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Clustering of Integral Membrane Proteins of the Human Erythrocyte Membrane Stimulates Autologous IgG Binding, Complement Deposition, and Phagocytosis*

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Damaged or old erythrocytes are cleared rapidly from circulation. Because several common biochemical lesions can induce the clustering of integral membrane proteins, we have proposed that formation of microscopic protein aggregates in the membrane might constitute a cell surface marker that promotes removal of the defective/senescent cells. We demonstrate here that treatments that cluster integral membrane proteins in erythrocytes (1 mM $ZnCl_2$, 1 mM acridine orange, and 0.35 μM melittin) induce autologous IgG binding, complement fixation, and phagocytosis by human monocytes *in vitro*. Removal of the clustering agents prior to incubation in autologous serum or cross-linking of cell surface proteins before addition of clustering agents prohibited the above response, while cross-linking after treatment with the clustering agents preserved the response even if the clustering agents were later removed. Furthermore, subsequent reversal of the chemical cross-link maintaining the clustered distribution also reversed the induction of IgG binding, complement deposition, and phagocytosis. Finally, by deleting or inactivating different steps in the phagocytosis pathway, the chronology of steps was shown to be: (i) integral protein clustering, (ii) IgG binding, (iii) complement deposition, and (iv) phagocytosis.

It is thought that near the end of an erythrocyte's lifespan, the cell binds antibodies (1-3) and complement (4) which in turn promote its recognition and phagocytosis by macrophages (3, 5). Because *de novo* protein synthesis has terminated by this time, the "senescence antigen" recognized by autologous antibodies must arise from changes in components already present in the cell's membrane. Modifications by which this signal might be generated include proteolysis (6) or denaturation (7) of membrane proteins, changes in glycosylation (8-10), exposure of internal leaflet lipids (11, 12), and oxidation of cell surface components (13-16).

Clustering of integral membrane proteins is one attractive mechanism of senescent cell recognition because it depends on the erythrocyte's biochemical integrity rather than on an unrelated cell surface event (17-19). Briefly, this hypothesis postulates that the normal distribution of band 3 and glycophorin in healthy erythrocytes is random, as noted by others (20). However, as the cell ages, one or more microscopic regions of integral membrane proteins are thought to collapse into an aggregate. Because these clusters are both structurally abnormal and highly uncommon in the circulating red cell population, they could be viewed as "nonself" by the immune system and consequently opsonized by antibodies. Since hemoglobin denaturation (21, 22), ATP depletion (23), malondialdehyde (16, 24), Ca^{2+} loading (25), and oxidative cross-linking (26, 27) can all lead to formation of integral membrane protein clusters, the above mechanism assures that an erythrocyte is removed as its biochemical systems begin to fail.

Although several lines of evidence currently support a clustering mechanism for senescent/abnormal cell recognition and removal, a direct and rigorous test of the hypothesis has never been conducted. Thus, acridine orange (28) and phenylhydrazine (21) have been reported to promote membrane protein clustering and autologous IgG binding (17), but a causal relationship between the two processes was never established. Similarly, diamide, a catalyst of disulfide bond formation, has been found to stimulate IgG binding, complement deposition, and phagocytosis (19), but the increase in IgG binding was small, and other potential side effects of the oxidant were not examined. Finally, integral membrane protein clusters or aggregates have been isolated from senescent normal cells (29) as well as short lived sickle cells (30) and have been shown to contain the majority of cell surface IgG, but whether the integral protein clustering was directly responsible for IgG binding or whether IgG binding induced protein clustering could not be deduced from the data. Thus, a direct test of the causal relationships among integral membrane protein clustering, autologous IgG binding, complement fixation, and phagocytosis is still lacking.

In the present study, we have isolated each stage of the proposed senescence pathway beginning with integral membrane protein clustering and culminating in phagocytosis by macrophages. Integral protein clustering was induced by chemical clustering agents that were carefully chosen to minimize side effects that could complicate interpretation of the data. Thus, reagents that clustered by oxidizing the membrane, aggregating the cytoskeleton, or hydrolyzing the lipid were avoided, while treatments that acted reversibly by presumably unrelated mechanisms were specifically selected. Such a screen yielded only the bivalent cation zinc (31), the peptide melittin (31, 32), and the hydrophobic dye acridine orange (28) as optimal treatments for promoting integral membrane protein clustering. With these clustering agents, we evaluate the linkage between protein clustering and sub-

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sequent events leading to recognition and phagocytosis of erythrocytes by macrophages.

EXPERIMENTAL PROCEDURES

Reagents and Materials—Reagents and media for monocyte isolation and culture were from Flow Laboratories (McLean, VA); IODO-BEADS, BS[3],[1] DSP, 3,3′-dithiobis(sulfosuccinimidylpropionate), and the bicinchoninic acid protein assay were from Pierce; acridine orange hydrochloride, BSA, 4-chloronaphthol, diisopropyl fluorophosphate, Ficoll, HEPES, luminol, MES, anti-spectrin antibodies, and phenylmethylsulfonyl fluoride were from Sigma; goat anti-human IgG (affinity-purified polyclonal) and melittin were from Boehringer Mannheim; protein A was from Pharmacia LKB Biotechnology Inc.; carrier-free $Na^{125}I$ was from Amersham International; anti-human C3c goat IgG ("Cyto grade," affinity-purified, solid phase-adsorbed polyclonal) was from The Binding Site (Birmingham, U. K.); electrophoresis reagents were from Bio-Rad; nitrocellulose (pore size 0.2 μm) was from Schleicher and Schuell; all other reagents were Sigma, Mallinckrodt, or Merck products of the highest purity available; sterile plastics were from Falcon Labware (Oxnard, CA). Affinity-purified antibodies to the cytoplasmic domain of band 3, ankyrin, glycophorin C, and band 4.1 were prepared and kindly provided by David Allen, Bernard Thevenin, Chris Lombardo, and B. M. Willardson in our laboratory. The specificities of the antibodies have been established elsewhere (22, 33).

Buffers—Buffers were prepared as follows: phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM Na_2HPO_4/NaH_2PO_4, pH 7.4); PBS-glucose (PBS supplemented with 5 mM glucose); and HEPES-saline (10 mM HEPES, 140 mM NaCl, 5 mM glucose, pH 7.4).

Isolation of RBC and Clustering of Integral Membrane Protein—Freshly drawn human venous blood was anticoagulated by the addition of preservative-free heparin (5 units/ml), the RBC were sedimented by centrifugation (2,000 × *g* for 10 min at room temperature), and the buffy coat and plasma were removed by aspiration. After three washes with HEPES-saline buffer, the RBC were sedimented and resuspended at 10% hematocrit in HEPES-saline buffer containing either no additive, 1 mM $ZnCl_2$, 1 mM acridine orange, or 0.35 μM melittin and incubated for 15 min at room temperature. When desired, a cross-linking agent (400 mM stock solution in dimethyl sulfoxide) was added directly to the RBC suspension containing the clustering agent to achieve a final concentration of 1 mM. After a 15-min incubation at room temperature, RBC were washed twice in HEPES-saline supplemented with 10 mM ethanolamine and then twice in HEPES-saline buffer supplemented with BSA (1% w/v). RBC cross-linking induced by DSP was reversed by treatment with 10 mM 2-mercaptoethanol for 30 min at room temperature followed by washing.

RBC Opsonization and Measurement of Bound IgG and C3c—Washed RBC were suspended at 33% hematocrit in autologous serum or diisopropyl fluorophosphate-treated serum (fresh autologous serum supplemented with 4 mM diisopropyl fluorophosphate and dialyzed in the cold against PBS (19)), diluted 1:1 (v/v) with PBS-glucose, and incubated at 37 °C for 60 min. After opsonization, RBC were washed three times with 100 volumes of HEPES-saline supplemented with 2% (w/v) BSA. Then, to 50 μl of washed and packed RBC were added 100 μl of HEPES-saline supplemented with 4% (w/v) BSA and containing 2.5 μg of affinity-purified, radioiodinated goat antibody to human IgG (specific activity, 8 μCi/mg of protein). A second aliquot of 50 μl of washed and packed RBC was treated similarly with 100 μl of HEPES-saline supplemented with 4% (w/v) BSA containing 85 μg of affinity- purified radioiodinated goat antibody to human C3c (specific activity, 9.9 μCi/mg of protein). Radioiodination of anti-human antibodies was performed with IODO-BEADS (Pierce) and $Na^{125}I$ (30). After a 120-min incubation at 37 °C, each sample was diluted by adding 1 ml of HEPES-saline supplemented with 4% (w/v) BSA, loaded onto 3 ml of a 19% (w/v) Ficoll cushion in HEPES-saline, and spun down at 2,000 × *g* for 15 min at 4 °C. Each cell pellet was transferred to a fresh tube, washed once with 4 ml of HEPES-saline supplemented with 4% (w/v) BSA, and counted. After counting, 5 ml of Drabkin reagent was added to each sample and hemoglobin concentration was measured spectrophotometrically. Hemoglobin concentrations were converted to RBC counts assuming a mean cell hemoglobin content of 29.5 pg (34).

Phagocytosis Assay—Adherent mononuclear cells were separated by Lymphoprep gradient from fresh human blood and plated as described elsewhere (5). Each well usually contained 50,000–70,000 monocytes in 1 ml of RPMI 1640 culture medium supplemented with 10% (v/v) fetal calf serum. For a typical experiment, 1 unit (450 ml) of blood produced 100–110 wells, allowing 5–6 repeats for each experimental point.

After opsonization, 5 μl of RBC suspension (33% hematocrit in 1:1 diluted serum, see above) were added to each well containing adherent monocytes at a ratio of about 300 RBC per monocyte. After a 60-min incubation in a humidified incubator (air, 5% CO_2 atmosphere, 37 °C), nonadherent/noningested RBC were removed by gentle aspiration followed by three washes with 1 ml of PBS each. Noningested, adherent RBC were then lysed by brief (20 s) treatment with distilled water, followed by an additional wash with 1 ml of PBS. The adherent macrophages were then dissolved in 1 ml of 0.5 N NaOH containing 0.025% (v/v) Triton X-100, and phagocytosis was quantified by measuring the peroxidase activity of protoheme (35). The protoheme of the ingested hemoglobin catalyzes the production of chemiluminescence by luminol and *tert*-butyl hydroperoxide at alkaline pH according to the reaction: luminol + 2 *tert*-butyl hydroperoxide → aminophthalic acid + N_2 + 2 butanol + light.

The intensity of the emitted light is proportional to the protoheme concentration (36, 37). In our assay,[2,3] chemiluminescence was elicited by injecting 100 μl of a *tert*-butyl hydroperoxide-EDTA solution (containing 3.7 mM *tert*-butyl hydroperoxide and 3 mM EDTA dissolved in 0.1 N NaOH) into a test tube containing 5 μl of solubilized macrophages in 100 μl of alkaline luminol-EDTA solution (containing 1 mg/ml luminol and 3 mM EDTA dissolved in 0.1 N NaOH). The injection of *tert*-butyl hydroperoxide triggered photon emission and counting. Since light emission reached its maximum after less than 1 s and did not decrease until ~2.5 s, integrated photon counting time was set at 2 s. Photon counting was performed with a Ciba Corning Magic Lite Analyzer (Ciba Corning Diagnostic Corp., Redfield, MA). Photon counts per macrophage were transformed into RBC ingested per macrophage by comparison with a calibration curve run for each experiment with known amounts of cells out of the same RBC sample utilized for that specific experiment. Different RBC treatments had no influence on the calibration curves. Reagent luminescence and eigenluminescence due to macrophage heme proteins were subtracted.

Characterization of Modified Membranes by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis—RBC ghosts were prepared from control as well as modified RBC by hypotonic lysis in 5 mM sodium phosphate, 1 mM EDTA buffer, pH 8.0 in the presence of phenylmethylsulfonyl fluoride (20 μg/ml final) according to the procedure of Dodge *et al.* (38). Protein content was determined by the bicinchoninic acid assay (Pierce), and membranes were solubilized and analyzed on 5–12% gradient polyacrylamide gels (39). For immunoblotting, the proteins were transferred to nitrocellulose paper using the buffer system of Towbin *et al.* (40) and blocked in PBS, pH 7.4, containing 3% gelatin. The nitrocellulose strips were rinsed three times for 15 min each time with PBS, pH 7.4, containing 0.05% Tween 20, then with PBS alone, and finally labeled with primary antibody (diluted in PBS, pH 7.4, containing 1% BSA). After further washing and labeling with the second antibody conjugated to horseradish peroxidase, the immunoblots were developed using 4-chloronaphthol as substrate.

Sucrose Density Gradient Ultracentrifugation of Detergent-solubilized KI-extracted Inside-out Erythrocyte Membrane Vesicles—Following treatment with clustering and/or cross-linking agents, RBCs were lysed at 4 °C and freed of extractable hemoglobin by washing four times in 5 mM sodium phosphate, 1 mM EDTA, pH 8.0. Spectrin-depleted and KI-stripped inside-out vesicles were then prepared as described by Bennett and Stenbuck (41). The samples were then suspended in PBS, pH 7.4, and solubilized with an equal volume of 2% $C_{12}E_8$ (octaethylene glycol dodecyl ether) to yield a final protein concentration of 0.5 mg/ml. After layering onto a 10–30% sucrose gradient prepared in the same buffer, the samples were centrifuged at 15 °C for 12 h at 25,000 rpm in a Beckman SW28.1 rotor. Fractions (0.9 ml) were collected by gravity from each tube after puncturing the bottom of the tube with a needle.

[1] The abbreviations used are: BS[3], bis(sulfosuccinimidyl)suberate; BSA, bovine serum albumin (fraction V); DSP, dithiobis(succinimidylpropionate); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; PBS, phosphate-buffered saline: RBC, human red blood cell(s).

[2] G. P. Pescarmona, personal communication.

[3] F. Turrini, H. Ginsburg, F. Bussolino, G. P. Pescarmona, and P. Arese, manuscript submitted for publication.

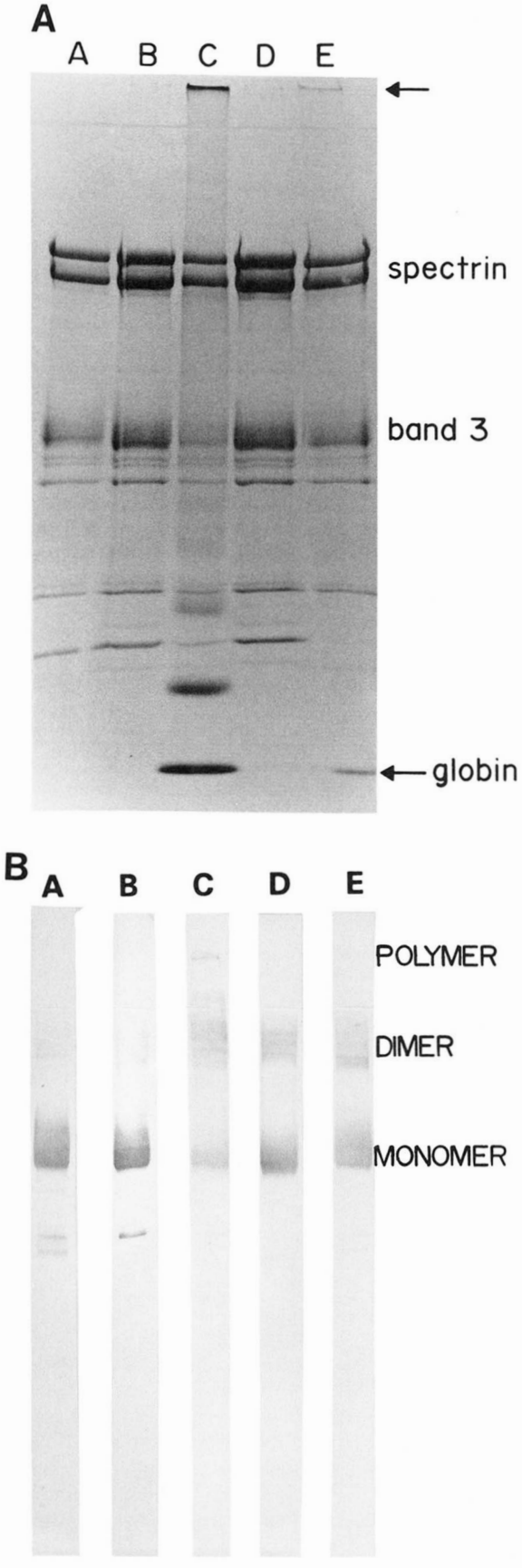

FIG. 1. **Evaluation of the capacity of various integral membrane protein clustering agents to stimulate band 3 cross-linking by BS^3.** Freshly drawn human erythrocytes were either left unmodified (*A*) or treated with 1 mM BS^3 following treatment with pH 7.4 buffer control (*B*), 1 mM $ZnCl_2$ (*C*), 0.35 μM melittin (*D*), or 1 mM acridine orange (*E*), as described under "Experimental Procedures." After neutralizing the unreacted BS^3 with ethanolamine, the cells were washed, and membranes were prepared. The membranes were solubilized in sodium dodecyl sulfate, separated electrophoretically on 5–12% polyacrylamide gels (39), and either stained directly with Coomassie Blue (*A*) or blotted onto nitrocellulose and visualized with rabbit antibodies to band 3 followed by horseradish peroxidase-linked goat anti-rabbit IgG (*B*). Staining was then conducted as described (30, 33).

RESULTS

Freshly drawn human erythrocytes were treated with 1 mM $ZnCl_2$, 1 mM acridine orange, or 0.35 μM melittin and then cross-linked with 1 mM BS^3 at room temperature for 15 min. After washing the cells and preparing membranes, the proteins were separated electrophoretically on sodium dodecyl sulfate-polyacrylamide gels and either stained directly or blotted onto nitrocellulose and stained with polyclonal antibodies to erythrocyte membrane proteins. As seen in *lanes C* and *E* of the Coomassie blue-stained gels of Fig. 1*A*, $ZnCl_2$ and acridine orange promoted BS^3 cross-linking of band 3 into high molecular weight aggregates too large to enter the stacking gels. Washing of the cells after addition of clustering agents but prior to treatment with BS^3 blocked formation of the high molecular weight aggregates (data not shown), suggesting that the effects of the clustering agents on membrane protein distribution are reversible. Immunoblots of the samples shown in Fig. 1*A* (Fig. 1*B*) not only confirmed the presence of band 3 in the plugs at the top of the gel, but also revealed the appearance of lower molecular weight cross-linked band 3 species in the region of the spectrin bands, as reported previously by Salhany *et al.* (42). Melittin, in contrast, did not promote significant cross-linking of band 3 into the very high molecular weight aggregates, yielding instead the less cross-linked forms that migrated in the spectrin region. Blots with antibodies to glycophorin C, spectrin, ankyrin, and band 4.1 revealed weak cross-linking of glycophorin C but no change in molecular weight of the other proteins, consistent with the impermeable nature of BS^3 (data not shown). In the case of $ZnCl_2$ at least, formation of high molecular weight species appears to be related to the concentration of clustering agent (Fig. 2), with measurable BS^3-stabilized aggregates appearing only at $ZnCl_2$ concentrations greater than 0.1 mM.

To test whether the higher molecular weight forms seen in Fig. 1 were due to cross-links between subunits of separate band 3 oligomers rather than cross-bridges within existing nonclustered oligomers (42–44), the modified membranes were solubilized in 1% $C_{12}E_8$ and centrifuged on a 10–30% sucrose gradient. As seen in Fig. 3, untreated oligomers of band 3 migrated in fractions 18–20. Only samples from cells treated with the clustering agent $ZnCl_2$ and subsequently stabilized with the cross-linker BS^3 yielded a large molecular weight pellet (fractions 1 and 2). Samples from cells treated with $ZnCl_2$ alone, 1 mM BS^3 alone, or even 5 mM BS^3 (which is known to cross-link subunits within existing band 3 oligomers (42–44)) also failed to penetrate the sucrose cushion. Gel filtration chromatography of the cross-linked aggregates indicated an apparent $M_r > 2 \times 10^6$ (data not shown).

Table I shows the consequences on autologous IgG binding, complement fixation, and phagocytosis of treating cells first with a clustering agent and then with BS^3. $ZnCl_2$, the strongest clustering agent, stimulated IgG binding, complement deposition, and phagocytosis by a factor of 6.5, 9, and 35, respectively. In contrast, melittin, the weakest clustering agent, enhanced the above events only by factors of 2, 2.5, and 15, respectively. Acridine orange was intermediate in potency as both a clustering agent and stimulant of the above immune

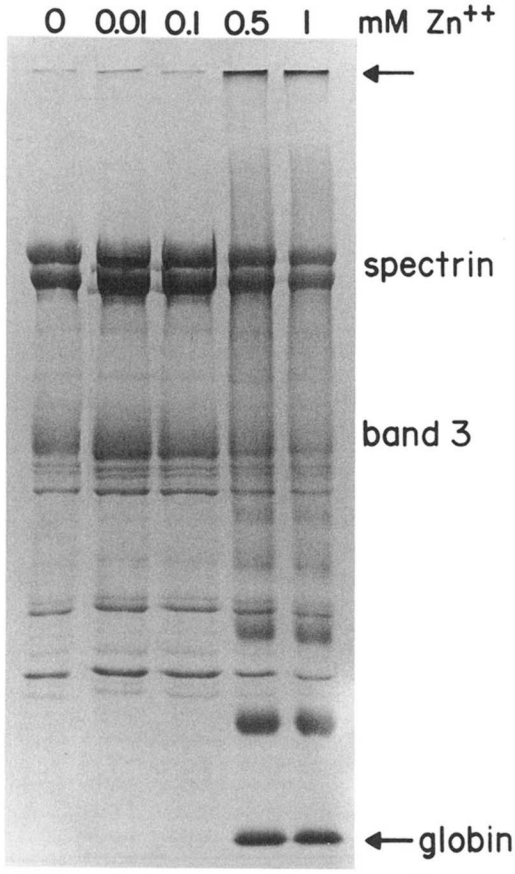

FIG. 2. **Effect of zinc on the ability of BS^3 to cross-link erythrocyte membrane proteins.** Erythrocytes were treated first with increasing concentrations of $ZnCl_2$ and then 1 mM BS^3, as described under "Experimental Procedures." The membranes were then separated electrophoretically and stained as described in Fig. 1.

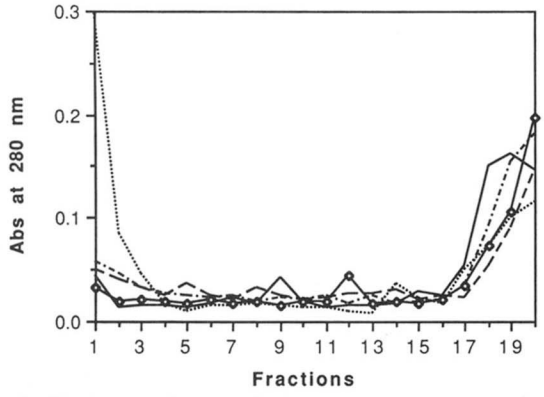

FIG. 3. **Sedimentation analysis of detergent extracts of membranes treated with $ZnCl_2$ and/or BS^3 on sucrose density gradients.** KI-extracted inside-out membrane vesicles were prepared from variously treated cells (*see below*) and solubilized in 1% $C_{12}E_8$ (final concentration). The samples were then loaded onto a 10–30% sucrose gradient and centrifuged for 12 h at 85,000 × *g*. Fractions collected from the bottom (fraction 1) to the top (fraction 20) of the gradient were examined at 280 nm to obtain an estimate of protein content. Washed cells from the same donor were treated with buffer (– — –), 1 mM $ZnCl_2$ (— — —), 1 mM BS^3 (——), 5 mM BS^3 (◇—◇), or 1 mM $ZnCl_2$ plus 1 mM BS^3 (-----) prior to preparation of the samples as described under "Experimental Procedures."

recognition/removal process. When the cells were treated with clustering agent and washed without treating with BS^3, none of the above events was stimulated to a significant extent (Table I), except possibly by melittin which can be removed only partially by washing (31). Control studies show that IgG binding, complement fixation, and phagocytosis are enhanced in the absence of BS^3 if the clustering agents are present during opsonization; thus, the clustering agents appear to initiate all three processes themselves.

To test whether cross-linking itself affected the events associated with erythrocyte phagocytosis, cells were treated with the irreversible (BS^3) or reversible (DSP) cross-linker and examined. At 1 mM concentration, neither BS^3 (Fig. 1*A*, *lane B*) nor DSP (not shown) promoted significant cross-linking of band 3 in the absence of a clustering agent. Under these conditions, no significant enhancement of any of the aforementioned processes was observed (Tables I and II and Fig. 4*A*). However, at 5 and 10 mM BS^3, where the reagent alone can cross-link band 3 (42–44), some IgG binding was measured (data not shown). For this reason, all further studies were conducted using 1 mM cross-linker.

To further test the role of membrane protein clustering in IgG binding, complement deposition, and phagocytosis, the sequence of clustering, fixation, washing, and opsonization was varied using $ZnCl_2$ as the clustering agent and the reversible cross-linker DSP as the fixative. As observed with BS^3, clustering and washing without fixation (Table II, row 1) and cross-linking without clustering (row 2) did not stimulate the events of the phagocytosis pathway, while fixation following the clustering treatment resulted in the anticipated expression of all three processes (row 3). Reduction of the disulfide bridge within the cross-linking agent, which presumably allowed dissociation of the clusters, prevented the activation of the entire recognition/removal sequence (row 4). Cross-linking with DSP prior to clustering was found to block the recognition/phagocytosis pathway (row 5), consistent with the suggestion that movement of the membrane proteins into clusters and not simply treatment with the above reagents promoted IgG binding.

The sequence of the IgG binding, complement fixation, and phagocytosis steps was further investigated by deletion/inactivation of specific steps in the pathway. When cells were treated with clustering agents, then fixed with cross-linker and directly subjected to macrophages without prior incubation in autologous serum, almost no complement could be detected, little nonspecific goat anti-human IgG binding was observed (15% of normal), and a rather consistent low level of phagocytosis (28% of normal) was measured (Fig. 4*B*). Furthermore, clustering and fixation followed by incubation in complement-inactivated serum, where IgG binding is normal but complement deposition is retarded, allowed little additional phagocytosis (Fig. 4*C*). When considered with the known dependence of complement deposition on prior IgG binding, these data and the data in Table II suggest that the sequence of events leading to phagocytosis follows the order: (i) integral protein clustering, (ii) IgG binding, (iii) complement fixation, and (iv) phagocytosis.

To determine how IgG binding, complement fixation, and phagocytosis might quantitatively interrelate in the recognition/removal pathway, erythrocytes were treated with increasing concentrations of $ZnCl_2$, fixed with BS^3, washed thoroughly to remove the $ZnCl_2$ and BS^3, and finally incubated in autologous serum. As shown in Fig. 5, elevation of Zn^{2+} concentration promoted a hyperbolic (saturating) increase in IgG binding. In contrast, deposition of new complement was low until 0.25 mM $ZnCl_2$, after which it rose abruptly. Importantly, phagocytosis was found to correlate mainly with complement fixation, reaffirming the enhancing effect of complement on the recognition and ingestion process (5, 45).

TABLE I

Analysis of the abilities of integral membrane protein clustering agents to stimulate autologous IgG binding, complement factor C3c deposition, and phagocytosis of human erythrocytes

Washed red cells were treated with clustering agent and then either treated with the cross-linker, 1 mM BS^3, or not modified further. The RBC were then washed three times in HEPES-saline, opsonized in autologous serum, and analyzed for IgG binding, complement deposition, and phagocytosis, as described under "Experimental Procedures."

Clustering treatment	Anti-human IgG[a]		Anti-human C3c[a]		Phagocytosis (RBC ingested/monocyte)	
	$-BS^3$	$+BS^3$	$-BS^3$	$+BS^3$	$-BS^3$	$+BS^3$
	molecules/RBC		*molecules/RBC*			
Control						
$\bar{x}$	215	988	1086	1958	0.091	0.12
S.D.	142	580	534	516	0.028	0.03
n	5	8	5	7	6	3
$ZnCl_2$ (1 mM)						
$\bar{x}$	520	6412	2261	17658	0.089	4.2
S.D.	335	1800	819	2421	0.019	0.24
n	4	8	4	7	3	7
Acridine orange (1 mM)						
$\bar{x}$	427	4479	1851	9826	0.036	3.5
S.D.	133	1105	486	3934	0.017	1.06
n	3	3	4	4	3	4
Melittin (0.35 μM)						
$\bar{x}$	740	1948	2314	4824	0.168	1.9
S.D.	39	660	819	2172	0.06	0.057
n	3	3	4	4	3	3

[a] Binding of autologous IgG and deposition of complement are expressed as the number of anti-human IgG and anti-human C3c radioiodinated antibodies bound per RBC, as described under "Experimental Procedures." In one experiment, cell surface IgG was quantitated using both ^{125}I-anti-human IgG and ^{125}I-protein A, and the ratio of the former measurement to the latter measurement was ~5. This suggests that roughly five anti-human IgGs can opsonize each cell surface autologous IgG. We have employed anti-human IgG to quantitate cell surface IgG since there appears to be less nonspecific binding than with protein A.

TABLE II

Analysis of the requirement of erythrocyte integral membrane protein clustering in the stimulation of autologous IgG binding, complement factor C3 deposition, and phagocytosis

Washed RBC were treated according to the sequence of steps listed in each box below, as described under "Experimental Procedures." Incubations were for 15 min at room temperature (30 min for 2-mercaptoethanol) and reagent concentrations were: 1 mM $ZnCl_2$, 1 mM DSP, 10 mM 2-mercaptoethanol, and 1 mM BS^3. The washing step consisted of resuspending the cells three times in HEPES-saline, pH 7.4, and pelleting. Following each sequence of treatments, the RBC were washed three times in HEPES-saline, opsonized in autologous serum, and analyzed for IgG binding, complement deposition, and phagocytosis, as described under "Experimental Procedures." Binding of autologous IgG and deposition of C3c are expressed as the number of goat anti-human IgG and anti-human C3c radioiodinated antibodies bound per RBC, as described under "Experimental Procedures" and in the legend to Table I.

Sequence of treatments		Anti-human IgG	Anti-human C3c	Phagocytosis (RBC ingested/monocyte)
		molecules/RBC	*molecules/RBC*	
1. $ZnCl_2$	$\bar{x}$	520	2261	0.089
2. Wash	S.D.	335	819	0.019
	n	4	4	3
1. DSP cross-link	$\bar{x}$	404	1744	0.11
2. Wash	S.D.	279	1015	0.024
	n	5	5	3
1. $ZnCl_2$	$\bar{x}$	4784	14934	4.2
2. DSP cross-link	S.D.	2572	4592	0.24
3. Wash	*n*	6	6	6
1. $ZnCl_2$	$\bar{x}$	1016	3969	1.47
2. DSP cross-link	S.D.	856	498	0.12
3. Wash	*n*	6	6	5
4. 2-Mercaptoethanol				
1. DSP cross-link	$\bar{x}$	941	1430	0.33
2. $ZnCl_2$	S.D.	171	630	0.05
3. BS^3 cross-link	*n*	3	3	3
4. Wash				

DISCUSSION

We have shown that an antigenic structure recognized by antibodies already present in the serum of all donors tested can be generated by the clustering of integral membrane proteins. Thus, treatment of cells with clustering agents promoted IgG binding only if the clustered distribution existed at the time of opsonization. Removal of the clustering agent prior to cross-linking, reversal of the cross-links maintaining

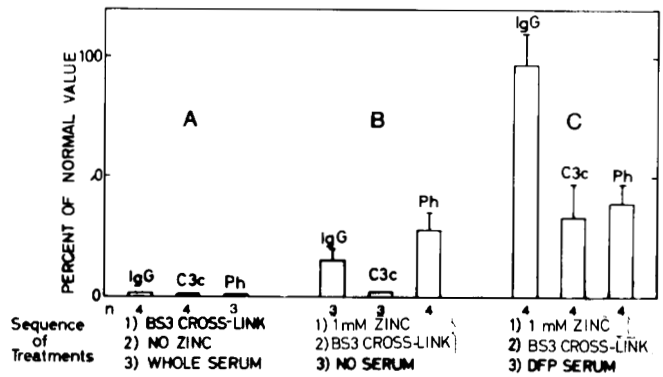

FIG. 4. **Evaluation of the interdependence of integral membrane protein clustering, autologous IgG binding, complement deposition, and phagocytosis of zinc-treated human erythrocytes.** Freshly drawn human erythrocytes were washed in HEPES-saline and either cross-linked directly with 1 mM BS^3 (*A*) or treated first with 1 mM $ZnCl_2$ and then cross-linked (*B, C*). After washing, the cells were incubated in whole serum (*A*), serum-free buffer (*B*), or diisopropyl fluorophosphate (*DFP*)-treated serum (complement-inactivated) (*C*), and then subjected to human monocytes for phagocytosis, as described under "Experimental Procedures." The various preparations were evaluated for autologous IgG binding (*IgG*), complement deposition (*C3c*), and phagocytosis (*Ph*). Each parameter is presented as a percent of the normal value obtained with cells treated with 1 mM $ZnCl_2$, cross-linked with 1 mM BS^3, and then opsonized in autologous serum. Mean values of *n* experiments ± S.D.

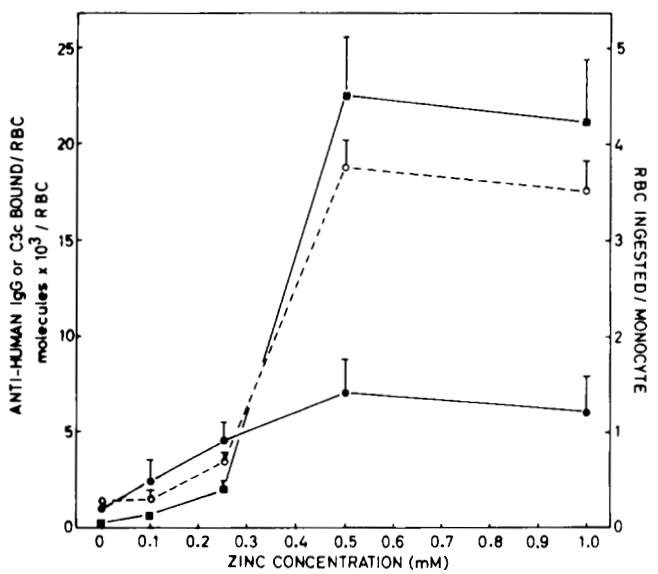

FIG. 5. **Dependence of autologous IgG binding (●——●) complement deposition (○– – –○), and phagocytosis (■——■) on treatment of erythrocytes with increasing concentrations of the clustering agent, $ZnCl_2$.** Freshly drawn erythrocytes were incubated with various concentrations of $ZnCl_2$, treated with 1 mM BS^3, washed to remove $ZnCl_2$ and unbound BS^3, incubated in autologous serum, and then analyzed for binding and phagocytosis. Bound IgG, C3c, and phagocytosis were quantitated as described under "Experimental Procedures." Mean values of four to six experiments ± S.D.

the clustered distribution, or treatment with cross-linking agents prior to clustering all inhibited antibody deposition. Because the above effects were independent of the clustering agent used, we conclude that clustering *per se* and not any side effect of the experimental protocol was responsible for antibody binding.

Since clustering *in vitro* stimulates autologous antibody binding, the question naturally arises as to whether clusters form during the course of erythrocyte aging *in vivo*. Recent data suggest that they do (29, 30). Thus, membrane protein aggregates can be isolated from the densest 1% of circulating erythrocytes (*i.e.* the fraction destined for immediate removal (46)), but not from the younger (lighter) populations of cells. Since these clusters represent less than 0.1% of the total membrane protein but contain 57% of the total cell surface antibody (*i.e.* an IgG enrichment of 680-fold over nonclustered regions of the membrane, Ref. 29), they must somehow exhibit a high affinity for circulating IgGs. Significantly, the data here suggest that membrane protein aggregates bind antibodies *because* they contain clustered proteins and not the converse.

What processes might drive the clustering reaction *in vivo*? Morrison *et al.* (47) have shown that large amounts of denatured hemoglobin bind to the red cell membrane immediately prior to erythrocyte removal, and we have demonstrated that denatured hemoglobin (hemichrome) avidly clusters band 3 both *in vitro* and *in vivo* (22, 48). Since the antibody-enriched membrane protein clusters from dense erythrocytes (29) as well as from short lived sickle (30) and thalassemic (49) cells are enriched in globin chains, hemichrome-induced band 3 clustering is one process that probably leads to protein aggregation *in vivo* (22, 50). Secondly, elevated production of oxidative species at the membrane surface, such as might occur when hemichromes localize on the cytoplasmic domain of band 3 (48), can also cluster band 3 and other proteins. Lutz *et al.* (19) have shown that diamide, a thiol oxidant, can cross-link band 3 and initiate recognition of erythrocytes by autologous IgG and complement. Turrini *et al.* (26)[4] have also shown that oxidized membrane protein aggregates exist in circulating glucose-6-phosphate dehydrogenase-deficient cells isolated during favism. Since oxidative damage can also be detected in older erythrocytes (1, 12, 13), oxidative cross-linking could conceivably generate the senescence antigen. Whether malondialdehyde production, another potential pathway for integral protein cross-linking (16, 24), occurs and contributes to IgG binding *in vivo* is still unclear.

Another unanswered question concerns why simple clustering of integral membrane proteins that have been present since the biosynthesis of the cell should create an antigenic site. One possibility is that clustering distorts one of the aggregated proteins causing it to display a conformation not found in healthy cells. Another explanation is that clustering removes an integral protein from a protected or occluded location, thus exposing a site not normally viewed by the immune system. The hypothesis we favor is that senescent cell antibodies have a low affinity for their epitopes on integral membrane proteins and do not form a stable monovalent attachment to their unclustered antigens (17). However, as cells become damaged or aged and protein clustering increases, the exponentially more stable (51) bivalent mode of IgG attachment would emerge and the cell would become opsonized.

We wish to point out that our protein clustering hypothesis is not necessarily exclusive of other current explanations of senescent cell recognition. Thus, opsonization of a cryptic α-galactosyl residue (8) or recognition of a deglycosylated (9, 10), cleaved (6), or oxidized (13–16) cell surface protein by autologous IgG may depend on its aggregation to allow the more avid bivalent attachment (18). Ion leakage (52, 53), loss of phospholipid asymmetry (11, 12), or oxidation of the membrane skeleton (54), *i.e.* defects commonly found in stressed and faltering cells, could also be linked indirectly to reorga-

[4] F. Turrini, S. Fasler, H. U. Lutz, G. Giribaldi, D. Alessi, and P. Arese, manuscript in preparation.

nization of membrane proteins.

The efficient initiation of complement fixation requires a cluster of bound IgG molecules (55, 56), and activation of one complement complex commonly stimulates the activation and deposition of others (55, 56). Thus, once a critical density of bound autologous IgG is reached at a localized site on the membrane, complement fixation should be autocatalytic, leading to the dense cap of IgG and complement necessary for erythrocyte docking with the clustered immunoglobulin and complement receptors on the macrophages (45).

In conclusion, we believe the basic elements of at least one senescent cell recognition pathway can be proposed. As a consequence of biochemical stresses such as hemoglobin denaturation, a few copies of band 3 and other integral membrane proteins are clustered into microscopic aggregates. These clusters are rapidly opsonized with autologous IgG. Once a sufficient density or size of IgG cluster is reached, complement is also deposited, marking the cell for removal by macrophages. In contrast to other models of senescent cell removal, this pathway allows the functional integrity of the cell to determine its own lifespan. Thus, glucose-6-phosphate dehydrogenase-deficient cells during favism (57), as well as sickle cells (58, 59) and thalassemic cells (60), can be removed rapidly, *i.e.* as their globin chains prematurely denature, while normal cells can circulate much longer, *i.e.* until their systems also begin to fail.

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