

HOMOGENEOUS RABBIT 7S ANTI-IgG WITH ANTIBODY SPECIFICITY FOR PEPTIDOGLYCAN*

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(Received for publication 6 July 1973)

Numerous studies have demonstrated that chronic bacterial infections in man and hyperimmunization of animals lead to the occurrence of anti-IgGs in serum (1-3). Rabbits immunized with streptococci produce both 19S and 7S anti-IgGs (4), which have specificity for the Fc piece of IgG. The immunological process which leads to the occurrence of these anti-IgGs under pathological and experimental conditions has long been the source of considerable speculation. One possibility is that anti-IgGs are antibodies to unidentified bacterial antigens which cross-react with Fc, but this notion has received little direct support. The studies reported here are concerned with IgG monoclonal components in rabbit antisera which react with IgG as well as bacterial cell wall peptidoglycan.

Peptidoglycan is a component of the bacterial cell wall composed of hexosamine polymers with pentapeptide side chains which are cross-linked through additional peptide chains (5). Previous studies have shown that peptidoglycans of the gram-positive cocci are immunogenic. Although both pentapeptide and the hexosamine polymer of the peptidoglycan are antigenic determinants, the former is the predominant one (6, 7). Furthermore, a recent report has shown that the C-terminal D-Ala-D-Ala is the immunodominant antigenic site of the pentapeptide (8). Immunologic cross-reactions which have been observed between the peptidoglycans of streptococci and staphylococci are due to the similarities of the peptide moieties of the two peptidoglycans (9).

Materials and Methods

Preparation of Streptococcal Vaccines, Immunization of Rabbits, Quantitative Precipitin Analysis for Antibodies to Group-Specific Carbohydrate, and Electrophoretic Methods.—These procedures have been previously described (4, 10, 11).

Cell Walls.—Cell walls of Groups A and C streptococci were prepared and purified by the described method (12).

* Supported by a National Institutes of Health Grant AI 08429 and in part by a Grant-in-Aid from the American Heart Association.

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Preparation of Peptidoglycan.—The peptidoglycan of *Staphylococcus epidermidis* and not that of streptococci was used in these studies, because the former has a larger percentage of non-cross-linked pentapeptide (13). For this reason it is more reactive as an antigen with the peptidoglycan antibodies in the streptococcal antisera. The peptidoglycans of both streptococci and staphylococci contain the same pentapeptide. The cell walls of *S. epidermidis* strain 24 were extracted with 10% trichloroacetic acid at 60°C for 8 h. The residue was termed peptidoglycan, and was solubilized by ultrasonic treatment (8).

Measurement of Anti-peptidoglycan Antibody.—The anti-peptidoglycan antibody was measured with a precipitin test employing peptidoglycan solubilized by ultrasonic treatment. To a series of tubes containing 0.1 ml of a 1:10 dilution of the antiserum were added increasing amounts of peptidoglycan of *S. epidermidis*. The precipitates were processed in a fashion similar to that used to perform the quantitative precipitin test for the group-specific carbohydrate (10). The amount of anti-peptidoglycan antibody in the precipitates was calculated by subtracting the amount of peptidoglycan from the amount of protein in the precipitate.

Peptides.—(D-Ala)₃ and (L-Ala)₃ were purchased from Cyclo Chemical Co., Los Angeles, Calif. The synthesized pentapeptides L-Ala-D-Glu-γ-L-Lys-D-Ala-D-Ala and Tyr-(Ala)₄-Tyr were kindly supplied by Dr. K.-H. Schleifer, Botanisches Institut der Universität, München.

Measurement of IgG and 7S Anti-IgG.—These methods were previously described in detail (4). The radial diffusion technique described by Mancini et al. (14) was used for the measurement of IgG. The 7S anti-IgG was quantitated with a coprecipitation assay which is based on the ability of 7S anti-IgG to coprecipitate with antigen-antibody complexes.

Isolation and Characterization of 7S Anti-IgG.—7S anti-IgGs were isolated on immuno-adsorbent columns and analyzed on alkaline urea polyacrylamide gels after reduction and alkylation according to described methods (15).

Radioactive Labeling of 7S Anti-IgG.—Isolated 7S anti-IgG was labeled with ¹²⁵I according to the method of McFarlane (16).

Inhibition of Coprecipitation Assay.—The inhibition of the coprecipitation assay to measure 7S anti-IgG was employed in studies concerning the specificity of the antibody-binding site of 7S anti-IgG. In these experiments (a) the inhibitor, (b) 60 μg of anti-Group A antibody, and (c) 10 μg of ¹²⁵I 7S anti-IgG were incubated for 2 h at 37°C. Subsequently, 2 μg of Group A streptococcal carbohydrate were added and the reaction volume was adjusted with phosphate-buffered saline (PBS) to 0.1 ml. After incubation overnight at 4°C, the tubes were centrifuged in a Beckman 152 microfuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), and 0.05 ml was removed with an Eppendorf pipette (Brinkmann Instruments, Inc., Westbury, N. Y.) and placed into another tube. Radioactivity was measured in both tubes in a γ-counter and the amount of precipitate was calculated by subtracting the counts in the second tube from those of the tube in which the reaction had been performed. The amount of radioactivity in the precipitate was expressed in percent of total amount added to the reaction mixture. Without addition of inhibitors, 25–35% of the radioactivity was found in the precipitate. This amount of coprecipitation was taken as 100% value in the inhibition experiments.

Inhibition of the Anti-Idiotypic Reaction.—Anti-idiotypic antibodies to 7S anti-IgG R3387 raised in guinea pigs were characterized in a previous report (15). The anti-idiotypic antiserum used in these studies maximally precipitated 65% of ¹²⁵I 7S anti-IgG R3387. For the inhibition assay, a reaction mixture with excess antigen was chosen. In these experiments the inhibitor was mixed with 50 μl of 1:5 diluted antiserum and incubated for 2 h at 37°C. Subsequently, 20 μg ¹²⁵I 7S anti-IgG was added, and the reaction volume was adjusted to 0.2 ml with PBS. Tubes were incubated overnight at 4°C. The zone of excess ¹²⁵I anti-IgG was selected to minimize the amounts of inhibitor required. This also increased the sensitivity of the assay. Under these conditions, without addition of inhibitors, 20% of 7S anti-IgG R3387 was found in the precipitate after centrifugation in a Beckman 152 microfuge. This amount of precipitate was taken as 100% value.

Allotypic Determination.—Quantitative measurement of allotypy on the isolated 7S anti-IgGs was done by a modification of the Mancini et al. (14) method, which employed specific allotypic antisera. The allotypic antisera were kindly supplied by Dr. Thomas J. Kindt, The Rockefeller University.

RESULTS

The four Group C streptococcal antisera listed in Table I were employed in these studies on the relationship between 7S anti-IgG and anti-peptidoglycan antibody. These antisera were selected because they all had greater than 6 mg/ml of 7S anti-IgG as detected by a coprecipitation test (4). A monoclonal 7S anti-IgG component could be isolated from each serum by the use of the IgG immunoabsorbent column (15). Two antisera had high levels of anti-peptidoglycan antibodies (6–15 mg/ml) and the other two had low concentrations (1–2 mg/ml). These four antisera contained between 5 and 23 mg/ml of antibody to Group C carbohydrate.

TABLE I
Serum Concentrations of Anti-Peptidoglycan Antibody, Anti-Group C Carbohydrate, and 7S Anti-IgG in Four Group C Antisera

Serum no.	Total IgG	Anti-group C antibody	7S anti-IgG	Anti-peptidoglycan antibody
	<i>mg/ml</i>	<i>mg/ml</i>	<i>mg/ml</i>	<i>mg/ml</i>
R3387	30	5	14.0	15
R3416	31	13	9.2	6
R4057	25	11	6.5	2
R3439	35	23	10.0	1–2

Peptidoglycan and the Group C carbohydrate are the two major antigens in the peptinized cell walls of Group C streptococci which were used to test for specific antibody activity. The 7S anti-IgG isolated from the four antisera gave no reaction with Group C carbohydrate in the capillary precipitin test. The 7S anti-IgG of antisera R3387 and R4057, however, gave strong capillary precipitin reactions with the peptidoglycan while that of antisera R3416 and R3439 did not. Thus, in the case of antisera R3387 and R4057, the peptidoglycan antibody was associated with the 7S anti-IgG fraction which was retarded on the IgG immunoabsorbent column.

Depicted in Fig. 1 are the quantitative precipitin tests for peptidoglycan utilizing whole antisera, and similar tests performed with the 7S anti-IgG components isolated from these antisera. The 7S anti-IgGs were used at concentrations equivalent to their concentrations in the antisera. Approximately 70% of 7S anti-IgG of serum R3387 and 40% of 7S anti-IgG R4057 precipitated with peptidoglycan at equivalence.

While these results suggest that the 7S anti-IgGs of sera R3387 and R4057 had antibody activity for peptidoglycan, an alternative explanation had to be

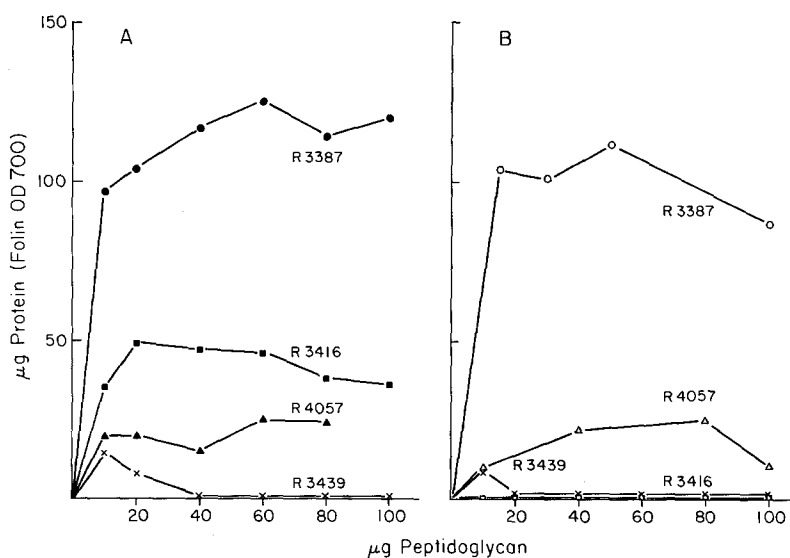


FIG. 1. Quantitative precipitin reaction between sonically disrupted peptidoglycan of *S. epidermidis* and four Group C antisera (panel A) and the 7S anti-IgGs isolated from these sera (panel B).

considered. It was possible, for example, that the 7S anti-IgG component which was isolated from these sera contained both antibodies to peptidoglycan and anti-Fc antibodies, especially since it has been shown that the latter may bind soluble IgG (4). This possibility appears less likely for the following reasons. First, as depicted in Fig. 2, polyacrylamide disk electrophoresis of the 7S anti-IgG components of R3387 and R4057 after reduction and alkylation showed only one major light chain band, suggesting the predominance of one antibody species. Second, the isolated 7S anti-IgG was allotype a2/b4 as determined by quantitative tests, although the rabbit R3387 was heterozygous at the *a* locus, a2, 3/b4. Exclusion of the second group *a* allele argues in favor of a single molecular species in the 7S anti-IgG component. Rabbit R4057 was homozygous at both the *a* and *b* loci, so the allotype of the 7S anti-IgG could not be used to judge restricted heterogeneity. Because the supply of 7S anti-IgG of serum R4057 was limited, only 7S anti-IgG of R3387 was employed for the following studies.

Specificity of the Binding Site of 7S Anti-IgG R3387.—Initial studies on the specificity of the 7S anti-IgG R3387 indicated that it reacted with the pentapeptide of the peptidoglycan and not with the hexosamine polymer. The reaction with pentapeptide was examined in greater detail by use of a synthetic pentapeptide, L-Ala-D-Glu-γ-L-Lys-D-Ala-D-Ala, which is identical with that in the peptidoglycan of streptococci. The synthetic pentapeptide, three other alanine-containing peptides, and IgG were used to inhibit the coprecipitation of

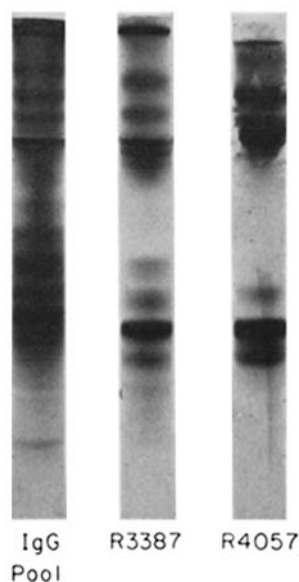


FIG. 2. Disk electrophoresis in urea polyacrylamide gel of reduced and alkylated 7S anti-IgG R3387, R4057, and of pooled rabbit IgG.

7S anti-IgG with antigen-antibody complexes. In these experiments, antigen-antibody complexes were formed at equivalence in the presence of the inhibitors and of ^{125}I 7S anti-IgG. All inhibitors were used at equal molar concentrations. The results are shown in Fig. 3.

The synthetic pentapeptide was as effective as IgG in inhibiting the coprecipitation between 7S anti-IgG R3387 and the immune complexes. It should be noted that $(\text{D-Ala})_3$ was inhibitory, whereas $(\text{L-Ala})_3$ was not. Furthermore, $\text{Tyr-(Ala)}_4\text{-Tyr}$, which is completely unrelated to the pentapeptide of peptidoglycan, was not inhibitory.

While definitive studies on the binding affinity of 7S anti-IgG for peptidoglycan must employ equilibrium dialysis experiments with radiolabeled pentapeptide, preliminary studies suggest that 7S anti-IgG R3387 may have a greater affinity for peptidoglycan than for the antigen-antibody complexes or for an IgG immunoabsorbent. To examine this question, the binding of ^{125}I anti-IgG R3387 to streptococcal cell walls (which have exposed antigenic determinants of the peptidoglycan moiety), antigen-antibody complexes, and an IgG immunoabsorbent was measured.

The first set of experiments determined the binding of ^{125}I 7S anti-IgG R3387, ^{125}I group C antibody, and ^{125}I preimmune IgG to Groups A and C cell walls. 0.2 ml of phosphate buffered saline (PBS) containing 20 μg of one of the different ^{125}I IgG preparations, and 0.1% human serum albumin were added to the sediment of 0.4 ml of a 10% cell wall suspension, and incubated overnight at

4°C with shaking. The cell walls were centrifuged, washed twice with 3 ml PBS and the radioactivity of the pellet was measured. The results of this experiment are summarized in Table II. 70–80% of the 7S anti-IgG R3387 was absorbed to both Groups A and C cell walls, even after the washing procedure, whereas less than 10% of the ^{125}I preimmune IgG was bound. The percent binding of the 7S anti-IgG R3387 to the cell walls compares favorably to the binding of ^{125}I group C antibody to the group C cell walls.

In a parallel set of experiments it was observed that ^{125}I anti-IgG R3387 was reversably bound to either preformed complexes consisting of Group A carbohydrate and anti-Group A antibody, or to IgG coupled to Sepharose. After two washes with 3 ml of PBS, less than 10% of the total ^{125}I 7S anti-IgG remained bound to the complexes or to the IgG Sepharose.

Inhibition of the Anti-Idiotypic Reaction with Pentapeptide.—Pentapeptide inhibits the reaction between 7S anti-IgG R3387 and its anti-idiotypic serum.

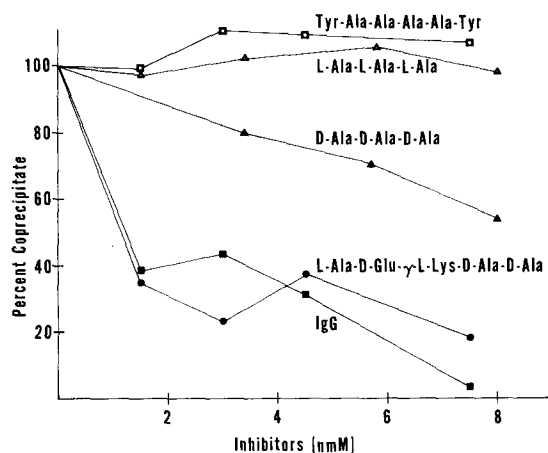


FIG. 3. Inhibition of coprecipitation of 7S anti-IgG R3387 with antigen-antibody complexes by pentapeptide, $(\text{D-Ala})_3$, $(\text{L-Ala})_3$, $\text{Tyr-(Ala)}_4\text{-Tyr}$, and pooled rabbit IgG.

TABLE II
Binding to Streptococcal Cell Walls of 7S Anti-IgG R3387 and Group C Antibodies Isolated from Antiserum

IgG	Streptococcal cell walls	
	Group A	Group C
	%*	%*
7S anti-IgG R3387	68	81
Anti-Group C antibody	12	91
Preimmune IgG	6.5	7

* The percent of total fraction added which was recovered from cell walls after washing. See text for details.

Anti-idiotypic antibodies were raised in guinea pigs and made monospecific by absorption with pooled rabbit IgG coupled to Sepharose. A precipitin curve was established by adding increasing amounts of anti-idiotypic serum to a series of tubes containing $10 \mu\text{g}$ ^{125}I -labeled 7S anti-IgG R3387. 65% of ^{125}I -labeled 7S anti-IgG R3387 precipitated even in the presence of excess antibody. Conditions of antigen excess were chosen for inhibition experiments. The pentapeptide and the $(\text{L-Ala})_3$ peptide were used as inhibitors. The results of these experiments are depicted in Fig. 4. The pentapeptide inhibited the anti-idiotypic reaction whereas $(\text{L-Ala})_3$ had no effect. This is additional evidence that the 7S anti-IgG R3387 has specificity for the pentapeptide of peptidoglycan.

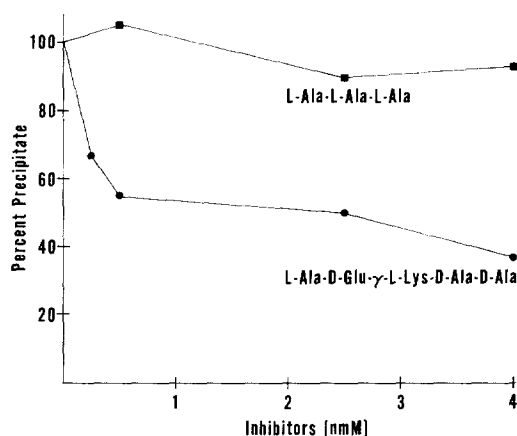


FIG. 4. Inhibition of the precipitin reaction between 7S anti-IgG R3387 and its anti-idiotypic antiserum by the pentapeptide.

DISCUSSION

The antisera of rabbits immunized with Groups A, A-variant, and C streptococci often contain 7S anti-IgGs and antibodies to the peptidoglycan of the bacterial cell wall, in addition to the antibodies to the group-specific carbohydrates. The specificity of the 7S anti-IgGs of four different Group C antisera, isolated by means of an IgG immunoabsorbent column, has been examined in greater detail here. From two of these antisera, the isolated component reacted with (a) antigen-antibody complexes in a coprecipitation test which is used to detect the 7S anti-IgGs and (b) the peptidoglycan of the cell wall. For the other two antisera, the isolated 7S anti-IgG components did not react with peptidoglycan; thus, the antibodies to peptidoglycan in the latter two antisera are distinct from 7S anti-IgG.

The 7S anti-IgG isolated from rabbit streptococcal antisera R3387 and R4057 was homogeneous by several criteria. Previous studies have shown that the activity of 7S anti-IgG was directed toward the Fc portion of IgG (15). In addition, these 7S anti-IgG components gave a precipitin reaction with strepto-

coccal cell wall peptidoglycan. Inhibition of the coprecipitation of 7S anti-IgG R3387 with antigen-antibody complexes by the pentapeptide, L-Ala-D-Glu- γ -L-Lys-D-Ala-D-Ala, of peptidoglycan and also by (D-Ala)₅ suggests that the 7S anti-IgG reacts with the immunodominant C-terminal D-Ala-D-Ala antigenic determinant of the pentapeptide just as do the previously described antibodies to peptidoglycan of streptococcal antisera (7, 8). The two specificities of this homogeneous 7S anti-IgG, one for the C-terminal sequence of pentapeptide in peptidoglycan, and the other for the Fc portion of IgG, are difficult to reconcile from an immunochemical point of view. Unusual cross-reactions of a similar sort have been reported by Hannestad (17) who described a polyclonal human 19S rheumatoid factor which reacted with TNP, and a Waldenström macroglobulin with anti-IgG activity which precipitated denatured DNA.

Additional support for the view that the homogeneous 7S anti-IgG component of serum R3387 may be an antibody to peptidoglycan stems from the idiotypic studies. The precipitin reaction between this antibody component and its idiotypic antiserum was inhibited by the pentapeptide. Such an observation is in keeping with the view that the idiotypic determinant includes the binding site of the antibody. Haptenic inhibition of the idiotypic reaction has been observed in other antigen-antibody reactions (18-20).

The occurrence of an antibody component in streptococcal antisera with specificity for both the Fc of IgG and the pentapeptide of peptidoglycan raises a question about the identity of the stimulating antigen. Is such an antibody component, which occurs as a result of this immunization procedure, primarily an autoantibody, or is it an antibody to the peptidoglycan of the bacterial vaccine, an antibody which incidentally cross-reacts with Fc? One possible approach to this question is to examine the binding affinity of this antibody for these two antigens. While this question requires further study, the preliminary results reported here raise the possibility that the 7S anti-IgG R3387 has a greater affinity for peptidoglycan than for antigen-antibody complexes or an IgG immunoabsorbent. Such a finding suggests that the term 7S anti-IgG is a misnomer for the antibody component isolated from antiserum R3387 by the IgG immunoabsorbent column. Rather, the evidence suggests this homogeneous IgG component was produced primarily as antibody to peptidoglycan. Such an interpretation based only on binding affinity data must, of course, be corroborated by additional approaches.

Several lines of evidence indicate that the occurrence of anti-IgGs in rabbits depends on the nature of the immunizing antigen. Whereas all rabbits immunized with streptococcal vaccines produced anti-IgGs, those immunized with pneumococcal vaccine were less likely to do so (4). Furthermore, the streptococcal vaccines vary in their efficacy in stimulating the occurrence of anti-IgGs. A survey of many antisera revealed that the concentration of 7S anti-IgGs in the Group A-variant sera were on the average higher than in the Groups A or C antisera.¹ In this connection, it should be noted that Group A-

¹ Bokisch, V. A., and R. M. Krause. Unpublished observations.

variant antisera usually have the highest titers of anti-peptidoglycan antibodies (21). Future studies on the origin of anti-IgGs may be facilitated by the use of A-variant rabbit antisera.

Other possible avenues should be explored for an association between antibodies to peptidoglycan and anti-IgGs. For example, Williams and Kunkel have noted the common occurrence of rheumatoid factors in patients with subacute endocarditis (22). Unusually high latex fixation titers were observed in patients infected with α -streptococci. The work presented here raises the possibility that such anti-IgGs may be cross-reactive with bacterial peptidoglycans. In another line of investigation, Braun and Holm (23), by use of Ouchterlony analysis, reported that 75% of the sera from patients with rheumatoid arthritis contained antibodies to peptidoglycan, whereas 16% of the sera from patients with acute poststreptococcal glomerulonephritis had detectable peptidoglycan antibodies. The influence of rheumatoid factors in the detection of these antibodies has yet to be assessed. These questions can be examined further by the measurement of peptidoglycan antibodies in sera of patients with a radioimmunoassay currently under development.

SUMMARY

The relationship between 7S anti-IgG and antibodies to streptococcal cell wall peptidoglycan was examined for four streptococcal Group C antisera. Homogeneous 7S anti-IgG components in these sera were isolated by means of an IgG immunoabsorbent column. For two of the four antisera, the anti-peptidoglycan activity of the 7S anti-IgG had specificity for the pentapeptide, L-Ala-D-Glu- γ -L-Lys-D-Ala-D-Ala, the antigenic determinant of peptidoglycan, as well as for the Fc of IgG. Detailed studies on the 7S anti-IgG from one of the antisera revealed that the pentapeptide inhibited the coprecipitation reaction of 7S anti-IgG R3387 with antigen-antibody complexes and the precipitin reaction between 7S anti-IgG R3387 and its anti-idiotypic serum.

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