SERUM i ANTIGEN: A NEW HUMAN BLOOD-GROUP GLYCOPROTEIN[1]

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Summary: A glycoprotein has been found in normal serum which inhibits the hemagglutination caused by human anti-i cold agglutinins. This glycoprotein binds to a Sepharose anti-i affinity column at 4°C and can be quantitatively eluted at 37°C. The eluted glycoprotein is specific in that it has potent hemagglutination-inhibition activity against anti-i antibodies but does not inhibit the hemagglutination by anti-I, -A, -B, -H or by the lectins Con A, PHA or Vicia graminea. The anti-i affinity method should provide a simple procedure for large scale purification and subsequent characterization of the i-antigen.

The I and i blood group antigens on human erythrocytes have been defined using human cold agglutinating antibodies (1,2). Some cold agglutinins (anti-I) preferentially agglutinate adult red cells, whereas others (anti-i) preferentially agglutinate cord red cells. The red cell antigens have not been characterized chemically but are thought to be related to the ABH and Lewis antigens (3). Recently soluble glycoprotein antigens which react with human cold agglutinins have been found in human saliva, milk and amniotic fluid (4-6), and these molecules are a logical source of material for chemical characterization. We here report the presence in normal human serum of a glycoprotein which strongly inhibits those cold agglutinins with the anti-i specificity. Using an affinity column consisting of anti-i covalently bound to Sepharose, we were able to purify the i glycoprotein by virtue of its binding at 4°C and elution at 37°C.

Materials and Methods: Fifteen different human cold agglutinin-containing sera were used in this study. Two were normal sera containing low-titer cold agglutinins and thirteen were from patients with chronic cold hemagglutinin disease (7) and contained monoclonal high-titered cold agglutinins. Two of this latter group (Mac and Dud, generously provided respectively by Mrs. Marie Crookston and Dr. R.

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Rosenfield) had previously been designated anti-i on the basis of hemagglutinin titers against cord and adult red cells. Hemagglutination and hemagglutination-inhibition (HAI) were performed with the Technicon AutoAnalyzer as this provided an automated, quantitative and highly sensitive assay (6). Using this method, aliquots of antibody are sampled and mixed with adult red cells at 4°C, and agglutinated red cells are removed from the continuous flow system. The remaining non-agglutinated cells are subsequently lysed with Triton and the hemoglobulin content measured optically (A550nm) and recorded. Agglutination by aliquots of antibody thus results in measurable peaks on the recording and the amount of agglutination is proportional to the height of the peak.

For each of the 15 cold agglutinins, a standard dilution was empirically chosen which gave agglutination of adult red cells corresponding to a peak height of 0.35 to 0.55 optical density units on the AutoAnalyzer recording. The relative i to I specificity of each cold agglutinin was determined by absorbing one aliquot of the standard dilution of each cold agglutinin with pooled group 0 adult red cells and another aliquot with an equal number of pooled group 0 cord cells. After mixing at 4°C for one hour, the aliquots were spun and the percent absorption of the antibody by each type of red cell was determined by measuring the residual cold agglutinin in the supernatant. Control absorptions at 37°C caused no diminution in the supernatant antibody. This method of determining the i to I specificity for each cold agglutinin was used rather than comparing agglutination of the two cell types because the absorption method gives a direct assessment of relative binding whereas agglutination depends on other factors as well as binding (8).

To measure HAI by soluble antigens, aliquots of the standard dilution of each cold agglutinin were made up containing different amounts of the potentially inhibiting substance. After incubation at 4°C for one hour, the aliquots were tested along with control uninhibited aliquots and serial dilutions of the standard controls. In this way, the percent inhibition of each cold agglutinin by the soluble antigen could be measured.

To look for an inhibiting antigen, samples of normal serum or plasma

were deproteinized at room temperature by drop-wise addition of perchloric acid (PCA) to a final concentration of 0.6M. The glycoprotein-rich supernatants were neutralized and dialyzed against pH 7.2 isotonic phosphate buffered saline and were then tested for inhibition of the different cold agglutinins.

Because preliminary results showed inhibition by the serum glycoprotein extracts of those cold agglutinins with the anti-i specificity, an affinity column was prepared by covalently coupling (9) to Sepharose 2B a purified cold agglutinin (10) which was shown to be inhibited by the serum extract. A PCA extract was prepared from 5 liters of outdated blood bank plasma (group O) and this extract was concentrated 10-fold (Diaflo UM 20E, Amicon) and dialyzed against pH 8.2 Tris-HCl buffered saline. The inhibitory substance could be purified by mixing batches of the plasma extract with the Sepharose-anti-i at 4°C, washing the gel with cold Tris-buffer, and eluting with 37°C buffer. Over 80% of the absorbed inhibitor could be recovered in the 37°C eluate. The details of the preparation of the affinity gel and the purification of the inhibitor will be published elsewhere.

Known amounts (based on lyophilized weights) of the purified inhibitor were then tested for percent inhibition of all 15 cold agglutinins to obtain a quantitative comparison of the affinity of the inhibitor for cold agglutinins of different i/I specificity. The purified substance was also tested for HAI of anti-A, -B, -H, -PR, Con A, Phaseolus vulgaris (PHA) and Vicia graminea antibodies and lectins using similar AutoAnalyzer techniques.

Results: Table 1 shows the results of differential absorption of each of the 15 cold agglutinins by cord and adult red cells. That dilution of each cold agglutinin which resulted in equivalent cold hemagglutination on the AutoAnalyzer is indicated. For the two normal donors (Coo and Wor) a small dilution was used. The dilutions used for the high titer antibodies varied depending on the potency of each serum. There was considerable variation in the effectiveness of absorption of each antibody by cord and adult cells. For the purpose of assigning relative i/I specificity, we ranked the cold agglutinins in order

Table 1

Determination of specificity of cold agglutinins by quantitative absorption with cord and adult red cells

Donor of cold agglutinin serum*	Dilution of serum tested	% cold agglutinin absorbed + By cord cells	By adult cells	% abs. by cord** / % abs. by adult
1) Dud	1/5,000	48%	20%	2.40
2) Mac	1/28,000	60%	30%	2.00
3) Tea	1/6,000	60%	30%	2.00
4) Maw	1/6,000	31%	21%	1.48
5) Col	1/60,000	78%	59%	1.32
6) Dun	1/125,000	47%	54%	0.87
7) But	1/30,000	52%	67%	0.78
8) Rei	1/125,000	33%	47%	0.70
9) Ser	1/10,000	28%	40%	0.70
10) Wil	1/30,000	52%	87%	0.60
11) Ada	1/150,000	12%	44%	0.27
12) Wor	1/6	12%	44%	0.27
13) Coo	1/6	10%	54%	0.19
14) Sim	1/4,000	0%	43%	0.00
15) Kah	1/50,000	0%	58%	0.00

*Donors Coo and Wor were normal adults having normal, low-titered, cold agglutinins. The other donors were patients with chronic cold hemagglutinin disease having high-titered antibodies.
+One ml of each diluted cold agglutinin was absorbed at 4°C with $5x10^6$ cord cells and with $5x10^6$ adult cells (except for the use of 10^7 of each cell type to absorb Sim and Kah). Following absorption, the residual peak heights of agglutination were measured with the Technicon AutoAnalyzer and the percent absorption was determined from the peak heights of unabsorbed and fractionally diluted unabsorbed standards.
**This ratio of percent absorption by cord to adult cells is an index of the relative i to I specificity of each cold agglutinin.

of the % absorption by $5x10^6$ cord red cells/% absorption by $5x10^6$ adult red cells (last column Table 1). Two of the high titer cold agglutinins (Sim and Kah) showed the greatest differential absorbability. Using 10^7 cord cells, no absorption could be demonstrated. However, some absorption by cord cells could be demonstrated when larger numbers of cells were used. In general, the relative percent absorption of different cold agglutinins by cord and adult cells paralleled the relative agglutination of cord and adult cells by the antibody, but this was not always true and we decided to rank the different antibodies by the binding criterion for reasons mentioned under Materials and Methods.

Examining the dialyzed PCA extracts of a number of serum and plasma samples, we found consistent inhibition of cold hemagglutination using those cold

agglutinins with a relative i specificity. This inhibitory activity was found in both adult and cord blood samples and was independent of ABO, Lewis, and secretor phenotypes. The inhibitory activity could also be detected in unextracted serum and plasma but we preferred to use the PCA extracts as this precluded any interference of the assay by normal cold agglutinins.

Using the cold agglutinin affinity column, we were able to obtain a highly purified inhibitor from the 5 liters of pooled plasma. Serial amounts of this purified inhibitor were mixed with 1 ml volumes of the standard dilutions of each cold agglutinin and the percent inhibition of each cold agglutinin was determined. Fig. 1 shows the results of inhibition studies using 10 nanograms (ng) of the inhibitor. There was selective inhibition of those five cold agglutinins which had been preferentially absorbed by cord red cells, i.e., the anti-i cold agglutinins. Similar results were obtained when these cold agglutinins were tested using cord red cells in the AutoAnalyzer. Using serial amounts of the inhibitor, the percent inhibition was proportional to the amount of inhibitor. Significant inhibition of the three cold agglutinins (Dud, Tea, Mac) with the strongest anti-i specificity was obtained with as little as 1 ng. In contrast, at the other end of the specificity range, even 500 ng of the inhibitor failed to inhibit Sim or Kah. As further proof of the specificity of our purified serum inhibitor, analogous AutoAnalyzer hemagglutination systems using anti-A, -B, or -H reagents as well as Con A, PHA and Vicia graminea lectins have been used and none of these agglutinators were inhibited by 1000 ng of our purified inhibitor. In addition, a cold agglutinin with the anti-PR specificity (11, 12) was not inhibited.

Preliminary tests on the i-inhibitor show it is excluded from Sephadex G-200, contains about 20% protein by Lowry determination and, on the basis of its PCA solubility and periodate sensitivity, is a glycoprotein. Extraction with chloroform-methanol failed to remove any of the inhibitory activity, indicating an absence of significant lipid content. Electrophoresis of the i-inhibitor on cellulose acetate and elution and testing of serial segments in-

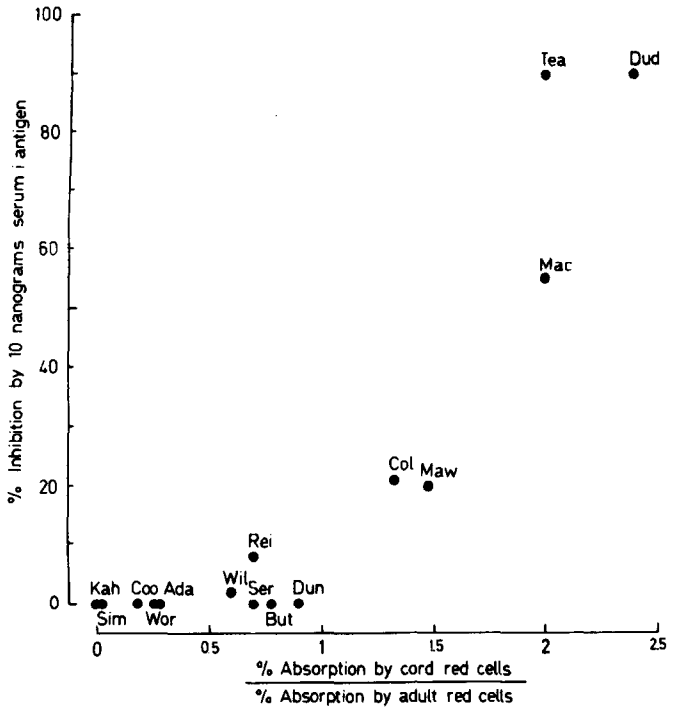

Figure 1: Percent inhibition of various cold agglutinins by 10 nanograms of purified serum i antigen. The soluble antigen inhibited those cold agglutinins which had been shown (Table 1) to be preferentially bound to cord (i) red cells.

dicates a beta mobility. The i-inhibitor is present in normal serum at a concentration of about 1 μg/ml and appears to be a previously unrecognized glycoprotein.

Discussion: We have demonstrated that normal serum contains a glycoprotein macromolecule capable of inhibiting preferentially those human cold agglutinins (anti-i) which bind to cord red cells better than to adult red cells. By covalently coupling one of the purified anti-i antibodies to Sepharose, we made an affinity column which selectively bound the inhibitor at 4°C and released it at 37°C. Using this affinity gel we were able to purify the serum inhibitor. As little as 1 ng of the purified glycoprotein gave significant inhibition of those cold agglutinins with the highest anti-i specificity, whereas much larger amounts failed to cause any inhibition of other antibodies and lectins. For these reasons we designate our serum inhibitor "i antigen".

Our working hypothesis about the Ii system, based on the findings reported

here are as follows. There are two forms of the antigen, I and i, which are probably portions of the carbohydrate side-chains of the glycoprotein antigens (3). The two forms of the antigen probably differ only slightly, e.g., by one hexose. There appears to be a wide range of cold agglutinins with slightly differing specificities. This could result from different cold agglutinins binding to slightly different portions of the Ii antigen. Some cold agglutinins would bind to that portion shared by the I and i antigens and these antibodies would not show much preferential binding to cord versus adult red cells. Other antibodies would, to varying degrees, bind to the shared portion of the antigen and also to that locus that distinguishes the I and i forms of the antigen. The soluble glycoprotein i-antigen described here would be able to effectively compete with the red cell antigens only for those antibodies (anti-i) whose combining site included recognition of that portion unique to that i-antigen.

It is probable that some binding to and agglutination of both cord and adult red cells occurs with all of these Ii cold agglutinins not only because of the shared determinants on the i and I antigens but also because both forms of the antigen exist, albeit in different ratios, on both types of cells. During infancy, a modification of some of the i-antigen sites to form I sites on the red cell occurs (13). We do not know the chemical basis of the difference between the i and I antigen, but consider that it consists of some small addition to the i molecules. This addition would be under enzymatic control and the change of the red cell antigen during infancy could be the result of expression of this converting enzyme. It is interesting that diseases causing peripheral destruction of red cells and increased rates of bone marrow production result in a relative increase in i to I antigen activity (14), so that these adult red cells more closely resemble the cord cell. This situation would be analogous to the "incompletion" of plasma membrane carbohydrate site-chains which often occur during malignant transformation (15). We believe that this serum glycoprotein will be of considerable interest: (1) it appears to be a monospecific i antigen and will provide a focal point for further definition of specificity in the Ii system; (2)

it provides an accessible source of readily purified soluble i antigen for chemical characterization and for studies on the marked temperature dependency of the binding to cold agglutinins; (3) it can be directly measured in minute amounts of serum, facilitating a search for altered levels in various disease states.

REFERENCES

1. Wiener, A.S., Unger, L.J., Cohen, L., and Feldman, J., Ann. Intern. Med., 44, 221 (1956).
2. Jenkins, W.J., Koster, H.G., Marsh, W.L., and Carter, R.L., Brit. J. Haematol., 11, 480 (1965).
3. Feizi, T., Kabat, E.A., Vicari, G., Anderson, B., and Marsh, W.L., J. Immunol., 106, 1578 (1971).
4. Marsh, W.L., Nichols, M.E., and Allen, F.H.Jr., Vox Sang., 18, 149 (1970).
5. Dzierzkowa-Borodej, W., Seyfried, H., Nichols, M., Reid, M., and Marsh, W.L., Vox Sang., 18, 222 (1970.
6. Cooper, A.G., Nature (London), 227, 508 (1970).
7. Cooper, A.G., and Hobbs, J.R., Brit. J. Haematol., 19, 383 (1970).
8. Pollack, W., Hager, H.J., Reckel, R., Toren, D.A., and Singher, H.O., Transfusion (Philadelphia), 5, 158 (1965).
9. Porath, J., Axen, R., and Ernback, S., Nature (London), 215, 1491 (1967).
10. Cooper, A.G., Clin. Exp. Immunol., 3, 691 (1968).
11. Cooper, A.G., and Worlledge, S.M., Nature (London), 214, 799 (1967).
12. Roelcke, D., Eur. J. Immunol., 19, 206 (1973).
13. Marsh, W.L., Brit. J. Haematol., 7, 200 (1961).
14. Giblett, E.R., and Crookston, M.C., Nature (London), 201, 1138 (1964).
15. Hakomori, S., in "The Dynamics of Cell Membranes," Wallach, D.F.H., and Fischer, H. (eds.), Springer-Verlag, Berlin/Heidelberg/New York, p. 65 (1971).