Agar-Gel Precipitin-Inhibition Technique for C-Reactive Protein Determinations

I. Preliminary Evaluation of Technique

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ABSTRACT


The capillary tube-precipitin test for C-reactive protein (CRP) has been adopted by many hospitals as a routine clinical test. It has aided the physician by supplementing the results attained with the erythrocyte sedimentation rate, the white blood cell count, differential smear, and the patient's temperature.

Although the CRP test is very specific in its action, certain pitfalls have been noted, among which is the variation in lots of commercially prepared CRP antisera.

The present investigation was undertaken to minimize this difficulty and, in addition, to attempt to improve the sensitivity of this important clinical test.

MATERIALS AND METHODS

Rabbit antiserum to CRP. The antiserum to the CRP was obtained commercially.

CRP. The source of CRP can be any human serum that shows at least a 4-mm capillary tube precipitation when the commercial CRP antiserum is used.

Agar-gel medium and plates. The test medium and plates were prepared as described by Ray and Kadull (1964).

Agar diffusion reading lamp. The reading lamp was the same as that used by Ray and Kadull (1964).

Source of sera for testing. Routine human sera from the hospital laboratory, representing 120 sera from the 1961-62 period and 20 known positive sera from the 1956-57 period, were used in this investigation.

Block titration of antigen-antibody system. Preliminary investigation by direct, or double, diffusion of positive serum against commercial CRP antisera in concentrated and diluted form led to the block titration in gel plates of positive serum containing CRP as the antigen versus CRP antiserum as the antibody. Five serial twofold dilutions of the CRP antigen and of the CRP antiserum were prepared in physiological saline, beginning with 0.5 ml of the antigen and 0.2 ml of the antiserum. To each dilution an equivalent volume of saline was added, which gave final dilutions ranging from 1:2 to 1:32. The center row of reservoirs (approximately 0.025 ml per reservoir) was filled with one of the prepared CRP antiserum dilutions, one plate being used for each dilution. Plates were incubated at room temperature for 30 min. After incubation, each of the two outer rows of wells (approximately 0.07 ml per well) was filled sequentially and in duplicate with the CRP antigen dilutions.

After remaining at 23 to 27 C for 24 hr, plates were observed with the agar diffusion reading lamp. The final reading was performed after 48 hr.

The antigen-antibody end point was determined as that combination of the highest dilution of antigen and antibody producing a visible line of precipitation in 48 hr. This initial titration in the agar-gel plates was essential to the establishment of maximum sensitivity and consistent reproducibility in subsequent tests with unknown sera. The end-point reading was arbitrarily assumed to represent a "minimal reacting dilution" (MRDₐ) of antigen and a "minimal reacting dilution" (MRDₐ) of antibody. This is illustrated in Table 1, wherein the MRDₐ of antigen was established as a 1:64 dilution and the MRDₐ of the commercial CRP antiserum-antibody as a 1:64 dilution.

Serum titrations. The inhibition, or indirect, method was used in titrating unknown sera. Serial twofold dilutions of 0.2 ml of unknown serum were made in saline. To each dilution 0.2 ml of a twofold
concentration (1:32) of the previously titrated antibody (1:64) was added, the final mixture thus containing a CRP antibody MRD₆ plus unknown serum dilutions ranging from 1:2 to 1:32. The mixtures were hand-shaken for 30 sec and incubated in a 37°C water bath for 30 min to permit antigen-antibody binding to proceed to completion.

Each of the wells in the center row of agar diffusion plates was filled with a 1:64 dilution (MRD₆) of the CRP-containing serum, and the plates were incubated at room temperature, 23 to 27°C, for 1 hr. Conclusion of incubation periods for the plates and for the antigen-antibody mixtures was timed to coincide and permit immediate accomplishment of the final step.

Outer rows of wells were filled sequentially and in duplicate with the incubated antigen-antibody mixtures. Thus, each well in one outer row contained the same mixture as the corresponding well in the opposite outer row. The end point was determined after plates remained at room temperature for 48 hr. A preliminary reading at 24 hr was generally made. The end point, or titer, of the CRP in the unknown serum was that dilution of the unknown serum that completely inhibited the formation of a visible line of precipitate with the block-titrated MRD₆ and MRD₆ in the agar-gel plates (Fig. 1).

**Fig. 1.** Serum titrations by indirect technique (AGPI). (A) Negative serum. Note undulating lines of visible precipitate between center and outer rows of wells, indicating absence of binding of MRD₆. (B) Positive serum. Note absence of visible line of precipitate between first four wells on the left of center and outer rows, indicating complete binding of MRD₆. End point is 1:16 dilution of serum.

**Fig. 2.** Direct, or double, diffusion in agar-gel. The smaller center row of wells contained undiluted CRPA, lot 201. Outer larger duplicate rows contained twofold dilutions of the tested serum. (A) Serum 3 (C.C., 11/7/56) shows lines of CRP precipitate in the 1:2 and 1:4 dilutions, respectively. (B) Serum 5 (normal human pooled, 12/21/59) shows lines of precipitate in the 1:2 dilution only.
Controls subjected to the same test procedures consisted of dilutions of the test antisera in saline and combinations of the predetermined MRD, of antibody with known negative and positive CRP sera.

Precipitin test of CRP. The precipitin test on patients' sera was conducted in capillary tubes, following the recommended procedures of Selman and Halpern (1956) and employing commercial antihuman CRP antiserum (Schieffelin and Co., New York, N. Y.). The results were recorded as 1 to 4 plus, depending on the amounts (in millimeters) of measured precipitate. This method is considered a semiquantitative estimation of CRP in the serum specimen.

**RESULTS**

Agar-gel inhibition test sensitivity. Ten known CRP positive sera and one normal human pooled serum sample were employed in a direct, or double, agar-gel diffusion test against undiluted CRP antiserum. Serial twofold dilutions, 1:2 through 1:32, of each serum specimen were added sequentially and in duplicate to the outer parallel rows of wells. The inner wells were filled with undiluted CRP antiserum (lot 201), and the plates were incubated at room temperature for 24 hr. Readings were made after 48 hr. Subsequent incubation up to 96 hr revealed no increase in titer over the 48-hr reading. Figure 2 illustrates comparable titer results. Serum 3 shows lines of precipitate in the first two series of wells, indicating a titer of 1:4. Serum 5 shows a faint line of precipitate in the first well series, indicating a titer of 1:2 by the gel-diffusion direct method. Those sera tested by the capillary-precipitin tube method revealed a 4-plus, or 4-mm, reaction of
CRP precipitate in serum 3 and a 1-plus, or 1-mm, reaction in serum 5.

When the above two sera were treated in a similar manner, with the exception that the CRP antiserum in the center wells was a 1:10 dilution, an increased titer of each serum was attained (Fig. 3). Serum 3 then had a titer of 1:16 and serum 5 a titer of 1:8.

The above results initiated the investigation of a block titration of serial twofold dilutions of CRP antiserum (lot 201) diffusing in agar-gel plates against serial twofold dilutions of a highly positive CRP human serum. The results are demonstrated in Table 1, producing evidence that there is a positive reaction occurring in a 1:64 dilution of each of the reagents in 48 hr. Therefore, the optimal CRP-CRPA (antigen-antibody) relationship, or minimal reacting dilution of each reagent, is a 1:64 dilution of antigen reacting with a 1:64 dilution of antibody.

As additional evidence of the sensitivity of the proposed agar diffusion test, six serum specimens previously assayed by the accepted capillary tube-precipitin technique were reassayed by this routine procedure and by the proposed agar-gel precipitin-inhibition (AGPI) technique. These results are tabulated in Table 2. It is interesting to note that serum 5 corresponds to serum 3 in Fig. 2 and 3. Serum 5 has a titer of 1:128, as compared to the previous titer of 1:4 and 1:16, respectively. Table 2 also illustrates the variance in the CRP capillary tube results when a serum is retested with a different lot of the commercially prepared antiserum, verifying the findings of Selman and Halpern (1956).

**Discussion**

Although an agar-gel precipitin test was described by Nilsson and Hanson (1962) and was shown to be more sensitive than the capillary-precipitin technique, the present investigation was undertaken in an effort to titrate the amount of CRP in human sera.

In the course of this investigation, experiments proved that a higher degree of sensitivity could be attained by application of the box titration to agar-gel. An end point was reached whereby the minimal reacting dilutions were obtained for CRP antigen and CRP antibody.

Since this appeared to be the most sensitive reaction between the two reagents, it was thought feasible that any inhibition of this relationship would be an index or titration of the unknown tested serum's ability to absorb the available CRP antibody and to prevent the diffusion and subsequent formation of a line of precipitate with the antigen reagent in the controlled system. This is the basis for determining the degree of sensitivity of this proposed new technique for the serological quantitation of the CRP in human serum. Further application of these minimal reacting dilutions in agar-gel resulted in the employment of the agar-gel precipitin-inhibition technique for CRP determinations and should negate the variability of repeated serum results due to different lot preparations of commercial CRP antiserum.

When the proposed technique is compared with the capillary tube-precipitin test, a higher degree of sensitivity is observed, and the unknown positive serum can be assigned a titer of its CRP content.

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**Literature Cited**

