Effects of Animal Sera and Serum Albumin on Latex-Fixation Test for Rheumatoid Arthritis.* (23395)

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The recently introduced latex-fixation test (1,2,3) provides a relatively simple and easily performed technic for the serologic study of rheumatoid arthritis. Thus it has some advantages over the more cumbersome, but widely employed, sheep cell agglutination tests(4,5,6,7).

Despite the technical problems associated with these latter procedures, they have proven invaluable for investigation of the factor or factors first reported by Waaler(8) in rheumatoid sera responsible for enhancing agglutination of sensitized sheep erythrocytes.

The use of human gamma globulin in the Heller FII test(5) and rabbit anti-sheep erythrocyte serum as the "antigen" source in the sensitized sheep cell test is well established. Others have reported guinea pig and horse sera also to be adequate(9,10).

It is the purpose of this report to show that, similarly, sera from several animal species can be substituted for human gamma globulin in the latex-fixation test. Furthermore, experiments will be described in which "antigen" reactivity of test sera was gained by either removal of albumin by Na_2SO_4 precipitation or by dilution.

Materials and methods. Latex-fixation test. The basic test as employed by these laboratories has been described previously (3) and is essentially the same as that suggested by Singer and Plotz(1). Human gamma globulin is mixed with a borate-buffered saline (pH 8.2) to which is added a measured quantity of latex particles. Two drops of this mixture are brought into the presence of 2 drops of the test serum dilutions (1:20 through 1:5120). After incubation for 30 minutes at 37°C or 56°C (water-bath), the tubes are centrifuged lightly and examined for evidence of flocculation. *Sera.* The human sera employed in this study were obtained for the Rheumatology Clinic of the University Hospital. Animal sera were collected at the Department of Bacteriology and from the clinics of the College of Veterinary Medicine. *Globulin.* In addition to the human gamma globulin (poliomyelitis immune globulin, 150 mg per ml) routinely used in these laboratories, porcine globulin commercially prepared by Armour and Co. was studied. *Serum fractionation.* Sera were fractionated according to the technic of Thurston, *et al.*(11). This is a Na_2SO_4 precipitation which required but small quantities of sera (1.0 ml or less). Albumin is effectively removed with this technic and no dialysis is necessary.

Results. Animal globulins as source of "antigen" in latex-fixation test. Nine sera from a variety of animal species were tested as possible sources of "antigen" by the latex-fixation technic. One-half ml of undiluted human or animal serum was substituted for 0.5 ml of 0.5% human gamma globulin in the preparation of the latex "antigens" (Table I). Each of these "antigens" then was tested with 1:20 dilutions of 3 standard sera: A, a strongly reactive rheumatoid serum; B, a rheumatoid serum of moderate reactivity; and C, a normal human serum. No flocculation was observed except in 2 instances (chicken serum and dog serum) and these proved to be non-specific inasmuch as the saline controls also showed evidence of flocculation. The sera from several chickens reacted similarly, while no other dog serum was tested.

Subsequently 1.0 ml of each serum was fractionated with Na_2SO_4 and 0.5 ml of the reconstituted globulin was used to prepare latex "antigens" as before. After ascertaining that the "antigens" were reactive with 1:20 dilutions of the standard sera, titrations were performed and the results compared

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TABLE I. Serum Globulins from Various Species as Source of "Antigen" in the Latex-Fixation Test.

Latex antigen prepared with:			Titer of standard sera, initial dilution 1:20		
			Rheumatoid arthritis		
Whole serum	Reconstituted globulins	Gamma globulin	Saline control	A	B	Normal C
Human			—	—	—	—
Rabbit			—	—	—	—
Porcine			—	—	—	—
Chicken			4+	4+	4+	4+
Guinea pig			—	—	—	—
Dog			1+	±	±	±
Cat			—	—	—	—
Horse			—	—	—	—
Bovine			—	—	—	—
Sheep			—	—	—	—
	Human		—	*5120	160	—
	Rabbit		—	1280	40	—
	Porcine		—	5120	80	—
	Chicken		4+	- - - - - - - - -		4+
	Guinea pig		—	640	40	—
	Dog		±	- - - - - - - - -		1+
	Cat		—	1280	160	—
	Horse		—	1280	80	—
	Bovine		—	640	80	—
	Sheep		—	1280	80	—
		Human	—	5120	160	—
		Porcine	—	2560	80	—

* Titers expressed as reciprocals of serum dilutions.

with those found by the conventional technic (see human gamma globulin). Although some variation in titers was noted, globulins from all species, other than chicken and dog, as noted previously, functioned as "antigen." Whether or not the variation in reactivity (3 tube dilution range) reflects quantitative differences in the "antigen" content of the sera was not determined. More likely, the precipitation procedure provided the source of variation since this technic was reported with rabbit and human sera only. Adjusting the percentage of the precipitant (Na_2SO_4) may have corrected these discrepancies.

The purified porcine and human gamma globulins behaved similarly as "antigens."

Diluting human sera as a means of gaining "antigen" activity. The results just described suggested that the albumin in undiluted sera, in some manner, interferes with the mechanics of the latex-fixation test since removal of the albumin by precipitation permitted the "antigenic" properties of the globulins to be demonstrated.

Ten human sera from apparently normal individuals were diluted by 2-fold increments from 1:20 through 1:320 with borate-buffered saline (pH 8.2). One-half ml of each dilution was used in the preparation of latex "antigen" as before. "Antigen" prepared from the undiluted serum was included in each "antigen" series. Two drops of a strongly reactive rheumatoid serum were mixed with an equal quantity of each "antigen" in all 10 series. After incubation, the resulting flocculation was recorded as 4+, 3+, 2+, 1+, ± or — (Table II). Since 9 of the 10 sera demonstrated reactivity as "antigens" when diluted, it seems probable that the interfering capacity of albumin is, at least in part, a function of its concentration. Diluting the majority of sera depreciated this property without completely eliminating the activity of the globulins. Differences in activity were noted among the series, as might be anticipated.

A similar experiment conducted with the animal sera listed in Table I yielded analogous results.

Singer and Plotz(1) reported that the con-

TABLE II. Dilution of Serum as a Means of Gaining "Antigen" Activity.

Normal human serum No.	Reactivity of positive rheumatoid serum tested with latex antigen containing dilutions of normal human sera				
	Undil.	1:20	1:40	1:80	1:160
1	—	—	—	—	—
2	—	1+	1+	±	—
3	—	2+	1+	—	—
4	—	±	1+	±	—
5	—	1+	±	±	—
6	—	±	1+	±	—
7	—	1+	1+	±	—
8	—	±	1+	±	—
9	—	1+	2+	±	—
10	—	±	—	—	—

centration of gamma globulin employed in the latex-fixation test could be varied over a wide range. Concentrations above 12,500 γ interfered with the test, while quantities of less than 25 γ failed to be fully reactive. The working concentration recommended by these authors is 250 γ of globulin per ml of latex-globulin antigen.

The following experiment was conducted (1) to determine if the same minimal concentration of globulin (25 γ) would be effective with the "drop" modification of the latex-fixation test employed here, and (2) to correlate this finding with the results of titrating the reactivity of human sera (Table II).

Three strongly reactive rheumatoid sera (I, II, III) were tested by the "drop" technic with latex antigens containing 4-fold decreases in globulin concentration as indicated in Table III. The results were in agreement with those described by Singer and Plotz(1), *i.e.*, maximum reactivity was observed when the globulin concentration of the "antigen" approximated 250 γ per ml. However, some reactivity resulted with "antigens" containing as little as 3.9 γ of globulin.

Since 8 of the 10 human sera demonstrated some level of "antigenicity" when diluted at 1:40 (Table II), the concentration of globulin in this dilution was calculated from data available in the Handbook of Biological Data (12). The figure of 11.2 γ is in keeping with the results shown in Table III. Seven of the 10 human sera reacted, although minimally, in even greater dilutions (1:80), which gamma globulin concentration also would correspond approximately to the least reactive quantity noted with the more pure globulin fraction (3.9 γ).

Depressant effect of gelatin on reactivity of human gamma globulin as "antigen" in latex-fixation test. The inhibiting effect of albumin was assumed to be due to its non-specific protective colloid properties. Since this property is also associated with gelatin and may be imparted or demonstrated simply, this substance was employed in the following tests.

Bacto-Gelatin was diluted in borate-buffered saline (pH 8.2) to 5.0% concentration by warming at 56°C. The working concentration of human globulin (0.5%) was prepared by mixing 0.5 ml of the original globulin stock (150.0 mg per ml) with 14.5 ml of the gelatin solution. The latex-globulin "antigen" containing 0.5 ml of this globulin-gelatin mixture was added to buffered saline and latex in the usual manner.

Eleven rheumatoid sera of differing reactivities and 4 negative sera (as measured with the conventional latex "antigen") were diluted 1:20 and screened with the new antigen. No reactivity was observed, thus substantiating the premise that a protective colloid can interfere with the "antigenicity" of globulin. Identical results were obtained with 3.0% gelatin.

One drop of each of the diluted sera was mixed with one drop of 5.0% gelatin. The resulting 1:40 dilutions of sera were tested with the conventional latex-globulin "antigen." For purposes of comparison, 1:40 dilutions of the same sera were prepared in saline only and examined simultaneously. The results (Table IV) illustrate the depressant effect of gelatin on the rheumatoid factor.

The influence of diluting the sera initially

TABLE III. Titers of Rheumatoid Sera Tested with "Antigens" Containing Decreasing Concentration of Globulin.

Serum No.	Concentration of globulin (γ) per ml of latex antigen				
	250	62.5	15.6	3.9	1
I	5120*	2560	2560	640	—
II	5120	5120	5120	1280	—
III	5120	5120	1280	80	—

* Titers expressed as reciprocals of serum dilutions.

TABLE IV. Depressant Effect of Gelatin on Reactivity of Rheumatoid Sera in Latex-Fixation Test.

Test serum No.	Reactivity when diluted 1:40 in: Saline	Equal parts of saline and 5% gelatin
1	3+	±
2	4+	1+
3	2+	—
4	3+	2+
5	3+	1+
6	1+	—
7	1+	—
8	2+	—
9	4+	±
10	2+	—
11	2+	—
12	—	—
13	—	—
14	—	—
15	—	—
16	—	—

with gelatin on titration endpoints also was tested. The gelatin influence was dissipated by the serial dilutions in saline since the titers were not materially affected, depending, of course, on the original reactivity of the serum being investigated.

Viscosity measurements of "antigens" prepared with and without gelatin failed to reveal differences which could account for the observed property of gelatin described above.

Discussion. The sensitization of erythrocytes with antibody-containing sera from several animal species, prior to demonstrating the agglutinability of such treated red cells upon admixture with rheumatoid sera is of considerable interest. Although not elucidating the component of serum globulin responsible for the "antigenicity" conferred on the sensitized red cells, the ubiquity of this component is established. In all such procedures, the albumin content of the anti-erythrocyte serum is of no consequence since this fraction is devoid of antibody and would not enter into the sensitization mechanism. The coating of tanned erythrocytes with the FII fraction (gamma globulin) of human serum also eliminates any possible influence of albumin in the Heller test(5). Similarly, employing human gamma globulin as the source of "antigen" in the more conventional latex-fixation technics (1,2,3) also excludes this factor from consideration.

In the studies discussed here, the sera of 7 animal species, in addition to human, were shown to contain the necessary "antigenic" component. The demonstration of this component, however, was dependent on the removal of albumin, either by precipitation procedures or by dilution. The fact that various animal sera can provide the "antigenic" component for the latex-fixation test likens it to the findings noted with the sensitized sheep cell technics.

The failure of the whole sera to function as "antigen" was attributed to some serum component or components acting as a protective colloid and, thus, effectively prohibiting the serologic reaction from attaining completion. The recognized property of gelatin to act nonspecifically as a protective colloid was employed to establish this fact. Attempts to simulate this property by adding albumin to either diluted but reactive sera, or to reactive globulins were not uniformly successful. Further efforts in this regard were abandoned inasmuch as the pepitization procedures indicated did not seem warranted and could only serve as substantiative evidence. Since gelatin had already been shown to prohibit gamma globulin from functioning as "antigen," it would seem that the interference shown with whole serum was, at least in part, associated with a more intimate property of albumin than can be demonstrated by mere admixture.

These findings may bear some significance to the fact that the activity of rheumatoid sera is best demonstrated in the latex-fixation test, if the sera are first diluted 1:20. Since both the "antigenic" component of globulin and the rheumatoid factor are thought to reside in the same or closely related serum fractions, dilution would in effect decrease the influence of albumin and the inhibitor to the rheumatoid factor (often considered to be synonymous with the "antigen"), as well as the rheumatoid factor. Should the latter be in sufficient concentration, a serum so diluted would still be reactive when brought into the presence of globulin and latex. This is in a sense the basis for the agglutination-inhibition test of Ziff(7). Some confirmation for this lies in the report of Singer and Plotz(1) who

found that 11% of the strongly positive sera reacted with latex and buffer, without benefit of added globulin.

The reactivity of adult chicken serum with latex and buffer is without explanation at present. Perhaps the relative concentration of albumin to globulin in chicken serum or the pH or ionic influence of this particular buffer is of significance. Embryonic sera did not behave in this manner.

Summary. The sera from 7 animal species were found to contain globulin components which would function as "antigen" for rheumatoid sera in the latex-fixation test. The effectiveness of these sera as "antigens" depended on removal of albumin by either precipitation or dilution. Nine of 10 human sera behaved similarly. The addition of gelatin to gamma globulin resulted in loss of "antigenicity."

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Elution of Pollen Antigens from Tannic Acid Treated Erythrocytes.* (23396)

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The use of Boyden's technic of adsorbing protein antigens(1) onto tanned red cells allowed several investigators(2-6) to measure antibody levels against pollen antigens in hayfever serum. Orlans *et al.*(2) were able to detect antibodies in the sera of treated cases and of some untreated cases, but not in the sera of non-hayfever persons. Feinberg *et al.* (6) described similar findings, but also showed that the hemagglutinating antibody detected in these hayfever sera is not identical with the reagin type of antibody. All of the previously reported work used a multi-component antigenic extract of various pollens. The present study is concerned with a crude fractionation of the whole saline extract of short ragweed pollen to reduce the number of antigenic specificities, and with the fact that some antigenic components of short ragweed extracts slowly and spontaneously elute from the surface of tanned sheep erythrocytes.

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Materials and methods. Extracts of short ragweed pollen were Seitz filtered and stored as stock antigen at 10°C(6). The whole saline extract containing 116 μg protein N/ml was designated Antigen #1.

An aliquot of the whole short ragweed extract (10 ml) was treated with 4 ml of sterile, cold, 10% trichloroacetic acid (TCA) and allowed to stand at 10°C for one-half hour. The precipitate was removed, washed 3 times with cold 10% TCA, dissolved with pH 7.2 buffered saline, and diluted to the original antigen volume. This was designated Antigen #2.

The TCA non-precipitable supernate was