Detection of Cold Agglutinin with Cross-Reactive Idiotype Produced by Pneumonic Patients

A. Morshedi*[1] and G.A. Smith [2]

1. Immunology Section, Veterinary Medicine, Urmia University, P.O.Box 165, Urmia, Iran
2. Wessex Regional Blood Transfusion Center, Southampton, University Hospital, UK

Summary

To detect the cold agglutinin antibody in patients with *Mycoplasma pneumoniae* an investigation was carried out on the 135 pneumonic patient's serum samples. The result indicated that there were 15 positive cases (11.1%) for *M.pneumoniae* antibody. Of these eleven positive cases (73.3%) for cold agglutinin with the titer of >1:16 showed anti-I specificity. It clearly demonstrated that *M.pneumoniae* was the major etiologic factor in cold agglutinin positive pneumonic cases. Competitive ELISA that reacted with 9G4 anti cross-reactive idiotype performed the frequency of cross-reactive idiotype level in cold agglutinin antibody molecule. As compared to the normal value there was an increase mean value (50.8±24.2) in 15 patient's serum samples. Hemagglutination inhibition test was performed on that 11 serum samples to investigate the relation between the cross- reactive idiotype positive serum and cold agglutinin antibody.

Key words: cold agglutinin, cross-reactive idiotype, antibody, *Mycoplasma pneumoniae*

Introduction

Autoantibodies against red cells are produced in variety of clinical conditions. Pathologic cold agglutinin (CA) antibody, that are specific for I or i carbohydrate antigens present on red cells (anti-I /i), are usually the product of a neoplastic B Cell clone (Roelcke 1989, Duggan & Schather 1986, Crisp & Pruzanski 1982). Although they can be detected in normal individuals and their titer increase during the course of certain viral and Mycoplasmal infections (Roelcke 1989, Chapman *et al* 1993). These CA antibodies tend to be lgM-K and they show preferential agglutination of adult (I) red cells at cold (Roelcke 1993). Such antibodies express cross-reactive idiotype (CRId) determinants, suggestive of common sequence in variable regions of Ig molecules (Williamas *et al* 1968, Feizi *et al* 1997). Similar findings have been

reported for other monoclonal IgM autoantibodies such as rheumatoid factor (Kunkel 1973, Fong *et al* 1988) or anti-DNA antibodies (Shoenfeld *et al* 1983). The monoclonal anti-CRId, 9G4, which recognizes CA antibodies with anti -I or -i specificity, was described (Stevenson *et al* 1989). The CRId was expressed by vast majority of Ig with anti-I/i activity but not by a panel of 74 myeloma proteins (Stevenson *et al* 1986). Serological evidence of anti-I and anti -F1 CA occurred in *M.pneumoniae* infection led to isolation of I/F1 glycoprotein from human red cells.

In this investigation the presence of shared idiotype CA with titer above normal value and their reactivity with 9G4 anti-idiotype by competitive ELISA and hemagglutination inhibition (HI) test were demonstrated.

Materials and Methods

Serum samples from patients with *M.pneumoniae* infection were obtained from Virology Dept., University Hospital, Southampton, UK, for detection of CAs. Normal sera were prepared from healthy blood donors.

Red cells for identification of CA specificity were used. A panel of red cell as untreated, papain-treated and sialidase-treated adult, and cord O cells (Decie & Lewis 1984) received from Wessex Blood Transfusion Center, Southampton, UK, and were kept at 4C.

Monoclonal antibody (MoAb). The production and characterization of 9G4 MoAb (CA-associated anti-idiotypic antibody) at the dose rate of 270mg/ml have been described in details by Champton *et al* (1993). It was the rat anti-idiotype antibody that was obtained from Dr.Stevenson, Tenevous Lab., Southampton, UK.

Hawkins idiotypic IgM. It was a human IgM, known to express the 9G4 epitope that were obtained from Dr. Stevenson.

Cold agglutinin assay. Sera from patients with *M.pneumoniae* were titerated against 2% human red cells of adult and cord group O with I, i and F1 antigenic specificity using routine procedure (Decie & Lewis 1984). Briefly, serial dilutions of sera were made in precipitin tubes and 2% red cells were added in equal volume. After hour incubation at 4C, the degree of agglutination (2+ to 4+) was estimated microscopically using pre-cooled slides. The last dilution of serum that showed agglutination of 2+ or more was considered as CA titer (Table 2). The specificity of CA antibodies i.e. anti-I, and-i and anti-F1 was determined by using of appropriate red cells, such as I, i and F1 red cells according to Roelcke (1989) and Decie & Lewis (1984).

Assay of CRId-positive Igs patients and normal serum. CRId-positive Igs (9G4 idiotypic Ig) was measured in the sera by competitive ELISA and HI assays (Thompson *et al* 1991).

Competitive inhibition ELISA for assay of CRId-Positive Ig. Microplate was coated with 100ml/well of Hawkins IgM, an idiotypic IgM (110 mg/ml), known to express the 9G4 epitope, using 40 ml/22 ml coating buffer (Stevenson *et al* 1986).

The plates were blocked with PBS/1%BSA and washed once with PBS/Tween. Serum sample dilutions were made in duplicate (1/15000-1/30000), 33ml of 9G4 MoAb (containing O.67mg/ml) was added and incubated for 30 min at room temperature before they were placed in the wells of microplate. The sample dilutions were added in the wells in duplicate and incubated for 90 min. Bound 9G4 MoAb was detected using peroxidase conjugated rabbit anti rat IgG, and the reduction in binding of 9G4 MoAb was then measured. The absorbency for each serum was calculated as the mean difference in optical density (OD) between the control wells, lonely with 9G4, and test wells. A known CRId positive IgM was used to used to established a standard curve and the percentage of inhibition in binding 9G4 MoAb by unknown samples were read off this (Stevenson *et al* 1986, Chapman *et al* 1993).

HI assay by 9G4 MoAb. This test which was based on inhibition of red cell agglutination was designed to assess directly the involvement of CRId positive CA in mediating agglutination by the 9G4 MoAb (Thompson *et al* 1991). For HI assay CA-positive sera initially were titerated in a doubling dilution series against a 2% red cells carrying appropriate antigen (Table 2). From this the dilution of each serum sample giving 2x minimum hemagglutination dose (MHD) was determined and aliquot 20ml of each dilution in to nine precipitin tubes. For determination of inhibition by 9G4 MoAb doubling dilutions of 9G4 were made in eight tubes, starting of 135mg/ml and were made in equal volume to test tubes. The tubes were incubated for one hour at 37C. Two volumes of 2% red cells were subsequently added to each test tubes and positive control tube (9th tube) and the tubes were incubated for 90 min at 4C. The last dilution of MoAb giving complete agglutination inhibition was recorded, and the CA antibodies of the serum were considered as CRId positive (Table 2).

Results

From 135 sera of patients with clinical pneumonic signs twenty patients showed positive CA titer (>1:16) and 15 out those (73.3%) were serologically proven to have

M.pneumoniae infection, that was a major cause in CA-positive pneumonic patients (Table 1).

Table 1. *Levels of idiotypic Ig and total Ig in sera from different patient groups*

Patient group	idiotypic Ig[a]		Total serum Ig (mg/dl)			
			IgG	IgA	IgM	
Normal	8.5±6.2	(15)[b]	1350±220	350±100	150±75	(15)
RA[c]	8.5±5.2	(21)	1590±750	350±120	195±150	(9)
SLE[d]	27±17.5	(25)	1260±450	270±50	150±90	(10)
IM[e]	48.4±33.4	(21)	1590±750	350±120	245±120	(20)
M.pneumonia Infection	50.8±24.6	(15)	1200±310	270±100	250±120	(15)

a. Values were measured as percentage of inhibition in the competitive ELISA
b. Numbers in parenthesis indicate serum samples analyzed
c. Rheumatoid Arthritis
d. Systemic Lupus Erythmatosis
e. Infectios Mononucleosis

All of the normal sera showed CA titer<1:8. The results of total amounts of CRId in patients serum with autoimmune RA (Schrohenloher *et al* 1990, Fong *et al* 1988), SLE (Stevenson *et al* 1993) and infectious mononucleosis (Chapman *et al* 1993) accompany to *M.pneumoniae* and normal sera are demonstrated on percentage of inhibition ELISA (Table 2). There is clear increase in mean value CRId (50.8±24.2)

Table 2. *Expression of the 9G4-epitope by CA-positive antibodies due to 11 M.pneumonia positive sera*

Patient	Specificity of CA	CA titer	Expression of the 9G4-epitope by competitive ELISA	Ability of 9G4 anti-idiotype to inhibit the agglutination reaction
PB11	anti-I	32	+(97)*	positive
PC4	anti-I/F1	64	-(18)	negative
PC10	anti-I/i	32	+(54)	negative
PF2	anti-I	64	+(91)	positive
PF3	anti-I	128	+(91)	positive
PF8	anti-I	32	+(50)	negative
QB12	anti-I	64	+(88)	positive
QC11	anti-I	32	+(41)	negative
QD7	anti-I	16	+(83)	positive
QH11	anti-F1	32	-(16)	negative
NB12	anti-I	64	+(81)	positive

*Numbers in paranthesis show % inhibition in competitive ELISA

of *M.pneumoniae* as compared to normal sera (8.5±6.5). Total serum Ig were measured on 15 patient sera and the result showed a significant increase in total IgM on compared with normal sera (Table 2). From 15 *M.pneumoniae* positive sera 11CA positive were found that their titer was between 1:16 to 1:128 (Table 1). Levels of CRId detected in normal sera in unit of percentage of inhibition by ELISA were 8.5±6.5%. The patient's sera showed the percentage of inhibition greater than this mean plus 2SD (>21.5%) were considered to be 9G4 idiotype positive.

In order to investigate association between the serum CRId and CA antibodies the HI assay was conducted on 11 CA positive titre sera (Table 1). Among the 11 CA positive sera nine (82%) were CRId-positive, significantly above the upper limit of normal range ($P<0.02$), and was found that 6 of these CRId-positive CA were also positive in HI assay.

Discussion

Cold agglutination syndrome is characterized by production of antibodies that bind to red cell at 4C, it could, therefore, clearly be considered to be an autoimmune disease. In many clinical cases, the pathologic antibody usually IgM is directed against a simple carbohydrate on the red cell surface (Chapman *et al* 1993). The monoclonal anti-idiotypic 9G4 have been used to identify common idiotypic structures in the variable regions of Ig molecules. The anti-CRId allowed more prices localization of the sequences involved in CRId expression and such reagents have proved useful for survey of presence of the CRId-serum and clonal antibodies from patient with certain infectious diseases that produce CA such as *M.pneumoniae* and infectious mononucleosis, and also for clonal Igs characteistic of B-cell lymphoma (Stevenson *et al* 1986,Stevenson *et al* 1989, Friedman *et al* 1991). Probing for use of the VH4-21 gene has been facilitated by the finding that the CRId recognized by the 9G4 anti-CRId is highly associated with this gene product, it has been reported to be expressed by 12/12 CRId positive Ig that utilize this gene (Silberstein *et al* 1992, Pascual *et al* 1992). Another interesting point is that the idiotype was expressed by autoantibodies that were specific for the I/i carbohydrate antigen on the red cell surface, it appeared that those autoantibodies were all encoded by the VH4-21 gene (Pascual *et al* 1991, Silberstein *et al* 1992). This degree of restriction of a VH gene for a particular antibody was unusual and might indicate a role for this particular Ig heavy chain in reaction with red cell surface; however, the VH4-21 gene was not

confined to anti- red cell antibodies because it has been found also to encode anti-DNA antibodies (Stevenson *et al* 1993).

The CA antibodies may be found in the serum of patients with *M.pneumoniae* infection, although it is not clear whether they arise due to B-cell genetic transformation or as a selective response. Analysis of serum samples from 15 patients with *M.pneumoniae* for the CA-associated idiotype revealed a marked increase as compared to normal. Further evidence for linking CA antibody activity to CRId-expression in serum has been obtained via several observation .In particular, the fact that 9G4 anti-CRId could specifically inhibit hemagglutination mediated by a postinfective serum, provided support for involvement of the CRId in agglutination.

Our results showed that many patients with *M.pneumoniae* developed CRId-positive CA with anti-I specificity against red cells.

It was shown that F1 and I glycoprotein antigens are receptors for *M.pneumoniae* (Hengge *et al* 1992). In present investigation we found only one anti-F1, but anti-I CA in majority of the patients. Anti-I CAs had the CRId with high frequency, whereas anti F1 CA considered CRId-negative. The MoAb 9G4 provides a convenient markers for locating normal counterpart of the autoantibody producing cells, the existence of which has been strongly suggested since CAs are detectable at low levels in normal serum (Feizi *et al* 1973). The fact that CRId/positive B cells are detectable throughout the normal adult lymphoid tissue and are stimulated by common infection might render them more susceptible to neoplastic transformation, and account for the fact that 12% of IgM-secreting B-cell tumors produce CA (Crisp & Pruzanski 1982).

Finally, if it would be shown that the IgM products of some or all of the normal B çells which bear the 9G4 idiotype could bind to red cells. It should then be possible to investigate the nature of these B cells which appears to be physiologically important, and which could in some circumstances give rise to autoimmune disease.

Acknowledgments

The authors wish to thank Dr.F.K. Stevenson for supplying the 9G4 anti-idiotype and Hawkins IgM cold agglutinin.

References

Chapman,C.J., Spellerberg, M.B., Smith, G.A., Carter, S.J., Hamblin,T.J. and Stevenson, F.K.(1993).*Autoanti-red cell antibodies synthesized by patients with*

Infectious Mononucleosis utilize the VH4-21 gene segment. *Journal of Immunology* 151:1051-1061.

Crisp,D., Pruzanski,W.(1982). B-cell neoplasms with homologous cold-reacting antibodies. *American Journal of Medicine* 72:915.

Decie,J.V., Lewis,S.M.(1984). Practical Haematology, (6th edn). Pp:337-357. Churchill Livingstone.

Duggan,D.B., Schather,A.(1986). Unusual manifestations of monoclonal gammopathies. *American Journal of Medicine* 81:864.

Feizi,T., Lecomte, J and Child , R.(1997).An Immunologbulin heavy chain variable region (VH) marker associated with cross-reactive idiotypes in man . *Clinical Experimental Immunology* 30:233 -241.

Feizi,T., Wernet,P., Kunkel,H.G. and Douglas,S.D.(1973).Lymphocyte forming red cell rossets in the cold in patients with chronic cold agglutinin disease. *Blood* 42:753-762.

Fong,S., Chen,P.P., Crowley,J., Silverman, G.J.and Crson,D.A.(1988). Idiotypes and the structural diversity of human rheumatoid factors. Springer Semin. *Immunopathology* 10:189-201.

Friedman,D., Cho,E.A., Goldman,J., Carmack,C.E., Beas,E.C. and Hardy, R.R.(1991). The role of clonal selection in the pathogenesis of an autoreactive human B cell lymphoma. *Journal of Experimental Medicime* 174:525.

Hengge,U.R., Krischfink,M., Konig, A.L., Nicklas,W. and Roelck,D.(1992). Characterization of I/F1 glycoprotein as a receport for *Mycoplasma pneumoniae* .*Infection & Immunity* 60 :79-83.

Kunkel,H.G., Angello,V., Joslin,F.G., Winchaster,R.J. and Capra,J.d.(1973). Cross-idiotypic specificity among monoclonal IgM with antigamma globulin activity. *Journal of Experimental Medicine* 137:331.

Pascual,V., Victor,K., Spellerberg,M., Hambiln,T.J., Stevenson,F.K. and Capra, J.D.(1992). VH restriction among human cold agglutinins : the VH4-21 gene segement is required to encide anti - I and anti-i specificities. *Journal of Immunology* 149:2337.

Pascual,V., Victor,K., Lelsz,D. and Spllerberg,M.B.(1991). Homologous of two IgM cold agglutinins. *Journal of Immunology* 146:4385.

Roelcke, D.(1989). Cold agglutination *Transfusion Medical Rev.* III.2:140.

Schrohenloher,R.E., Accavitti,M.A., Bhown,A.S. and Koopman,W.J.(1990). Monoclonal antibody 6B6.6 defines a cross-reactive Kappa light chain idiotype on

human monclonal and polyclonal rheumatoid factor . *Arthritis Rheumatoid.* 33:187-198.

Shoenfeld,Y., Isenberg,D.A., Rauch, J., Madaio,M.P., Stollar,B.D. and Schwartz, R.S.(1983). Idiotypic Cross-reaction of monoclonal human lupus autountibodies. *Journal of Experimental Medicine* 158:718.

Silberstein, L.E., Jefferies,L.C., Goldman, J., Friedman, D., Moore,J.S., Norwell, P.C., Roelck,D., Pruzanski,W., Roudier,J. and Silverman,G.(1992).Variable region gene analysis of pathologic human autoantibodies to the related I and I red blood cell antigens. *Blood* 78:23372.

Stevenson,F.K., Longhurst,C., Chapman,C.J., Ehrenstein,M., Spellerberg,M.B., Hamblin, T.J., Ravirajan,C.T., Latchman,D. and Isenberg,D.(1993). Utilization of VH4-21 gene segment by Anti-DNA antibodies from patients with systemic Lupus Erythematosus. *Journal of Auto immunity* .6:809-825.

Stevenson, F.K., Smith,G.J., North, J., Hamblin,T.J and Glennie,M.J (1989). Identification of normal B-cell counterparts of neoplastic cells which secrete cold agglutinin of anti-I and anti-i sepecifictity. *British Journal of Haematology* 72:9.

Stevenson,F.k., Wrightham,M., Glennie, M.J., Jones, D.B., Cattan, A.R., Feizi,T. and Hamblin,T.J.(1986). Antibidies to shared idiotypes as agents for analysis and therapy for human B cell tumors. *Blood* 68:430

Thompson,K.M., Suterland,J., Barden,G., Melamed,M.D., Random,I., Natnig,J.B., Pascal,V., Capra,J.D. and Stevenson,F.K.(1991). Human MoAbs against blood group antigens preferentially express a VH4-21 variable region gene-assoeiated epitope. *Scandinavian Journal of Immunology.* 34,509-518.

Wiliamas,R.C., Kunkel,H.G. and Capra,J.D.(1968).Antigenic specificites related to the CA activity of gamma M globulins. *Science* 16:379.