Possible role of Cold Agglutinins in Pathogenesis of Acquired Autoimmune Hemolytic Anemia

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Abstract
Present study relates to the results of detection of high-titer cold anti I antibodies in sera of 3 patients with anemia. Age of the patients ranged from 15 to 67 (median 30) years. Characterization of immune complexes on sensitized erythrocytes revealed presence of IgM, IgG₁, IgG₃, IgA, C3c and C3d in a patient, suggesting a diagnosis of cold agglutinin disease (CAD). Another case had IgG₁, IgG₃, IgA, C3c and C3d, suggesting a diagnosis of paroxysmal cold hemoglobinuria (PCH). Another patient had C3d alone on their erythrocytes, suggesting dissociation of immunoglobulins from red cell membranes. Findings of this study suggest possible role of cold agglutinins in pathogenesis of autoimmune hemolytic anemia.

Keywords: Heterophil anti I antibody, IgM, IgG.

Introduction
Cold agglutinins are known to develop following certain microbial infections, e.g. mycoplasma pneumonia, infectious mononucleosis and ancylostomiasis due to heterophil antibody formation. Microbial heterophil antibody is polyclonal IgM (κ and λ). Specificity of auto reactive cold agglutinins may be directed against I or i antigens. I antigens are expressed on adult human erythrocytes while i antigens are mainly expressed on cord-blood red cell membranes. CAD is a direct extravascular anti-human immunoglobulin positive hemolysis¹. IgM may activate and fix complement components. Later, monocyte-macrophages may eliminate C3b coated erythrocytes, resulting in mild anemia. Rarely, the cold autoantibody in CAD may be related to Waldenstrom’s macroglobulinemia and IgMκ antibody may be produced. The prevalence of primary CAD was 16 cases per million inhabitants and the incidence rate was 1 per million per year². Conversely, cold agglutinin positive tests are much more frequent in hospital patients as evidenced by anti C serum³. Present study relates to the results of estimation of cold agglutinins in 3 patients with anemia. In addition, we also
characterized immune complexes on surface membranes of patients’ erythrocytes. As it will appear, composition of immune complexes was different in 3 patients.

**Material and Methods**

Age of the patients ranged from 15 to 67 (median 30) years. Male female ratio was 1:2. Venous blood samples were collected in 2 tubes. First sample was collected in a warm (37°C) plain tube and serum was separated. Second sample was collected in cold EDTA tube (+4°C) and red cells were washed in plenty of cold (+4°C) normal saline. Serum sample was doubly diluted in saline. Later, 1% human blood group O RBC (I\(^+\)) were added at +4°C overnight and examined. Cold agglutinin titer was obtained\(^3\). Normal titer was taken as <64 at +4°C as described earlier\(^4\). Pathologic cold agglutinins have titres well over 512.

Patient’s washed RBCs were also used for characterization of immune complexes on sensitized erythrocytes by direct antiglobulin test (DAT). A card of several microtubes having columns of solid phase matrix mixed with anti-human immunoglobulins was used. Later, patient’s sensitized RBCs were added. Tubes were centrifuged in 1D-centrifuge at +4°C for agglutinination\(^4\). Unagglutinated cells pass to the tip of the tube but agglutinates do not pass through the gel. As the antiglobulin serum is present in the microtubes, washing was not required. Later DAT was done to investigate the class of immunoglobulins and complement components on erythrocytes. Kits were purchased from Bio-Rad.

**Results**

Cold agglutinin titers of 3 patients were 128, 256 and >512 (table 1). Results of DAT revealed presence of IgM, IgG1, IgG3, IgA, C3c and C3d on erythrocyte surface in a patient (no.2). The patient was diagnosed as a case of cold agglutinin disease (CAD). Another patient (no.1) had IgG1, IgG3, IgA, C3c and C3d on red cell membranes, possibly due to paroxysmal cold hemoglobinuria (PCH). Another patient (no.3) had C3d and low-titer anti-human Ig on patient’s RBC (table 1). Later finding suggested deagglutination or dissociation of immunoglobulins from patient’s erythrocytes.

**Table 1** : Characterization of immune complexes of cold haemagglutinins by direct antiglobulin test (DAT)

| No. | Patient ID | Age in yrs/gender | Anti I titer | DAT | Anti Hlg DAT | Anti HlgM DAT | Anti HlgG DAT | Anti HlgA DAT | Anti C3c DAT | Anti C3d DAT | Anti H IgG1 DAT | Anti HlgG3 DAT | Interpretation |
|-----|------------|------------------|--------------|-----|--------------|---------------|---------------|---------------|---------------|---------------|----------------|---------------|----------------|
| 1   | 11801190163 | 67 M             | >512         | +   | 30           | -             | +             | +             | +             | +             | 100            | 100           | Paroxysmal cold hemoglobinuria (PCH) |
| 2   | 11712200070 | 15 F             | 128          | +   | 1000         | +             | +             | +             | +             | +             | 100            | 100           | Cold agglutinin disease (CAD) |
| 3   | 11801210121*| 30 F             | 256          | +   | <10          | -             | -             | -             | +             | -             | -              | -              | Cold agglutinin disease (CAD) |

Abbreviations : PCH = paroxysmal cold hemoglobinuria, CAD = Cold agglutinin disease. H Ig = human immunoglobulin, + positive * Last patient had mild anemia (Hb 12.5 g/dl) and mild neutrophilia (neutrophils 9216 cells/mm\(^3\)) suggesting transient cold agglutinin hemolytic anemia.

**Discussion**

Most important feature of this study was the detection of high-titer anti I antibody (>512) in serum of one of the patients. In addition, this patient (no.1) also had IgG1, IgG3, IgA, C3c and C3d on patient’s erythrocytes. These findings suggested a diagnosis of PCH. Similar high-titer cold agglutinins (titer 1024 at 4°C) were reported earlier\(^5,6\). It results from the formation of polyclonal IgG antibody directed against P antigen and may induce antibody-dependent complement-mediated hemolysis. PCH is known to cause acute intravascular hemolysis. It may contribute as 10% of autoimmune cold hemolytic anemias. It may occur either as a primary idiopathic disease or secondary to viral infection. Another case had above immune components along with IgM. Presence of IgM suggested a diagnosis of CAD which is known to cause mild anemia. Our third patient (no.3) was different...
from previous 2 patients. Erythrocytes from this patient had low-titer anti human Ig and C3d, suggesting deagglutination or dissociation of immunoglobulins from patient’s erythrocytes. Anti C3d activity is important for the direct antiglobulin test in the investigation of acquired immune haemolytic anemia. Low-titer cold agglutinins may be present physiologically in normal healthy individuals. However, high-titer narrow-thermal-range cold agglutinins may contribute to anemia. Results of characterization of immune complexes suggested pathological role of cold agglutinins in haemolytic anemia. Retuximab (anti CD20) therapy may decrease the levels of cryoglobulins and may also improve complement levels. Though cryoglobulins are different from cold agglutinins, Rituximab may be useful in the treatment of cold agglutinin-induced haemolytic anemia. In addition, EBV transformed B-cell lines/clones may produce anti-Pr2 cold agglutinins similar to the pathogenic cold auto antibodies in patients’ plasma. Some patients with CAD may have a relative deficit of complement in their sera. An increase in complement production occurs during an acute phase reaction which may enhance haemolysis.

Conclusion
Cold agglutinin test for anti I antibody was positive in 3 patients. In addition, IgM, IgG, IgA and complement components (C3c and C3d) were detected on a patient’s erythrocytes. Another patient had C3d on erythrocytes. These 2 cases were diagnosed to have cold agglutinin disease. Another patient had IgG on erythrocytes, suggesting Paroxysmal cold hemoglobinuria. Results of this study suggested possible role of cold agglutinins in pathogenesis of autoimmune hemolytic anemia. Treatment consisted of keeping the patient warm and use of Rituximab.

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