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Naturally occurring anti-band-3 antibodies and complement together mediate phagocytosis of oxidatively stressed human erythrocytes

(diamide/protein oligomers/cellular senescence/complement component C3/alternative complement pathway)

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ABSTRACT **Treatment of erythrocytes with the thiol-specific oxidant azodicarboxylic acid bis(dimethylamide) (diamide) enhances their phagocytosis by adherent monocytes. Phagocytosis of diamide-treated erythrocytes required that the cells were opsonized with whole serum, since complement inactivation abolished phagocytosis. Opsonization with whole serum containing 20–100 times the physiological concentration of naturally occurring anti-band-3 antibodies enhanced phagocytosis of diamide-treated erythrocytes. High inputs of anti-band-3 also restored phagocytosis of erythrocytes that had been incubated with complement-inactivated serum. Elevated concentrations of anti-spectrin antibodies were ineffective in whole and complement-inactivated serum. Specific recognition of diamide-treated erythrocytes by anti-band-3 antibodies may be due to generation of anti-band-3 reactive protein oligomers on intact diamide-treated erythrocytes. Generation of such oligomers was dose-dependent with respect to diamide. Bound anti-band-3 alone was not sufficient to mediate phagocytosis. It resulted in deposition of complement component C3b on the cells through activation of the alternative complement pathway in amounts exceeding that of bound antibodies by two orders of magnitude. Thus, anti-band-3 and complement together mediate phagocytosis of oxidatively stressed erythrocytes, which simulate senescent erythrocytes with respect to bound antibody and complement.**

Antibodies to erythrocyte membrane constituents are present in normal human serum (1–5). Some of them opsonize senescent or damaged erythrocytes and induce engulfment of the cells by phagocytes (3–5). One of them, a naturally occurring autoantibody to band 3 protein of erythrocyte membranes (anti-band-3), reacts with exoplasmic and cryptic domains of this protein (2). This antibody appears to be involved in clearance of senescent human erythrocytes, since it has the same specificity on blots as IgG eluted from senescent cells (6) or IgG precipitated from extracts of senescent erythrocytes (7). This evidence is, however, not conclusive because the amount of IgG bound to senescent erythrocytes (8–10) is not sufficient to elicit IgG Fc receptor-mediated phagocytosis, which requires thousands of IgG per cell (11, 12). Furthermore, antibodies with specificities other than anti-band-3 also recognize senescent erythrocytes (4, 5). The functional role of naturally occurring anti-erythrocyte antibodies has been studied by opsonizing erythrocytes with purified antibodies in the absence of serum (3–5). These conditions cannot differentiate between erythrophilic (13, 14) and antigen binding of added IgG. We, therefore, studied the effect of naturally occurring antibodies in the presence of serum. This avoids erythrophilic binding of added antibody and allows us to investigate the role of complement. Probing the effect of such antibodies on erythrocytes that had been in contact with these antibodies while in circulation requires manipulations, including elution of cell-bound antibodies or exposure of additional antigenic sites. Since antibody elution is incomplete and perturbs the membrane structure in an uncontrolled manner, young erythrocytes were *in vitro* "aged" by storage in the absence of serum (15). Although this classical manipulation resulted in an IgG requirement for efficient phagocytosis, it did not allow us to identify the antigenic sites exposed in the course of storage. Therefore, erythrocyte aging was mimicked by oxidative stress, which is known to accelerate erythrocyte clearance *in vivo* (16–18) and to induce membrane protein oligomerization (17, 19–22) and band 3 protein clustering (22). Band 3 clustering enhances binding of antibodies from autologous IgG, whether studied on skeleton-free vesicles (8) or on erythrocytes containing Heinz bodies (22, 23). This suggests, as claimed earlier (24), that anti-band-3 antibodies specifically recognize oligomerized band 3 protein. We therefore asked if a selective oxidation of membrane SH groups by azodicarboxylic acid bis(dimethylamide) (diamide) (19, 25) enhances phagocytosis of human erythrocytes *in vitro* and if phagocytosis was stimulated by anti-band-3 in the presence of serum.

MATERIALS AND METHODS

Diamide Treatment of Erythrocytes. Human blood, type O Rh^+, collected in heparin was freed from leukocytes (26). Erythrocytes were either used as such or were separated into subpopulations by density centrifugation on Percoll gradients (27) as modified (2). Erythrocytes were washed three times with phosphate-buffered saline (PBS)/glucose (10 mM phosphate/150 mM NaCl/5 mM D-glucose, pH 7.4). They were then treated with 50–100 μCi (1 Ci = 37 GBq) of [^{14}C]cyanate per ml (1 mM) at 50% hematocrit for 30 min at 37°C (28), if used for phagocytosis experiments. Erythrocytes used for binding studies were treated similarly with unlabeled cyanate (1 mM). After incubation, cells were washed three times with PBS/glucose and resuspended at 25% hematocrit in Tris buffer (40 mM Tris·HCl/5 mM KCl/116 mM NaCl/0.2 mM $MgCl_2$/5 mM D-glucose, pH 7.6). These suspensions were incubated with or without diamide (Sigma) at the indicated concentrations for 60 min at 37°C. Cells were washed three times in Tris buffer and were added to sera.

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Abbreviations: iPr_2P-F, diisopropylfluorophosphate; C3, complement component C3.

Opsonization of Erythrocytes in Sera Supplemented with Naturally Occurring Antibodies. Naturally occurring anti-band-3 (2) and anti-spectrin antibodies (1) were purified from polyspecific human IgG (Sandoglobulin) by immunoadsorption on immobilized antigens as outlined (2). Where indicated, autologous sera were supplemented with purified anti-band-3 or anti-spectrin dimer antibodies. Antibody-supplemented sera were dialyzed at 4°C against saline and PBS/glucose containing gentamycin (Sigma). They were used to opsonize erythrocytes at 33% hematocrit for 30 min at 37°C.

Phagocytosis Assays. Phagocytosis was carried out with adherent peripheral monocytes. Lymphocytes and monocytes were isolated (29) and plated (1–2 × 10^6 cells per well) on multidish Nunclon plates in RPMI 1640 (Flow Laboratories) containing 10% heat-inactivated calf serum. RPMI used for adhesion of monocytes was adjusted to pH 8.1 (30). After adhesion in 5% CO_2 for 2–16 hr at 37°C, nonadherent cells were removed by three washes with RPMI (pH 7.4). Plates were incubated with RPMI (pH 7.4) for 3–4 hr prior to addition of 2–4 × 10^7 opsonized, [^{14}C]cyanate-labeled erythrocytes per well. The extent of phagocytosis was determined as outlined (30). Results are given as the mean + 1 SD from a given number of independent experiments using 1–3 wells for each assay.

Binding of Anti-Band-3 Antibodies. Purified anti-band-3 antibodies were labeled by using either the chloramine T method (50–65 × 10^6 cpm/μg) or Enzymobead reagents (BioRad) (4–12 × 10^6 cpm/μg) (2). Fixed amounts of labeled and increasing amounts of unlabeled anti-band-3 were added to diisopropylfluorophosphate (iPr_2P-F)-treated serum to yield the final concentrations given. Anti-band-3 binding was studied as follows: Erythrocytes with a creatine content ranging from 20 to 40 μg per 10^{10} red cells were treated as outlined and incubated with 70% iPr_2P-F-treated serum containing a fixed amount of ^{125}I-labeled anti-band-3 (0.4–1.2 × 10^6 cpm in 50 μl) and the given final concentration of anti-band-3. After opsonization, the suspensions were diluted 10-fold with cold PBS/glucose and layered on 11 ml of 21.5% Ficoll in PBS/glucose to determine bound antibody by pelleting erythrocytes (14 min; 4300 × *g* at the bottom of the tube) at quasi-equilibrium conditions. The tubes were frozen, and the bottom containing the pelleted erythrocytes was cut to determine bound radioactivity. Controls were run in the absence of erythrocytes. Bound label was normalized to the number of opsonized erythrocytes. Results are given as the ng of anti-band-3 bound per 10^{10} erythrocytes.

Binding of Anti-Band-3 to Blotted Erythrocyte Polypeptides. Formation of anti-band-3 reactive protein oligomers was analyzed on blotted polypeptides from diamide-treated erythrocytes as follows: Membranes were prepared (2) from erythrocytes treated with or without diamide. Membranes were solubilized with 2% $NaDodSO_4$, and S—S-containing polymers were enriched by adsorption of SH-containing polypeptides on thiopropyl-Sepharose (Pharmacia), essentially as outlined elsewhere (31). The unbound material from 1.2 × 10^9 erythrocytes was electrophoresed with or without reduction and alkylation on $NaDodSO_4$/PAGE and was blotted (32). Blots were treated as outlined (2), except that they were incubated with ^{125}I-labeled anti-band-3 (0.5–1 × 10^6 cpm/ml) at 100 ng/ml in 10 mg of IgG per ml absorbed on band 3 protein in 2% milk powder instead of gelatin. Anti-band-3 reactive polypeptides were visualized by autoradiography.

Binding of Complement Component C3 (C3) to Erythrocytes. Human C3 was purified as described (33, 34), followed by absorption on protein A-Sepharose or anti-human IgG. C3 was ^{125}I-labeled using Enzymobead reagents (10–12 × 10^6 cpm/μg). Labeled C3 that was not frozen showed a similar susceptibility to trypsin as native C3 (35). Whole serum or iPr_2P-F-treated serum was supplemented with a fixed amount of ^{125}I-labeled C3 (up to 800,000 cpm in 50 μl) and increasing concentrations of antibodies, as indicated. Erythrocytes were treated as outlined and were opsonized with antibody-supplemented 70% whole serum or iPr_2P-F-treated serum that contained labeled C3. After opsonization, samples were diluted 10-fold, and bound label was determined as outlined for anti-band-3 binding. Bound label is given in micrograms of C3 per 10^{10} erythrocytes, assuming that normal serum contains 1.2 mg of C3 per ml and that bound material was either bound as C3 or deposited in the form of C3b and was not further processed. The total amount of C3d-containing C3 fragments on erythrocytes was assessed by goat anti-C3d (Nordic, Tilborg, The Netherlands) and an affinity-purified, ^{125}I-labeled second antibody (rabbit anti-goat IgG; Nordic). The number of C3b receptors on erythrocytes was determined by binding of an ^{125}I-labeled monoclonal anti-CR1 antibody as described elsewhere (36).

RESULTS

Anti-Band-3 Enhances Phagocytosis of Diamide-Treated Erythrocytes. Human erythrocytes treated with diamide were phagocytized by adherent monocytes following incubation with autologous serum, whereas cells treated with no diamide were not (Fig. 1). Phagocytosis of diamide-treated erythrocytes was dose-dependent at diamide concentrations from 5 to 1000 μM when phagocytosis was carried out with the same batch of monocytes (not shown). Diamide had the same effect at 20 and 200 μM when results were averaged from several experiments (Fig. 1). The extent of phagocytosis was comparable to that obtained with erythrocytes opsonized by anti-Rh D antigen at 2 μg/ml (Fig. 1).

Pretreatment of serum with iPr_2P-F, which prevents complement activation (37), abolished phagocytosis of diamide-treated erythrocytes (Fig. 1). Heat treatment of the serum decreased phagocytosis by 76 ± 16% (mean ± SD; n = 9). Thus, phagocytosis of diamide-treated erythrocytes was dependent on an intact complement system. Phagocytosis of cells opsonized in serum that had been dialyzed against a Mg^{2+}/EGTA buffer reached two-thirds the value observed with serum dialyzed against a Ca^{2+}/Mg^{2+} buffer (not shown), which indicates a predominant involvement of the alternative complement pathway (38).

Anti-band-3 added to serum enhanced the extent of phagocytosis of diamide-treated erythrocytes in a concentration-dependent manner (Fig. 2*A*). Moreover, addition of anti-band-3 at 50- to 100-fold its physiological concentration (0.1–0.2 μg/ml; ref. 2) to complement-inactivated serum

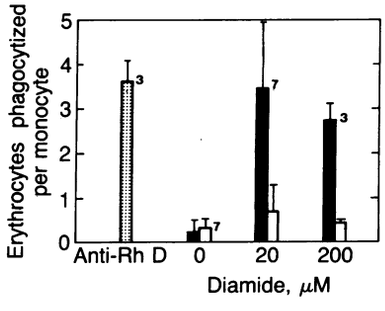

FIG. 1. Effect of diamide on phagocytosis of erythrocytes. Human erythrocytes were pretreated and ^{14}C-labeled. They were opsonized with either 2 μg of anti-Rh D per ml in buffer (stippled bars), whole serum (solid bars), or iPr_2P-F-treated serum (open bars). Opsonized erythrocytes were added to adherent monocytes, and the extent of phagocytosis was expressed as the number of erythrocytes engulfed per monocyte. Results are given as the mean + 1 SD from the indicated number of independent experiments (1–3 wells for each assay).

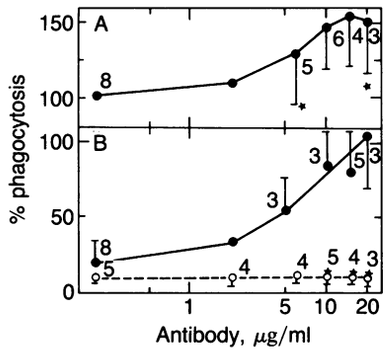

FIG. 2. Effect of naturally occurring antibodies on phagocytosis of diamide-treated erythrocytes. Labeled erythrocytes, treated with or without diamide, were opsonized with antibody-supplemented 70–80% whole serum or iPr_2P-F-treated serum. Opsonized erythrocytes were diluted 3-fold, pelleted, and resuspended in 70–80% serum (*A*) or iPr_2P-F-treated serum (*B*) to remove extra amounts of naturally occurring antibodies prior to phagocytosis. The extent of phagocytosis was the same whether or not the supplemented antibodies were removed after opsonization. Results are given as a percent of the controls. Diamide-treated erythrocytes opsonized in 70–80% whole serum served as controls with an average extent of phagocytosis (100%) of 4.1 ± 1.7 erythrocytes per monocyte (n = 8). The results are given as the mean of two independent experiments or as the mean + 1 SD from the number of independent experiments (given near each point) with 1–3 wells for each assay. (*A*) Phagocytosis of red cells treated with 20 μM diamide and opsonized with whole serum supplemented with anti-band-3 (●) or anti-spectrin (★). (*B*) Phagocytosis of erythrocytes treated without (open symbols) or with 20 μM diamide (filled symbols) and opsonized with iPr_2P-F-treated serum supplemented with anti-band-3 (○, ●) or anti-spectrin (★).

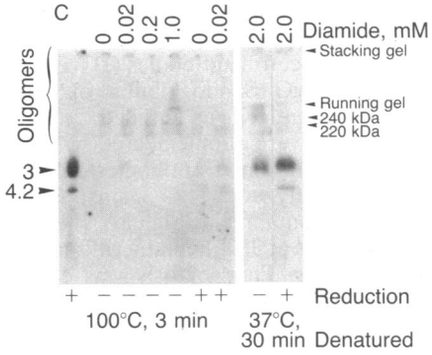

FIG. 3. Anti-band-3 reactive protein oligomers in diamide-treated erythrocytes. S—S-linked polypeptides from erythrocytes treated with increasing concentrations of diamide (0–2 mM) were enriched by adsorption of SH-containing polypeptides. Samples were denatured at 37 or 100°C with (+) or without (−) reduction for the indicated length of time, electrophoretically spread, and blotted. Blots from both the running and the stacking gel were incubated with ^{125}I-labeled anti-band-3. Lane C: 20 μg of erythrocyte membranes. Apparent molecular mass of 240 and 220 kDa refers to the spectrin bands as detected on stained blots from gels run in parallel with membranes. An autoradiograph of the gel is shown. The arrowheads at the left indicate the positions of band 3 and band 4.2.

restored phagocytosis to that of normal serum (Fig. 2*B*). In contrast, supplementation of serum or iPr_2P-F-treated serum with anti-spectrin did not enhance the extent of phagocytosis (Fig. 2 *A* and *B*), further indicating that the diamide-induced modification was specifically recognized by elevated concentrations of anti-band-3.

Diamide Generates Band 3 Protein Oligomers. Treatment of cells with 1–2 mM diamide generated band 3 oligomers, which were detected as anti-band-3 reactive species on blots from whole membranes (not shown). Since they comprised less than 1% of total band 3 protein, S—S-linked polypeptides containing oxidized band 3 oligomers were enriched (31) from $NaDodSO_4$-solubilized membranes of cells that had been treated with 0–2 mM diamide. Anti-band-3 reacted specifically with high molecular weight protein oligomers from fractions enriched in S—S-linked polypeptides (Fig. 3). Reduction of S—S bonds with dithiothreitol dissociated the oligomers and generated anti-band-3 reactive material in the position of band 3 protein and in the band 4.2 protein region (70 kDa). Oligomer formation was dose-dependent with respect to diamide; at 20 μM diamide, oligomer formation was already greater than in the controls.

Binding of Anti-Band-3. Diamide treatment also increased binding of anti-band-3 to erythrocytes (Fig. 4*A*). In the range of 2–15 μg of anti-band-3 per ml, more ^{125}I-labeled anti-band-3 bound to diamide-treated erythrocytes than to control cells. For erythrocytes treated with 200 μM diamide, differences were significant between 4 and 10 μg of anti-band-3 per ml and corresponded to less than 15 additional IgG molecules bound per erythrocyte. The increment in bound antibody was not due to a systematic error in counting diamide-treated erythrocytes, since a similar increase in antibody binding was found with unoxidized erythrocytes, to which diamide instead of buffer was added during the binding assay (Fig. 4*B*). The small increment in the number of bound antibodies on diamide-treated erythrocytes is real and occurred at antibody concentrations that also enhanced phagocytosis (Fig. 2). Neither this increment nor the total amount of cell-associated anti-band-3 could cause phagocytosis alone if it occurred by Fc receptors alone and were dependent on as many IgG as with anti-Rh antibodies (11). A few hundred antibodies could initiate phagocytosis if antibodies were clustered (12) or if a concomitant C3b deposition allowed cooperative recognition by Fc and complement receptors (11).

Anti-Band-3 Elicits C3 Binding. Efficient phagocytosis required primarily complement at physiological concentrations of anti-band-3 (Fig. 1). Elevated anti-band-3 concentrations not only enhanced phagocytosis but also restored phagocytosis of diamide-treated cells in complement-inactivated serum (Fig. 2). The latter finding does not imply that phagocytosis was exclusively dependent on IgG Fc receptor interactions, since C3 fragments acquired while in circulation were not eluted before diamide treatment. Correspondingly, erythrocyte-bound C3 fragments, as measured by anti-C3d, were lower by only 10 ± 2% (mean ± SD; n = 3) when cells

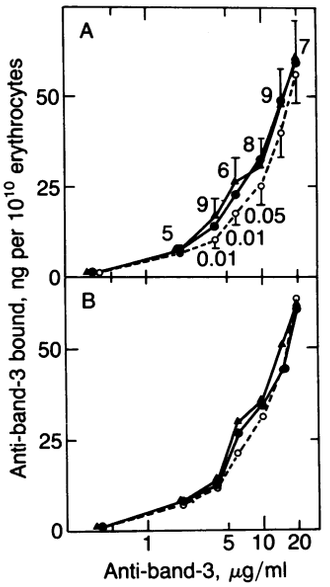

FIG. 4. Erythrocytes were pretreated with unlabeled cyanate, with or without diamide. Binding of ^{125}I-labeled anti-band-3 to erythrocytes was studied by opsonizing them with 70% iPr_2P-F-treated serum supplemented with the given concentration of antibody. (*A*) Binding of ^{125}I-labeled anti-band-3 to erythrocytes treated with 0 (○), 20 (●), or 200 μM diamide (▲). Results are given as the mean + 1 SD and, where appropriate, the P value for the given number of experiments. (*B*) The conditions were the same as in *A* except that erythrocytes were incubated with ^{125}I-labeled anti-band-3 in iPr_2P-F-treated serum to which 0, 20, or 200 μM diamide was added during the binding assay. Results are the mean of two experiments.

were opsonized in iPr_2P-F-treated serum instead of whole serum. Hence, the total amount of C3d-containing fragments is of little help in elucidating whether anti-band-3 stimulated C3b deposition in whole serum. Therefore, we measured binding of ^{125}I-labeled C3. At physiological anti-band-3 concentrations, binding of C3 to diamide-treated erythrocytes exceeded that to control cells by 10% at 20 μM diamide and 20% at 200 μM diamide ($P < 0.05$; P values in the text were calculated from normalized data, which are shown in absolute terms in Fig. 5). Moreover, binding of C3 to diamide-treated erythrocytes first increased with anti-band-3 concentrations, then reached a maximum, and finally declined again (Fig. 5*A*). Maximum C3 binding was 65 ± 15% greater at 20 μM and 235 ± 94% greater at 1 mM diamide ($P < 0.01$) than in controls without added anti-band-3. Thus, anti-band-3 significantly enhanced C3 binding in whole serum, whereas it only slightly enhanced binding of C3 in iPr_2P-F-treated serum (Fig. 5*B*). Thus, in whole serum, a large portion of C3 binding was due to generation and deposition of C3b and/or its fragments. Binding of C3 to erythrocytes was further dependent on whether glucose was present during pretreatment of cells with or without diamide. Omission of glucose, which leads to ATP-depletion, enhanced anti-band-3 dependent C3 binding (Fig. 6). This was most prevalent for control cells and thereby diminished the dose-dependent effect of diamide.

C3 binding to erythrocytes was not due to binding of C3b-containing soluble immune complexes since (*i*) diamide-treated and control cells were opsonized with the same serum and (*ii*) contained the same number of C3b receptors (CR1) (39), as was evident from unaltered binding of an ^{125}I-labeled monoclonal anti-CR1 antibody (36) (not shown). C3 binding is most likely due to anti-band-3 mediated C3b deposition. In the case where diamide induced partial lysis, the effect could have been due to other naturally occurring antibodies [e.g., anti-spectrin (1)]. This is unlikely because serum supplemented with anti-spectrin instead of anti-band-3 did not measurably contribute to C3 binding. C3 bound from anti-spectrin supplemented serum was subtracted from that recorded for anti-band-3 supplemented serum in Fig. 7*A*. Thus, anti-band-3 antibodies specifically enhanced binding of C3. Its binding was two orders of magnitude greater than that of anti-band-3 (for limitations see Fig. 7).

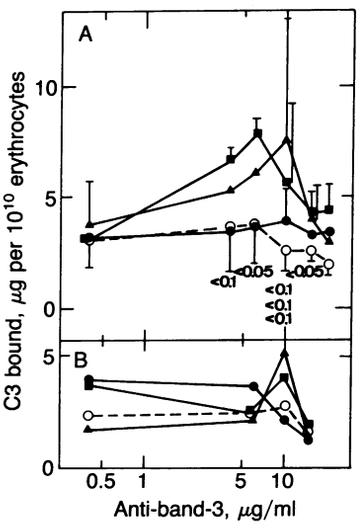

FIG. 5. Binding of ^{125}I-labeled C3 to diamide-treated erythrocytes is enhanced by anti-band-3 in sera. C3 binding to diamide-treated erythrocytes is shown for 70% whole serum (*A*) and for 70% iPr_2P-F-treated serum (*B*) that were supplemented with ^{125}I-labeled C3 and increasing concentrations of anti-band-3. Results are either the mean from two independent experiments or the mean + 1 SD from 3–4 independent experiments. The P values for the absolute values are given below the curves in *A*; the corresponding P values for the normalized data (as percent of controls) are given in the text. C3 binding was studied to erythrocytes treated with 0 (○), 20 (●), 200 (▲), or 1000 (■) μM diamide.

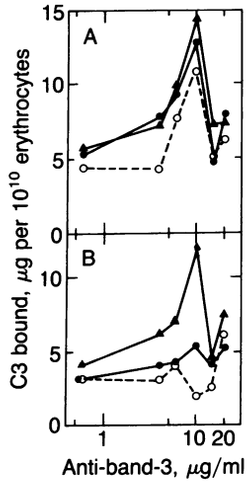

FIG. 6. Binding of ^{125}I-labeled C3 to erythrocytes that were pretreated with or without diamide in the absence (*A*) or presence (*B*) of glucose. Erythrocytes were pretreated as outlined, except that half of the cells were pretreated in a buffer without glucose. All erythrocytes were opsonized with 70% whole serum containing increasing concentrations of anti-band-3, identical amounts of ^{125}I-labeled C3, and glucose. The results are the mean of two independent experiments with erythrocytes treated with 0 (○), 20 (●), or 200 (▲) μM diamide.

DISCUSSION

Diamide accelerated *in vivo* clearance of dog erythrocytes at 200–400 μM as shown by others (17). This study demonstrates that diamide enhanced phagocytosis of human erythrocytes *in vitro* at concentrations ≥20 μM. Phagocytosis of diamide-treated erythrocytes required opsonization with serum. The most effective opsonin was C3b as judged from the 80–90% inhibition (controls subtracted) of phagocytosis by complement inactivation. Binding of C3 to diamide-treated cells was 10–20% higher than that to controls cells, whereas binding of anti-band-3 was not enhanced in whole serum containing physiological concentrations of anti-band-3 antibodies. The small extent of phagocytosis remaining after complement inactivation made it technically unfeasible to differentiate further between the effect of complement-inactivated serum that contained anti-band-3 or was depleted

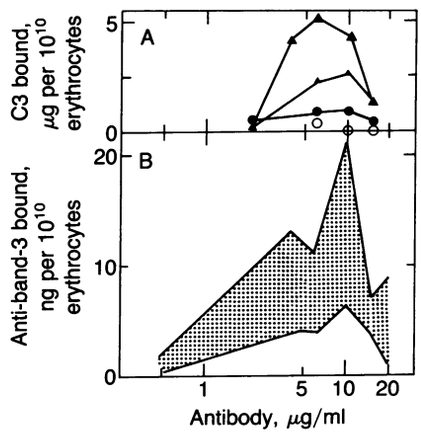

FIG. 7. C3 binding is anti-band-3 dependent and not anti-spectrin mediated. Binding of ^{125}I-labeled C3 to diamide-treated erythrocytes was determined by opsonizing them with 70% serum supplemented with labeled C3 and with either anti-band-3 or anti-spectrin. (*A*) The difference in bound C3 is plotted. Results shown are from two experiments using erythrocytes treated at 200 μM diamide (▲, ▲) and from one in which erythrocytes were treated with 0 (○) or 20 (●) μM diamide. (*B*) The diamide-induced increment in bound anti-band-3 was determined in five experiments by subtracting the values obtained without diamide from those recorded for cells treated with 200 μM diamide (Fig. 3). The range of values (±2 SD) is shown.

of it. Thus, we could not elucidate the type of antibody that initiates C3b binding at physiological concentrations of naturally occurring antibodies. Since the binding assay exclusively assessed binding of added antibody and thereby did not reveal bound IgG acquired while in circulation, it is possible that previously acquired IgG initiated C3b deposition. Since erythrocyte-bound IgG has anti-band-3 specificity (6, 7), it is conceivable that low concentrations of diamide preferentially cross-linked those band 3 molecules that carried antibodies. On the other hand, evidence is shown for a specific effect of anti-band-3 antibodies at elevated concentrations. Phagocytosis of diamide-treated erythrocytes that were opsonized in whole serum was stimulated by added anti-band-3, and high concentrations also compensated for inhibition by complement inactivation. Likewise, anti-band-3 binding was significantly higher to diamide-treated erythrocytes than to control cells at concentrations that also stimulated phagocytosis. The extent of binding was small, but specific, because it occurred in the presence of a 500- to 1000-fold excess of other serum IgG and its effect was not mimicked by increasing the concentration of anti-spectrin antibodies.

Added anti-band-3 evidently recognized a diamide-induced modification on erythrocytes. Diamide oligomerizes spectrin within intact erythrocytes, as is well established (17, 19). Diamide concentrations ≥ 20 μM also generated anti-band-3 reactive protein oligomers as shown here. Anti-band-3 reactive protein oligomers with apparent molecular mass values ≥ 200 kDa were verified by enrichment and antibody binding. They contained band 3 protein as well as anti-band-3 reactive material in the region of 70 kDa. The latter component may represent a band 3 breakdown product (40) or a cross-reacting membrane protein (2). Formation of anti-band-3 reactive oligomers on intact erythrocytes is probably responsible for enhanced anti-band-3 binding, since antigen oligomerization increases antibody binding (8, 22, 41). This process may explain the small increment in anti-band-3 bound to treated cells. It may also explain why anti-band-3 antibody binding and complement deposition reached a maximum and then declined. The high-affinity binding sites, most likely represented by band 3 oligomers, were saturated at 6–10 μg of anti-band-3 per ml. Beyond this point, binding of a bivalent antibody is primarily monovalent (42) and, thus, less firm and undistinguishable from that to the unsaturable number of band 3 molecules. Erythrocyte-bound anti-band-3 mediated C3b deposition on diamide-treated erythrocytes. The effect of anti-band-3 cannot be explained by generation of a fluid phase convertase with anti-band-3 and binding of C3b to innocent bystanders (43). This would have resulted in equal C3b binding whether or not cells had been treated with diamide. The amount of bound C3b exceeded that of bound anti-band-3 by two orders of magnitude. This, as well as the independence from added Ca^{2+}, strongly suggests that cell-bound anti-band-3 triggered C3b deposition through activation of the alternative complement pathway, as is known for another naturally occurring antibody, to a yet uncharacterized rabbit erythrocyte surface component (44). This property is of particular relevance because it renders anti-band-3 antibody considerably more effective than if it induced classical pathway C3b deposition, which lacks a positive feedback. Complement requirement was either not apparent or not considered in earlier studies of phagocytosis of senescent (3–6) and oxidatively damaged red cells (15–17), though erythrocyte senescence (45) and shortened viability of stored erythrocytes (46) correlated with increased deposition of C3 fragments. The mechanism by which anti-band-3 stimulated C3 binding may involve enhanced convertase activity of C3b bound to antibody (ref. 47; S.F. and H.U.L., unpublished results).

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