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# COMPETITION BETWEEN, AND EFFECTIVENESS OF, IgG AND IgM ANTIBODIES IN INDIRECT FLUORESCENT ANTIBODY AND OTHER TESTS

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By using an indirect fluorescent antibody (IFA) procedure, we were recently able to demonstrate that a significant portion of natural antibody reactivity to Gram-negative bacteria resided in the IgG class as well as in the IgM class of antibodies (1). The magnitude of this IgG activity had apparently eluded detection in previous studies that used bactericidal, agglutination and other tests (2, 3). This suggested that the relatively greater sensitivity of these tests for IgM antibodies (4, 5) may have caused the substantial contribution of IgG antibodies to be overlooked. In order to examine this hypothesis, the reactivities of antibodies in purified 7S and 19S fractions of serum were studied in bactericidal, agglutination and IFA assays. The sensitivity to IgG antibodies of the IFA procedure and the relative insensitivity to IgG antibodies of bactericidal and agglutination tests were confirmed.

In the course of these studies it was observed that IgM antibodies in some whole sera had less IFA reactivity at low dilutions than was present at higher dilutions. It was found that this IgM prozone effect in the IFA assay could be explained by a competitive inhibition exerted by IgG antibodies. It was further found that high concentrations of IgG antibodies partially inhibited the reactivity of IgM antibodies in the bactericidal reaction. These findings are also presented in this article.

## MATERIALS AND METHODS

*Bacteria.* Virulent T1 *Neisseria gonorrhoeae* (6) of strain F62 was kindly supplied by Dr. D. S. Kellogg, Jr. Bacteria were grown on agar plates made from Bacto-G C Medium Base[2] (Difco) plus a defined supplement (7). Bacteria were cultivated in candle jars at 36° to 37°C for 16 to 18 hr before use. Cells were heated for 2 hr at 121°C (somatic antigens), or were treated with formalin (3%) for 10 min (surface antigens) as described (8), or used live.

*Serum fractions.* Whole adult or cord sera, or human Cohn fractions II or III-1 (Hyland), were fractionated into 19S and 7S peaks (9) by gel filtration through Sephadex G-200 (Pharmacia) as described (10). A mixture of 0.1 M Tris-1.0 M NaCl, pH 8.0, buffer was used for elution. The purity of the 19S and 7S fractions was confirmed in studies utilizing an analytical ultracentrifuge (Spinco model E) and standard methods, kindly performed by Dr. Edward B. Wylie and Mr. O. Max Kroeger of the Communicable Disease Center. Sera and fractions were diluted in phosphate buffered saline (PBS), pH 7.2, (Bacto Hemagglutination Buffer, Difco) before use.

*Quantitation of immunoglobulins.* The concencentrations of immunoglobulins G and M were measured in a semiquantitative fashion on Immunoplates (Hyland), using a radial diffusion precipitin technique similar to that described by Fahey and McKelvey (11).

*Indirect fluorescent antibody (IFA) procedure.* The IFA procedure using fluorescein-conjugated goat antisera specific for human IgG (IFA-IgG) and IgM (IFA-IgM) (Hyland) was performed as described (8). The specificity of the fluorescent antibody reagents in our test system was confirmed by specific IFA staining of purified antibody fractions, by cross-absorption with such fractions, and by comparison with reference antisera (10). We are indebted to Dr. J. Fahey, Dr. J. Vaughan and Drs. R. Williams, Jr. and F.

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[2] Trade names are used for identification only and do not represent an endorsement by the Public Health Service or the United States Department of Health, Education, and Welfare.

Wollheim for supplying reference antisera. Fluorescein-conjugated antiserum specific for human complement (C′3) was kindly supplied by Dr. C. G. Cochrane.

*Bactericidal test.* In order to circumvent the anti-complementary effects of purified whole 19S and 7S fractions, bactericidal tests of purified fractions were performed in the following manner: A 0.1-ml suspension containing approximately $2 \times 10^6$ living bacteria in PBS was incubated for 20 min at 37°C with 0.1 ml of an appropriate serum fraction or combination of fractions. Bacteria were then separated from the fraction by sedimentation at 10,000 rpm for 2 min and washed once in 0.5 ml of fresh PBS. The bacteria were resuspended in 0.1 ml PSB, and 0.15 ml of fresh guinea pig serum (diluted 1:10) was added as a source of complement. After further incubation at 37°C for 30 min the suspension was diluted $10^{-1}$ and $10^{-2}$ in a broth made according to the formula for G C Medium Base, minus agar. A volume of 0.05 ml of each dilution was spread over the surface of an agar plate. The colonies were counted after 18 hr incubation at 37°C in candle jars and the per cent killing was computed. Controls consisted of tubes containing guinea pig serum plus PBS, and G C broth plus PBS.

Bactericidal tests of whole sera were performed by adding 0.1 ml of diluted test serum to 0.3 ml of guinea pig serum (1:10) and 0.1 ml PBS containing approximately $2 \times 10^6$ bacteria. After incubation for 60 min at 37°C, the suspensions were diluted and plated as described above. The maximum dilution of serum or fraction producing at least 50% killing was considered to be the bactericidal titer.

*Agglutination test.* Whole sera and fractions were tested with heat-killed (121°C for 2 hr) *N. gonorrhoeae* in a microagglutination test (Microtiter). A mixture of 0.025 ml of serum or fraction was serially diluted in PBS, and 0.025 ml of $2 \times 10^9$/ml bacteria were added to each dilution. After incubation at 50°C for 2 hr and overnight at room temperature, the maximum dilution producing obvious agglutination was considered to be the agglutination titer.

## RESULTS

*Assay of 7S and 19S antibody reactivities by agglutination, bactericidal and IFA procedures.* Purified 19S fractions of human sera were found to have antibody activity to *N. gonorrhoeae* which was readily demonstrable by agglutination, bactericidal and IFA-IgM reactions. Antibodies to *N. gonorrhoeae* in purified 7S fractions of human sera, in contrast, were demonstrable by the IFA-IgG procedure, but not by agglutination or bactericidal tests. Figure 1 illustrates the results of testing the 19S and 7S fractions of the serum of a normal adult (P.E.M.). The elution volumes comprising the 19S and 7S protein peaks were pooled. The protein concentration prior to dilution of the 19S fraction was approximately 16 mg/ml and that of the 7S fraction was 24 mg/ml. These fractions were reacted with heat-killed *N. gonorrhoeae* in the agglutination and IFA tests, and with living *N. gonorrhoeae* in the bactericidal test. It can be seen that the 19S fraction demonstrated a positive agglutination reaction at a titer of 1:40. The bactericidal and IgM-IFA tests were both positive at a titer of 1:80. The 7S fraction of P.E.M. was without detectable bactericidal or agglutinating activity at a 1:5 dilution. The IgG-IFA activity of this 7S fraction, on the other ahdn, was positive at a titer of 1:160.

Table I compares the amounts of purified 19S and 7S fractions of P.E.M. needed to produce positive reactions against *N. gonorrhoeae* in IFA, bactericidal and agglutination tests. It was found that the IFA-IgM, bactericidal and agglutination reactions were almost equally sensitive to antibodies in the 19S fraction reactive with *N. gonorrhoeae*. However, the IFA-IgG procedure was at least 20 to 80 times more sensitive to antibodies in the 7S fraction than were agglutination and bactericidal tests. These findings indicated that the IFA procedure was at least as sensitive an assay of IgG as it was of IgM antibody activity, while bactericidal and agglutination reactions were relatively insensitive to IgG antibodies.

In order to minimize possible artifacts due to the fractionation procedure, whole cord serum was studied by bactericidal, agglutination and IFA procedures. Since the fetus receives only maternal IgG antibodies in quantity, compared to adult serum, cord serum is naturally deficient in IgM antibodies (12). Table II illustrates the bactericidal, agglutination and IFA reactivities of whole pooled cord serum compared with the same reactivities found in an adult serum. Also included in Table II are the serum concentrations of IgG and IgM antibodies. A deficiency of bactericidal and agglutinating power of the cord serum was found to correlate with the relative

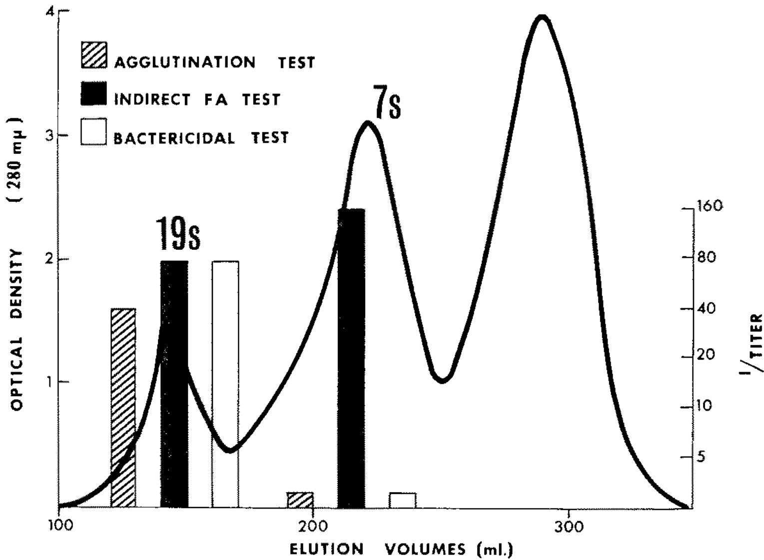


*Figure 1.* Antibodies to *Neisseria gonorrhoeae* in the 19S and 7S fractions of a normal adult human serum (P.E.M.) detected by several procedures. The serum was fractionated by gel filtration through Sephadex G-200 and the resulting 19S and 7S fractions were assayed for antibody reactivity with *N. gonorrhoeae* by agglutination, bactericidal, and indirect fluorescent antibody (IFA) procedures. Note that agglutination and bactericidal tests were relatively insensitive to 7S antibodies.

TABLE I

*Quantities of purified 19S and 7S human serum fractions producing positive reactions in indirect fluorescent antibody (IFA), bactericidal, and agglutination tests*

| Test Procedure[a] | 19S | 7S |
|---|---|---|
| | *mg* | *mg* |
| IFA | 0.008 | 0.006 |
| Bactericidal | 0.020 | >0.480 |
| Agglutination | 0.010 | >0.120 |

[a] Approximately 0.04 ml of fraction was used in the IFA, 0.1 ml in the bactericidal, and 0.025 ml in the agglutination procedures. The 19S and 7S fractions were obtained by gel filtration of the whole serum of a normal adult (P.E.M.).

deficiency of cord IgM antibody. The IgG concentration of the cord serum and its IgG-IFA titer were approximately equal to those of the adult serum. The antibody activities in cord serum, therefore, paralleled the results found in testing purified 7S fractions (Fig. 1).

*Complement binding by 7S and 19S fractions.* The bactericidal reaction of normal serum antibodies against gram-negative bacteria depends on the binding of complement (13). The poor bactericidal power of IgG antibodies in 7S fractions might therefore be due to a deficiency in the binding of complement to the bacterial surface by these antibodies. In order to study this possi-

TABLE II

*Agglutination, bactericidal, and indirect fluorescent antibody (IFA) activities of human adult and cord sera against* Neisseria gonorrhoeae

| Serum | Immunoglobin Concentration[a] | | Antibody Reactivity (1/Titer)[b] | | | |
|---|---|---|---|---|---|---|
| | IgG | IgM | Agglutination | Bactericidal | IFA | |
| | | | | | IgG | IgM |
| | *mg/100 ml* | | | | | |
| Adult | 1500 | 200 | 32 | 40 | 64 | 32 |
| Pooled Cord[c] | 1500 | 24 | <2 | <2 | 64 | <2 |

[a] Immunoglobulin concentration of normal adult (I.R.C.) and pooled cord sera was estimated on Immunoplates (Hyland) using a radial diffusion precipitin method similar to that employed by Fahey and McKelvey (11).

[b] See Materials and Methods.

[c] A cord serum pool was obtained by combining aliquots of cord sera from 10 apparently normal newborn babies.

TABLE III

*Binding of complement (C'3) to Neisseria gonorrhoeae by 7S and 19S fractions of normal human serum and Cohn fractions*

| *N. gonorrhoeae* Cells Incubated with:[a] | | IFA Reactivity[b] | | |
|---|---|---|---|---|
| Fraction | Complement | C'3 | IgG | IgM |
| 19S | ABS[c] | 3+ | | |
| | ABS-heated | 1+ | | |
| | None | 1+ | | 3+ |
| 7S | ABS | 3+ | | |
| | ABS-heated | ± | | |
| | None | 1+ | 4+ | |
| Phosphate buffered Saline only (control) | ABS | ± | – | – |
| | ABS-heated | ± | – | – |

[a] *N. gonorrhoeae* (formalin-fixed) was incubated first with phosphate buffered saline or 19S fraction (3.5 mg protein/ml) isolated by Sephadex G-200 from Cohn fraction III-1 or 7S fraction (6.8 protein/ml) from Cohn fraction II of normal human serum (Hyland). The bacteria were then incubated with a fresh human serum which served as a source of complement. This human serum had been absorbed in the cold with *N. gonorrhoeae* (ABS).

[b] The binding to the bacteria of antibodies (IgG, IgM) or complement (C'3) was detected by specific fluorescent antibody reagents. Where no score is reported the reaction was not studied.

[c] "ABS" indicates that the fresh human sera had been pre-absorbed in the cold with *N. gonorrhoeae*. Control portions of the complement source were heated (ABS-heated) at 56°C for 30 min.

bility, human 7S or 19S fractions were incubated with formalin-treated *N. gonorrhoeae* on glass slides. The sensitized bacteria were then incubated with fresh human serum as a source of complement. This serum had been preabsorbed in the cold with *N. gonorrhoeae*. The binding of complement was detected by use of a fluorescent antiserum to human complement (C'3). It was found (Table III) that complement (C'3) was bound to *N. gonorrhoeae* cells by both 7S and 19S fractions of pooled normal human serum. Since the 7S and and 19S fractions contained an unknown number of antibody molecules it was not possible to compare the complement-binding ability of IgG to that of IgM in a quantitative manner.

*Competitive inhibition of IgM by IgG antibodies in the IFA test.* An IgM prozone was observed in testing a number of whole sera. An example of this phenomenon is illustrated in Table IV. It was found occasionally that the IFA activity of IgM, but not IgG, increased as whole sera were made more dilute. This was usually noted in sera which had high titers of IgG reactivity. Purified 19S fractions did not show this effect, even when the 19S fractions were concentrated. This suggested that the presence of a high concentration of IgG antibodies might inhibit the reactivity of of IgM antibodies. In order to study this possibility, aliquots containing an increasing concentration of a purified 7S serum fraction were mixed with aliquots containing a constant amount of a 19S serum fraction and the IgM and IgG reactivities of the mixtures were tested in the IFA procedure. Table V illustrates these results. It was found that the IFA-IgM reactivity of the 19S fraction was inhibited by the presence of antibodies in the 7S fraction. An increase in IFA-IgG

TABLE IV

*The IgM prozone of whole human serum in the indirect fluorescent antibody (IFA) procedure*

| Specificity of Fluorescent Antibody Reagent | IFA Reactivity with *Neisseria gonorrhoeae*[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Serum dilution | | | | | | |
| | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 |
| IgM | 1+ | 3+ | 4+ | 3+ | 2+ | 1+ | – |
| IgG | 4+ | 4+ | 3+ | 3+ | 3+ | 2+ | ± |

[a] The whole serum of a patient with gonococcal arthritis was diluted in phosphate buffered saline and each dilution was tested in the IFA procedure against *N. gonorrhoeae* (formalin-fixed) using fluorescein-conjugated reagents specific for IgG or IgM.

reactivity was correlated with a decrease in the IFA-IgM reactivity of the 19S fraction, even though the concentration of 19S antibodies was not decreased.

*Competitive inhibition of 19S bactericidal activity by 7S antibodies.* In view of the finding that IgG antibodies appeared to inhibit IgM reactivity in the IFA procedure, it was decided to study the effect of 7S antibodies on the agglutinating and bactericidal activity of the 19S fraction. Although, as shown in Figure 1, the 7S fraction of serum had relatively little intrinsic agglutinating or bactericidal activity against Gram-negative bacteria, the positive IFA-IgG results indicated that IgG antibodies in the 7S fraction did bind to the bacterial surface. IgG antibodies, therefore, might compete with IgM antibodies for antigenic sites and inhibit the reactivity of the 19S fraction in these tests, too.

It was not possible to demonstrate this inhibitory effect in agglutination tests. The prior incubation of heat-killed *N. gonorrhoeae* cells with 7S fractions had no demonstrable effect on the subsequent agglutination caused by the addition of 19S fractions.

Inhibition of 19S reactivity by 7S antibodies was noted, however, in the bactericidal reaction. Table VI illustrates the results of addition of the 7S fraction to the 19S fraction of P.E.M. serum (see Fig. 1). Although the 7S fraction had no bactericidal power by itself, it partially inhibited the killing effect of the 19S fraction. The addition of the 7S fraction, shown to contain IgG activity by the IFA test, reduced the killing power of the 19S fraction at least threefold; 6 to 8% survival was increased to 25% survival. The inhibiting effect of the 7S fraction was removed by absorbing the 7S fraction with the test bacteria. This suggested that IgG antibodies (7S) competed with IgM antibodies (19S) in the bactericidal as well as in the IFA reaction.

TABLE V

*The effect of an increasing concentration of human IgG antibodies on the reactivity of IgM antibodies in an indirect fluorescent antibody (IFA) system*

| Specificity of Fluorescent Antibody Reagent | IFA Reactivity with *Neisseria gonorrhoeae* | | | |
|---|---|---|---|---|
| | Concentration of 7S stock solution in an 0.1-ml aliquot which was added to an 0.1-ml aliquot of a stock solution of 19S fraction[a] | | | |
| | 0% | 4 0% | 100% | 100% (7S-ABS)[b] |
| IgM | 2+ | 1+ | − | 2+ |
| IgG | ± | 3+ | 4+ | − |

[a] To 0.1-ml aliquots of a 19S fraction (3.5 mg protein/ml) were added 0.1-ml aliquots containing phosphate buffered saline or increasing concentrations of a 7S fraction (6.8 mg/ml). These 19S and 7S fractions were derived from Cohn fractions III-1 and II, respectively, of pooled normal human serum. The mixtures of the 19S and 7S fractions were then tested against *N. gonorrhoeae* (heat-killed) in the IFA procedure.

[b] The 7S fraction absorbed with heat-killed *N. gonorrhoeae* (10% by volume) for 60 min at 37°C. The absorbed 7S fraction (7S-ABS) was then added to an equal volume of the 19S fraction and tested.

TABLE VI

*The inhibition of the 19S-mediated bactericidal reaction by 7S antibodies*

| Type of Human Antibody Preparation[a] | Viable Bacteria in Bactericidal Test |
|---|---|
| | % |
| 19S | 6 |
| 7S | 100 |
| 19S + 7S | 25 |
| 19S + 7S-ABS | 8 |
| Broth control | 100 |

[a] "19S" indicates that the 19S fraction (16 mg protein/ml) of a normal adult human serum (P.E.M.) was diluted 1:80 in phosphate buffered saline, and "19S + 7S" indicates that the 19S fraction was diluted 1:80 in a 1:5 dilution of the 7S fraction (24 mg protein/ml) of this same serum.

"19S + 7S-ABS" indicates that the 19S fraction was diluted 1:80 in a 1:5 dilution of P.E.M. 7S fraction which had itself been absorbed (60 min, 37°C) with living *Neisseria gonorrhoeae* (10% by volume).

"7S" indicates that the 7S fraction was diluted 1:5 in phosphate buffered saline.

## DISCUSSION

The sensitivity of indirect fluorescent antibody (IFA) procedures in the detection of IgG antibodies (1) and the relative insensitivity of agglutination and bactericidal tests for IgG antibodies reactive with *N. gonorrhoeae* were confirmed by assaying purified 7S and 19S fractions in these reactions. Approximately 0.006 mg of a purified 7S fraction produced a positive reaction for IgG antibody in the IFA procedure, while 20 to 80 times this amount of 7S fraction was inactive in agglutination and bactericidal tests (Table I).

Whole cord serum, which was deficient in IgM antibody but not in IgG antibody concentrations, was also deficient in bactericidal, agglutination and IFA-IgM tests. The IFA-IgG titer of cord serum, on the other hand, was comparable to the IFA-IgG titer in adult serum.

The IFa test results indicated that IgG antibodies were bound to the bacterial surface but apparently were not in great enough concentration to produce agglutination or bactericidal reactions. Robbins, Kenny and Suter (4) found that purified rabbit IgM antibody eluted from *Salmonella typhimurium* was 22 times more active/mole of antibody than purified IgG antibody in producing agglutination, and 120 times more potent in the bactericidal reaction. Since we did not study eluted specific antibodies but whole fractions, we could not compare the activities of human IgG and IgM antibodies to *N. gonorrhoeae* in these tests on a molar basis. Our results, however, are consistent with those of Robbins and associates.

The factors responsible for the relative insensitivity to IgG antibodies of agglutination and bactericidal reactions were not identified in the present study. Borsos and Rapp (14) have shown that 19S antibodies fix complement much more efficiently than 7S antibodies. The greater sensitivity to IgM of the complement-dependent bactericidal test could be explained on this basis. However, by the use of fluorescent antiserum to human C′3, we were able to show (Table III) that 7S antibodies, as well as 19S antibodies, bound complement to the bacterial surface. Our fluorescent antibody assay could not quantitate or reflect the dynamics of complement-fixation, and these factors might be more important than the static binding of complement observed in the IFA reaction. Perhaps natural IgG and IgM antibodies combine with different gonococcal antigens (15) and those with which IgG antibodies react may not be as sensitive to the lytic activity of complement. The greater size of IgM antibody molecules may enhance both the agglutination and bactericidal reactions. The bias in favor of IgM inherent in these tests has thus tended to obscure the presence of natural IgG antibodies reactive with Gram-negative bacteria (1).

The detection of antibodies in the IFA procedure depends upon the avidity or stability of the antibody-antigen combination. Antibodies which have high dissociation constants would therefore be at a disadvantage in producing positive IFA reactions. It has been shown (4) that IgG antibodies are more avid and maintain a more stable combination with Gram-negative bacterial antigens than do IgM antibodies. This might explain the inhibition of IFA-IgM reactivity in the presence of high concentrations of IgG antibodies.

The inhibition of the bactericidal activity of 19S fractions by 7S fractions might also be explained by a more avid combination of 7S antibodies with the bacterial surface. IgG antibodies binding to the bacterium may be poorly bactericidal in themselves, but at the same time hinder the access to the cell of reactive IgM antibodies. Perhaps the two-step bactericidal procedure which we used in the testing of purified fractions hindered the reactivity of IgM antibodies and exaggerated the inhibitory effects of IgG antibodies. It is also possible that the observed inhibition was due to other unknown factors and did not result from antibody interactions. It may be that techniques which yield antibodies purified by absorption and subsequent elution from cell surfaces might select only antibodies which do not compete with one another. Therefore, such methods may not reveal a competitive interaction of IgG and IgM antibodies.

It appears that IgM and IgG antibodies are not equally capable of producing positive reactions in certain antibody test systems. The bias inherent in each method, as well as possible interactions between antibodies themselves, must be considered in interpreting antibody test results. This is important both in clinical diagnostic serology and in basic studies of immune systems. For example, Freeman and Stavitsky (16) have recently suggested that concepts regarding the temporal sequence of IgM-IgG antibody production should be reexamined in the light of conflicting results obtained using different test systems.

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## SUMMARY

Natural human 19S (IgM) and 7S (IgG) antibodies reactive with *Neisseria gonorrhoeae* were assayed by agglutination, bactericidal and indirect fluorescent antibody (IFA) procedures. Fluorescent antibody reagents specific for IgG and IgM were used in the IFA procedure. It was

found that purified 19S serum fractions contained antibodies which were readily detected by agglutination, bactericidal and IFA techniques using an anti-IgM reagent. However, bactericidal and agglutination tests appeared to be relatively insensitive to natural IgG antibodies in purified 7S fractions. In contrast, the indirect fluorescent antibody technique using an anti-IgG reagent could detect high titers of IgG antibodies within the 7S fractions.

A prozone phenomenon was observed in the IFA reactivity of IgM antibodies in whole serum. It was found that the addition of purified 7S antibodies to purified 19S antibody fractions decreased the ability of the 19S fraction to react with the anti-IgM reagent. This suggested that IgG antibodies inhibited IgM antibody reactivity in the IFA procedure. A partial inhibition of 19S bactericidal activity by 7S fractions was also found.

This study indicates that the unequal capacity of different antibody classes to produce reactions, and the interactions between antibodies themselves in various test systems, must be taken into account in the interpretation of the results of immunologic tests.

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