Studies in Latex Agglutination: An Approach to the Determination of Optimum Conditions for Discrimination between Rheumatoids and Normals

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Optimum conditions for latex agglutination by rheumatoid factors have been investigated with respect to pH, ionic strength, gamma globulin concentration and heat of inactivation. A large number of sero-negative cases of rheumatoid arthritis was studied. Best discrimination between rheumatoids and normals was found at pH 8.4 instead of at pH 8.2, with 0.6 per cent instead of 1.0 per cent sodium chloride, without gamma globulin and without heat except for 15 minutes inactivation at 56 C.

Le optime conditiones pro le agglutination de latex per factores rheumatoide esseva investigate con respecto a pH, fortia ionic, concentration de globulina gamma, e thermo-inactivation. Un grande numero de sero-negative casos de arthritis rheumatoide esseva studiate. Le melior differentiation inter rheumatoides e normales esseva obtenite a pH 8,4 (in loco de 8,2) con 0,6 pro cento de chloruro natrium (in loco de 1,0) e sin calor con le exception de 15 minutas de inactivation a 56 C.

SINCE 1956 (Singer and Plotz)[1] little has been published on the systematic investigation of optimum conditions under which latex particle agglutination can be used for discrimination between patients with rheumatoid arthritis and normals. Based upon the observations of Singer et al.,[2] work on the reactivity of latex particles has proceeded chiefly in two directions: serum fractionation and the use of "coating" substances other than gamma globulin. Increased sensitivity has been achieved with the use of complicated time-consuming procedures. Ease of performance has been accompanied by decreased specificity.[3-5,8]

Analysis of the latex methods used reveals up to 35 per cent false negatives in patients with definite and classical rheumatoid arthritis and up to 8 per cent false positives in normals.[3-8] It seemed that a search for optimum conditions for producing latex agglutination emphasizing this false negative group would lead to the development of tests with increased sensitivity as well as to some insight into reasons for their false negativity. A systematic exploration was decided upon aimed at developing a rapid easy method for use in field surveys, where convenience in large scale operations, as well as sensitivity and specificity, is essential.

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Material and Methods

Materials

1. Plastic plates—Disposo-Tray Model 96U-CV. Linbro Chem. Co., New Haven, Conn.
2. Svedmyr pipettes—Kirurgiska Instrument Co., Stockholm, Sweden.
3. Human gamma globulin—Pentex, Inc., Kankakee, Ill., Lot No. H28.
4. Latex particles—.81 micra-LS-449-E, Dow Chemical Co., Midland, Mich.

Methods

1. Latex agglutination in plastic plates (PLAT): A method was devised (Brooks and Cobb, 1960)[9] for producing latex agglutination in plastic plates. Using this method, optimum conditions were sought in regard to gamma globulin coating of latex particles, hydrogen ion concentration, sodium chloride concentration, and serum preheating or inactivation which in combination would produce maximum specificity and sensitivity. The density of the latex suspension was held at a concentration such that a 1:10 dilution shows a 20 per cent transmittance at 650 mμ in the Coleman Universal Spectrophotometer using a 16 mm. cuvette. This was selected because several observers in our laboratory agreed that it provided easy readability in the plastic plates. The following instructions were followed routinely.

a. Bring serum to room temperature. Heat 0.5 ml. serum in a Wasserman tube in a water bath at 56 C.
b. With a Svedmyr pipette make progressive twofold dilutions of 0.2 ml. heated serum, from 1:10 to 1:640, in the appropriate 0.1 M glycine buffer in the depressions of the plastic plate.
c. To each 0.2 ml. of diluted serum, add 0.2 ml. latex particles prepared by dilution in the appropriate 0.1 M glycine buffer.
d. Rotate plastic plates for 15 minutes at 180 rotations per minute at room temperature on an Esbach rotator.
e. Using a desk lamp, read agglutination for each dilution. Record degree of agglutination from 0 to ++++.

2. Latex fixation (IRLF): The refrigerated method described by Singer et al. (1960)[2] was used. We modified the method only to include 30 minutes of serum inactivation at 56 C. before adding latex particles. Unheated gamma globulin in a concentration of 100 μg./ml. latex particles was utilized. Agglutination of one plus or more at serum dilution 1:160 or greater was considered positive.

Populations Tested

I. Patients with definite or classical rheumatoid arthritis who are positive on the IRLF from the private practice of two colleagues (*RA's* LF_+).

II. Patients from the same source with definite or classical rheumatoid arthritis who are negative on the IRLF at a dilution of 1:20 (*RA's* LF_0).

III. Randomly selected individuals from industrial populations (controls).

Thirty individuals were used in each group for each experiment except for one step in the experiment on sodium chloride concentration for which only 15 in each group were available. In reviewing our results it is important to note the population number with which each chart is labeled and to make comparisons only on the same populations. The reason for this is that the variations between samples of this size are sufficient to lead to confusion if this point is not considered. Figures 1 and 2 relate to population I and are therefore comparable. No other "between figure" comparisons can be made and conclusions can only be drawn from the trends observed within each.

For each experiment the percentage of individuals positive at serum dilution 1:80 has been plotted. This point was selected because it turned out to be convenient for showing

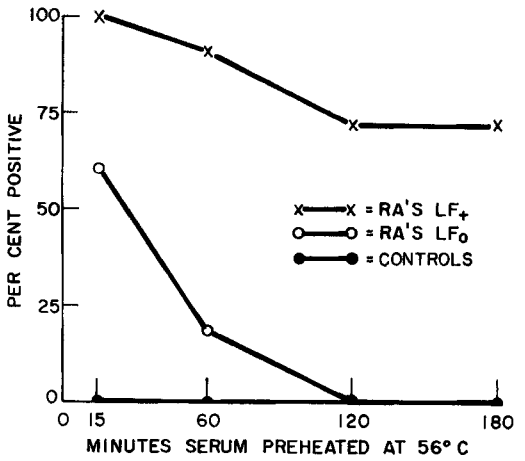

Fig. 1.—The effect of serum preheating at 56 C. on latex agglutination. (Population I: N = 30, pH = 8.2, NaCl = 1.0 per cent, gamma globulin, 15 μg./ml., serum dilution 1:80.)

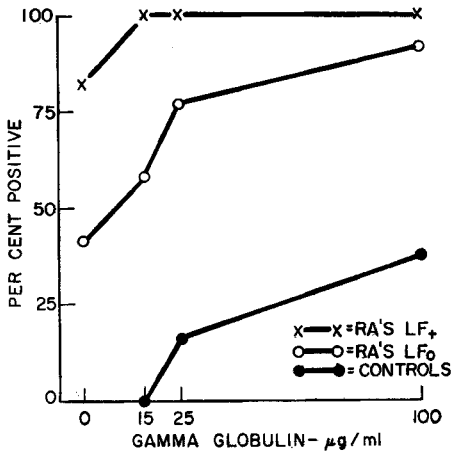

Fig. 2.—The effect of gamma globulin concentration on latex agglutination in rheumatoids and normals. (Population I: N = 30, pH = 8.2, NaCl = 1.0 per cent, heated 15 min. at 56 C., serum dilution 1:80.)

the relationships simply. Copies of the full data are available from the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C.*

Results

Preheating for inactivation. It has been recognized (Brine et al., 1958;[10] Schubart et al., 1959;[11] Brooks and Cobb, 1960[9]) that the sera of many patients with rheumatoid arthritis contain thermolabile inhibitors. If not inactivated these may produce prozone effects and, in the case of low titer sera,

*Copies may be secured by citing Document No. 7483 and by remitting $1.25 for photoprints or microfilm.

actual false negative results. "Rheumatoid factor" has been shown to maintain agglutinating activity at temperatures up to 78 C. (LoSpalutto and Ziff, 1956).[12] In this light, figure 1 is of considerable interest, for it indicates that the "rheumatoid factor" detected by the IRLF is considerably more heat stable than that found in the sera of those who are negative on this test. As can be seen from the figure, 100 per cent of the RA's LF_+ group and 57 per cent of the RA's LF_0 group were positive at the end of 15 minutes of serum heating at 56 C., compared with 93 per cent positive in the RA's LF_+ and only 20 per cent positive in the RA's LF_0 at the end of 60 minutes.

Heat destruction of rheumatoid factor within the two groups was measured by the loss in titer of the reactive sera. It was calculated that during the 45-minute period following inactivation, average loss in titer within the RA's LF_0 was almost twice as great as within the RA's LF_+ group, It is, therefore, concluded that in order to obtain maximal ability to detect these cases with a single test, it is important to use only the minimum amount of heat necessary for inactivation of the inhibitors. Present indications are that this is about 15 minutes at 56 C. but further experiments may reveal the optimum to be less than this.

Gamma globulin concentration. The effect of varying the globulin concentration is depicted in figure 2. Similar data were obtained after varying length of time of preheating at 56 C. and the relationships were generally the same.

At first look it might seem that the increasing agglutination with increased globulin is in conflict with the contention of Singer et al. (1960)[2] that the concentration of gamma globulin does not matter in the range between 10 and 100 μg. per cc. In their system the continuous heating, 90 minutes at 56 C., probably counteracts this increasing agglutination.

As can be seen from the figure the best discrimination was obtained at 15 μg. per cc., at which point all the controls were negative; all the RA's LF_+ were positive; and 56 per cent of the RA's LF_0 were positive. This seemed like a promising result, but it was found that under these conditions (inactivation 15 minutes at 56 C.; gamma globulin 15 μg. per cc.; pH 8.2; NaCl conc. 1.0 per cent) 56 out of 60 hypertensives were positive.[9] This led to explorations with the uncoated particle.

Salt concentration. Figure 3 shows the effect of varying the salt concentration on the agglutination of uncoated particles, using serum inactivated for 15 minutes at 56 C. It is important to note that the studies from 1.0 to 0.5 per cent were done on population II and those from 0.5 to 0 per cent were done on population III. Hence the apparent discontinuity in the middle of the figure It is apparent that the optimum salt concentration lies close to 0.5 per cent.

Hydrogen ion concentration. Using the above determined optima of 15 minutes inactivation at 56 C., uncoated particles, and a salt concentration of 0.5 per cent, the effects of pH were explored. Figure 4 shows a clear optimum at pH 8.4.

Final explorations. A systematic exploration of the area pH 8.4–8.6 and salt concentration 0.4–0.6 per cent was undertaken using a group of hypertensives in addition to the population controls, for comparison with the rheuma-

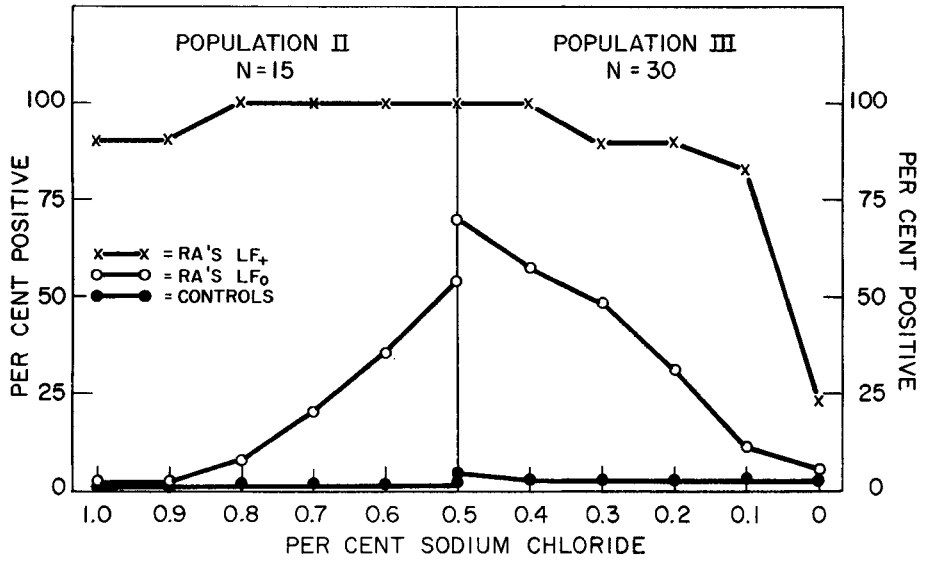

Fig. 3.—The effect of salt concentration on latex agglutination. (pH = 8.2, no gamma globulin, heated 15 min. at 56 C., serum dilution 1:80.)

toids. This confirmed the fact that optimum agglutination by the sera of rheumatoid arthritics is obtained at pH 8.4 in 0.1 M glycine buffer with addition of 0.5 per cent salt. However, it was found that 17 per cent of the hypertensives were positive at this point but that they were all negative at pH 8.4 and 0.6 per cent NaCl. Since this change in salt concentration entailed only a small loss in sensitivity for rheumatoid arthritis, it was deemed wiser to work at this point for the sake of high specificity, i.e., a low frequency of false positives.

It seemed likely that this new set of conditions, inactivation 15 minutes at 56 C., uncoated particles, pH 8.4, 0.6 per cent NaCl, would prove appreciably more sensitive and specific than other existing tests. The next step was to examine a sizeable sample of cases and controls by both PLAT and IRLF. This sample consisted of sera from 300 classical or definite rheumatoid arthritics from the same two private practices and a randomly selected group of 300 employees in a large chemicals plant. As shown in table 1, 92 per cent of the sera from these cases gave positive tests at 1:80 while only 4 per cent of the controls showed up as positive. Since these controls were selected without respect to any medical examination it is quite possible that a few of them are in fact suffering from rheumatoid disease.

Table 1.—*Comparison between PLAT and IRLF: Sensitivity and Specificity for Rheumatoid Arthritis*

Population	N	PLAT % pos.	IRLF % pos.
Rheumatoids	300	92	73
Controls	300	4	8

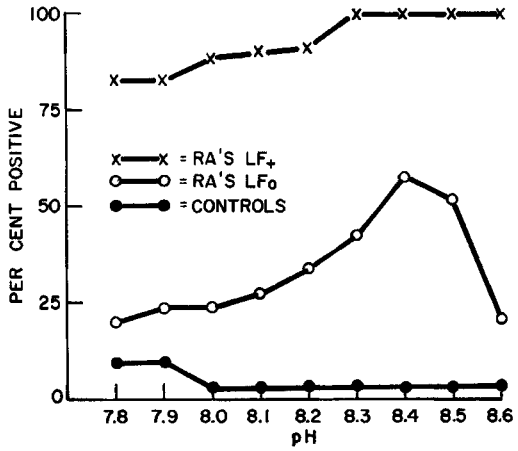

Fig. 4.—The effect of pH on latex agglutination. (Population III: N = 30, NaCl = 0.5 per cent, no gamma globulin, heated 15 min. at 56 C., serum dilution 1:80.)

It is felt that 92 per cent is about as high a sensitivity as can be reached in a group of cases such as this for it is entirely possible that, in line with the experience of Dixon (1960),[13] some of the negatives will eventually turn out to have some disease other than rheumatoid arthritis. This compares favorably with the highest sensitivities achieved by other particle or red cell agglutination tests for rheumatoid factor. This sensitivity is accompanied by a frequency of false positives that is comfortably within the 5 per cent level specified in the ARA criteria. If greater specificity is desired, it is obtainable at higher titers (fig. 5). It is also important to note from table 1 that this sensitivity is accomplished on a population of rheumatoids, only 73 per cent of whom are positive on the IRLF.

Comment

The success of this empirical development is in considerable part due to the emphasis on the study of cases negative on one of the commonly used forms of the latex test. Without this emphasis much larger samples would have had to be studied in order to get significant results. Another part of the success of the development is due to the systematic approach. It must be admitted, however, that even though some 7,000 tests were done in the course of this work, there remain large areas to be explored. Particularly, it would be useful to know more about the effect of pH and ionic strength in gamma globulin containing systems. It would also be interesting to study the effect of polyvalent ions.

One difficulty with the PLAT is that dilutions between 1:1280 and 1:20,000 may demonstrate nonspecific agglutination. The exact reason for this phenomenon is not known (Heimer,[14] Winblad[15]). However, it can be eliminated, if these dilutions are needed, by the addition of a 1:640 dilution of normal serum heated at 56 C. for 3 hours to eliminate agglutinators.

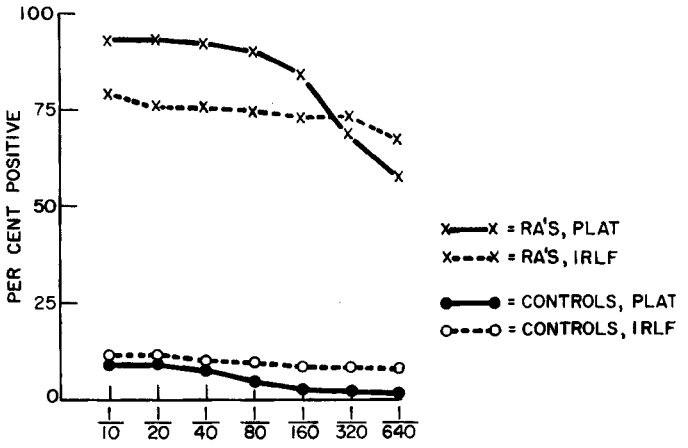

Fig. 5.—Per cent of rheumatoids and controls positive at each dilution of the PLAT and IRLF.

This raises the whole question of whether it is important to be able to measure titers. Some have contended that rheumatoid factors are either present or absent and that the titer is of no diagnostic significance. On the other hand, the usefulness in field studies of describing the full frequency distribution of titers has been emphasized (Ball and Lawrence, 1961;[16] Cobb, 1961[17]). The proper approach to this matter is not yet apparent and probably will remain obscure until tests become available which can determine small amounts of the rheumatoid factors despite the presence of interfering substances. On the whole we lean towards the view that this phenomenon is, like most others in biology, subject to a gradient and that the real questions are "How steep is this gradient?" and "What proportion of people are entirely free of all of the rheumatoid factors?" For the time being we are satisfied that for clinical purposes and for most epidemiologic purposes there is no need to determine titers beyond 1:640. If research needs require end-point titers at high levels, heated normal serum can be added to the system.

The greatest value of the PLAT lies not so much in its excellent sensitivity and specificity characteristics as in its ease of performance in quantity. A single technician can do 100 of these tests in a day and have all the glassware clean for use again the next day. The reproducibility is excellent for 96 per cent of duplicate determinations of 50 sera give the same end point and none differ by more than one tube.

As a clinical tool this test may well prove useful, but prior to extensive clinical trial its reactivity in other diseases should be examined. It was developed primarily for epidemiologic purposes, i.e., for convenience in large-scale operations and to help detect mild forms of rheumatoid arthritis. The success of this second operation will be detailed in a separate report.

From a theoretical standpoint a number of interesting points have come out of this research. First, the fact that increased sensitivity and specificity is found in the PLAT as compared with the IRLF, where the patient supplies his own 7 S gamma globulin for the reaction, gives encouragement to the notion

that rheumatoid factors may be autoantibodies. The specificity of rheumatoid factors for genetically determined 7 S gamma globulins has not been demonstrated using latex particles (Fudenberg and Kunkel, 1961).[18]

Second, the observation that some of the rheumatoid factors are more heat labile than others lends further support to the notion of their multiplicity. Since some of the factors are destroyed fairly rapidly by heat, it is clear there has been a ceiling on the achievable sensitivity of tests that are incubated at 56 C. for long periods. It would be interesting to characterize further the various members of the rheumatoid factor family in terms of their heat sensitivity.[9]

Third, the clear demonstration that pH and salt concentration are critical factors in relation to the sensitivity and specificity of this test and that the optimum point for agglutination is well out of the physiologic range, pH 8.4, and salt concentration, 0.6 per cent, gives us an insight into the nature of the phenomenon that should be helpful in further studies. It should be pointed out that there is no reason to suppose that the optima determined for this system will prevail in systems using other kinds of particles. It does mean that pH and ionic strength optima should be sought for every system. In this connection it is interesting to note that Valkenburg (1962),[19] working independently, arrived at the same conclusion as ours with regard to the ionic strength optimum.

Finally, the exciting discovery that hypertensives have agglutinating substances similar to rheumatoid factors seems likely to open up a whole new field for investigation. This will, of course, be pursued vigorously.

Summary

1. The effects of gamma globulin concentration, hydrogen ion concentration, sodium chloride concentration and serum preheating on latex agglutination have been investigated in an orderly approach to determine optimum conditions for discrimination between rheumatoids and normals.
2. "Rheumatoid factors" are gradually destroyed by heating at 56 C., some rheumatoid factors are more heat labile than others; therefore, the minimum heat necessary to inactivate inhibitors is desirable.
3. Increasing the concentration of gamma globulin reduces the discrimination between rheumatoids and normals. Under suitable conditions there is no need for added gamma globulin.
4. pH and sodium chloride levels are critical variables.
5. To date, best discrimination between rheumatoids and normals has been under the following conditions:
    a. No gamma globulin
    b. pH 8.4 in 0.1 M glycine buffer
    c. Sodium chloride concentration 0.6 per cent
    d. Serum inactivation for 15 minutes at 56 C.
6. Under these conditions (PLAT), 92 per cent of 300 definite and classical rheumatoids were positive compared to 73 per cent positive on IRLF; 4 per cent of 300 randomly selected controls were positive compared with 8 per cent on IRLF.

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