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Editorial note. The article is of a general importance since it reviews a domain in which the hematologist uses modern methods of membrane investigation. In presence of a functional deficiency of the red cell efforts are made to localize, at a molecular level, the component responsible for impaired membrane function.

Hemolytic Disorders Associated with a Primary Red Cell Membrane Defect

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Introduction

In the past few years we witnessed a soaring interest in the study of the structure and function of biological membranes. Of all plasma membranes, the red cell membrane has been one of the first to be isolated, fractionated and analyzed. To day we are in the possession of a large body of information concerning the detailed arrangement of the main components within the red cell membrane and progress has been made in assigning them specific functions. From the study of the red cell membrane emerged the general principles of membrane organization crystallized in a membrane model[1]. Moreover, deciphering of the membrane structure in normal red cells initiated a new trend in the investigation of the pathological one. The hematologist, when faced with a hemolytic condition, might look at the membrane for an answer.

Anemia due to increased red cell destruction can be caused by a variety of red cell abnormalities. In a number of hemolytic disorders the cell abnormality seems to derive from a primary defect of the cell membrane. The membrane, which forms a boundary between the inside and the outside of the cell, is responsible for a number of vital functions such as selective permeability, transport, support for the enzymes and site of the immune reactions. In the modern concepts the red cell membrane is represented as a fluid lipid bilayer in which the fatty acid chains stretch inwards facing each other and the polar groups oriented outwards. According to their affinity for lipid, proteins are either penetrating the bilayer or coating its inner and outer surfaces. It is obvious that an abnormality in either the protein or the lipid components may lead to impaired membrane function and red cell destruction. The hemolytic disorders known as hereditary spherocytosis (HS), hereditary elliptocytosis (HE) and paroxysmal nocturnal hemoglobinuria (PNH) seem to be associated with abnormal membrane protein. For most of the hemolytic disorders which are associated with abnormal lipid composition the primary defect is in the serum. Thus, hyperlipemia secondary to liver disease, hypercholesterolemia secondary to hereditary defects in plasma cholesterol-lecithin transferase or to abetalipoproteinemia are leading to red cell disorders that can be partially corrected by incubation in normal serum. Since this survey is limited to primary membrane defects, such disorders will not be included.

The evidence for a primary membrane defect in a number of hemolytic disorders is of a relatively recent date and its presentation forms the main object of this review article.

Hereditary spherocytosis and elliptocytosis

These two hemolytic conditions present many common features such as inheritance, abnormal red cell shape and destruction of red cells in the spleen. HS, the most common of the hereditary hemolytic disorders among people of Northern European descent, has been studied in more detail. Hereditary spherocytosis[2-4] is clinically manifested by anemia, jaundice and splenomegaly. Red cells with a spheroidal shape and decreased diameter, microspherocytes, are present in various amounts in the peripheral blood. In hereditary elliptocytosis more than 75% of the cells are elliptic but the majority of the patients manifest only mild hemolysis and in only 10–15% of the cases is hemolysis pronounced. The spleen being the site of red cell destruction, splenectomy is usually improving the clinical symptoms without however

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correcting the red cell defect. Both HS and HE are inherited as an autosomal dominant trait.

Microspherocytes from HS patients present a number of abnormalities, one of them being a characteristic increase in osmotic fragility, i.e., when suspended in hypotonic solutions, they lyse at higher molarities than normal cells[2–4]. The normal bidiscoidal erythrocyte has an excess of surface area for a given cell volume and, when in hypotonic media, is able to take up considerable amounts of water until the limiting spherical shape is reached at a volume increase of 60–70%. Any further increase beyond this critical hemolytic volume stretches the membrane and induces hemolysis.

HS cells have usually a normal corpuscular volume but a much smaller surface area and as a consequence, when suspended in hypotonic media, the critical hemolytic volume is reached at a volume increase of only about 20%. The cells appear therefore much more vulnerable to osmotic hemolysis[5]. HE cells have increased osmotic fragility only in patients with overt hemolysis.

JACOB[4] attributes the spherical shape of HS cells to a deficiency in surface area, in other words, the cells would have less membrane material. Reports on a decreased total lipid content in HS cells[6] support this view but since that would only partially account for the loss in area, membrane protein is probably also decreased. As yet there are no available data on the amount of membrane protein in HS cells. The tendency of HS cells to loose pieces of membrane during in vitro incubation might be significant. When incubated in the absence of glucose, HS cells hemolyze sooner than normal cells. Prior to hemolysis the membrane is budding assuming a blistered appearance and whole pieces of membrane are eventually lost[2]. The loss of lipid is symmetrical involving all lipid classes. There are no data as to the extent of loss in membrane protein. As for the osmotic fragility, the incubation fragility of HS cells is increased only in the hemolytic form of the disease. Another characteristic abnormality of HS erythrocytes is their diminished deformability, this being a critical property in assisting the passage of red cells through narrow capillaries and openings in the basement membranes.

There are more than one known causes leading to diminished red cell deformability. One is a corollary of the spheroidicity or decreased surface area/volume ratio[7]. Another cause might be the alteration of a membrane component causing intrinsic rigidity as induced by ATP depletion or by calcium[8]. Since red cell ghosts from HS cells are intrinsically stiffer than normal[4] the rigidity of HS cells might be the result of both mechanisms. It is noteworthy that also stromas from HE cells remain elliptical in shape[9].

In addition to the defects described above, a basic functional deficiency of HS cells is their increased permeability to Na ions as demonstrated by a significant Na^+ accumulation during incubation. In normal cells permeability to Na^+ is low enough to allow maintenance of an important chemical gradient against the high plasma concentrations by means of active Na^+ outward transport. HS cells oppose the equilibration of Na^+ by an increased rate of active transport coupled with increased ATP generation by glycolysis. The survival of HS cells is therefore critically dependent on the energy supply. When glucose supply is limited and ATP becomes depleated, Na^+ accumulates within the cell and colloid-osmotic hemolysis follows. Such a situation may occur in the stagnant circulation through the spleen or upon in vitro incubation.

Investigating the metabolism of HS erythrocytes in peripheral blood and in splenic pulp MAYMAN and ZIPURSKY[10] demonstrated that the red cells in peripheral blood have a normal Na^+ pump with increased activity as a response to changes in environmental ion distribution. The pump ATPase of these cells is also normal. The splenic cells however have a decreased Na^+ pump as well as ouabain sensitive ATPase activity. It is of interest that permeability to Na^+ does not increase in either splenic or in vitro incubated HS cells as compared to the peripheral ones. The failure of the Na^+ pump in splenic cells was accompanied by alteration in glycolysis but the levels of ATP were found normal. Thus, the authors support the assumption of a reduced Na^+ pump activity in these cells being the consequence of structural changes in the membrane.

The HS characteristics such as spheroidicity, increased osmotic fragility, autohemolysis and excessive permeability to Na^+ point to a primary structural defect of the membrane. The earliest investigations on the defective HS membrane focused on the lipid components. Their exclusive association with the red cell membrane and a well developed methodology allowed their study well before that of the membrane proteins. The lipid distribution in HS cells was found normal, but the total lipid content slightly decreased[11,12]. Splenectomy rises the total lipid content to normal values which however are lower than the post-splenectomy levels in patients without HS[6]. The fact that HS cells continue to present even after splenectomy their specific characteristics in spite of a normal lipid content, pointed to a factor other than the lipid

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deficiency per se as a primary defect. However, a difference in lipid composition has been recently demonstrated in HS cells, namely the disappearance of the long chain fatty acids in the lecithin, sphyngomyelin and phosphatidyl serine fractions[13]. This difference in fatty acid composition has been linked with the increased osmotic fragility of HS cells[13].

For the study of the membrane proteins a number of difficulties such as preparation of hemoglobin-free membranes, solubilization and fractionation of the material had to be solved. One of the first such attempts has been made by SCHNEIDERMANN[14]. Stromas were solubilized in a mixture of Triton x-100, urea and β-mercaptoethanol and subjected to electrophoresis on polyacrylamide gels at basic pH. Staining for protein revealed a number of bands which, in the case of stromas derived from HS and HE cells, deviated grossly from the normal pattern. A different conclusion was reached by ZAIL and JOUBERT[15]. Analyzing partially solubilized stromas by starch-gel electrophoresis they observed no qualitative differences between the patterns of normal, HS and PNH cells. However, total solubilization of membranes by a mixture of phenol, urea and acetic acid followed by electrophoresis on acrylamide gel without sodium dodecyl sulfate (SDS) led LIMBER et al.[16] to the finding that a specific protein band which is constant in normals, varies greately in HS cells. Similar results were reported by HAYASHI et al.[17] following analysis of the erythrocyte membrane proteins from 15 patients with hereditary spherocytosis. Polyacrylamide gel electrophoresis in 0.1% SDS buffer revealed almost complete deficiency in protein band IVb of a Mol.wt. $\sim$75,000 in 4 cases and a small but significant decrease in most of the other cases. Because of differences in gel composition it is difficult to ascertain whether the band IVb of HAYASHI and the band C of LIMBER represent the same or different membrane proteins. NOZAWA et al.[18] who studied a HS patient with severe anemia confirmed the absence of protein band IVb and clarified the conflicting results reported from various laboratories who used acrylamide gel electrophoresis in presence of SDS. It appears that the absence of band IVb can be demonstrated only in 0.1% SDS while in the presence of 1% SDS there is no difference between normal and pathological membranes. The IVb protein of hereditary spherocytes might be more resistant to dissociation by SDS than that of normal cells.

Further proof for an abnormal protein in membranes of HS erythrocytes has been provided by the studies of GOMPERTS et al.[19,20]. These authors compared results obtained by using two different solubilization techniques: an acetic and extraction, which solubilizes about 30% of the membrane protein, and a butanol/water partition which yields about 80% of the protein in solution. Protein characterization was achieved by starch and polyacrylamide gel electrophoresis in the presence of urea. Whereas following the more extensive butanol solubilization, there were no differences in the pattern of extracted protein from normal and HS membranes, the protein extracted by acetic acid showed that two of the slowest moving bands were absent in all the HS cases investigated. Applying the acetic acid extraction to HE membranes, the authors found that a protein pattern alteration consisting in the absence of either 2 or 4 of the slowest moving bands appeared only in those cases associated with severe hemolysis. The nonhemolytic HE cases had a normal protein pattern. Protein patterns similar to HS were also found in cells from autoimmune hemolytic anemias.

The authors further found that both the sulfhydril inhibitor PMB applied to red cells and the reducing agent 2-ME applied to the membrane protein affected mainly the slowest moving group of protein bands, suggesting that they have a significant sulfhydril component.

The findings of GOMPERTS et al.[19,20] strongly suggest that the abnormal electrophoretic patterns in HS and HE may not result from the in vivo absence of certain membrane proteins but rather from their different solubility properties. The same possibility was also considered by LIMBER et al.[16]. In confirmation of this view are the results of KITAO et al.[21] and of BOIVIN and GALAND[22]. Using a standard method of ghost preparation which minimizes the loss of water soluble membrane protein followed by total solubilization of the membranes, the protein pattern of HS membranes on SDS-acrylamide gel electrophoresis in 1% SDS buffer appeared normal. Moreover, proteolytic digestion of the major protein components revealed no significant difference in electrophoretic pattern between normal and HS cells[23]. Interestingly, a patient suffering from a mild hemolytic anemia of unknown causes presented an altered protein pattern indicating extensive proteolysis.

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In conclusion, all the apparently conflicting results reported above suggest that membranes from HS cells possess all the major polypeptides identified in normal membranes. However, because of an as yet ill defined alteration in their structure, their behaviour toward a number of reagents differs from that of normal membrane proteins. One of the consequences is the occasional disappearance of some protein bands which can be due to either loss during preparation or solubilization of the ghosts, or failure to dissociate and separate in electrophoresis, all depending on the specific method which has been employed. Of a particular relevance to this matter are the studies of JACOB et al.[24,25]. Their basic assumption was that a specific fibrillar membrane protein is responsible for the biconcave shape and deformability of the normal erythrocyte. Indeed, MARCHESI and STEERS[26] have described a protein located on the membrane interior and able to form fibrils when treated with divalent cations and ATP. The studies of JACOB et al.[24,25] revealed an abnormal behaviour of the red cell membrane protein from HS patients such as a lack of increase in sedimentation rate upon addition of cations and a decreased susceptibility to precipitation by vinblastine. Since the cation induced increment in sedimentation of normal membrane protein is taken to mean alignement of small subunits into fibrils, and since vinblastine specifically precipitates microfilamentous protein, the above findings were interpreted to indicate that in HS a membrane protein is genetically altered in such a way as to inhibit its attaining the proper fibrillar conformation. The authors found further confirmation of their theory in the fact that treatment of normal erythrocytes by vinblastine, colchicine or strychnine induced all the characteristics of HS cells.

The conclusions based on the effects of vinblastine are however challenged by SEEMAN et al.[27] who consider the action of the alkaloids as non-specific and resulting from their membrane expanding property, similar to the effect of other lipid-soluble drugs and of the anesthetics.

Studying protein phosphorylation in membranes of normal and HS erythrocytes, GREENQUIST and SHOHET[28] propose an entirely different mechanism to account for a defective protein conformation. Their data show that endogenous protein kinases in erythrocyte membranes induce phosphorylation of the protein doublet known as spectrin and of the band III proteins, the process being stimulated by cyclic AMP. In membranes of defective cells, phosphorylation of all substrates is much lower than in normals both in presence and in the absence of cyclic AMP. The defect in the filamentous protein, supposedly spectrin, described by JACOB might thus be secondary to a reduced protein kinase activity which fails to maintain it in the phosphorylated state essential for a normal configuration. That the primary defect is associated with the enzyme and not the substrates is suggested by the fact that several different polypeptides are simultaneously affected.

A similar approach which attempts to explain the altered configuration of HS membrane proteins as a result of primary enzyme deficiency is found in a study by FEIG and GUIDOTTI[29]. These authors demonstrate that spherocytic ghosts, when compared with age-matched controls, show a significantly decreased Ca^{2+}-dependent ATPase activity. This enzyme is known to be involved in the active transport of Ca^{2+} across red cell membranes. A relationship between increased cell Ca^{2+} content, increased permeability and decreased deformability, probably through the intermediate of fibrous membrane proteins, has also been documented[8].

There are therefore two general ways of interpreting the nature of the defect occurring in the HS membrane proteins: a) a genetic alteration in one or more membrane proteins including those involved in membrane deformability and b) a primary deficiency in membrane enzymes which in turn affects the state of the membrane proteins.

Experimental spherocytosis

We mentioned before that red blood cells subjected to various chemical treatments can aquire the properties of HS cells[19,20]. In addition, a spherocytic hemolytic disease has been experimentally induced in rats maintained on a Mg^{2+}-deficient diet[30]. The erythrocytes from Mg^{2+}-deprived rats showed a decreased deformability and spherocytosis, but, unlike the HS cells, they had a decreased glucose utilization and diminished ATP and 2,3-DPG levels. The mechanism inducing this experimental spherocytosis seems to involve both a decreased ATP production and possibly a defective membrane construction due to the Mg^{2+}-deficient environment.

Paroxysmal nocturnal hemoglobinuria (PNH)

In PNH, a membrane abnormality renders the red blood cells extremely sensitive to the lytic action of complement. The incidence of PNH is about 2 per million and it appears in all races and both genders with equal frequency. The clinical manifestations of this disease,

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which is acquired and not inherited, are varied. The classical presentation of the disease, which is however observed in a minority of cases, is hemoglobinuria following sleep. The cause of the nocturnal variation, which was thought to be the acidification of the serum during sleep, was not completely confirmed.

If the red cells from patients with PNH are subjected to a test for hemolysis in presence of antibody and normal serum as source of complement, two cell populations are revealed: the complement sensitive cells which are 25–30-fold more sensitive than normal cells, and the complement insensitive ones which are slightly more sensitive than normal cells. The complement sensitive cells are also lysed by normal acidified serum.

The nature of the membrane defect in the sensitive cells is largely unknown. An alteration of the red cell lipids has been postulated[31–36] and a greater tendency of the lipids to form peroxides upon exposure to ultraviolet light or H_2O_2 has been reported[35,37]. However, it seems that the cause of the red cell abnormality is connected with a change in the membrane protein.

The possibility of obtaining PNH-like cells by means of chemical treatments has been largely used in the studies on the nature of the PNH defect. Thus, Atlas et al.[38] studied the surface properties of PNH and of PNH-like cells produced by treating normal cells with reduced glutathione (GSH). They found that lactoperoxidase iodination yields more ^{125}I per cell in PNH cells and less in PNH-like cells as compared to normal cells. Intact cell acetylcholinesterase activity was found lower in PNH cells but the same or higher in PNH-like cells as compared to normal cells. PNH-like cells also different from PNH cells in exhibiting non-specific hemolysis and a greater increase in Na^+ uptake. PNH cells differed from normal cells in both protein distribution pattern as shown by SDS-polyacrylamide gels and in sulfhydril content of the intact erythrocytes. The authors concluded that the surface of PNH cells differs significantly from that of normal cells and that GSH-treated cells are not a satisfactory model for PNH cells. Among the sulfhydril compounds examined, the radio-protector AET (2-amino ethylisothiouronium bromide) has been shown to be the most effective in producing PNH-like abnormality, i.e. cell which lyse in slightly acidified normal serum and in a medium of low ionic strength if complement is present, and display a low acetylcholinesterase activity[39]. The mechanism of action of the sulfhydril compounds on the red cell membrane is believed to consist in splitting of membrane S–S bonds with formation of a mixed disulfide and a membrane –SH group[40]. (–S–S– membrane. S–S – membrane → membrane – S–S–G + membrane –SH).

A comparative study of the PNH and PNH-like cells produced by AET shown further similarities with respect to a membrane protein alteration[41]. Electrophoresis of membranes on SDS-acrylamide showed that in PNH cells and AET-modified cells, a glycoprotein band of molecular weight 77.100 replaces the glycoprotein band of mol. wt. 82.000 present in normal cells. The authors rule out the possibility of proteolytic degradation during the procedure and consider that the degradation of the 82.000 glycoprotein to a 77.100 chain is a characteristic in vivo feature of the PNS cells. The mechanism by which AET induces cleavage of the same glycoprotein is unknown.

A new property of the PNH erythrocyte membrane has been recently reported, namely an abnormality in solubilization of membrane components by the non-ionic detergent Triton X-100[42]. Detergent concentrations which cause partial solubilization of membrane components eluted less cholesterol and hexoses from PNH than from normal membranes while protein was dissociated from both normal and pathological membranes in equal amounts. In addition, immunoelectrophoresis of PNH membranes revealed the absence of one precipitation line. The authors suggest that the major defect in PNH is an altered organization of the membrane constituents mediated by an as yet undefined anomaly in a membrane glycoprotein.

Acetylcholinesterase in PNH

The enzyme acetylcholinesterase is an integral component of the human erythrocyte membrane, located at its outer surface. Although its function in the red cell membrane is obscure, the study of acetylcholinesterase in PNH might be relevant as to the state of the surface membrane proteins. The total acetylcholinesterase activity in a PNH cell population is decreased. The pattern of acetylcholinesterase activity in PNH red cells which have been separated according to their density indicates normal enzyme levels in the older cells, and a very decreased enzyme level in the younger

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ones[43]. This correlates with the existence of two cell populations in PNH, a short lived, deficient one and another with longer survival. A striking difference appears in this respect between PNH cells and cells from patients affected by autoimmune hemolytic anemia. In the latter case the cells are also ACHE deficient, but the enzyme activity declines sharply with the cell age[43].

JACKSON and WHITTAKER[44] confirmed both the low acetylcholinesterase activity of PNH cells, as well as their abnormally low density previously observed by LEWIS and VINCENT[45]. The low density could not be correlated with either a change in lipid content, which was found normal, or with increased permeability, their osmotic fragility being also normal[45].

An abnormal membrane protein pattern obtained by SDS-polyacrylamide gel electrophoresis was found in a PNH patient which was severely aplastic. Other PNH cases, as well as the same patient in a hyperplastic phase, gave normal protein patterns[44].

Electron microscopy in PNH

Numerous efforts have been made to visualize the initial acquired lesion in PNH cells by electron microscopy, but these attempts have produced conflicting results with some investigators claiming to find lesions and others finding essentially normal red cell membrane ultrastructure. WEINSTEIN and WILLIAMS[46] criticize the technique used in these studies as producing drying artifacts, and studied the membrane in intact PNH cells and in the ghosts derived from them by the freeze cleaving technique. Their results failed to confirm previously reported lesions, and the authors suggest that they were either artifacts of drying, or they reflect structural differences revealed by drying.

Hemolysis associated with altered phospholipid composition of the erythrocyte

A familial nonspherocytic hemolytic anemia associated with abnormalities in membrane lipids was described by JAFFE and GOTTFRIED in 1968[47]. The patient's erythrocytes showed an absolute increase in lecithin content while the plasma lipid distribution was normal. The unusual lipid abnormality seemed to be related to the hemolysis. Later, SHOHAT et al.[48] studied the mechanism of lecithin accumulation in the erythrocytes of such patients and concluded that lecithin increases because a defect in the catabolism of actively incorporated lecithin fatty acids. This defect appears to be a block in the transfer of fatty acids from lecithin to phosphatidyl ethanolamine prior to final release from the cell. The passive exchange pathways and the active anabolic acylase in the erythrocytes of such patients were not abnormal[48].

This familial hemolytic disease with abnormal lipid composition results from an inherent membrane defect and differs from other similar states in which the primary defect is in the serum.

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Some Aspects of the Early Development and Implantation of the Mammalian Egg

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Summary. The early development and implantation of the mammalian egg is described for various species and the differing and often contradictory solutions proposed by different authors for the many problems arising from their investigations are exposed, compared and discussed.

The early development and implantation of the mammalian egg has received much attention and an immense quantity of literature has been accumulating, continuously increasing during the last decades. Nevertheless many problems are still not clear and for most of them the solutions proposed by different investigators differ widely and are often contradictory. It would be quite impossible to give an overall survey of the work dealing with the subject. The present review, which is far from complete, attempts to expose some of the problems and their solutions.

There are great differences between species, and even between strains, and although the mouse has been my main experimental animal, observations in other species will be cited when necessary.