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Characterization of I/Fl Glycoprotein as a Receptor for *Mycoplasma pneumoniae*

U. R. HENGGE,[1]† * M. KIRSCHFINK,[1] A. L. KÖNIG,[1] W. NICKLAS,[2] AND D. ROELCKE[1]

Institute of Immunology, University of Heidelberg,[1] and German Cancer Research Center,[2] Heidelberg, Germany

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Serologic evidence of anti-I and anti-Fl cold agglutinins occurring in mycoplasma infections led to the isolation of I/Fl glycoprotein from human erythrocyte membranes. *Mycoplasma pneumoniae* bound to purified I/Fl glycoprotein in a dose-dependent fashion depending on sialylated carbohydrate determinants. This was shown by the decreased binding of mycoplasmas to either sialidase-treated I/Fl glycoprotein (dot blot analysis) or sialidase-treated erythrocytes (hemagglutination test). Structural properties of the receptor for optimal binding could be explored by hemagglutination inhibition assays. Glycophorins were excluded as receptors. These results indicate that Fl (and I) antigens are receptors for *M. pneumoniae*.

Para- and postinfectious cold agglutinins (CA) are immunoglobulin M (IgM) autoantibodies against erythrocytes (RBC) occurring during or following certain infections. It is well known that certain infectious agents induce CA with distinct specificities (12, 14, 17, 22, 35, 39, 40). The specificity of mycoplasma-induced CA was thought to be exclusively anti-I (7, 8, 21). Following these observations, it was suggested that I antigen (branched poly-*N*-acetyllactosamine chains [Fig. 1]) served as a receptor (6, 23, 24). However, the sialylated I sugar sequence gave optimal binding of *Mycoplasma pneumoniae* (6, 23). The binding of mycoplasmas to the receptor is thought to render the receptor autoimmunogenic and induce CA production against the basic structure (anti-I) and against a related sialylated sugar chain (anti-Fl) (16, 30).

To evaluate the interaction of mycoplasma with host cells carrying I and Fl antigens (e.g., respiratory epithelium, RBC) (1, 2, 13, 15, 20, 25, 38), CA specificities in sera of mycoplasma-infected patients were determined. In addition, binding studies with mycoplasmas and RBC or mycoplasmas and purified I/Fl glycoprotein were performed.

MATERIALS AND METHODS

RBC. Adult RBC (O Rh^+) and newborn RBC (O Rh^+) from adenine-citrate-dextrose-anticoagulated blood were each pooled from several donors and stored at 4°C. O_h (Bombay type) RBC with high I-antigen expression were kindly provided by E. Gallasch, Göttingen, Germany; these RBC were stored in liquid nitrogen and thawed in glycerol (in sequence, 12, 8, 4, and 2% glycerol). Type i-adult RBC were obtained from Immucor (Norcross).

Enzymatic treatment of RBC. Protease (papain) and sialidase treatments were performed by the methods in reference 33. Endo-β-galactosidase treatment was carried out as described elsewhere (18, 19).

Mycoplasmas. *M. pneumoniae* FH/PH was grown in a modified bouillon described by Tully and Razin (42). Growth was initiated with 1 ml of sterile mycoplasma suspension (10^8 CFU/ml). Incubation was carried out for 5 to 6 days at 37°C. Then bouillon was centrifuged for 30 min at 15,000 × *g* (Sorvall RB-5B; Du Pont Instruments), and the pellet was washed three times in sterile 0.9% saline. The suspension was controlled for sterility and adjusted to 10^8 CFU/ml (= 100 μg of protein per ml). Finally, the mycoplasma suspension was aliquoted and stored at −80°C.

Patient sera. Two hundred twenty-three serum samples containing CA of anti-I specificity (titers ranging from 32 to 32,000) were subjected to absorption-elution experiments, using sialidase (RDE)-treated RBC to test for additional CA of anti-Fl specificity. Clinical information was obtained for 178 patients by reviewing patients' records, when available, or by sending questionnaires to the treating physicians. Moreover, 22 serum samples from patients with an acute mycoplasma infection were kindly provided by H. Hof, Institute of Hygiene and Microbiology at the Klinikum Mannheim, University of Heidelberg, and were tested for anti-I and anti-Fl CA.

Characterization of CA. CA specificities were determined by agglutination studies, using untreated, papain-treated, and sialidase (RDE)-treated adult and newborn RBC (31, 32).

Serologic test for acute mycoplasma infection. Sera were screened for IgM antibodies to *M. pneumoniae* by an indirect immunofluorescence test (Zeus). False-positive results due to rheumatoid factor (RF) were excluded by preabsorption of sera with RF absorbens (no. OUCG 14/15; Behring, Marburg, Germany).

Hemagglutination test. A 2% suspension, 50 μl of native or enzyme-treated RBC, was incubated with 50 μl of serial twofold dilutions of serum for 1 h at 0°C for cold agglutination. For hemagglutination by mycoplasmas, 50 μl of a 2% suspension of native or enzyme-treated RBC was incubated with 50 μl of mycoplasma suspension at 37°C. After a short centrifugation, strength of hemagglutination was quantitated as titers and as hemagglutination scores by the method of Race and Sanger (13a, 29). The hemagglutination score was obtained by adding the score of each agglutinate of the serial twofold dilutions. A high hemagglutination score represents strong agglutination of mycoplasmas by RBC or by CA.

Hemagglutination inhibition test. Serial twofold dilutions, 50 μl, of substances studied for inhibition were incubated in U-bottom microtiter plates (no. 650101; Greiner, Nürtingen,

* Corresponding author.

† Present address: Universitätsklinikum Essen, Klinik und Poliklinik für Dermatologie und Venerologie, Allergologie, Hufelandstr. 55, 4300 Essen 1, Germany.

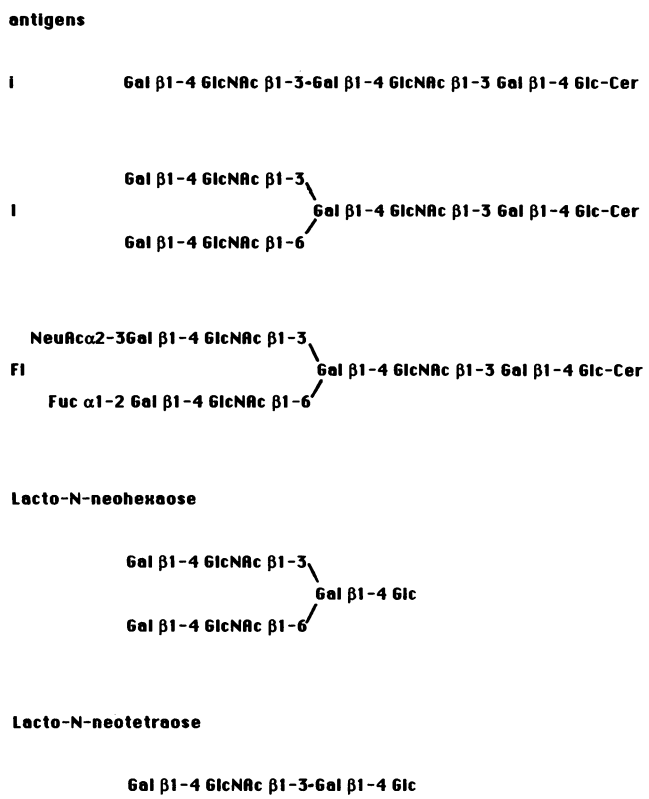

FIG. 1. Structures of polylactosamine-containing determinants important for the binding of *M. pneumoniae*. Gal, galactose; GlcNAc, *N*-acetylglucosamine; Glc, glucose; Cer, ceramide.

Germany) with 50 μl of mycoplasma suspension. After incubation for 30 min at 37°C, 50 μl of 2% RBC was added. Results were read after sedimentation of the agglutinates. The following substances were tested for inhibition: lacto-*N*-neohexaose, lacto-*N*-neotetraose, glycophorin A (Bio Carb, Stolberg, Germany), sialolactose (Boehringer, Mannheim, Germany), lactose (Serva, Heidelberg, Germany), *N*-acetylneuramic acid (Boehringer), D-glucosamine, *N*-acetylglucosamine (Serva), albumin, and α_1-acid glycoprotein (Sigma Chemical Co., St. Louis, Mo.).

Preparation of Fab fragments of CA. Fab fragments of CA were prepared by the methods in references 27 and 36; they were affinity purified by binding to RBC at 0°C and eluted with phosphate-buffered saline (PBS) at 37°C.

Isolation of I/Fl glycoprotein. I/Fl active glycoprotein was extracted from membranes of protease-treated RBC by a modified phenol-saline extraction in accordance with reference 5. The isolated glycoprotein was subjected to anion exchange (MonoQ) on fast protein liquid chromatography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the isolated glycoprotein was carried out by the method of reference 19. Staining by the method of reference 41 revealed two bands with molecular weights of 72,500 and 75,000, both reactive with anti-Fl and anti-I in Western immunoblot analysis (41) at 0°C.

Enzyme treatment of I/Fl glycoprotein. A 200-μl portion of sialidase (Behring) in 0.8 ml of PBS was added to 100 μg of I/Fl glycoprotein and incubated for 30 min at 37°C. The reaction was terminated by the addition of enzyme inhibitors (50 mM ascorbate, 50 mM EDTA) and substrate (25 mM *N*-acetylneuramic acid).

TABLE 1. RBC-mycoplasma hemagglutination[a]

RBC	*M. pneumoniae* agglutination		Anti-I CA titer	Anti-Fl CA titer
	Titer	Score		
Untreated	8	31	5,000	1,000
Protease treated	32	45	10,000	1,000
Bombay type (O_h)	32	16	32	10,000
Sialidase treated	4	24	20,000	0
Endo-β-galactosidase treated	4	16	4	1,000
i-adult	2	14	512	16
Newborn	4	23	512	64

[a] RBC containing different I/Fl determinants (shown as titers with anti-I and anti-Fl) were agglutinated by the addition of a mycoplasma suspension. The agglutination is expressed as titer and as a hemagglutination score. The experiments were run in triplicate seven times. Titers are shown as geometric means; hemagglutination scores are mean values. Hemagglutination scores of untreated, protease-treated, endo-β-galactosidase-treated, and Bombay-type RBC were statistically significant.

Endo-β-galactosidase treatment of 100 μg of I/Fl glycoprotein in 4 ml of PBS was carried out with 100 mU of enzyme (Boehringer) in 1 ml of NaCl-$NaCH_3COO$ buffer (70 mM NaCl, 100 mM $NaCH_3COO$). The pH was adjusted to 5.8 with 0.1 N HCl. After incubation (2 h at 37°C), 100 μl each of 1 mM Cu^{2+} and Fe^{2+} in solution were added to stop enzymatic digestion.

Dot blot analysis. Two milliliters of mycoplasma suspension was added to serial twofold dilutions of I/Fl glycoprotein immobilized on nitrocellulose (45 μm; Schleicher & Schüll, Dassel, Germany). After a washing, bound mycoplasmas were detected by anti-mycoplasma (IgM) (E. Jacobs, Institute of Microbiology, Freiburg, Germany) and peroxidase-conjugated anti-IgM antibodies (Dianova, Hamburg, Germany).

RESULTS

CA specificities in infections by *M. pneumoniae*. In 43 (19%) of 223 anti-I-containing serum samples, additional anti-Fl antibodies could be detected; 180 serum samples contained anti-I alone. Some 51% of patients with both anti-I and anti-Fl CA suffered from an acute mycoplasma infection compared with about 13% of patients with only anti-I, as published earlier (18). Anti-Fl titers ranged from 1 to 64 (mean, 8), and anti-I titers ranged from 1 to 512 (mean, 32).

We have extended the study group by including 22 patients with an acute mycoplasma infection. Nine (40%) of these 22 patients' sera contained both anti-Fl and anti-I CA. Another 45% of these sera contained only anti-I. These results suggest that CA arise in about 85% of acute mycoplasma infections and frequently appear as a combination of anti-I and anti-Fl CA.

Hemagglutination between RBC and *M. pneumoniae*. Binding of *M. pneumoniae* to RBC was tested by hemagglutination assays. To evaluate the importance of I and Fl antigens, different types of RBC (Bombay and i-adult) and enzyme (protease, sialidase, and endo-β-galactosidase)-treated RBC were used.

As shown in Table 1, there is an obvious correlation between I/Fl antigen expression on RBC and hemagglutination scores with one major exception. Sialidase-treated RBC were only slightly agglutinated by *M. pneumoniae* despite their increased I-antigen expression. Endo-β-galactosidase treatment of RBC strongly reduced I determinants, whereas

TABLE 2. RBC-mycoplasma hemagglutination inhibition[a]

Inhibitory substance	MIC (μg/ml) after inhibition	
	Mycoplasma	Anti-I CA
Untreated I/Fl glycoprotein	0.19	5.9
Sialidase-treated I/Fl glycoprotein	1.5	3.2
Endo-β-galactosidase-treated I/Fl glycoprotein	24.8	750.0
Lacto-*N*-neohexaose	50.0	
Lacto-*N*-neotetraose	202.0	
Glycophorin A	9,000	
Sialolactose	225.0	
Lactose	450.0	
N-Acetylneuramic acid	450.0	

[a] Mycoplasmas were preincubated with different substances and tested for inhibition of RBC-mycoplasma hemagglutination. The results of 10 experiments are shown. MICs of untreated I/Fl and sialidase-treated I/Fl glycoproteins are statistically significant ($P = 0.01$).

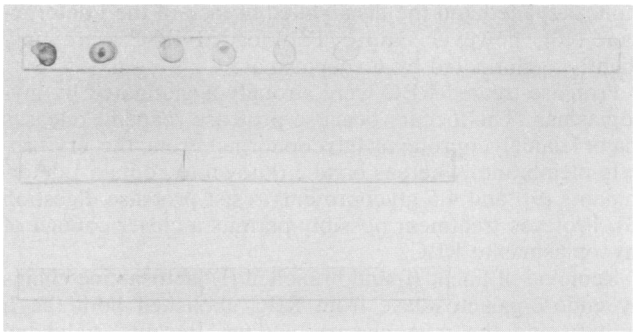

FIG. 2. Binding of mycoplasma to I/Fl glycoprotein. Mycoplasmas which bound to I/Fl glycoprotein (top) and to sialidase-treated I/Fl glycoprotein (middle) were detected by anti-mycoplasma antibodies. The I/Fl glycoprotein concentration was 29.4 μg on the first dot and was diluted twofold at each step. As negative controls for binding, glycophorin A and α_1-acid glycoprotein were used. Without mycoplasmas the reaction was negative for binding (bottom).

Fl determinants were not affected. This might be due to reduced accessibility of Fl determinants on its carriers in the RBC membrane for the enzyme. RBC of the Bombay type (O_h) had a low Fl antigen titer but were strongly agglutinated by mycoplasmas. RBC of the Bombay type do not have fucosylated lactosamine chains and therefore lack remarkable Fl antigenicity (fucosylated and sialylated I determinants). *M. pneumoniae*, however, does not require fucosylation as a condition for binding. This finding clearly indicates that sialylated sugar sequences of the I-antigen type optimize binding of mycoplasmas.

Hemagglutination inhibition. Different substances were tested for inhibition of hemagglutination between mycoplasmas and untreated RBC. The strongest inhibition occurred with untreated I/Fl glycoprotein (Table 2). Desialylated I/Fl glycoprotein inhibited the agglutination of RBC by mycoplasmas eightfold less than native glycoprotein, although the I-antigen expression increased. On the other hand, endo-β-galactosidase treatment, which removes polylactosamine chains by splitting internal β-galactosidic linkages, abolished the inhibition of mycoplasma binding to RBC as well as the ability to inhibit cold agglutination by anti-I CA. D-Glucosamine, *N*-acetylglucosamine, and α_1-acid glycoprotein did not show any inhibition at a concentration of 9 mg/ml. Inhibition should occur if I (and Fl) antigens on RBC are blocked by fragments of anti-I CA. Therefore, Fab fragments of CA were required because RBC-mycoplasma agglutinates could not be distinguished from cold-agglutinated RBC when native (pentameric IgM) anti-I CA were used. The hemagglutination score decreased from 31 to 7 ($P = 0.01$) when RBC were preincubated with anti-I (Fab) prior to the addition of mycoplasmas. To rule out a possible steric hindrance of the binding sites by adjacent anti-I (Fab), control experiments were carried out with anti-D (IgG), anti-glycophorin A (IgG), anti-A (IgG), and anti-Duffy[a]. None of the controls influenced the hemagglutination score.

Binding of mycoplasmas to I/Fl glycoprotein. To prove direct binding of *M. pneumoniae* to the putative receptor molecule I/Fl glycoprotein, dot blot experiments were performed. I/Fl glycoprotein was immobilized on nitrocellulose and was overlaid with a suspension of mycoplasmas. In comparison, sialidase-treated I/Fl glycoprotein was used as receptor. As negative controls for binding, glycophorin A and α_1-acid glycoprotein were used. After incubation, binding of mycoplasmas to purified I/Fl glycoprotein could be demonstrated in a dose-dependent fashion (Fig. 2).

DISCUSSION

For 20 years it has been well known that CA of anti-I specificity may occur during mycoplasma infections (7, 21). Because of the detection of CA against related sialylated sugar chains by Roelcke and coworkers (16, 30), we reinvestigated 223 anti-I-containing serum samples for the presence of additional anti-Fl. We found coexisting anti-I and anti-Fl CA in the sera of patients suffering from infections with mycoplasmas as reported previously (18). These results were confirmed by serologic data of patients with fresh mycoplasma infections, presented in this paper. Our serological results support the hypothesis that sialylated sugar sequences of the I-antigen type, called Fl, are receptor molecules for mycoplasmas (18).

Specific interaction between mycoplasmas and human host cells (e.g., respiratory epithelium, fibroblasts, and RBC) depends on sialoglycoproteins and gangliosides (4, 10, 11, 23, 24, 26). Glycophorin A has been proposed as a receptor for mycoplasmas (1, 4). Glycophorin A is indeed highly sialylated, but lacks any I/Fl activity, in accordance with the absence of poly-*N*-acetyllactosamine sequences (data not shown). We were able to demonstrate that preincubation of RBC with anti-glycophorin A antibodies had no effect on mycoplasma-induced hemagglutination. We first demonstrated that sialylated sugar sequences of the I-antigen type (Fl) serve as receptors for *M. pneumoniae* by hemagglutination studies with RBC and mycoplasmas and by direct binding of isolated I/Fl glycoprotein with mycoplasmas (dot blot analysis).

Sialidase treatment of the receptor molecules revealed a reduction in binding of mycoplasmas to I/Fl glycoprotein (dot blot analysis) and to RBC (hemagglutination test), despite their increased I-antigen expression. Therefore, the conclusion was drawn that the (nonsialylated) I antigen is of minor importance as a receptor for mycoplasma binding.

To support the importance of Fl determinants as receptors for mycoplasmas, binding experiments with anti-Fl (Fab)-loaded RBC should be performed, but we did not have enough monoclonal antibody of the very rare Fl type to prepare Fab fragments. Since anti-I may also react with the

monosialylated and the disialylated branch of the I determinant (16), however, anti-I (Fab)-loaded RBC were only slightly agglutinated by mycoplasmas.

Protease-treated RBC were strongly agglutinated by mycoplasmas. This occurs because protease (papain) releases major sialoglycoproteins (glycophorins) from the erythrocyte membrane, whereas band 3 (known to contain I determinants [9]) and 4.5 glycoproteins resist protease digestion (3). Protease treatment possibly permits a closer contact of mycoplasmas to RBC.

Removal of linear (i) and branched (I) lactosamine chains by endo-β-galactosidase from RBC abolished both the Ii activity and the mycoplasma binding. Because sialylated branched lactosamine chains, i.e., Fl determinants, are predominantly responsible for binding of *M. pneumoniae*, Fl antigen expression on RBC should follow mycoplasma binding with RBC. There are two exceptions. (i) Fl determinants were not released from the RBC membrane by endo-β-galactosidase. This might be caused by hampered accessibility of the Fl determinant in the RBC membrane (34). (ii) Fl antigen was weakly expressed on O_h RBC, which bound mycoplasmas strongly. It has been shown that the Fl determinant is a biantennary structure requiring sialylation of one and fucosylation of the other branch (16). Since O_h cells are not fucosylated, Fl antigens are highly decreased on O_h RBC. In contrast, the binding of *M. pneumoniae*, requiring only sialylation, is not affected in O_h cells.

In contrast to results of other investigators (24), who found that *M. pneumoniae* sheet cultures bound equally well to linear (RBC of i-antigen type) and branched (RBC of I-antigen type) poly-*N*-lactosamine chains, our hemagglutination studies with RBC of different I expression (low, i-adult; high, Bombay type) clearly demonstrate a preferential binding of mycoplasmas to branched rather than linear lactosamine chains. The significance of the repetitive lactosamine chains beyond the branched I structure was well recognized by the weak inhibition of RBC-mycoplasma hemagglutination by lactoneohexaose (the branch of the I antigen without repetitive lactosamine units) or by sialolactose.

These results prove that optimal binding of *M. pneumoniae* requires sialylated and branched sugar chains of the poly-*N*-lactosamine type. We identified the I/Fl active glycoprotein as a receptor for *M. pneumoniae*.

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