Malaria and the red cell

Ciba Foundation symposium 94

1983

Pitman
London
Malaria and the red cell
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Introduction

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This meeting was first suggested to the Ciba Foundation by Neil Brown. I think that there are excellent reasons for holding it at this particular time. In the last few years there has been a remarkable increase in knowledge about the physiology and pathology of the red cell, and in particular we are starting to gain some insight into the structure and function of the red cell membrane. At the same time a great deal of new and fascinating information has appeared about the immunological aspects of malarial parasites and how various antigens appear during different specific phases of development. Even more important for the subject of this meeting are the recent observations which suggest that these changes may be associated with specific alterations in the red cell itself.

On the broader biological front, there is no question that the high frequency of many of the common single-gene disorders in the world population, sickle cell anaemia and thalassaemia for example, is due to the protection which is afforded to heterozygous carriers against malaria. I was thinking about this recently when some of my colleagues in Papua New Guinea asked us to look at the globin genes in a population that is still highly malarious. We found that approximately 80% of the population are missing one or two $\beta$ globin genes. If, as seems likely, this extraordinarily high gene frequency reflects protection against malarial infection, the malarial parasite is a remarkably sophisticated organism. Certainly no haematologist can identify a red cell which is missing one $\beta$ globin gene! During the meeting we shall hear about attempts to identify some of the factors both in the red cell and on its surface which may make these genetically variant cells less attractive to the parasite. The feedback from that information may help us to understand a little bit more about parasite interactions with red cells.

Although this is not a clinical meeting on malaria we should keep in mind that a lot of what we shall be talking about has important clinical relevance. We shall have vaccines in the back of our minds, of course, but beyond that, my feeling is that much of what we will discuss will underline the need to rethink a lot of the pathophysiology of malaria over the next year or two. Thinking about some of

the recent studies on cerebral blood flow in cerebral malaria, and about our old ideas about the cerebral microcirculation being cramped with parasitized red cells, my guess is that one of the most exciting areas for study over the next few years will be the way in which changes on the surface of parasitized red cells cause them to interact with vascular endothelium. I hope that we shall be able to keep all these different aspects of this fascinating subject in mind over the next couple of days.
Erythrocyte membrane structure and function

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Abstract The structure and function of the proteins of the human erythrocyte membrane are discussed. The major integral proteins comprise the anion transport protein (band 3), the glucose transporter and four sialic acid-rich polypeptides. The anion transport protein equilibrates Cl\(^-\) and HCO\(_3\)\(^-\) between the plasma and red cell and also provides an anchorage site for peripheral proteins including those in the red cell cytoskeleton. The sialic acid-rich proteins are predominantly exposed at the surface of the cell. The bulk of the peripheral proteins are organized in a complex fashion to form a skeletal meshwork at the cytoplasmic surface of the membrane which maintains the shape and deformability of the red cell. The changes in membrane components during differentiation of the red cell are discussed.

The erythrocyte is highly specialized for the transport of oxyhaemoglobin complexes and carbon dioxide between the lungs and tissues. Many of the special properties of the cell are attributable to proteins associated with the flexible membrane surrounding the cell. This paper briefly reviews the biochemistry of the erythrocyte membrane proteins.

Figure 1 identifies the major proteins present in the human erythrocyte membrane after separation by sodium dodecyl sulphate (SDS)–gel electrophoresis and detection with a protein stain (Fig. 1a) or the periodic acid–Schiff’s base (PAS) carbohydrate stain (Fig. 1b). The conventional nomenclature for the protein-staining bands of the human erythrocyte membrane is based on that of Steck (1974); however, since the functional roles of the major proteins have now been assigned, they are often referred to by more informative names. The nomenclature of the PAS-staining bands is discussed below.

The integral membrane proteins
The proteins involved in anion transport (band 3), proteins in the band 4.5 region which include the glucose transport protein, and the PAS-staining...
glycoproteins (Fig. 1b) are the only major integral membrane proteins that are detectable on SDS–gel electrophoresis. These proteins all interact with the hydrophobic interior of the membrane since they can be solubilized only after disruption of the membrane by detergents. All these proteins are glycoproteins and contain regions which together substantially contribute to the surface structure of the erythrocyte.

The anion transport protein (band 3)

This protein increases the capacity of blood to carry CO$_2$ (in the form of HCO$_3^-$) from tissues to the lungs by allowing HCO$_3^-$ to equilibrate between the intraerythrocytic and plasma phases of the blood. The equilibration occurs through a very rapid one-for-one exchange transport of cell HCO$_3^-$ for plasma Cl$^-$ and vice versa. The transport process has been shown to involve the band 3 protein and has been extensively studied (see Knauf 1979 for a detailed review). This protein is the predominant integral polypeptide of the membrane and makes up about 25% of the membrane protein, which is equivalent to $1.2 \times 10^6$ copies per red blood cell (Steck 1974).
The band 3 protein is made up of two domains of clearly different character. The N-terminus of the protein consists of a 40,000 $M_r$ portion located entirely within the cytoplasm which can be readily rendered water-soluble by mild proteolysis, while the C-terminal domain is intercalated into the membrane (Fig. 2). The cytoplasmic domain can associate with many of the peripheral proteins found in red cell membranes (see review by Gillies 1982). The glycolytic enzymes aldolase and glyceraldehyde-3-phosphate dehydrogenase bind electrostatically to a highly acidic segment of polypeptide close to the N-terminus (Murthy et al. 1981) and the extent of this association is influenced by the presence of metabolites which bind to these enzymes. This domain also binds band 4.2 and additionally forms part of the linkage between the membrane and the red cell cytoskeleton by association with ankyrin (band 2.1) (Bennett & Stenbuck 1980). Other proteins, such as phosphofructokinase and haemoglobin, can probably also bind to this region of the band 3 protein. Because of the relative abundance of the anion transport protein the individual protein ligands each bind to only a small
fraction of the total number of anion transport protein molecules present. For example, only about 10% of the band 3 molecules are complexed with ankyrin (Bennett & Stenbuck 1980). It is not known whether all these components bind to the same anionic binding site at the extreme N-terminus of the anion transport protein molecule.

The C-terminal portion of the band 3 protein ($M_r$ of approximately 55,000) carries out the anion exchange and is membrane bound. The polypeptide chain in this region traverses the membrane several times to form the

![Diagram of band 3 and band 4.5 oligosaccharide.](image)

FIG. 3. Structure of band 3 and band 4.5 oligosaccharide. Suggested structures for the N-linked oligosaccharide containing repeating N-acetyllactosamine disaccharide units (Fukuda & Fukuda 1981). (a) General structure of lactosaminoglycan oligosaccharide. (b) Structure of I antigen found on adult erythrocytes. The antigenic determinant is the terminus of branched chains ending in galactose residues. (c) Structure of i antigen found on fetal erythrocytes. The antigenic determinant is the terminus of linear chains ending in galactose residues.

The band 3 protein carries a single N-linked oligosaccharide which is heterogeneous in size and gives rise to the diffuse character of the band on SDS gel electrophoresis (Fig. 1a). This oligosaccharide contains a variable number of repeating N-acetyllactosamine disaccharide units (Gal β1→4 GlcNAc)$_n$β1→4 which are branched in adult erythrocytes and are substituted into the N-linked oligosaccharide core. The structure suggested for these lactosaminoglycan chains by Fukuda & Fukuda (1981) is shown in Fig. 3a. The branched chains found in adult erythrocytes terminate in galactose
residues and give rise to the blood group I antigenic determinant (Fig. 3b). These chains can also carry blood group ABH antigens (Finne 1980). In fetal erythrocytes the oligosaccharide is not branched (it lacks the R₂ substituents in Fig. 3a) and the linear chains which terminate in galactose residues give rise to the i blood group antigenic determinant (Fig. 3c). There is a further level of heterogeneity in this oligosaccharide in that a proportion of the protein molecules appear to have no repeating N-acetyllactosamine units, but only the core structure mannose residues substituted to different extents with GlcNAc and Gal-GlcNAc structures (Tsuji et al 1981). In erythrocytes which lack the major sialoglycoprotein α (En(a−) erythrocytes) (see below), the anion transport protein has a significantly increased relative molecular mass \( M_r \) which appears to result from an increase in the average number of (Gal β1→4 GlcNAc)β1→3 repeating units present in the oligosaccharide (Tanner et al 1976).

The band 4.5 region and glucose transport

The band 4.5 region contains a diffuse band of protein which has a similar heterogeneous lactosaminoglycan oligosaccharide to that found on the anion transport protein. Studies using reconstitution and the binding of cytochalasin B, the glucose transport inhibitor, have shown that one of the components in this band is involved in transporting glucose into the erythrocyte (see Baldwin & Lienhard 1981 for a recent review). Immunological studies suggest that this component is distinct from the anion transport protein and its fragments. However, this protein is likely to be of a similar structural type to the anion transport protein, although it is not clear whether this protein associates as extensively with peripheral membrane proteins as the anion transport protein.

The PAS-staining glycoproteins

Four sialic acid-rich glycoproteins are present in the human erythrocyte membrane (denoted α, β, γ, δ; Fig. 1b, see Anstee et al 1979 for nomenclature). These sialoglycoproteins give a complex pattern of bands on SDS-gel electrophoresis because two of them, α (glycophorin A) and δ (glycophorin B), form complexes with themselves and each other (Fig. 1b). The major sialoglycoprotein, α, is a well-characterized amphiphilic membrane protein orientated in the membrane with the N-terminus extracellular and C-terminus on the cytoplasmic side of the membrane (Fig. 2). The complete amino acid
sequence (Fig. 4) of the protein is known (Tomita et al. 1978). It contains a 23-residue hydrophobic segment which forms an apolar \( \alpha \)-helix that traverses the membrane (Schulte & Marchesi 1979). The extracellular N-terminal domain is densely glycosylated and contains 15 O-linked sialotetrasaccharides (with the structure shown in Fig. 5a; Thomas & Winzler 1969), most of which are located close to the N-terminus of the protein. A single larger N-linked oligosaccharide is present at asparagine-26. The structure shown in Fig. 5b predominates but related structures are also present (Yoshima et al. 1980). The blood group M, N antigens are located on \( \alpha \). Although the antigenic determinants involve both the polypeptide chain and part of the carbohydrate on the protein, the blood group M and blood group N antigenic forms of the protein differ only in the amino acids present at residues 1 and 26 (Fig. 4; and see review by Anstee 1981). The \( \alpha \) and \( \delta \) sialoglycoproteins have unusually closely related amino acid sequences (Fig. 4). The sequence of the N-terminal 26 residues of \( \delta \) is identical to the same portion of blood group N type \( \alpha \), and so, as might be expected, \( \delta \) carries blood group N antigen activity. The glycosylation sites of both \( \alpha \) and \( \delta \) in this N-terminal region are also similar except that \( \delta \) lacks the complex mannose-containing oligosaccharide at asparagine-26. The remaining nine residues of

FIG. 4. Amino acid sequences of sialoglycoproteins \( \alpha \) and \( \delta \). The partial amino acid sequence of \( \delta \) (Furthmayr 1978, Dahr et al 1980a,b) and the complete sequence of \( \alpha \) (Tomita et al 1978) are shown. The hydrophobic membrane-penetrating portion of \( \alpha \) is between residues 73 and 92. The regions of homology between \( \alpha \) and \( \delta \) (apart from the N-terminal identity between residues 1 and 26) are shown underlined (Dahr et al 1980b). The amino acids in the sequence of \( \delta \) that are shown in parentheses are those found by Furthmayr (1978), who also suggests a slightly different sequence for residues 28–31 of \( \delta \). *indicates the presence of an O-linked oligosaccharide; † indicates the location of the N-linked oligosaccharide in the sequence of \( \alpha \).
the sequence of δ which have so far been determined (residues 27–35) show homology with two sequences at residues 56–64 and 59–67 of α (Dahr et al 1980a,b). The δ sialoglycoprotein carries blood group S and blood group s antigen activity, which is associated with the presence of methionine and threonine respectively at residue 29 (Fig. 4; Dahr et al 1980b).

(a) \[
\text{NANα2} \quad \downarrow 6 \\
\text{NANα2→3Galβ1→3GalNAcβ1→Ser/Thr}
\]

(b) \[
\text{NANα2→6Galβ1→4GlcNAcβ1→2Manα1} \\
6(3) \quad 3(6) \\
\text{Galβ1→4GlcNAcβ1→2Manα1}
\]

\[
\text{GlcNAcβ1} \quad \downarrow 4 \\
\text{Manβ1→4GlcNAcβ1→4GlcNAc→Asn} \\
6 \\
\text{Fucα1}
\]

FIG. 5. Structure of the oligosaccharides present in α. (a) Structure of the O-linked sialotetrasaccharide (Thomas & Winzler 1969). (b) Structure of the N-linked oligosaccharide (Yoshima et al 1980). The major structure is shown but a related structure in which both peripheral chains terminate in sialic acid was also found.

The N-terminal sequence of the sialoglycoprotein β (glycophorin C) differs completely from that of α (Furthmayr 1978, Dahr et al 1980b). This protein has been called ‘glycoconnectin’, since there is evidence that it may be associated with the erythrocyte cytoskeleton network by attachment to band 4.1 (Mueller & Morrison 1980). The sialoglycoprotein γ is poorly characterized at the present time.

Sialoglycoprotein variants

Several different types of sialoglycoprotein variants occur which involve alterations in the α and δ sialoglycoproteins (see Anstee et al 1982a for a review). Table 1 shows some of the variants that have been studied. As might be expected, most of these are associated with altered blood group MN and Ss antigens. The M8 and Mc variants contain rare simple alleles of α and involve changes in the N-terminal five residues of the protein which appear to be immunologically dominant. The Mc form of α appears to be intermediate between the M and N forms, since it contains the serine-1 characteristic of blood group M-type α as well as the glutamic acid-5 characteristic of N-type
TABLE 1 Sialoglycoprotein variants of human erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Cell type&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Alteration&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Variants involving α</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Altered α</td>
<td>M&lt;sup&gt;§&lt;/sup&gt;</td>
<td>Thr&lt;sup&gt;+&lt;/sup&gt;-4 → Asn-4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>M&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Res. 1 → Ser. Res. 5 → Glu</td>
</tr>
<tr>
<td>Loss of α</td>
<td>En(a−)</td>
<td>? Deletion</td>
</tr>
<tr>
<td><strong>Variants involving δ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Altered δ(s)</td>
<td>MiIII</td>
<td>Allelic forms of δ with approx. 15 K increase in M&lt;sub&gt;f&lt;/sub&gt;</td>
</tr>
<tr>
<td>Altered δ(S)</td>
<td>MiIV</td>
<td></td>
</tr>
<tr>
<td>Loss of δ</td>
<td>S−s−</td>
<td>? New component also present</td>
</tr>
<tr>
<td><strong>Variants involving both α and δ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Loss of α and δ</td>
<td>M&lt;sup&gt;§&lt;/sup&gt;</td>
<td>? Deletion</td>
</tr>
<tr>
<td>(δ−α) hybrid with α and δ still present</td>
<td>Ph</td>
<td>Anti-Lepore type hybrid resulting from cross-over event&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>St&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>(α−δ) hybrid with loss of α and δ</td>
<td>MiIV</td>
<td>Lepore-type hybrid with deletion of α and δ genes resulting from cross-over event</td>
</tr>
</tbody>
</table>

<sup>a</sup> MiIII, MiIV, MiV: Miltenberger classes III, IV and V.

<sup>b</sup> See review by Anstee et al (1982a) for a more comprehensive bibliography and discussion of the variants described in this table.

<sup>c</sup> Thr<sup>+</sup> denotes the replacement of an O-glycosylated threonine residue in the M<sup>§</sup> variant (Furthmayr et al 1981).

<sup>d</sup> The St<sup>a</sup> variant is discussed by Anstee et al (1982b).
ERYTHROCYTE MEMBRANE STRUCTURE AND FUNCTION

α. The Lepore-like and anti-Lepore-like variants (Ph, Sta and MIV) probably result from mispairing between δ and α genes and cross-over within the sequences coding for residues 27–35 of δ and the internal homologies at residues 56–64 or 59–67 of α (see Anstee et al 1982a for a more detailed discussion). There are no known deleterious effects on the health of the individuals carrying any of the variant erythrocytes shown in Table 1, and the functional roles of α and δ are not at all clear. In contrast, erythrocytes lacking β (and also band 4.1) appear to have defective cytoskeleton networks (see review by Cohen & Branton 1981).

The discussion so far concerns the most predominant of the integral proteins of the erythrocyte membrane. Many less abundant integral proteins are also present. These include transporters such as the Na+, K+-ATPase, enzymes like acetylcholinesterase, and several blood group antigenic proteins such as Rh. At present there is little structural information available on most of these components.

The peripheral membrane proteins and the red cell cytoskeleton

Many of the ‘peripheral’ proteins of the red cell membrane appear to be involved in the formation of an extensive submembrane reticulum which is usually called the red cell cytoskeleton (see Lux 1979, Gratzer 1981, Cohen & Branton 1981 for recent reviews). The major components of this cytoskeletal network are bands 1 and 2 (spectrin), band 2.1 (ankyrin), band 4.1, and band 5 (actin). This cytoskeleton is believed to control the shape, integrity and flexibility of the erythrocyte membrane. Removal of this complex from erythrocyte membranes causes the membranes to fragment into small vesicles. Treatment of erythrocyte membranes with non-ionic detergent (which solubilizes the lipid bilayer and the integral membrane proteins) leaves a structure containing the cytoskeletal proteins which also retains the discoid shape of the red cell.

The spectrin bands 1 and 2 are both fibrous molecules about 100nm long which associate with each other at one end to form a heterodimer. The open ends of two dimers associate tail-to-tail to give the spectrin tetramer (see inset to Fig. 2), which appears to be the building block of the cytoskeleton. Electron microscopy has shown that band 4.1 associates with the ends of the tetramer, while band 2.1 binds to the band 2 chain nearer to the centre of the spectrin tetramer. The association between spectrin tetramers to form a two-dimensional network appears to involve both band 4.1 and short actin ‘protofilaments’ in the formation of a polyvalent linkage between spectrin tetramers. This meshwork is connected to the lipid bilayer by the association of band 2.1 with the N-terminal region of the anion transport protein, and
perhaps also by the association of band 4.1 with the C-terminal cytoplasmic portion of the sialoglycoprotein \( \beta \) (glycoconnectin). A schematic view of the overall structure of the cytoskeleton is shown in Fig. 2 (p 5).

The importance of the cytoskeleton in erythrocyte viability is emphasized by increasing evidence that several types of haemolytic anaemia (which usually involve misshapen erythrocytes) are associated with different molecular defects in the cytoskeleton system (see Cohen & Branton 1981). Whether the cytoskeleton system simply provides a mechanical restraint to stabilize the membrane against excessive deformation and vesicularization, or whether more subtle mechanisms operate, is not at all clear.

**Changes in membrane components during erythrocyte differentiation**

The earliest recognizable cell of the erythroid line, the pronormoblast, matures by division through three successive nucleated cell stages. These stages, the basophilic, polychromatic and orthochromatic normoblast, all normally remain in the bone marrow. The orthochromatic normoblast then expels its nucleus and the anucleate reticulocyte is subsequently released from the bone marrow into the circulation, where it matures into the erythrocyte.

The erythrocyte membrane proteins are made at different times during erythropoiesis. Spectrin and the other high \( M_r \) proteins are synthesized during the nucleated cell stages in the bone marrow (Chang et al 1976). The synthesis of these is essentially complete by the reticulocyte stage, with only peripheral membrane proteins being synthesized in reticulocytes (Light & Tanner 1978). The surface of the developing erythrocyte undergoes its most marked changes during the nucleated cell stages between the pronormoblast and orthochromatic normoblast. Both the major sialoglycoprotein \( \alpha \) (Gahmberg et al 1978) and the anion transport protein (Foxwell & Tanner 1981) appear at the cell surface in increasing amounts during these stages, and their incorporation is complete by the time the cells mature to the reticulocyte. Interestingly, the membrane loss which occurs during the decrease in size of the reticulocyte as it becomes an erythrocyte appears to occur by endocytosis of spectrin-free areas of the plasma membrane (Zweig et al 1981). During enucleation of the orthochromatic normoblast, spectrin is also excluded from the membrane surrounding the expelled nucleus. Zweig et al (1981) suggest that loss of spectrin-free membrane occurs until the density of spectrin in the cytoskeleton reaches a sufficiently high concentration for the membrane to reach the stable state in the erythrocyte. Perhaps the predilection of certain malaria parasites for young cells results from the natural instability and tendency towards endocytosis of the membrane of the immature cells.

The invasion of the erythrocyte by the malarial parasite involves both
interaction with an erythrocyte surface receptor and the disruption, or
dissociation from the membrane, of the erythrocyte cytoskeleton. The
detailed biochemical information available on these components and on the
structure of the red cell membrane should make it possible to test specific
hypotheses about the way in which the parasite gains entry into the red cell.
For example, is it possible that the histidine-rich protein, which is released by
the parasite during invasion, enters the cell and because of its strongly
cationic nature binds to the strongly anionic N-terminus of the anion
transport protein? This might cause the displacement of ankyrin from the
anion transport protein with the dissociation of the cytoskeleton from the
membrane, so destabilizing the local area of membrane and rendering it more
susceptible to endocytosis. Clearly, there are many other ways in which the
parasite could achieve the same end, but specific hypotheses like these are
directly amenable to experimental test and should help to increase our
understanding of the invasion process.

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DISCUSSION

Walfach: It has recently been shown that hereditary elliptocytosis is due to a defect in the ability of spectrin to form tetramers (Liu et al 1982). This has always been considered a possibility, but now this has been shown not only for spherocytosis but for elliptocytosis. I am interested in the loss of red cell membrane during maturation. Is the membrane exocytosed, or endocytosed?

Tanner: It seems that the lost membrane goes into the cell in reticulocytes. Zweig et al (1981) have observed vesicles in reticulocytes which are spectrin-free and derived from the plasma membrane. The reticulocyte is rapidly degrading its intracellular structures, so presumably there is a membrane-degrading system for dealing with the endocytosed membrane.

Pasvold: Is the endocytosed membrane glycophorin-free as well?

Tanner: Zweig et al (1981) did not determine whether there was any exclusion of integral membrane proteins from the endocytosed membrane. However, if the cytoskeleton limits membrane protein mobility in the reticulocyte as it does in the intact red cell, even if only by simple entrapment of the proteins, the absence of spectrin from the endocytosed membrane makes it likely that the major integral membrane proteins are also absent.

Pasvold: In your hypothesis about the histidine-rich protein and its ability to dissociate cytoskeleton from membrane, would there be a requirement on the part of the red cell for either the uncoupling process or re-instating the status quo? Can the fact that young red cells are more likely to be invaded than older cells depend on the red cell’s metabolic activity, rather than the parasite’s predilection for young cells or other structural aspects of the cell, such as the availability of ‘receptors’?

Tanner: Several different factors may determine the overall likelihood of invasion of a cell. The endocytosis that occurs in immature cells may tip the balance in favour of a younger cell being invaded because its membrane is intrinsically less stable, so that parasite endocytosis is helped by the natural endocytic mechanism present in the immature cell.

Weatherall: What do you really mean by saying that the young cell is more susceptible to endocytosis?

Tanner: The interpretation at the molecular level is that there is insufficient spectrin present to form a cytoskeleton which will cover the surface of the additional membrane that is present in immature cells. As a result there will be spectrin-free areas of the immature cell membrane which may then spontaneously be endocytosed (Zweig et al 1981).

Weatherall: So the final cell size is related to the density of spectrin?

Tanner: Yes, cell size is related to the amount of cytoskeleton present. When the ratio of cytoskeleton to membrane surface area reaches a critical value, the membrane becomes stable. Presumably this is when all the membrane is covered by cytoskeleton.