IDENTIFICATION OF HUMAN ERYTHROCYTE BLOOD GROUP ANTIGENS ON DECAY-ACCELERATING FACTOR (DAF) AND AN ERYTHROCYTE PHENOTYPE NEGATIVE FOR DAF

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Decay accelerating factor (DAF) is a glycoprotein present on the surfaces of many types of cells in contact with plasma, including erythrocytes, leukocytes, and platelets (reviewed in reference 1). A small amount of DAF is also present in serum. Numerous investigators have demonstrated that DAF inhibits the action of C3 convertases on cell surfaces, and its absence has been shown to be at least partially responsible for the abnormal sensitivity to lysis by complement exhibited by erythrocytes of patients with the acquired stem cell disorder paroxysmal nocturnal hemoglobinuria (PNH) (2). Hereditary absence of DAF has not been previously described.

Tca and Cr are high-frequency human erythrocyte antigens. These antigens are part of a family of blood group antigens, designated Cromer related, which are all absent from the null phenotype cell IFC−, or Inab (3). Recently, Spring and colleagues (4) have identified two monoclonal antibodies which bound to high frequency red cell antigens absent from the Inab phenotype. They also demonstrated that these antibodies, as well as several human antisera to Cromer-related antigens, bound to a 70-kD glycoprotein when used to stain immunoblots of human erythrocyte membrane proteins. Because the wide tissue distribution of mAb reactivity, along with some of the biochemical characterization and immunoblotting data, was similar to that of DAF, we investigated whether the Cromer-related antigens Cr and Te resided on the DAF molecule.

Materials and Methods

DAF was purified from random donor human erythrocytes by the method of Nicholson-Weller (5), as modified by Sugita et al. (6). Resultant material was homogeneous as analyzed by SDS-PAGE and silver staining, and C3 convertase inhibitory activity was intact, as measured by the ability of purified DAF to inhibit lysis of sheep erythrocytes sensitized with antibody and bearing human complement components 1 and 4b (EAC1, 4b) (3). This material...
was used to raise a rabbit polyclonal antiserum to DAF (5); the resulting antiserum was non-reactive with PNH type III cells, indicating that it did not bind to non-DAF erythrocyte surface proteins.

Antibody reactivity with random donor as well as PNH and phenotypically rare erythrocytes was measured by RIA as previously described (7), except that radiolabeled Staphylococcal protein A (SPA) was used to detect binding of rabbit antiserum, and F(ab')2 sheep antibody to human Ig (heavy and light chains, Amersham Corp., Arlington Heights, IL) was used when human blood group antisera were tested. Competitive inhibition assays were performed as previously described (8), except that purified proteins were solubilized in 0.1% NP-40 at ~700 µg/ml and further diluted in PBS supplemented with 10% BSA. Tc and Cr' antisera were incubated with purified proteins for 1 h at ambient temperature before addition of erythrocytes. All Cromer-related human antisera were reacted with erythrocytes at 37°C.

Erythrocytes from PNH patients were obtained and separated into populations of type I (normal) and type III (markedly sensitive to complement) cells as previously described, using cobra-venom lysis of type III cells to obtain type I cells (9) and anti-acetylcholinesterase affinity chromatography to isolate type III cells (10). None of the patients studied had appreciable numbers of type II cells (with intermediate sensitivity to complement).

Immunoblotting of erythrocyte proteins was performed as previously described (11), except that binding of human blood group antibodies was detected using 125I-labeled F(ab')2 sheep antibody to human Ig (~100,000 cpm/100 µl) and PBS, 0.2% Tween 20 buffer, pH 7.4. Reactivity of rabbit antibodies was detected using radiolabeled SPA.

Results

Reactivity of Cromer-related Antibodies with Purified DAF in Solution. Purified DAF was tested for its ability to inhibit binding of antisera against the high frequency Cromer-related antigens Tc' and Cr' to random donor erythrocytes. Prior incubation of antisera with purified DAF was able markedly to inhibit subsequent binding of both Tc' and Cr' antibodies to erythrocytes. This effect demonstrated a clear dose-response relationship (Fig. 1). In contrast, substitution of purified In(Lu)-related
Binding of Cromer-related Antibodies to PNH Erythrocytes. Isolated PNH I and PNH III erythrocytes were tested, along with unseparated cells and cells from normal donors, for their ability to bind antisera to the Cromer-related antigens Tc and Cr. For all patients and antibodies tested, PNH I cells bound antibodies to Cromer-related antigens as well or nearly as well as cells from normal donors, while PNH III cells showed reduced or no binding of anti-Tc and anti-Cr (Table I). Some Cromer-related antibodies reacted weakly with PNH III cells; these results could have been due to contamination of the PNH III cells by small numbers of PNH I cells or to the presence of small amounts of antibody to other antigens in the antisera used. Unseparated PNH erythrocytes showed binding of anti-Tc and anti-Cr intermediate between that obtained with purified PNH I and PNH III cells, as would be expected.

PNH I and PNH III erythrocytes were also tested by standard agglutination techniques for reactivity with antisera to Cromer-related antigens. Antisera to Cr, Tc, IFC, Wes, Dr, and Es all agglutinated PNH I cells but failed to agglutinate PNH III cells.

Immunoblotting. When antisera to Tc were used to stain immunoblots of purified DAF, these antisera showed clear reactivity with DAF protein, while no reactivity was detected using nonreactive human serum (Fig. 2) or using purified In(Lu)-related p80 (12), a protein unrelated to DAF (data not shown). Similar data were obtained with anti-Cr (not shown).

Expression of DAF by Inab Phenotype Erythrocytes. When rabbit polyclonal antiserum to DAF was tested for reactivity with Inab phenotype erythrocytes, these cells showed

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### Table I

**Reactivity of Cromer-related Antisera with PNH Erythrocytes**

| Cell type       | Anti-Tc* | Anti-Tc** | Anti-Cr* |
|-----------------|----------|-----------|----------|
|                 | %        | %         | %        |
| Patient 1       |          |           |          |
| Unseparated     | 22       | 59        | ND       |
| Type I          | 81       | 119       | ND       |
| Type III        | 0        | 27        | ND       |
| Patient 2       |          |           |          |
| Unseparated     | 56       | ND        | 82       |
| Type I          | 83       | ND        | 97       |
| Type III        | 21       | ND        | 51       |
| Patient 3       |          |           |          |
| Unseparated     | ND       | 31        | 63       |
| Type I          | ND       | 106       | 105      |
| Type III        | ND       | 2         | 39       |

*Antisera from different donors.
minimal reactivity (mean of three experiments = 1.1 × background cpm, range = 0.6-1.3) compared with Cr(a+) Tc(a+) cells from six donors, which bound anti-DAF strongly (5.7 × background cpm, range = 3.5-6.6). Immunoblotting of Inab erythrocyte membrane proteins with anti-DAF also failed to show reactivity of antibody with a specific protein band consistent with the known molecular weight of DAF (Fig. 2).

Discussion

These studies have demonstrated that antibodies to the Cromer-related human blood group antigens Cr a and Tc a reacted with purified DAF, a glycoprotein that regulates the activation of the complement component C3 on the surface of hematopoietic cells. Soluble purified DAF was able to competitively inhibit binding of Cromer-related antibodies to erythrocytes, and anti-Tc a and anti-Cr a bound to purified DAF but not to other proteins in immunoblots. Moreover, PNH III erythrocytes, which lack DAF, demonstrated reduced or absent reactivity with human antisera to the Tc a and Cr a antigens, as well as with antisera to other Cromer-related antigens. In contrast, PNH I erythrocytes from the same donors reacted normally with these antisera. These data all demonstrate not only that expression of these antigens is dependent on expression of DAF but also that these antigens reside on the DAF molecule. However, our data do not provide information regarding the topographic relationship of these two epitopes to each other or to the functional domain of the DAF protein. Preliminary studies have shown that binding of Cromer-related antibodies to DAF protein does not inhibit activity of DAF, as measured by the complement lysis sensitivity test (13).

Identification of DAF as the molecule that carries these Cromer-related blood group antigens is largely consistent with the biochemical characterization of the protein identified by Spring and colleagues (4). However, they were unable to demonstrate
the presence of N-linked glycans by endoglycosidase F treatment of erythrocyte membrane proteins under nonreducing conditions, while DAF has been shown to bear \( \sim 3,000 \) daltons of such oligosaccharides (14). Most likely, the N-linked oligosaccharides of the DAF protein are not available to enzymatic digestion unless the intrachain disulfide bonds are disrupted. DAF is known to have multiple intrachain disulfide bonds (15), consistent with observations by Spring and colleagues that Cromer-related antigens are destroyed by reagents that denature such bonds (4). The known variation in molecular weight of DAF from various cell lineages (15) is also consistent with the data obtained with Cromer-related antibodies (4).

Polymorphisms in the Cromer-related antigens are rare, with the most common being expression of the Tc\( ^{b} \) antigen, an antigen that occurs in \( \sim 5\% \) of blacks (reviewed in reference 16). Since none of the rare Cromer-related antigen phenotypes have been reported to be associated with hemolytic diseases, such polymorphisms most likely do not affect function of the DAF protein as an inhibitor of C3 convertases. However, direct study of erythrocytes with rare Cromer-related phenotypes is needed to confirm that such polymorphisms are not associated with subclinical disease states. Recently, Stafford and colleagues (17) have presented data describing restriction fragment length polymorphisms of the gene encoding DAF. Also, Sugita et al. (6) described amino acid sequence polymorphisms in the first and fourth amino acids of DAF. However, these polymorphisms are most likely not directly related to Tc and Cr blood group antigen polymorphisms, since the latter are of quite low frequency.

Cells of the very rare Inab phenotype bound little or no rabbit polyclonal antibody to DAF. This extremely rare null phenotype is therefore likely to represent an inherited inability to express normal DAF. Lack of expression of DAF by Inab cells could be due to defective DAF genes or to a processing or insertion defect similar to that proposed for PNH. Although persons with the Inab phenotype have not been described to have hemolytic disease, cells with an isolated DAF defect would be expected to behave like PNH II cells and thus suffer little hemolysis in vivo (1). However, two of the four persons described to have had this phenotype have also had severe protein-losing enteropathies, suggesting that lack of DAF or lack of another protein genetically linked to expression of DAF and Cromer-related antigens may play a critical role in human gastrointestinal pathophysiology.

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