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## The Role of Hemoglobin Denaturation and Band 3 Clustering in Red Blood Cell Aging

Abstract. *As hemoglobin begins to denature, it forms hemichromes that cross-link the major erythrocyte membrane–spanning protein, band 3, into clusters. These clusters provide the recognition site for antibodies directed against senescent cells. These antibodies bind to the aged red cell and trigger its removal from circulation.*

The homeostasis of the circulatory system depends on the removal of aged and damaged cells from the blood (*1*). Autologous antibodies [immunoglobulin G (IgG)] present in healthy human serum selectively bind to senescent red blood cells (RBC's) and initiate the removal of the senescent cells by phagocytes and macrophages (*2–6*). The age-specific antigen on senescent RBC's is the predominant membrane protein, band 3 (*4, 7–9*), but since band 3 is present on both young and old cells, the molecular change in band 3 that marks the erythrocyte as "aged" is unclear. Three hypotheses have been offered to explain this change in band 3: (i) aged band 3 is cleaved or covalently modified, (ii) a cryptic antigenic site on band 3 is exposed on aging, or (iii) the lateral distribution of band 3 in the membrane in aged cells is altered (*6, 9*). We now report that as hemoglobin begins to denature, it cross-links band 3 into aggregates, and these cross-linked aggregates constitute the antigenic determinant recognized by the antibodies to senescent cells. Thus, RBC's are removed from circulation and destroyed when they begin to falter in their primary function of $O_2$ transport.

The initial stages of hemoglobin denaturation involve the formation of hemichromes, which remain oligomeric but do not carry oxygen because of a conformational change in the vicinity of the heme (*10, 11*). We reported earlier that these hemichromes display a high affinity for the cytoplasmic domain of the membrane-spanning protein, band 3 (*10*). The association between hemichromes and the cytoplasmic domain of band 3 does not terminate with a single biomolecular complex but propagates with a 2.5:1 (hemoglobin to band 3) stoichiometry indefinitely into a tightly aggregated copolymer (*10*). Since a change in the distribution of band 3 in the membrane has been postulated as a possible indicator of erythrocyte senescence (*9*), we decided to investigate whether the clustering of band 3 by copolymerization with denatured hemoglobin might promote autologous antibody binding to whole RBC's.

Hemichromes were artificially induced in normal RBC's by treatment of the cells with low concentrations of phenylhydrazine, a reagent that catalyzes the denaturation of hemoglobin (*11, 12*). This drug-induced denaturation appears to produce an alteration in hemoglobin similar to that of normal hemoglobin denaturation in vivo (*11, 12*). After treatment with phenylhydrazine, the RBC's were washed, incubated in whole human serum to allow autologous IgG binding, rewashed to remove unbound IgG, and analyzed for adhering IgG by measuring the radioactivity of tightly absorbed $^{125}$I-labeled protein A. Figure 1a shows that phenylhydrazine-promoted hemoglobin denaturation does, in fact, lead to enhanced binding of IgG to erythrocytes. The autologous IgG obtained by stripping the treated RBC's with 0.1*M* glycine buffer (*p*H 3) cross-reacted with intact band 3 in Western blots (*13*) of erythrocyte membranes, but not with the isolated cytoplasmic domain of band 3 prepared as described elsewhere (*14*) (not shown). This indicates, as recently demonstrated by Kay (*15*), that the bound IgG was directed against a determinant in the integral or external part of the band 3 molecule. With 16 m*M* phen-

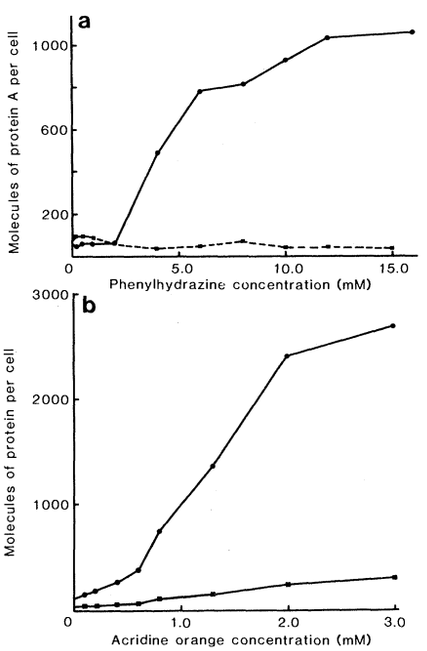
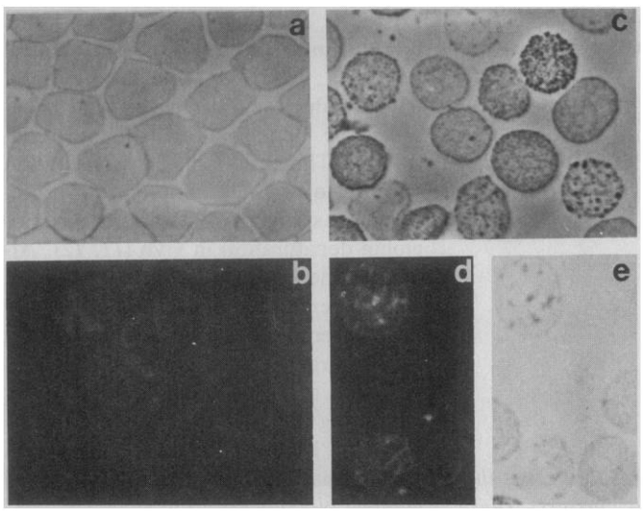



Fig. 1. (a) Binding of IgG to phenylhydrazine-treated cells. Whole blood was separated into fractions by the method of Murphy (*23*), and the upper (youngest or least dense) 10 percent was collected and washed. Portions of these cells were suspended at 5 percent hematocrit in phosphate-buffered saline (PBS), *p*H 7.4, 10 m*M* glucose, and 2 m*M* adenosine (buffer A) containing the indicated amounts of phenylhydrazine, incubated for 2 hours at 37°C, and then pelleted and washed three times in 20 volumes of buffer A. The cells were suspended at 5 percent hematocrit in the donor's own serum (●) or in incubated buffer A as a control (■) for 3 hours at room temperature, and then pelleted and washed five times with 40 volumes of buffer A, the last wash containing bovine serum albumin (1 mg/ml) (buffer B). The IgG-coated cells were then incubated at 4 percent hematocrit in buffer B containing $^{125}$I-labeled protein A (approximately 0.4 μg/ml) for 1 hour at room temperature. Cells were then pelleted and washed six times with 40 volumes of buffer B, and the amount of radioactive label was determined. (b) Binding of IgG to acridine orange–treated cells. Red cells were separated as in (a), washed three times in 20 m*M* sodium phosphate and 125 m*M* NaCl (PBS), *p*H 7.3, and suspended at 5 percent hematocrit in PBS, *p*H 7.3, containing the indicated concentrations of acridine orange. Each sample was then incubated for 0.5 hour at room temperature, pelleted, and suspended either in the donor's own serum containing the same concentration of acridine orange (●) or in PBS containing acridine orange as a control (■). After 2 hours of incubation at room temperature, the cells were pelleted, washed five times in 20 volumes of PBS plus the desired concentration of acridine orange, and suspended at 4.5 percent hematocrit in the same acridine orange buffer containing $^{125}$I-labeled protein A (approximately 0.23 μg/ml). Incubation then continued for 1 hour at room temperature, after which the cells were pelleted and washed five times with 20 volumes of the acridine orange buffer, and the amount of radioactive label was determined. Acridine orange was kept in all solutions, since band 3 clustering rapidly reversed when it was removed.



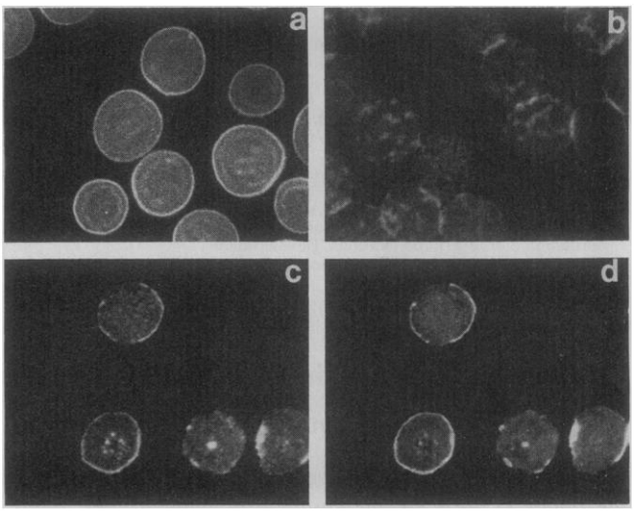




Fig. 2 (left). The effect of phenylhydrazine treatment on the subcellular location of denatured hemoglobin aggregates (Heinz bodies) and autologous IgG in erythrocytes. (a and b) Control erythrocytes in PBS, *p*H 7.4. (c, d, and e) Red blood cells treated for 2 hours at 37°C with 15 m*M* phenylhydrazine. Red blood cells were viewed by phase-contrast microscopy (a, c, and e) or by immunofluorescence microscopy (b and d) after being fixed and labeled with goat IgG to human antibody to visualize the location of the bound autologous IgG (magnification, ×1600). Fig. 3 (right). Immunofluorescence comparison of the location of band 3 and autologous IgG in phenylhydrazine-treated RBC's after incubation in human serum to allow autologous antibody binding. (a) Control erythrocytes in PBS, *p*H 7.4. (b, c, and d) Phenylhydrazine-treated RBC's, as in Fig. 2. Cells were fixed, permeabilized with acetone, and treated with rhodamine-labeled IgG directed against the cytoplasmic domain of band 3 (a, b, and d) or with fluorescein-labeled IgG against human antibody (c). Since the bound autologous antibody could be labeled with antibody to human IgG, it must include IgG (magnification, ×1600).

ylhydrazine (Fig. 1a), a concentration that leads to significant Heinz body formation in RBC's, the bound autologous IgG has increased one to three orders of magnitude above the background. To demonstrate that band 3 aggregation and not hemoglobin denaturation as such was responsible for the enhanced antibody binding, band 3 was aggregated in situ by treatment with acridine orange. This lipophilic drug induces the reversible aggregation of band 3 without altering the properties of hemoglobin or the structural intactness of the membrane (*16*). As shown in Fig. 1b, acridine orange concentrations that reversibly cluster band 3 can promote the binding of autologous antibody without causing denaturation of hemoglobin. Thus, the clustering of band 3 must constitute a recognition site for the binding of antibodies directed against senescent cells.

More direct evidence for the link between denatured hemoglobin aggregation of band 3 and autologous IgG binding can be obtained by comparing the locations of denatured hemoglobin, clustered band 3, and bound IgG in the same cells. Normal RBC's in human serum (Fig. 2a) were either fixed and stained with a fluorescent antibody to human IgG (Fig. 2b) or treated with 15 m*M* phenylhydrazine to promote hemoglobin denaturation (Fig. 2, c, d, and e), and after incubation in serum, fixed and stained with fluorescent antibody to human IgG (Fig. 2d). Although little autologous IgG was detected in untreated RBC's (Fig. 2b), significant IgG staining was evident in the phenylhydrazine-treated RBC's. Comparison of the same field of treated cells by phase-contrast (Fig. 2e) and immunofluorescence (Fig. 2d) microscopy shows that the clusters of membrane-bound autologous IgG precisely overlie the dense aggregates of denatured hemoglobin.

To demonstrate the involvement of aggregated band 3 in the senescent cell IgG clusters, we compared the location of the autologous IgG and band 3 by immunofluorescence staining. A monospecific antibody to the cytoplasmic domain of band 3 (*14*) was used to compare the distribution of band 3 in permeabilized control and phenylhydrazine-treated RBC's (*13*). Band 3 was evenly distributed in control cells and clustered in the 15 m*M* phenylhydrazine-treated cells. A similar but less dramatic redistribution of band 3 was also observed in RBC's treated with 5 m*M* phenylhydrazine (not shown). To determine whether these clusters of band 3 coincide with the sites of autologous IgG binding, we incubated phenylhydrazine-treated RBC's in normal serum to allow IgG binding, washed the RBC's to remove loosely bound IgG, fixed and permeabilized them, and then relabeled them with the IgG directed against the cytoplasmic domain of band 3. The distribution of band 3 and autologous IgG are shown for the same field of cells in Fig. 3, c and d. The sites of band 3 clustering match precisely the sites of autologous IgG binding. The distribution of autologous IgG and aggregates of band 3 also coincided in acridine orange–treated cells (not shown). Therefore the clustering of band 3, whether by denatured hemoglobin or acridine orange, promotes autologous IgG binding to the membrane. Other treatments that promote IgG binding, such as adenosine triphosphate depletion, desialylation of the cell surface, and generation of oxidizing species (*3, 6, 17, 18*), may also act by inducing the aggregation of band 3.

The mechanism by which band 3 clustering enhances senescent cell antibody binding is likely very simple: the prior aggregation of band 3 by denatured hemoglobin pays for the decrease in entropy that would otherwise occur when the bivalent IgG clusters its receptor, that is, band 3. Brandts and Jacobson (*19*) showed that the ligand-receptor binding enhancement (*T*) due to prior aggregation of the receptor in the plane of the membrane can be quantitatively evaluated with the expression

$$T = \frac{1}{m}\left[\frac{2}{X_o}\right]^{m-1}$$

where $m$ is the valence of the ligand (2 in the case of IgG) and $X_o$ is the two-dimensional mole fraction of the receptor in the membrane. Assuming there are $3 \times 10^5$ copies of tetrameric band 3 per membrane (*20*) and that the exterior poles of the tetramer are too far apart to be bridged by an IgG in the absence of clustering (*21*), we find that prior clustering of band 3 by any mechanism will

enhance senescent IgG binding by more than three orders of magnitude. Therefore, expression of the senescence marker requires neither covalent modification of band 3, nor exposure of a cryptic antigenic site as previously suggested; the clustering of a few copies of band 3 results in a substantial increase in IgG binding to senescent cells. We have shown that hemoglobin denaturation is one mechanism by which this clustering can occur. The near absence of Heinz bodies in RBC from normal individuals and the abundance of Heinz bodies in RBC from normal individuals after splenectomy (that is, after a major site of senescent cell phagocytosis has been removed) indicates that this mechanism is operative in vivo (*12*). Immunofluorescence results from our laboratories demonstrate that the sites of Heinz body binding in untreated cells from splenectomized patients coincide with the sites of both band 3 clustering and autologous IgG binding (*22*).

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## Insulin Mediators from Rat Skeletal Muscle Have Differential Effects on Insulin-Sensitive Pathways of Intact Adipocytes

Abstract. *The effects of partially purified insulin-generated mediators from rat skeletal muscle were compared to those of insulin on intact adipocytes. Insulin and insulin mediator stimulated both pyruvate dehydrogenase and glycogen synthase activity of intact adipocytes. In contrast, insulin stimulated glucose oxidation and 3-O-methylglucose transport, whereas insulin-generated mediators had no effect. Insulin-generated mediators cannot account for all the pleiotropic effects of insulin, especially membrane-controlled processes.*

Insulin, as the major anabolic hormone in mammals, is responsible for a variety of effects on numerous metabolic pathways. These pleiotropic responses occur in seconds, minutes, and hours after the interaction of insulin with target cells. The metabolic pathways affected by the hormone involve reactions that are located on the plasma membrane, in cytoplasmic compartments, and in the nucleus. The molecular mechanisms by which insulin causes its effects have not been elucidated. Recent studies have indicated that the interaction of insulin with its receptor causes the release from the plasma membrane of several related, low molecular weight, acid- and heat-stable molecules that appear to function as mediators or second messengers for many of insulin's intracellular effects (*1, 2*). These mediators can be generated by insulin in subcellular systems (*3–11*), from intact cells (*12–14*), and in vivo (*15, 16*). In vitro, these mediators stimulate pyruvate dehydrogenase (*4–7, 9–13, 17*), glycogen synthase (*2, 15, 18*), adenosine 3′,5′-monophosphate (cyclic AMP) phosphodiesterase of low Michaelis constant (*19*), and acetyl coenzyme A carboxylase (*20*) and inhibit adenylate cyclase (*21*), similar to insulin's effects on these same enzymes in vivo.

Recently it was shown that insulin mediator preparations from rat adipocyte and liver plasma membranes affect a variety of metabolic pathways in isolated intact adipocytes (*22*) and hepatocytes (*23*), respectively. In the former system the mediator preparations stimulated lipogenesis and decreased hormonally elevated lipolysis and cyclic AMP levels. In cultured hepatocytes the mediators stimulated lipolysis and caused down-regulation of the insulin receptor. Our laboratory has found that mediator prepared from skeletal muscle of insulin-treated rats stimulated pyruvate dehydrogenase activity of intact adipocytes and that the mediator was degraded by the adipocyte with time (*24*). The ability of these mediator preparations to act on intact cellular systems opens new avenues for investigating the effects of these mediators on various metabolic pathways not approachable in subcellular systems.

In this study we compared the effect of insulin and of partially purified preparations of insulin mediator from rat skeletal muscle on glucose oxidation, 3-*O*-methylglucose transport, pyruvate dehydrogenase activity, and glycogen synthase activity by isolated rat adipocytes. This approach allows investigation of insulin-sensitive membrane processes, such as glucose transport, that cannot be studied in subcellular systems.

The mediators used were extracted from freeze-clamped skeletal muscle from control and insulin-treated rats with a mixture of 0.2*M* sodium acetate buffer (*p*H 3.8), 1 m*M* EDTA, and 0.1 m*M* dithiothreitol (*16*). The extract was centrifuged in the cold to remove cellular debris, boiled, and ultracentrifuged to remove denatured material. Nucleotide impurity was extracted with charcoal and the supernatant was sized by ultrafiltration. The fraction of molecular weight 500 to 2000 was lyophilized and taken up in 1 m*M* formic acid (4 ml per 10 g of original tissue weight). This procedure provided a 500-fold purification from starting material on the basis of protein measurement. Adipocytes were prepared by a modification of the method of Rodbell (*25*). The cells were incubated and enzyme activity or glucose oxidation was measured as described in the legends to Figs. 1 to 3.

The effects of insulin and mediators from muscle of control or insulin-treated rats on intact rat adipocyte pyruvate dehydrogenase are illustrated in Fig. 1. Insulin at 100 μU/ml stimulated pyruvate dehydrogenase activity twofold, which was the maximal response to the hormone. This stimulation is comparable to that measured by other investigators us-