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membrane structure and function of HUMAN BLOOD CELLS

1976

Membrane Structure and Function of Human Blood Cells

A Symposium

Presented at the
Twenty-Ninth Annual Meeting
of the
American Association of Blood Banks
November 4, 1976

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PREFACE

THE NOTION that morphologic appearance of various cells is determined by a rigid cell-wall enveloping the intracellular contents of living cells has given way to the definite recognition that biologic membranes are intrinsically fluid structures, dynamic in their physical, biochemical, and functional organization. Several models of the structure of biologic membranes have evolved in the past decade. The concept that asymmetric lipid bilayers have dissolved in them the proteins synthesized by the cell cytoplasm has gained support from extensive studies with the red cell membranes. Membrane lipids and proteins together with their prosthetic carbohydrate groups are germane to our interest in the alloantigens in the biologic membranes. Basic understanding of the properties of natural membranes has been advanced by the studies with artificial liquid membranes and experimental manipulations of the liposome models. Multi-disciplinary investigations of biologic membranes not only have elucidated the composition and structure of the membranes but also have provided new insights into the functions of energy capture, cell water organization, ion binding and transport, cell-cell interaction, complement lysis, various receptors, and the transmission of specified hormonal signals.

With unprecedented activity, the cell biologists have focused their attention on the structure and function of intracellular membranes of various cell organelles and extracellular plasma membranes. "Suddenly it is all membranes," says Gerald Weismann in his eloquent foreword to a recent popular volume. Despite fierce controversies, enough information has crystallized to review this subject with particular emphasis on its scientific and education relevance to our mundane daily functions of providing human blood for transfusion. Unlike basic membrane biologists, the immunohematologists need to understand the pathophysiologic role of biomembranes of the various cellular components used in transfusion therapy.

In composing the scope of this symposium and selecting the respective speakers, the Scientific Program Committee had to forego several stimulating possibilities. The constraints of time demanded considerable ingenuity on the part of both the symposium sponsors and the distinguished speakers. Personally, I am most grateful to the Scientific Program Committee for entrusting this responsibility to me, and to the speakers for their judicious compliance with my stipulations for the scope of their own manuscripts included in this volume.

I am certain that the audience present at this convention and the extended audience who will read this monograph will share my excitement in bringing to fruition a review of the Membrane Structure and Function of Human Blood Cells presented by recognized investigators with exceptional teaching skills.

Girish N. Vyas, Ph. D.
University of California

San Francisco, California
November 4, 1976

CONTENTS

CHAPTER IV

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CHAPTER V

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Professor of Microbiology,
University of Texas Southwestern Medical School, Dallas, Texas

CHAPTER VI

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Chapter I

STRUCTURE OF BIOLOGICAL MEMBRANES

Vincent T. Marchesi

I. *Lipids are arranged as a dynamic asymmetric bilayer*

ONE OF THE most remarkable structural principles of biological membranes was first clearly described in the mid 1920's when Gorter and Grendel suggested that the lipids of the red cell membrane might be arranged in the form of a double layer of phospholipid molecules. This remarkably simple idea was based on the fact that the surface area of red cell ghosts contained approximately twice as many lipid molecules as was needed to make monomolecular film surrounding the cell. This lipid bilayer hypothesis soon received a certain amount of support from early x-ray diffraction studies carried out on model membrane systems and on myelin membranes; however, there was no universal agreement that this was in fact the correct structural arrangement of membrane lipids.

Electron microscopic studies of both model membranes and biological membranes seemed to provide support for the bilayer hypothesis. Thin sections of cell membranes examined after standard heavy metal staining techniques produced the well known "railroad track" image which initially was thought to represent the selective deposition of heavy metal stains at the polar regions of phospholipid molecules. Although the "railroad track" image obtained by thin sectioning techniques seemed to support the idea that lipids were arranged as a single bilayer, several investigators found that the image did not change significantly after the phospholipid molecules had been removed from the membranes by organic solvent extraction.

The ambiguity created by these studies was dispelled when other physical techniques and more modern microscopic methods were reapplied to the study of membrane structure. The development of freeze etching techniques was a significant step forward in the analysis of membranes by electron microscopy. Using this approach, it was discovered that cell membranes cleaved roughly in half along planes which correspond to the middle of the hydrocarbon regions of the lipid bilayers.[1] At the same time that these new electron microscopic studies were carried out, more definitive x-ray and other physical analyses provided convincing proof

that the bulk of the lipids of cell membranes are arranged in a bilayer configuration.

Studies with some of the newer physical techniques also established the fact that lipid molecules in bilayers appear to be in very active motion. By studying the mobilities of electron spin resonance probes placed on different segments of phospholipid molecules which sample different layers of the hydrocarbon matrix, it has been shown that the hydrocarbon chains of fatty acids of the phospholipids are rapidly flexing. In addition, it has been found that individual phospholipid molecules also move at significant rates within the lateral plane of the lipid bilayer. Hence, it is now recognized that the individual phospholipid molecules are in a dynamic state within the intact membrane.[2]

These new discoveries have resulted in the development of the concept of membrane fluidity. In general terms, fluidity refers to the degree of viscosity objects would encounter if they were able to float freely within the interior of the bilayer. The fluidity of a membrane is determined by the types of lipids and fatty acids, which make up the bilayer and the temperature of the system.

As rough generalizations, it may be stated that fatty acids having greater degrees of unsaturation are more fluid at lower temperatures than fatty acids which are more fully saturated. Also, shorter chain fatty acids are more fluid than longer chain fatty acids. Recent studies on the role of sterols and their affects on membrane fluidity are interesting in that sterols have a somewhat paradoxical affect in that they can have different affects on the fluidity of membranes, either increasing or decreasing the relative fluidity, depending upon the types of phospholipids and the concentrations of sterols in the preparations.

One additional recent modification of the bilayer hypothesis is the fact that the individual classes of lipid molecules appear to be arranged asymmetrically within the bilayers of intact cells. In the case of red blood cells, it appears that the phosphatidylcholine and sphingomyelin in which are choline-containing phospholipids, seem to be concentrated in the external leaflet of the bilayer, while phosphatidylserine and phosphatidylethanolamine are concentrated in the cytoplasmic half of the bilayer.[3] Now that the concepts of membrane fluidity and lipid asymmetry are more or less established, investigators are actively pursuing the biological meaning of these remarkable properties.

II. *"Integral" proteins interact with lipids via hydrophobic associations; "Extrinsic" proteins (may) interact electrostatically*

Approximately one-half of the mass of most mammalian cell membrane fractions is made up of proteins, and the number of types of different polypeptide chains seems to vary with the cell type. Human red blood cells have at least seven major polypeptide chains as determined by SDS gel electrophoresis (Fig. 1) and, in addition, there are an indeterminate number of minor protein species.

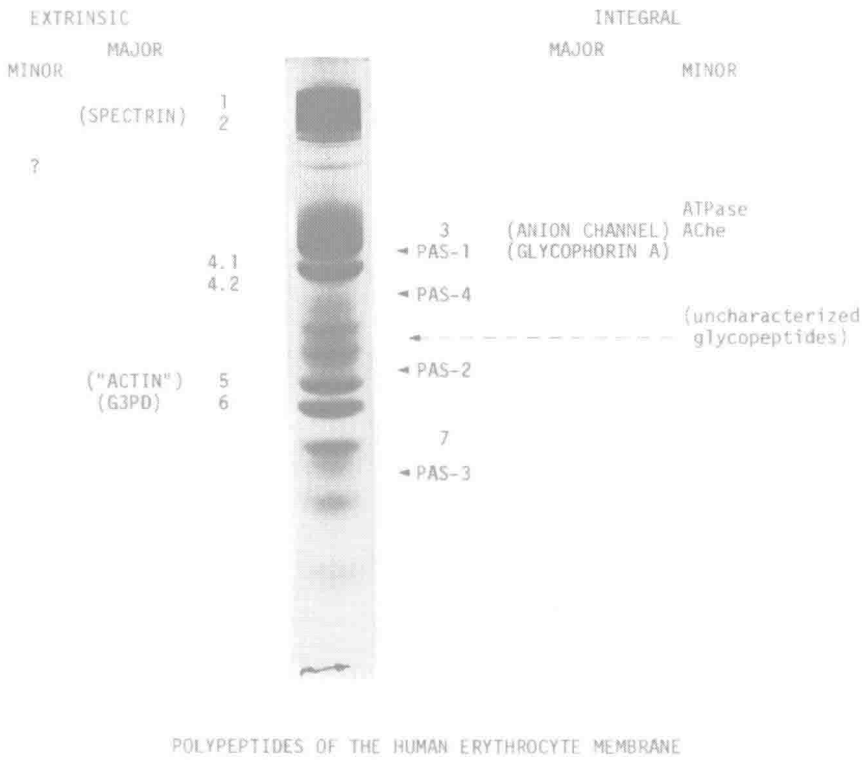

Fig. 1. Electrophorogram of the proteins of human red cell membranes in SDS-polyacrylamide gels detected by staining with Coomassie brilliant blue. The numerical designation for the major polypeptides is adopted from Fairbanks et al.[4] The bands indicated as 4.1 and 4.2 are not separated under the conditions used (1% SDS). For the sake of simplicity, designations for minor components are not included. The positions where the four sialoglycopeptides migrate on appropriately stained gels (PAS) are indicated by arrows.

As a result of the initial attempts to extract proteins from cell membrane fractions, it became evident that some of the polypeptide chains were easily detached from membrane elements, while others seemed resistant to all but the most disruptive solubilizing agents. On the basis of such experiences from many different laboratories, Singer[5] suggested that perhaps polypeptide chains of red cell membranes might be operationally classified into two types, based on the apparent mode by which they associate with the membrane lipids. He suggested that the polypeptide chains which are relatively loosely bound to the membrane and can be removed by manipulating the pH or ionic strength of solubilizing media, be called extrinsic membrane proteins. One of the most characteristic of the extrinsic membrane proteins is the set of polypeptide chains called spectrin which appear as bands one and two on SDS gels.

Many investigators discovered that the spectrin polypeptides could be rapidly and quantitatively eluted from red cell ghosts by simply immersing the membranes in low ionic strength media containing a dilute chelating agent.[6] Although red cell membranes which are treated this way fragment into small vesicles as a result of the spectrin being released from the membrane, it is generally felt that the spectrin polypeptides probably do not intercalcate within the lipid bilayer, but instead associate electrostatically either with other proteins of the membrane or, possibly, with the polar groups of the phospholipids.

In contrast, the polypeptide chains, which resist solubilization from membranes by simple buffer washings, represent an entirely different class of macromolecules. These proteins are called integral proteins largely because they cannot be dissociated from the lipid elements of the membrane without the use of detergents or harsh denaturing agents. Hence, it is considered likely that these proteins form an integral part of the membrane structure. One of the most characteristic of the integral membrane proteins is the major sialoglycoprotein of the human red cell membrane called glycophorin A.

Glycophorin A has been isolated by a number of different laboratories using a wide variety of protein solvents (see reviews 7 to 9 for references), and as the result of an intensive amount of study, it is now possible to construct a provisional model of its primary structure and offer some ideas as to how and where it is oriented in the intact red cell membrane.

Recently, the complete amino acid sequence and the sites of oligosaccharide attachment have been determined,[10] and this is shown in Figure 2.

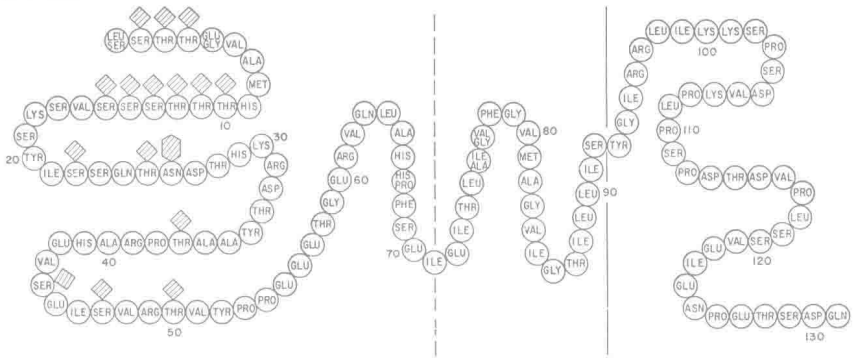

Fig. 2. The amino acids of glycophorin A are arranged in this diagram to simulate, in a very general way, the positions they might have if the glycophorin molecule were to be arranged perpendicular to the lipid bilayer of the membrane. The limits of the bilayer are defined by the two vertical lines. The solid vertical line, which passes between residues 92 and 93, should be the approximate location of the polar groups of the inner half of the phospholipid bilayer. This assignment is based on the results of enzymatic iodination of tyrosine 93 and the distribution of ferritin-antibody conjugates directed against antigenic determinants defined by residues 102 to 118. Since we do not have comparable data with regard to the amount of the N-terminal end of glycophorin A which is buried with the lipid bilayer, we can only guess at the location of this outer lamella of the bilayer relative to the glycophorin molecule; hence, the outer edge of the bilayer is defined by the dashed vertical line.

The polypeptide portion of glycophorin A, which comprises approximately 40% of the total dry weight, is made up of 131 amino acids. The distribution of different amino acids is rather interesting in that there is a very high concentration of threonine and serine residues located near the N-terminal end of the polypeptide chain. There is also a striking concentration of nonpolar amino acids located approximately midway between the N-terminal third of the polypeptide and the C-terminal third. In the diagram shown in Figure 2, the segment starting from amino acid 71 and extending through amino acid 90 is depicted between two vertical lines, and this is thought to be the segment of polypeptide which is buried within the lipid bilayer. Reasons for assigning this orientation will be described below.

It is also evident from this diagram that there are 16 different oligosaccharide chains attached to the polypeptide chain, and their positions

are assigned to the threonine and serine residues shown. It is significant that the glycosylated residues start from the N-terminal end of the molecule and extend to residue 50, but no additional sugar residues have been detected in amino acids beyond residue 50. One of the oligosaccharide chains which is linked to asparagine is of the complex variety[11] while the remaining 15 seem to be of the tetrasaccharide type originally described by Winzler and Thomas.[12]

Since all of the glycosylated residues are concentrated at one end of the molecule and some of these sugars probably represent receptor sites for lectins and, perhaps, for some blood groups antigens, this end of the molecule has been provisionally designated as the receptor domain.

The amino acid sequence of the C-terminal 40 amino acids is striking in that this portion of the polypeptide chain contains a large number of charged amino acids with a peculiar clustering of acidic amino acids at the very C-terminal segment of the chain. This portion of the polypeptide chain also contains a substantial number of prolines which may play a major role in determining the conformation of this part of the molecule.

The 22 amino acid stretch of peptide which connects the glycosylated receptor region with the C-terminal end (Fig. 2) is composed solely of non-polar residues, and it is logical to suggest that this segment is the part of glycophorin that interacts with the lipids of the membrane.

Glycophorin A Spans the Lipid Bilayer

There is a considerable amount of experimental data in support of the idea that glycophorin A, and probably other integral membrane proteins of the red cell, are in a transmembrane configuration. This idea was originally based on the attempts to differentially label different portions of the polypeptide chain in intact cell membranes versus membrane preparations which had been prepared by osmotic lysis. Bretscher originally showed that a segment of the polypeptide chain of glycophorin A was not accessible to labeling by a radioactive isotope introduced outside intact cells; however, this inaccessible segment could be labeled when the cell membranes were damaged or rendered permeable to the reagent.[13] One interpretation of this experiment was that part of the polypeptide chain of the molecule extends outside the intact red cell and another part is either buried within the membrane or

situated in the cytoplasmic compartment of the cell. This experiment was repeated in several laboratories using a more gentle labeling procedure, lactoperoxidase iodination, with comparable results.[14,15] Under ideal conditions lactoperoxidase should selectively iodinate tyrosine residues.

In theory, lactoperoxidase iodination should be an effective probe for analyzing the orientation of glycophorin A since this molecule has four tyrosines, three of which are located in the glycosylated portion of the polypeptide chain, while the fourth is close to the C-terminal end. Thus, three tyrosines should be labeled when intact cells are incubated with lactoperoxidase, while the fourth, located at position 93, should not be labeled unless the permeability of the membrane is broken. Several investigators have reported the expected results,[14,15] although other investigators were not able to reproduce these findings.[16,17]

Some investigators also consider the positive findings to be ambiguous and suggest that they could be the result of any one of a number of technical artifacts. For example, it has been suggested that rearrangements of membrane proteins might take place as a result of osmotic lysis. It has also been suggested that chemical modifications of membrane proteins which result from the labeling procedures themselves could also lead to rearrangement of the proteins, or changes in membrane permeability, or both. In either case, any one of these changes could serve to block or modify the reactivity of certain exposed proteins, or alternatively expose other proteins which normally are not exterior to the lipid barrier.

Other investigators also have pointed out the need to use mild labeling reagents at saturating levels to lessen the likelihood of selectively labeling either the most accessible or the most reactive groups of proteins rather than sampling the complete spectrum of externally arranged molecules. In addition to these many theoretical caveats, some investigators have failed to differentially label the C-terminal segment of the glycophorin A molecule when labeling reagents were introduced inside the cell. Thus, they argue that some of the earlier studies may have been artifactual. For all of these reasons, it must be admitted that the evidence for the transmembrane orientation of glycophorin A produced by labeling studies is not definitive.

In the light of the controversy surrounding the results of radiolabeling, we recently reinvestigated this problem and attempted to study

the orientation of glycophorin A in intact red cells using ferritin-conjugated antibodies directed against specific polypeptide segments of this molecule.[18] We have immunized rabbits with the cyanogen bromide fragment containing residues 82 through 131 and have found that this segment elicits good antibodies. A radioimmuno assay was developed in order to assess the specificity of the antisera and to determine what segments of the polypeptide chain bore the determinants. By a series of purification steps we have determined that the antibodies which were used to prepare ferritin conjugates were directed specifically against a 17 amino acid peptide fragment, residues 102 to 118, which are just adjacent to the C-terminal end of the polypeptide chain. Antibodies were further purified by immunoadsorption with a small tryptic peptide also derived from the C-terminal end of the molecule. These antibodies were found to be unreactive with intact cells, and were unreactive to peptides derived from the N-terminal or glycosylated end of the polypeptide chain. These antibodies also showed remarkable specificity in that they did not react with other sialoglycopeptides isolated from human red cell membranes nor with comparable glycoproteins isolated from a variety of different animal red cell membranes.

Since there was good reason to suspect that the antigenic determinants assigned to residues 102 to 118 probably were on the cytoplasmic side of the red cell membrane, the only available technique for applying labeled antibodies to the inner surfaces of intact cell membranes is to expose conjugated sera to thin sections of red cells which are either fixed and embedded in albumin or rapidly frozen in sucrose media. Such thin sections permit complete access of antibody-conjugates to subcellular sites without having to resort to osmotic lysis or other tricks to break down membrane barriers. Appropriately prepared frozen thin sections are also suitable for high resolution transmission electron miscroscopy.

Ferritin-antibody conjugates of such sera were found to localize exclusively to sites which were distributed uniformly along the inner surfaces of the intact red cell membranes. No staining was seen on sections prepared from red blood cells from other species, consistent with the immunochemical findings, nor on sections of human red blood cells which were pretreated with unconjugated blocking antisera. Thus, these results provide the first direct evidence that glycophorin A has a transmembrane orientation in intact human erythrocytes.

Glycophorin A May Exist as Multimeric Complexes in the Membrane

Recent studies from this and other laboratories suggest that glycophorin A has a remarkable capacity to form multimeric complexes in the presence of SDS.[19, 20] Almost all glycophorin preparations which are analyzed by SDS acrylamide gels contain multiple PAS-staining bands, usually designated PAS-1, -2, and -3. These bands correspond to molecular weights of 83,000, 45,000, and 25,000 when they are compared with the mobilities of standard proteins. However, we are not sure that these values represent the true molecular weights of the glycoproteins, since they are known to migrate anomalously on SDS gels.[21] PAS-1 is the predominant form and represents approximately 75% of the total.

When this pattern was first obtained, it was not clear whether the multiple bands represented a series of chemically-distinct polypeptide chains, or whether the smaller PAS staining bands represented degradation products of the larger component. In a series of recent studies we have shown that two of the PAS staining bands (PAS-1 and PAS-2) are interconvertable and their relative amounts depend on the conditions used to prepare the glycoproteins for SDS electrophoresis. Glycophorin A appears to exist as a dimeric form, which corresponds to PAS-1, and this dimeric form seems to be stabilized by noncovalent associations between hydrophobic segments of its polypeptide chains.[20] This association can be disrupted by heating the glycoproteins in SDS or by modifying the methionine located in position 81 by specific alkylation in the presence of denaturing agents.[22]

The conditions for the selective alkylation are extremely stringent in that high concentrations of alkylating agents must be used in the presence of either urea or guanidine, and the glycoprotein must have been previously delipidated with organic solvents. Since the presence of either lipid or SDS interferes with the alkylation of methionine 81, and in so doing renders the molecular aggregate less susceptible to dissociation, it is conceivable that when the molecule is oriented in the intact cell membrane, the surrounding membrane lipids serve a similar function. On the basis of this, we might speculate that glycophorin A actually exists as dimeric, or, possibly, even multimeric forms, *in situ*. If this proves to be true, then the possibility exists for multimer-monomer association and dissociation reactions to take place in membranes, which could play a role in regulating the functions of these macromolecules. This hypothetical idea is consistent with recent studies on

the apparent mobility of membrane components and the important role the lipid matrix may play in regulating this process.

III Proteins Are Distributed in a Mosaic: Some May Be More Mobile Than Others

One of the most intriguing, and still largely unanswered, questions in membrane biology concerns the three-dimensional arrangement of proteins at the cell surface. Do membrane proteins occupy fixed positions relative to some internal cytoplasmic structure, or are they in continuous motion, constantly changing their positions within the bilayer? One of the earliest suggestions concerning the arrangement of membrane proteins was the idea that monomolecular sheets of polypeptide coated the phospholipid polar groups of the lipid bilayer. This idea now seems unlikely for many reasons. Most of the evidence suggests that the bulk of the membrane proteins of the red cell membrane exist as discrete macromolecular complexes which are attached to the lipid bilayer either by insertion of hydorphobic segments into the hydrocarbon interior (like glycophorin A) or bound to polar groups of lipids electrostatically.

Our conceptions about the arrangement of proteins in membranes have also been heavily influenced by the spectacular experiments demonstrating membrane fluidity and phospholipid mobility described above. Since the lipid matrix in which the protein molecules must be inserted is in a constant state of flux, many have reasoned that it is likely that protein molecules are also in a mobile state, although it is not clear whether they are simply floating passively in the lipid sea, or are directed by some other agents independent of the surrounding lipids.

A vivid demonstration of the fact that some membrane proteins are mobilizable by external forces has been provided by the results of applying multivalent ligands to lymphocytes and other cells in suspension. Appropriately labeled ligands form patches and caps on such cells within minutes after their application. Other evidence for the mobility of surface components has been provided by cell fusion studies in which it has been shown that surface antigens can freely intermix over the combined surfaces of hybrid cells at a remarkably rapid rate.[23] Both sets of observations demonstrate rather unequivocally that certain types of surface macromolecules are capable of moving within the plane of the membrane. Unfortunately, the dramatic nature of these experiments may be overemphasizing the potential importance of the