgG (μg) was adjusted so that it was the same both for absorbed and unabsorbed IgG, as determined by radial immunodiffusion.
[b]Mean ± the standard error of the mean of quadruplicate cultures.

TABLE V. Phagocytosis of Neuraminidase-Treated Young RBC Incubated With IgG Eluted From RBC Aged In Situ

IgG source	% Phagocytosis ± SEM
None	0
Autologous	35 ± 2
Allogeneic	46 ± 6

Absorption of the eluted IgG with stored RBC, but not with freshly isolated young RBC, abolished its phagocytosis-inducing ability (Table III). Likewise, absorption of pooled normal human IgG with stored RBC abolished its phagocytosis-inducing ability (Table IV).

To confirm these findings and to determine the nature of the membrane molecules that are altered during RBC aging in situ, young RBC were treated with neuraminidase, a microbial enzyme that cleaves sialic acid groups from glycoprotein molecules. Neuraminidase was selected for this purpose because it is known that: 1) approximately 10% of the RBC membrane is composed of glycoproteins, 2) removal of sialic acid from circulating glycoproteins can result in their removal, 3) transient autoimmune hemolytic anemias sometimes follow respiratory infection with viruses such as influenza which contain neuraminidase, and 4) IgG will bind to neuraminidase-treated RBC [10]. Neuraminidase-treated RBC incubated in medium alone were not phagocytized, whereas those incubated in autologous and allogeneic IgG eluted from old RBC aged in situ were phagocytized (Table V). IgG was observed with scanning immunoelectron microscopy on the surface of neuraminidase-IgG-treated RBC, but not on RBC treated with neuraminidase alone.

The following experiments were performed in order to determine whether the Fab region of IgG binds to old RBC, thus indicating an immunological receptor binding, or whether old RBC simply develop Fc receptors. One group of stored RBC was incubated first with human Fab, then with goat antihuman Fab, and finally with rabbit antigoat IgG conjugated to ferritin. An identical group of stored RBC were incubated with human Fc,

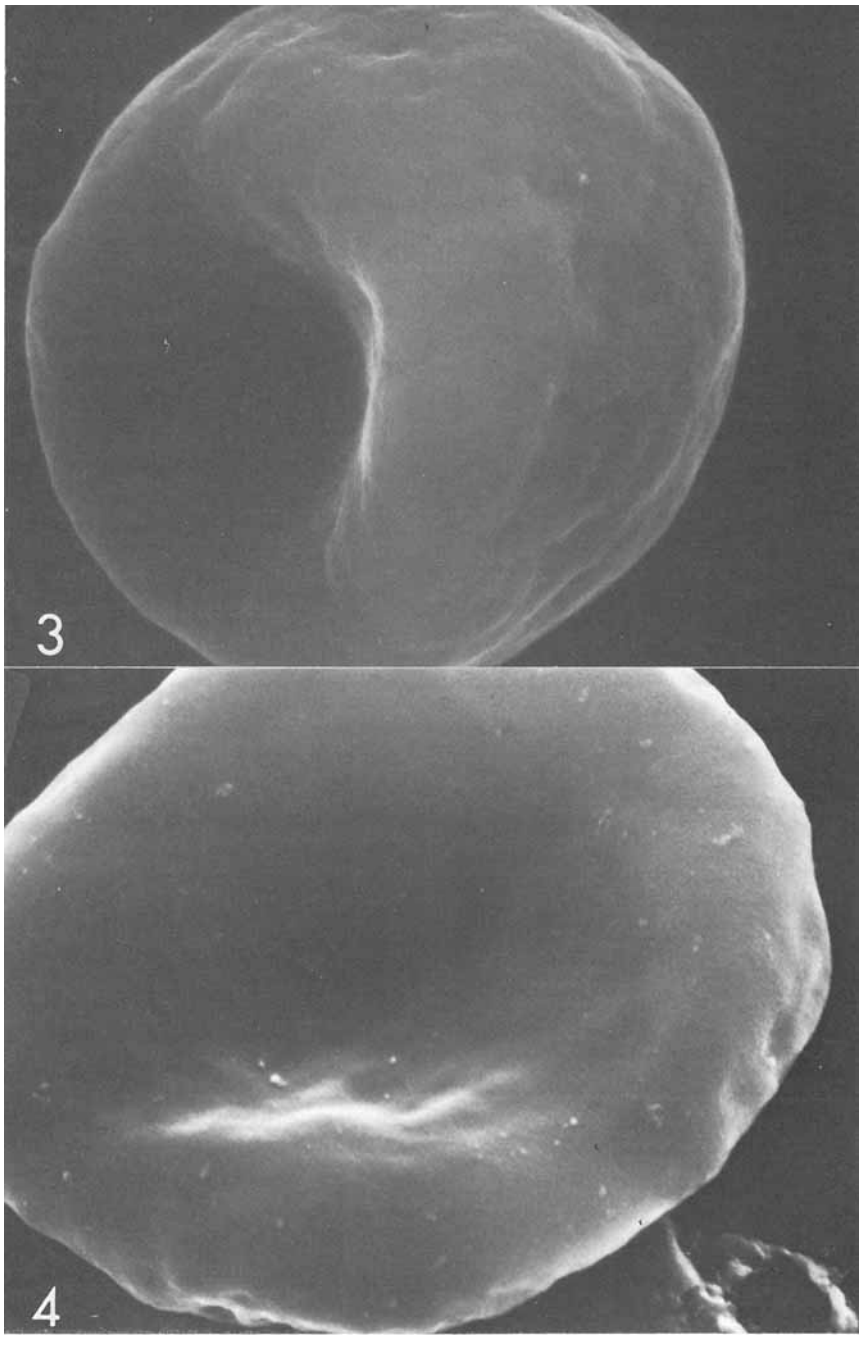

Fig. 3. Scanning electron micrograph of a typical stored RBC, incubated with human Fc, then goat anti-human Fc followed by rabbit antigoat conjugated to ferritin. RBC is essentially unlabeled (× 23,500).

Fig. 4. Scanning electron micrograph of a typical stored RBC, incubated with human Fab, then goat anti-human Fab followed by rabbit antigoat conjugated to ferritin. RBC is labeled with ferritin (× 22,979).

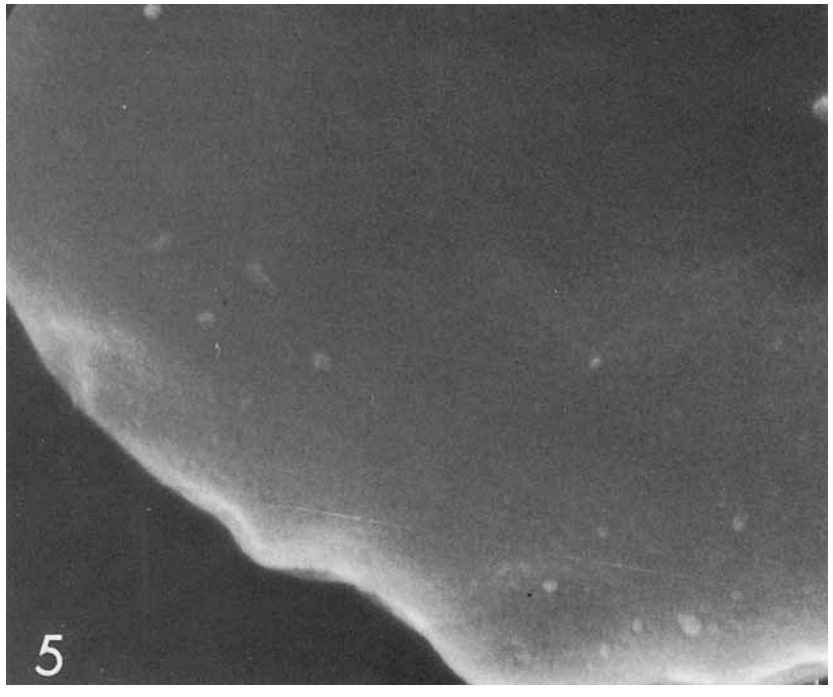

Fig. 5. Higher magnification of RBC from same preparation as that shown in Figure 4. Ferritin label indicating Fab is easily visible (× 57,065).

then with goat antihuman Fc, and finally with rabbit antigoat IgG conjugated to ferritin. Controls consisted of untreated RBC incubated with either goat antihuman Fab or anti-Fc, and then with rabbit anti-goat IgG conjugated to ferritin. As an additional control, stored RBC were incubated with either Fab and goat antihuman Fc, or Fc and goat antihuman Fab, followed in both cases by incubation with rabbit antigoat IgG conjugated to ferritin. Cells were then viewed with scanning electron microscopy. The results of these scanning experiments showed that the Fab but not the Fc region of IgG binds to stored RBC (Figs. 3–5).

To determine whether the Fab or Fc portion of IgG attaches to RBC, by a method independent of scanning immunoelectron microscopy, receptor blockade studies were performed. The results are summarized in Figure 6. Stored RBC were incubated for 30 min with either pooled normal human IgG, its Fab fragment, or its Fc fragment. The RBC were washed, and all three groups were incubated with IgG for another 30 min. RBC were washed again and incubated with autologous mononuclear phagocytes (Fig. 6A). For the control cultures, stored RBC were treated with IgG, Fab, or Fc before they were exposed to mononuclear phagocytes (Fig. 6B). Control results show that phagocytosis was achieved by exposing the RBC to IgG. Treatment with Fab or Fc did not promote phagocytosis. Experimental results show that treatment of RBC with Fab prior to incubation with IgG reduced the phagocotyosis to essentially 0, whereas pretreatment with Fc did not inhibit phagocytosis (Fig. 6A). Thus, these blockade studies also demonstrate that IgG binds old RBC via its Fab region.

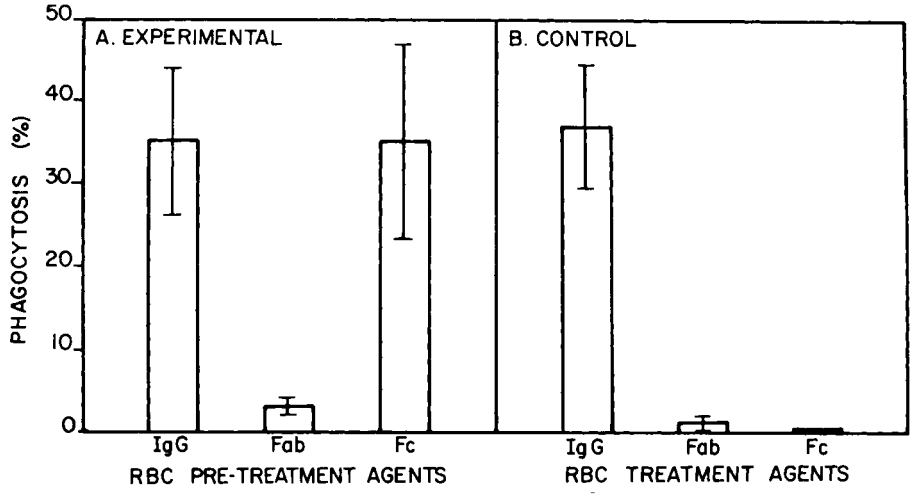

Fig. 6. A. Susceptibility to phagocytosis of aged RBC as influenced by their pretreatment with either IgG, Fab, or Fc before exposure to IgG. Stored RBC were incubated with either IgG, Fab, or Fc for 30 min, washed, and incubated with IgG for 30 min. The RBC were washed and incubated with mononuclear phagocytes for 3 h. B. Susceptibility to phagocytosis of aged RBC as influenced by their exposure to either IgG, Fab, or Fc. Stored RBC were incubated with either IgG, Fab, or Fc for 30 min, washed and then incubated with mononuclear phagocytes for 3 h. Bars indicate standard error of the mean; sample size was 6.

DISCUSSION

An earlier study from this laboratory [10] revealed that stored RBC were phagocytized by mononuclear phagocytes only after they had been exposed to IgG. The same study demonstrated that freshly isolated RBC aged in situ had IgG, but not IgA or IgM, on their surface. These RBC were phagocytized by mononuclear phagocytes, whereas young RBC were not. The experiments reported here confirm and extend this earlier study by showing that the Ig isolated from senescent cells is an IgG that is polyclonal with respect to light chains.

The binding and specificity experiments indicate that 1) IgG is required for the phagocytosis of stored autologous and allogeneic RBC; 2) nonspecific binding of IgG does not play a major role in these experiments because absorption of both pooled normal human IgG and IgG eluted from senescent cells with stored RBC abolishes its phagocytosis-inducing activity; 3) IgG eluted from senescent RBC is reactive against stored, but not young, cells; and 4) IgG eluted from senescent RBC cannot discriminate between autologous and allogeneic cells. The last two findings suggest that the receptor site appearing on the surface of cells aged in situ and that appearing on stored cells is the same, or closely related, for all individuals. This was confirmed by binding experiments utilizing neuraminidase-treated RBC, which showed that the IgG eluted from RBC aged in situ binds to neuraminidase-treated young autologous or allogeneic RBC, initiating their phagocytosis by mononuclear phagocytes. The observation that the IgG eluted from RBC aged in situ recognizes the receptor exposed by removal of sialic acid groups suggests that the receptor exposed as cells age naturally in situ may be the same as, or analogous to, that exposed by the removal of sialic acid in vitro. This would suggest that membrane glycoproteins may

be losing sialic acid as cells age, thus exposing the molecular determinants to which IgG binds. Another possibility is that the structural association between glycophorin and integral membrane proteins may be disrupted during aging, leading to exposure of cryptic antigens [23]. In such a case, one could speculate that neuraminidase acts, not by exposing new sugars, but by changing glycophorin–protein interactions through removal of sialic acid.

Although the antibody eluted from senescent cells has been found to bind only to senescent RBC aged in situ and to stored RBC, it is possible that it also binds and initiates the removal of damaged cells. Thus, this antibody could be participating in a generalized mechanism by which altered cells are removed from the circulation.

The evidence that indicates that the Fab region of the IgG molecule binds to aged RBC is consistent with an immunological binding of IgG with a surface receptor. Preliminary experiments suggest that the IgG eluted from senescent RBC belongs to the IgG_1 and IgG_3 subclasses. It is interesting that reports in the literature show that macrophages have receptors for the Fc region of complement-binding IgG_1 and IgG_3 subclasses [24].

The IgG eluted from senescent RBC may be considered an autoantibody, as it specifically reattaches to homologous RBC and initiates their selective destruction by mononuclear phagocytes. Indeed, this IgG may be a "physiologic" autoantibody. It appears to contribute to the maintenance of homeostasis by collaborating with macrophages in the removal of sensecent and damaged cells. This collaboration may permit the more efficient mature cells to carry out their vital functions without hindrance from less efficient cells, or by pathological reactions that could arise as a consequence of cells dying or decaying within the organism. Since the IgG autoantibodies may be part of the normal immune mechanism for removing cells, the data allow for the suggestion that the B cell clones producing these antibodies are not "forbidden" [25]. Further, the presence of these autoantibodies need not be attributed to an age-related decrease in suppressor cell function [26, 27].

Greater than 90% of the Ig molecules in human serum are of the IgG class, which supports the concept that IgG may perform homeostatic functions. Human serum contains an average of 12.1 mg/ml of IgG, 2.5 mg/ml of IgA, 0.93 mg/ml of IgM, 0.023 mg/ml of IgD, and 0.0003 mg/ml of IgE [28]. The total mass of IgG in the extravascular fluids is about the same as that in blood [28]. Thus, if one uses the averages figure for the plasma volume of a 70 kg male (ie, 3.5 liters) and the average figure for the extracellular fluid of a 70 kg male (ie, 10.5 liters), one arrives at the startling amount of 42.35 g of IgG in the plasma and 127.06 gm in the extracellular fluid, for a total of 169.40 gm or 1.13×10^{-3} moles of IgG in the average adult male. By comparison, the plasma mass of IgM is approximately 3.26 gm, or 3.6×10^{-6} moles. This can be considered the total body mass of IgM, as there is no evidence that IgM enters the extravascular fluids under normal conditions.

In view of these considerations, it may be appropriate to speculate that a collaboration between "humoral factors" and phagocytes may have evolved as a "safeguard" to ensure the specificity of surveillance mechanisms.

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