Evaluation of Micro Complement Fixation Tests for Antibodies Against Group A Streptococcal M and M-Associated Antigens in Rabbit and Human Sera

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Variables involved in micro complement fixation (CF) tests for type-specific (TSM) and non-type-specific (NTSM) antibodies against preparations of streptococcal M protein were studied. Sera of rabbits immunized with purified M protein which contained high titers of anti-TSM and low titers of anti-NTSM antibodies reacted type specifically in the CF tests when relatively low concentrations of M protein were employed. In contrast to these artificially induced TSM antibody responses, the sera of rheumatic fever patients demonstrated elevated CF antibody titers (1:80 to 1:320) in the presence of both high and low concentrations of M antigen. The CF test cannot distinguish TSM and NTSM antibodies when the latter predominate. The CF test can be employed with confidence for the detection of NTSM antibodies, provided that the test sera are known to lack type-specific antibodies against the M protein used in the test.

The importance of the type-specific M (TSM) antigen in virulence of, and immunity against, group A streptococci has been established. Various methods have been used to measure the antibody response to this antigen in man and in animals after natural infections or artificial immunization (8, 9, 14). The only in vitro method, however, that has been widely accepted as capable of consistently providing evidence for the presence of type-specific protective antibody has been the opsonophagocytic test (8, 9, 11). All of the other in vitro methods except the long-chain test (10) employ M protein extracts in various states of purification and are hampered by cross-reactions to non-type-specific M-associated antigens (NTSM) (1, 2a, 3, 5, 12, 13).

Wittner and Fox (14), however, devised a sensitive micro complement fixation (CF) test to measure type-specific M antibody. They suggested that under special conditions such CF tests might replace the more cumbersome opsonic test in measurements of the immune response to M protein vaccines.

In contrast, Widdowson et al. (13) recently described a similar CF test for measuring non-type-specific antibodies directed against a unique M-associated protein which they designated MAP. Concomitantly, we found that antibodies detected by such CF tests in sera of rheumatic fever patients were directed against at least two NTSM antigens present in the partially purified M protein preparations (2a). The issue of whether or not such NTSM antibodies play a role in the pathogenesis of post-streptococcal sequelae via their toxic properties has not been resolved (2a, 4, 13). Therefore, we studied the variables involved in the CF test which must distinguish such potentially nontype-specific toxic antibodies from those that are type specific and involved in protective immunity.

MATERIALS AND METHODS

M protein was prepared from types 12, 24, and 30 group A streptococci by extraction with either HCl at pH 2.0 or hydroxylamine at pH 10.0 and was purified as previously described (1, 2, 6). Antisera to whole streptococci were produced by immunizing rabbits by the method of Lancefield (7). Antisera also were prepared by immunization with single 1-mg doses of purified M protein emulsified in complete Freund adjuvant. Rabbits were injected in five or six intracutaneous and subcutaneous sites with a total of 1 ml of the emulsion. The latter rabbits were bled at 2-week intervals after immunization. The source of the sera from patients with rheumatic fever and the methods for performing CF and opsonophagocytic tests also have been described in detail previously (2a, 3). Complement-fixation titers were recorded as the reciprocal of the highest dilution of serum that fixed complement (50% hemolysis) in the presence of antigen.
RESULTS

When serial dilutions of a 1.0-mg/ml solution of type 30 M protein were tested against twofold dilutions of homologous unabsorbed rabbit antiserum against whole group A streptococci in the CF test, several antibody titer levels were observed (Fig. 1). These results indicated the presence of more than one antigen-antibody system (2a). The peak titer (1:640) obtained at antigen concentrations of 4.0 to 6.0 μg/ml was seen only with the homologous M30 and not with the heterologous M12 preparation. Moreover, at such low concentrations of antigen, M30 failed to react in any detectable titer in the presence of rabbit antiserum against heterologous serotypes (not shown in figure). High CF titers (1:160 to 1:320) in the range of 40 to 100 μg of antigen per ml, on the other hand, were observed both in the homologous (M30-anti-M30) and the heterologous (M12-anti-M30) systems (Fig. 1). The peak titers obtained at the lower antigen dose range, therefore, represent TSM-anti-TSM reactions, whereas those at higher dose ranges represent NTSM-anti-NTSM reactions.

The effect of antigen concentration on the specificity of the CF test was further confirmed by using sera from rabbits immunized with partially purified M protein rather than whole streptococci (Table 1). At the lower concentration of each antigen, high CF titers (1:80 to 1:160) were obtained only with the homologous type antiserum. In contrast, low non-type-specific titers in the range of 1:10 to 1:40 were obtained when the dose of each antigen was increased 5- to 10-fold. At even higher (antigen excess) or lower (antibody excess) antigen concentrations, the CF titers were less than 10 in all cases (not shown in Table 1).

In studies of rheumatic fever sera, M protein derived from the rare type 30 group A streptococcus was used to avoid possible complement fixation by TSM-anti-TSM reactions. The absence of type-specific M30 antibodies in these sera was further ascertained by negative opsonization (bactericidal) tests against type 30 streptococci by the methods previously described (2a). All four of the rheumatic fever sera showed peak CF titers at relatively high doses of antigen (Table 2). At lower dose ranges, these titers fell gradually, but only one serum showed a greater than one tube fall (1:80 to 1:10). In contrast, the homologous M30 rabbit antiserum showed a one tube rise (from 1:320 to a peak titer of 1:640) with a corresponding decrease in antigen dose (Table 2).

Opsonic antibodies appeared in the second week after immunization of rabbits with M24 protein (Table 3). These type-specific opsonic antibodies were used in a complement-fixation test to determine the presence of low and high concentrations of M protein test antigens.

Table 1. Complement-fixing antibody titers in the presence of low and high concentrations of M protein test antigens

| Rabbit antiserum | Low doses a of: | High doses a of: |
|-----------------|----------------|-----------------|
|                 | M12 | M24 | M30 | M12 | M24 | M30 |
| Anti-M12        | 80  | <10 | <10 | 20  | <10 | <10 |
| Anti-M24        | 10  | 160 | 10  | 20  | 40  | 40  |
| Anti-M30        | 10  | <10 | 160 | 20  | 20  | 40  |

  a Low doses of test antigen: M12 and M30, 0.78 μg/ml; M24, 1.56 μg/ml.
  b High doses of test antigen: M12 and M24, 12.5 μg/ml; M30, 6.25 μg/ml.

Table 2. CF antibody titers of rheumatic fever sera in the presence of various concentrations of M protein

| Rheumatic fever serum | Conc of type 30 M protein (μg/ml) |
|-----------------------|----------------------------------|
|                       | 0.2 | 4-16 | 31-125 | 250-1,000 |
| No. 1                 | <10 | 160  | 320    | <10        |
| No. 2                 | 30  | 80   | 160    | <10        |
| No. 3                 | ND  | <10  | 80     | <10        |
| No. 4                 | ND  | 40   | 80     | <10        |
| Normal human serum    | <10 | <10  | 30     | <10        |
| (pool)                |     |      |        |            |
| Rabbit anti-M30 p     | 10  | 640  | 320    | 20         |

  p ND, Not done.
  p Rabbit immunized with whole type 30 streptococci.

Fig. 1. Complement-fixing antibody titers of type 30 whole streptococcal antiserum against various concentrations of either homologous M30 or heterologous M12 antigen preparations.
antibodies increased to titers of 1:16 during the following 2 weeks. At that time type-specific CF titers of 1:40, using low antigen concentration, were observed. By the eighth week, however, the opsonic antibodies fell fourfold, whereas CF antibodies rose fourfold to a titer of 1:160 with a homologous antigen; that with the heterologous antigen rose to 1:40. Thus, the rise in CF titer against the homologous antigen was due to TSM-anti-TSM as well as NTSM-anti-NTSM reactions.

**DISCUSSION**

Several important conclusions can be made concerning the utility of the complement-fixation test for M antibody in clinical serological studies. First, the use of the CF test as a substitute for the more cumbersome type-specific opsonophagocytic tests must take into account which antibodies (NTSM versus TSM) predominate in the sera to be tested regardless of the antigen concentrations that are employed. Our data indicate that the source of the serum to be tested is an important determinant of which antibody (anti-TSM or anti-NTSM) predominates. Sera from rabbits immunized with purified M protein contained high titers of TSM antibodies. As might be expected, the CF tests showed a high degree of type specificity in the presence of optimally low concentrations of M protein. These results are in agreement with those of Wittner and Fox (14), who measured type-specific CF antibodies in sera from humans immunized with purified M protein. Although the amount of NTSM antigen in the purified M protein is sufficient to produce positive reactions with non-type-specific antibodies, it apparently is not sufficient to produce a significant immune response in the vaccinated rabbits or humans.

In contrast to these artificially induced TSM antibody responses, rheumatic fever patients tend to develop high titers of NTSM antibodies as detected by the CF test (2a, 13). In the present study, we observed elevated CF antibody titers in the presence of both high and low concentrations of test antigen. Low antigen concentrations which are optimal for the detection of TSM antibody therefore cannot discriminate NTSM antibodies when the latter predominate.

The second conclusion that can be made concerning the utility of the complement-fixation test for M antibody is that the CF test can be used confidently for detection of NTSM antibodies, provided that higher antigen concentrations are employed and the test sera are known to lack type-specific antibodies against the M protein used in the test. In the case of immune rabbit sera, TSM reactions can be excluded simply by using antigen derived from any M type that is heterologous with respect to the immunizing serotype. In human sera, M30 can be used as antigen because infections with this serotype are extremely rare. Alternatively, as described in the present study, type-specific opsonization (bactericidal) tests of the human sera insure that type-specific M antibody is not involved in the CF test.

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**Table 3. Comparison of type-specific opsonic and complement-fixing antibody responses of rabbit immunized with type 24 M protein**

| Weeks after immunization | Antibody titers against homologous (M24) antigen | Antibody titers against heterologous (M30) antigen |
|--------------------------|-----------------------------------------------|-----------------------------------------------|
|                          | Complement fixation* | Opsonization | Complement fixation* | Opsonization |
| Preinjection             | <10            | <1           | <10            | <1           |
| 2                        | 20             | 1            | 10             | <1           |
| 4                        | 40             | 16           | 20             | <1           |
| 6                        | 80             | 16           | 20             | <1           |
| 8                        | 160            | 4            | 40             | <1           |

*Antigen concentration: M24 1.56 μg/ml; M30 12.5 μg/ml.
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