Inhibition of Cold Agglutinins (Anti-I) by *M. pneumoniae* Antigens[1] (36167)

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Cold agglutinins (CAI) with specificity to the I-erythrocyte antigen are found in the sera of approximately half of patients with *M. pneumoniae* (MP) infections (1). Although this association in man has conclusively been demonstrated, considerable controversy exists as to the immunochemical relationship between the etiologic agent (MP), human erythrocyte antigens and CAI synthesis. Various hypotheses have been offered to explain the emergence of CAI in patients with MP infections: CAI may be produced in response to I-antigen enzymatically cleaved by MP from the erythrocyte surface (2, 3) or the interaction between the I-antigen with MP leads to the formation of an immunogenic complex which triggers the synthesis of CAI (4).

We have previously reported that rabbits and several strains of mice challenged intravenously with MP, *Listeria monocytogenes* or *Streptococcus MG* synthesize CAI (5). The CAI produced by rabbits react not only with the I-erythrocyte antigen but also with the immunogen responsible for their production (5). On the other hand, CAI from sera of patients with MP infections do not react with MP (6).

We now present evidence that the antigen responsible for synthesis of CAI in experimental animals is a component of the lipopolysaccharide fraction of the microorganism and is hidden and unavailable, in the intact microorganism, for reaction with CAI.

Materials and Methods. Preparation of antigens. MP (Pl-1428) was grown in broth according to the method of Somerson *et al.* (7). The microcolonies adhering to glass were harvested after 6 days, washed in phosphate buffered 0.15 *M* NaCl (pH 7.3) and the final suspension adjusted to a turbidity corresponding to tube No. 2 of the McFarland nephelometric scale. For these experiments MP was killed by freezing and thawing prior to injection.

Streptococcus MG (American Type Culture Collection, *Streptococcus* species 9895) (MG) was grown in brain heart infusion (Difco) for 16 hr at 37°. The culture was centrifuged at 12,000*g* for 10 min and the sediment washed thrice and resuspended in 0.15 *M* NaCl. The microorganisms were killed by incubation at 70° for 1 hr.

L. monocytogens serotype 4b. (LM) was prepared as previously described (8). The suspensions of MG and heat killed LM were adjusted to contain 5×10^9 microorganisms per ml.

Lipopolysaccharide fractions of MP, LM or MG were prepared by a modification of the method of Ribi and Milner (9). Briefly, to 1 volume of MP, LM or MG suspensions was added 1 volume of phenol. The mixture was vigorously shaken and incubated at 68° for 60 min. The mixture was cooled to 4°, centrifuged and the aqueous phase was collected. The sediment was again extracted with saline and the supernate added to the aqueous phase previously collected. Cold acetone (10–15 volumes) was added to the pooled supernates and incubated at −20° overnight. The precipitate contained the crude lipopolysaccharide (LP).

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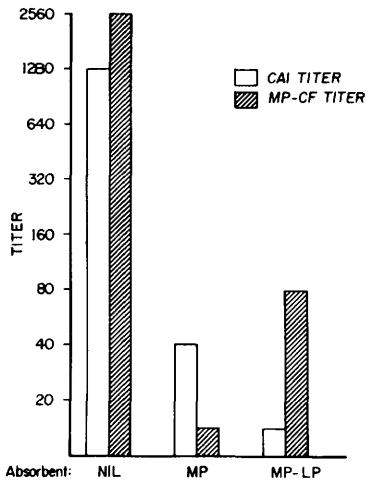

FIG. 1. Typical *M. pneumoniae* complement fixing (MP–CF) and cold agglutinin (CAI) titers before (NIL) and after absorption of serum of rabbits immunized with *M. pneumoniae*; (MP) absorption with *M. pneumoniae*; (MP–LP) absorption with *M. pneumoniae* lipopolysaccharide.

Immunization and serologic methods. Volumes of 2.5 ml suspensions of MP, LM or MG were separately injected intravenously into groups of 6 albino rabbits. Each rabbit was bled from the retroorbital venous sinus before immunization and periodically thereafter. The serum of these animals was tested for the presence of CAI as previously described (8).

Serum containing CAI was obtained from 12 patients with MP infections, 5 patients with chronic cold agglutinin disease and 1 with macroglobulinemia of Waldenstrom and cold agglutinin hemolytic anemia.

CAI and bacterial antibody titrations were done as previously described (8). Complement fixing antibodies to MP were measured by the standard Kolmer ⅕ method (10). The MP antigen used for complement fixation was prepared as described by Somerson *et al.* (7). Antibody inhibition studies were done as previously reported (5) using sera obtained at the peak of antibody response (days 5–7) (8).

Results. Six rabbits immunized with MP antigen produced, in addition to complement fixing antibodies (mean $\log_2$ titer 9.8) to this microorganism, high titers of CAI (mean $\log_2$ titer 9.2). Incubation of sera from these animals with intact MP resulted in greater than 95% reduction of both complement fixing antibody and CAI. Figure 1 shows typical results of absorption of rabbit serum with these antigens. Similar reduction in titer was observed when sera of rabbits immunized with MP were incubated with MP–LP (Fig. 1).

Sera from 6 rabbits immunized with LM contained CAI (mean $\log_2$ titer 9.0) and agglutinating antibodies to LM (mean $\log_2$ titer 9.6). These antibodies were inhibited equally by LM and LM–LP resulting in a greater than 97% reduction of titer (Fig. 2). CAI (mean $\log_2$ titer 8.8) and MG agglutinating antibodies (mean $\log_2$ titer 8.6) synthesized by 6 rabbits injected with MG were neutralized (greater than 90%) by incubation with both MC and MG–LP (Fig. 3).

In contrast to the finding that intact MP, LM and MG or their LP inactivated CAI produced in rabbits in response to these microorganisms, the titer of CAI synthesized by 12 patients with MP infections was not significantly affected by incubation with MP (2-fold or less reduction of titer) (Fig. 4).

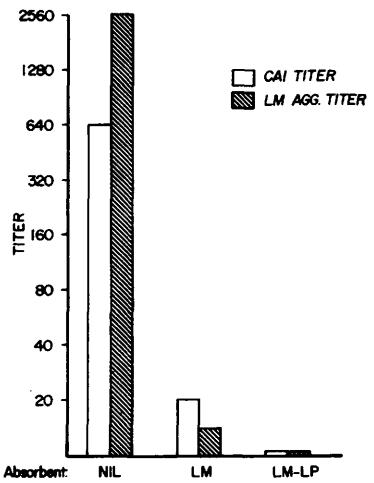

FIG. 2. Typical *L. monocytogenes* agglutinin (LM AGG.) and cold agglutinin (CAI) titers before (NIL) and after absorption of serum of rabbits immunized with *L. monocytogenes;* (LM) absorption with *L. monocytogenes;* (LM–LP) absorption with *L. monocytogenes* lipopolysaccharide.

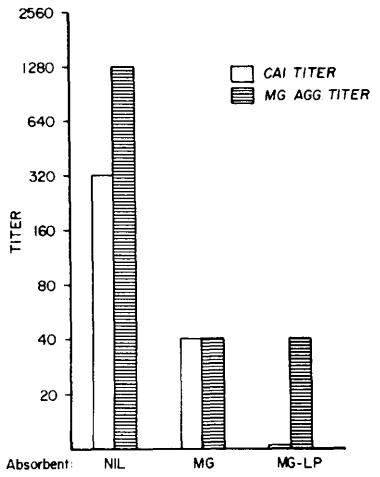

FIG. 3. Typical *Streptococcus MG* agglutinin (MG AGG.) and cold agglutinin (CAI) titers before (NIL) and after absorption of serum of rabbits immunized with *Streptococcus MG*; (MG) absorption with *Streptococcus MG*; (MG–LP) absorption with *Streptococcus MG* lipopolysaccharide.

On the other hand, a marked reduction of CAI (greater than 6-fold) was observed following incubation of these 12 sera with MP–LP (Figs. 4 and 5). The titer of complement fixing antibody to MP was reduced to a lesser degree (4-fold) following incubation

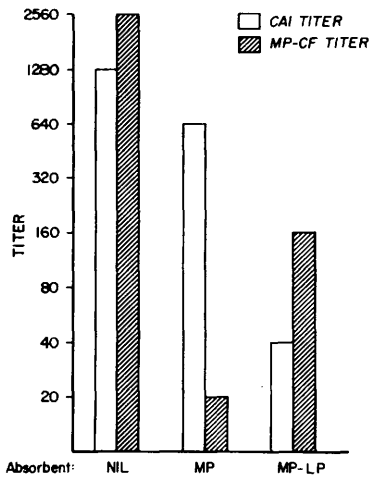

FIG. 4. Typical *M. pneumoniae* complement fixing (MP–CF) and cold agglutinin (CAI) titers before (NIL) and after absorption of serum from patients with *M. pneumoniae* infection; (MP) absorption with *M. pneumoniae*; (MP–LP) absorption with *M. pneumoniae* lipopolysaccharide.

with MP–LP (Fig. 4). CAI from patients with chronic cold agglutinin disease and macroglobulinemia of Waldenstrom were not affected by incubation with MP or MP–LP (Fig. 5).

Discussion. We have presented evidence that CAI produced in man infected with MP can be inhibited by a crude lipopolysaccharide fraction isolated from this microorganism. The observation that CAI from patients with malignant lymphoproliferative disorders were not inhibited by this lipopolysaccharide indicates that the reaction between CAI synthesized during MP infections and MP–LP is specific. The results of these

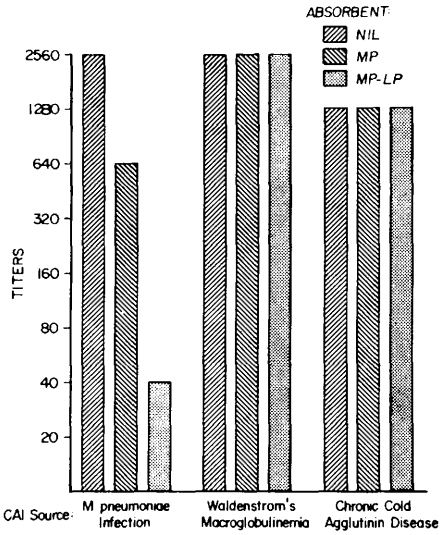

FIG. 5. Typical cold agglutinin (CAI) titers before (NIL) and after absorption of serum of patients having cold agglutinins; (MP) absorption with *M. pneumoniae*; (MP–LP) absorption with *M. pneumoniae* lipopolysaccharide.

experiments support our hypothesis (5) that CAI produced in man and experimental animals are cross-reacting antibodies and that their synthesis does not require the formation of neoantigens (4) or cleavage of the I-antigen from the red cell membrane (2, 3).

Razin *et al.* conclusively demonstrated that the determinant haptens of MP were associated predominantly with its glycolipids (11). The crude lipopolysaccharide we have utilized in our absorption studies probably con-

tained such glycolipids since it inhibited to some extent the complement fixing ability of sera containing antibodies to MP.

While MP inactivates CAI of rabbits immunized with this microorganism intact MP does not appreciably affect the titer of CAI of human origin (6). This observation and the results of our experiments suggest that CAI of human origin are directed against antigenic structures hidden in the limiting membrane of MP.

The emergence in man and experimental animals of CAI appears to be the consequence of immunization with cross-reacting antigens carried by a variety of microorganisms (12). Whether or not this mechanism is operative in other autoimmune phenomena remains to be determined.

Summary. CAI produced by rabbits challenged with *M. pneumoniae* (MP), *Streptococcus MG* (MG) or *L. monocytogenes* (LM) were inactivated by absorption with those microorganisms or a lipopolysaccharide (LP) fraction thereof. CAI from sera of patients with MP infections were not inactivated by the intact microorganism, but by the LP fraction of MP. CAI from patients with malignant lymphoproliferative disorders was not absorbed by either. An antigenic component of MP is responsible for CAI in MP infections.

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