
To the editor:

New and old integral proteins of the human erythrocyte membrane

Salzer et al, from the University of Vienna, have recently described that vesicles released from Ca++/Ca++ ionophore-treated erythrocytes are enriched in lipids and proteins that are typically found within lipid microdomains of the parent cell’s plasma membrane, the so-called “lipid rafts.” The microvesicles shed from Ca++-loaded erythrocytes, processed for the separation of lipid domains, appear to contain the conventional markers of lipid rafts, cholesterol and ganglioside GMI. The novelty of the data is in the large number of membrane-associated proteins, most of which were previously undetected, found by Salzer et al in the human erythrocytes: stomatin, flotillin-1, flotillin-2, synexin, and sorcin. These proteins appear to be associated with lipid rafts, and, most important, some of them are enriched in the vesicles, relative to the parent cell’s membrane. They share this property with the family of exofacial proteins inserted in the membrane via a glycosyl phosphatidylinositol (GPI) anchor. In erythrocytes, the most famous member of this family is acetylcholinesterase (AChE), whose enrichment in Ca++-dependent vesicles was known well before the structure of the GPI anchor was elucidated.

If one takes the amount of membrane phospholipids as a measure of membrane surface extension (as is common and correct practice) and then normalizes the amount of AChE, as enzymatic activity, over membrane phospholipids, a 3- to 4-fold enrichment of AChE in microvesicles, with respect to erythrocytes, is observed. This figure, supported by several independent reports in the past, was easily confirmed in our lab. For reasons that were not explicitly stated in their paper, and that therefore remain obscure to the reader, Salzer et al chose to normalize AChE activity to hemoglobin content of vesicles and cells, and were able to calculate a different figure. Thus, the amount of AChE in vesicles, when referred to hemoglobin, is “roughly 80 times” the amount in the parent cells. This figure may seem confusing for a reader more accustomed to the old notion of a 3- to 4-fold enrichment. However, we must say that this result is correct. A 185-nm spherical vesicle has a volume of approximately 0.0033 μm³, and a surface area of approximately 0.108 μm². The surface-to-volume ratio (S/V) is therefore approximately 33 μm⁻¹. The measured S/V of erythrocytes (total population of cells) is approximately 1.59 μm⁻¹. Thus, the S/V in vesicles is roughly 20 times the S/V in erythrocytes. A 3- to 4-fold enrichment in AChE in vesicles, calculated by normalization of AChE activity over membrane surface extension (phospholipids), becomes a 60- to 80-fold enrichment when normalizing to cell volume (hemoglobin): a good fit. However, it was probably not necessary to introduce a new procedure for quantifying AChE enrichment in vesicles, since it does not constitute an original finding. However, Salzer et al go one step further and claim that band 3 protein and glycophorins are strongly decreased in vesicles compared with cells. The way they demonstrate this is clearly incorrect: sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels were loaded with equivalent amounts of erythrocyte membranes and vesicle membranes by taking AChE as the normalizing parameter. Then, band 3 was stained (glycophorins were not, however) and shown to be decreased in vesicles. But since AChE is enriched in the membrane of vesicles with respect to cells, this method will always detect a decrease of anything that would otherwise keep constant in the membrane of vesicles. Therefore, we would prefer to adhere to the well-documented notion that band 3 is equally represented, per unit surface area, in vesicles and cells, as we have found the same result by direct measurements of properly loaded gels in our lab. Salzer et al conclude that, “interestingly, only trace amounts of the flottilins are found in the vesicles [italics added].” Maybe flottilins are indeed present in more than trace amounts in vesicles. It is only a matter of watching more carefully.

Giampaolo Minetti and Annarita Ciana

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Response:

Segregation of lipid raft proteins during calcium-induced vesiculation of erythrocytes

The erythrocyte membrane contains lipid rafts, with stomatin, the flotillins, and glycosylphosphatidyl-inositol (GPI)–linked proteins as the major integral proteins, whereas band 3 protein and the glycophorins are largely absent. As microvesicles released from erythrocytes after Ca++ treatment are specifically enriched in the GPI-linked enzyme acetylcholinesterase (AChE), it is likely that
lipid rafts are involved in the vesiculation process. We showed that microvesicles in fact contain lipid rafts; however, the relative amounts of the raft proteins differ between microvesicles and the erythrocyte membrane. These results suggest a calcium-induced segregation of different types of lipid rafts, with stomatin-specific lipid rafts, synexin, and otillin-2 are major integral proteins of erythrocyte lipid rafts. Blood. 2001;97:1141-1143.

The letter of Minetti and Ciana refers to a paragraph in "Results" in our study, in which we discuss the relative amounts of certain membrane proteins in the erythrocyte membrane compared with whole microvesicles and nanovesicles (and not to vesicle membranes as stated by the authors). There are 2 misunderstandings in the comments of Minetti and Ciana. First, we did not “normalize AChE activity to hemoglobin content.” We mentioned the enrichment of AChE relative to hemoglobin in microvesicles. Second, as stated in “Results,” we used the raft marker AChE for normalization to compare the relative amounts of other raft proteins, particularly stomatin and the flotillins, and contrasted these findings to nonraft proteins. The term “relative” in the criticized statement (“the relative amounts of the major integral membrane proteins band 3 and glycoporphins are diminished [italics added]”) should be understood as relative to AChE and not relative to phospholipid/cell surface. However, in contrast to the unpublished results of Minetti and Ciana (the Butikofer et al’ reference cited by the authors does not contain original data on the band 3 distribution), Hagelberg and Allan reported that “band 3 and glycoporphins are depleted from microvesicles” using phospholipid content for normalization. They found that only 40% of the band 3 protein is present in the microvesicles when compared with erythrocyte membranes.

To address the question of membrane protein dynamics during microvesiculation in more detail, we compared erythrocyte membranes and increasing amounts of microvesicles by quantitative immunoblotting, thereby assessing the protein distribution relative to AChE (data not shown). As an example, we show the relative abundance of the respective proteins at a 4-fold AChE activity in the microvesicle sample (Figure 1A). Whereas similar fractions of the total erythrocyte AChE, stomatin, flotillin-2, and aquaporin-1 content are found in lipid rafts (Figure 1B), the relative amounts of these proteins in microvesicles are quite different, thereby demonstrating the segregation of erythrocyte raft proteins during vesiculation. A small fraction of band 3 protein is also found in the lipid rafts; however, the significance of this finding remains to be evaluated. Taking the well-supported factor of 3 for the AChE enrichment in microvesicles relative to phospholipid, we calculated the enrichment/depletion of the following proteins in microvesicles relative to membrane surface: stomatin (1.7), flotillin-2 (0.2), aquaporin-1 (0.4), glycoporphin C (0.5), and band 3 (0.4; in agreement with Hagelberg and Allan6).

In accordance with our previous study, these data clearly show the segregation of lipid raft proteins during calcium-induced vesiculation of erythrocytes. Whereas AChE and stomatin are enriched in the released microvesicles, the flotillins and aquaporin-1 are depleted. In line with the interpretation of Hagelberg and Allan,6 we assume a partial cytoskeletal association as the cause for the depletion of the respective membrane proteins in microvesicles. The mechanism of the enrichment of AChE and stomatin in microvesicles remains to be elucidated; however, the membrane aggregating and fusogenic properties of synexin, which is present in vesicular rafts, may play an important role in this process.

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References

To the editor:

**Acquired high-titer factor VIII inhibitor: fatal bleeding despite multimodal treatment including rituximab preceded by multiple plasmaphereses**

Acquired factor VIII (FVIII) inhibitors can cause life-threatening bleeding. Rapid restoration of coagulation is vital. Therapeutic approaches include factor substitution, immunosuppression (eg, steroids, cyclophosphamide), and plasmapheresis. A novel treatment option is rituximab, a chimeric monoclonal antibody targeting the CD20 antigen and blocking proliferation of normal B cells.5

Recently, Wiestner et al reported on the reduction of acquired FVIII inhibitors in 4 patients by an immunosuppressive regimen including rituximab.6 Patients presented with FVIII activity (FVIIIc) ranging from less than 1% to 4% (normal range, 70%-200%) and inhibitor titers ranging from 5 to 60 Bethesda units (BU). In 3 patients, FVIIIc normalized after the first of 1 to 4 treatment courses. The inhibitor became undetectable within 3 to 12 weeks. Plasmaphereses were not necessary.

Here, we describe the clinical course of a patient suffering from acquired idiopathic FVIII inhibitors with extraordinarily high titer. The 71-year-old male was admitted for the development of a large painful mass in his left gluteal region. He had received an intramuscular injection for lumbalgia 4 days prior. Patient history included years of chronic obstructive pulmonary disease but was otherwise unremarkable, particularly for allergic diathesis. There was no family history of autoimmune diseases, bleeding disorders, or neoplasias. Clinical examination revealed a large painful mass in his left gluteal region and diffuse mucosal bleeding. Respiratory sounds were slightly prolonged; liver and spleen were not enlarged. Laboratory work-up demonstrated pathologic coagulation studies with a markedly prolonged activated partial thromboplastin time (aPTT) of 80 seconds, decreased FVIIIc of less than 1%, and high FVIII inhibitor titers of 633 BU. Extensive laboratory exams did not reveal further pathologic results.

After a 2-week treatment with steroids he was transferred to our unit with persistent bleeding (day 0, Figure 1). Here, the patient received one dose of FVIII inhibitor bypassing activity (FEIBA, Baxter BioScience, Heidelberg, Germany), followed by recombinant FVIIa (NovoSeven, NovoNordisk, Mainz, Germany) given for 3 days, which did not improve the clinical course. In need of rapid intervention, cyclophosphamide and vincristine were applied twice. At this point, as inhibitor titer had even increased to 19 800 BU, plasmapheresis was started. A dramatic decline in inhibitor titers was observed immediately thereafter (Figure 1). Neverthe-

In 30% of patients, spontaneous resolution of acquired FVIII inhibitors has been described after an average of 21 months.7 However, in the case of bleeding and high antibody titers, rapid restoration of coagulation is required. This often is not achieved by current immunosuppressive regimen. With regard to novel treatment options, the successful application of 2-chloro-deoxyadenosine has recently been reported.8 Here, the median time to reach nadir inhibitor titers was 137 days; the median time for a 50% increase in FVIIIc was 117 days. Concerning efficacy of rituximab, data of Wiestner and colleagues suggest a faster FVIII recovery (3–12 weeks). Despite the promising treatment results with rituximab in several immunoglobulin-mediated disorders,9 it remains a concern whether the nadir of FVIII inhibitors can be achieved fast enough in high-risk cases.

To maximize treatment efficacy in our critically ill patient, we combined standard immunosuppressive therapy with plasmapheresis and rituximab. Plasmapheresis was intended to rapidly reduce autoantibody levels and allow for infusion of large amounts of plasma with procoagulant activities. Indeed, we experienced a decline in inhibitor titers after initiation of plasmapheresis. Within 25 days, a 200-fold reduction of inhibitors was achieved. Yet, it is...