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Research Article

AGGLUTINATION OF SENSITIZED SHEEP ERYTHROCYTES AND COLLODION PARTICLES BY TUBERCULOUS AND NORMAL SERA [1]

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The agglutination of sensitized erythrocytes by antisera directed against the sensitizing substance was first applied to a study of tuberculous sera by Middlebrook and Dubos (1). Its dependability for diagnosis of the disease was immediately explored by a number of investigators. Scott and Smith (2) reported remarkable specificity of the reaction using a special preparation of Old Tuberculin concentrated to four times standard strength, as did Rothbard, Dooneief and Hite (3). The latter authors state that the test is a valuable diagnostic tool, titers of 1:8 and over being indicative of tuberculous activity. Others (4–6), including Smith and Scott (4) in a second more extensive study, found less correlation between hemagglutination titer and either the presence or the activity of the tuberculous process. The present report is a further attempt to evaluate the usefulness of the hemagglutination test in the detection of specific antibody for the tubercle bacillus or its fractions and to compare results with those obtained using collodion particles instead of sheep erythrocytes as carriers for antigens (7–9).

METHODS

Antigens. Sheep erythrocytes were sensitized with either Old Tuberculin, four times standard strength (2),[2] or with an aqueous extract of strain H37 Rv tubercle bacilli obtained by distilled water refluxing of dried, washed bacilli which had been grown on glycerol beef extract broth and briefly extracted with acetone. The main part of the bacillary debris in the aqueous extract was removed by centrifugation. The remaining opalescent solution, which gave positive color reactions with both Molisch's and Millon's reagents, was used as the antigen. Antigen prepared in this manner was originally used in the complement fixation test for tuberculosis (10). O.T. in our hands was not satisfactory for sensitizing collodion particles since it caused non-specific agglutination of particles; hence, only the aqueous extract was used in collodion particle agglutination tests. Polysaccharide from the Olson strain of tubercle bacillus [3] was found useful in the agglutination-inhibition reaction (1), but neither erythrocytes nor collodion particles treated with this polysaccharide in the concentrations used were agglutinated by human tuberculous sera.

Sera. Blood serum was obtained from (*a*) tuberculous human patients and (*b*) non-tuberculous individuals. The non-tuberculous group consisted of 21 apparently healthy, supposedly normal adults plus seven children who had visited the out-patient department of The Childrens Memorial Hospital for various reasons. Mantoux reactions made two months or more prior to obtaining blood samples were negative in 17 and positive in 11 of these individuals. Tests were performed either within a few days of drawing the blood or else on sera which had been kept frozen. Sera were inactivated at 56° C. for 30 minutes immediately before use. Two-fold serial dilutions beginning with 1:4 were made in either phosphate buffered or unbuffered physiological saline for hemagglutination tests and in 1:100 normal rabbit serum in 1.1% NaCl (11) for collodion particle agglutination tests. Controls consisted of sensitized carrier plus diluent, 1:4 dilution of serum plus unsensitized carrier, and unsensitized carrier plus diluent. Known agglutinating and non-agglutinating human serum controls were set up each time tests were run.

Hemagglutination test technique. Sheep erythrocytes were collected in modified Alsever's solution (10) and washed three times with either physiological saline or buffered saline solution. Adsorption of aqueous extract was in the proportion of 0.1 ml. packed washed red cells to 2 ml. of isotonic antigen. In the case of four-times concentrated O.T., 1 volume of washed, packed sheep cells was adsorbed either with 48 volumes of 1:11 dilution of antigen as described in the report of the Laboratory Committee of the V.A. Central Office Streptomycin Committee (June 2, 1950), or with 60 volumes of 1:15 O.T. as described by Scott and Smith (2). Two-fold

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[2] Lot No. 2725–12, kindly supplied by Dr. Piersma of the Lederle Laboratories.

[3] Supplied through the kindness of Dr. W. J. Nungester, Department of Bacteriology, University of Michigan, Ann Arbor.

serial dilutions of sera previously absorbed with washed unsensitized sheep cells to remove heterophile antibody were set up in 0.4 ml. amounts to which an equal volume of sensitized cells was added. Sensitized cells were first used in 0.5% suspension, later changed to 0.2% to facilitate reading of tests. Readings were made after two hours' incubation in a 37° C. water bath followed by overnight incubation at room temperature. Tubes were read, after noting pattern formed by settling of the cells, following gentle resuspension and the amount of agglutination estimated.

An analysis of hemagglutination test results indicated that in our hands it was not a satisfactory diagnostic test. The hemolytic modification (12) was then used with a number of sera to determine whether or not it would detect tuberculous antibody with greater specificity.

Technique for the hemolytic modification of the hemagglutination test. The hemolytic modification was performed as described by Middlebrook (12). An excess of complement which had been previously absorbed twice with unsensitized sheep erythrocytes at refrigerator temperature was added to each tube of the regular hemagglutination test. Appropriate controls were included. Tests were read for resulting hemolysis after one hour's incubation in a 37° C. water bath.

Collodion particle agglutination technique. Equal portions of antigen and concentrated collodion particles prepared by Cavelti's method (11) were mixed and allowed to stand for 30 to 40 minutes at room temperature, with occasional shaking, and then stored overnight in the refrigerator. Before use, they were diluted with 1:100 normal rabbit serum in 1.1% NaCl solution, centrifuged lightly (1,400 r.p.m.) in an International No. 2 SB centrifuge for five minutes; the supernate was then decanted and centrifuged similarly. Centrifuging in this manner was repeated until the supernate was clear (four to six times). Sediments were resuspended, pooled, and diluted to the density of a No. 2 McFarland standard with 1:100 normal rabbit serum in 1.1% NaCl. Normal rabbit serum in 1.1% NaCl was used following Cavelti's suggestion that it helped eliminate non-specific agglutination of particles by human sera (11). Equal portions of unsensitized particles and three-times distilled water (instead of antigen) were similarly prepared for serum controls for non-specific agglutination.

Equal portions of sensitized collodion particles and serum dilutions were incubated at room temperature for one to two hours. Following centrifugation at low speed (1,400 r.p.m.) for three minutes, preliminary readings were made against a dark background, using a bright light source and a concave mirror for magnification. Final readings were made in a similar manner after tests had stood overnight in the refrigerator.

RESULTS

Hemagglutination and collodion particle agglutination. Results are summarized in Table I. It will be noted that the percentage of high titers (1:8 and over [3] or 1:16 and over [12]) in the tuberculous group was higher than in the non-tuberculous group by both methods and with both antigens. Differences between non-tuberculous and tuberculous sera were greater if 1:16 were accepted as the "significant" titer. The range of titers of tuberculous sera was one two-fold dilution higher than the range with non-tuberculous sera; although this difference was slight, it was consistent with both sensitizing substances and both carriers. Significant differences (*i.e.*, at least a four-fold dilution) in average titers (modal and median) of tuberculous and non-tuberculous sera occurred with aqueous extract-sensitized erythrocytes; there was less difference in average titers of the two groups of sera using O.T.-sensitized red cells and aqueous extract-sensitized collodion particles. The high percentage of non-tuberculous sera which agglutinated both types of sensitized carriers in dilutions of 1:16 and over (28% to 39%), although lower than the percentage of tuberculous sera (54% to 74%), appeared to pre-

TABLE I

Hemagglutination and collodion particle agglutination titers of non-TB and TB sera compared

	Hemagglutination Ag:* Aqueous extract		Hemagglutination Ag:* O. T.		Collodion particles Ag:* Aqueous extract	
	Non-TB† sera	TB sera	Non-TB† sera	TB sera	Non-TB† sera	TB sera
No. of individuals	28	43	21	46	28	43
Median titer reciprocal	4	32	8	16	8	16
Modal titer reciprocal	4	32	0 or 8	16	8	16
Range of titers	0 to 64	0 to 128	0 to 32	0 to 64	0 to 64	0 to 128
Titers 1:8 and over	15 or 53%	35 or 81%	12 or 57%	34 or 73%	18 or 64%	34 or 79%
Titers 1:16 and over	11 or 39%	32 or 74%	6 or 28%	25 or 54%	10 or 35%	28 or 65%

* Ag: = Antigen.
† Non-TB = 17 Mantoux neg. and 11 Mantoux pos. (see Table II).

TABLE II

Non-TB sera: Hemagglutination titers of Mantoux negative and Mantoux positive individuals compared

	Hemagglutination Ag:* Aqueous extract		Hemagglutination Ag:* O. T.		Collodion particles Ag:* Aqueous extract	
	Mantoux neg.†	Mantoux +	Mantoux neg.†	Mantoux +	Mantoux neg.†	Mantoux +
No. of individuals	17	11	11	10	18	10
Median titer reciprocal	4	16	8	4	8	4 to 8
Modal titer reciprocal	4	16	8	0	8	4
Range of titers	0 to 64	0 to 64	0 to 32	0 to 32	0 to 32	0 to 64
Titers 1:8 and over	8 or 47%	7 or 63%	8 or 72%	4 or 40%	13 or 72%	5 or 50%
Titers 1:16 and over	5 or 29.4%	6 or 54%	4 or 36%	2 or 20%	6 or 33%	4 or 40%

* Ag: = Antigen.
† Negative to at least second strength PPD.

clude the use of tests as employed in this work for diagnostic purposes.

Even though correlation of antibody titer and skin reactivity to tuberculin have not previously been observed, it is conceivable that serum of individuals having positive tuberculin skin tests might, as a result of previous infection, contain specific circulating antibody. Lack of correlation between skin reactivity to tuberculin and agglutinating titer, however, is evident in Table II, in which the non-tuberculous sera were separated into two groups: those from positive and those from negative reactors (tested with at least 0.005 mg. PPD). Only with aqueous extract-sensitized erythrocytes was the percentage of "significant" titers (1:16 and over) (12) somewhat lower in the Mantoux negative than the Mantoux positive group. The lowest per cent of titers of 1:16 and over occurred with O.T.-adsorbed erythrocytes (Mantoux positive group); however, it will be noted in Table I that the lowest percentage of high-titered tuberculous sera also occurred with O.T.-sensitized erythrocytes. There is little difference in average titers or range of titers between the two groups of non-tuberculous sera.

Further evidence of failure of correlation of skin reactivity to tuberculin and titer of sensitized particle agglutination was obtained in the following manner. Repeated intracutaneous tuberculin tests were made on five normal, supposedly healthy individuals of the tuberculin negative group after drawing blood for agglutination tests. Successive injections of 1:10,000, 1:1000, 1:100 and 1:10 O.T. were made; as soon as tests with one dilution were read as negative, the next strongest concentration of O.T. was injected.[4] None of the five reacted to 10 mg. O.T. Sera taken before tuberculin tests had the following titers: with O.T.-sensitized red cells, 0, 1:4, 1:8, 1:8, and 1:16; with aqueous extract-sensitized red cells, 0, 1:4, 1:8, 1:16 and 1:32; sensitized collodion particles were agglutinated by dilutions of 1:4, 1:8, 1:16 and 1:32 of these five sera.

Blood serum was again obtained from these five individuals within four to seven days after the last tuberculin injection to determine whether or not repeated injections of tuberculin (four injections in two weeks' time) had resulted in increased titers. Dilutions of serum which agglutinated both O.T.-adsorbed erythrocytes and aqueous extract-adsorbed collodion particles were the same for each individual as before the repeated injections of tuberculin.

Inasmuch as negative skin reactions in children constitute more conclusive evidence than in adults of the absence of tuberculous processes, blood serum was obtained from eight patients under 12 years of age from The Childrens Memorial Hospital. Mantoux tests of seven were negative. Serum from one of the seven agglutinated aqueous extract-adsorbed erythrocytes in a dilution of 1:32. Two sera agglutinated sensitized collodion particles in dilutions of 1:16. Unfortunately the concentrated O.T. was not available at that time.

Titer and disease activity. Results with tuberculous sera were studied for possible correlation of titer with activity of the disease, graphically presented in Figure 1. It is evident that most of the

[4] We are indebted to Dr. Jennings Fershing for the tuberculin injections.

sera from patients with moderate or severe symptoms (3 or 4 activity) had high hemagglutination titers with both antigens and all of these sera agglutinated collodion particles in high dilution. Correlation of less disease activity with low titers is poor, however. Most sera from cases with little disease activity had low titers, but serum from one quiescent case agglutinated both bacillary extract-sensitized red cells and collodion particles in a dilution of 1 : 32 and O.T.-sensitized red cells in a dilution of 1 : 8. Sera from other quiescent cases agglutinated O.T.-sensitized red cells in dilutions as high as 1 : 32. Titers of sera from cases whose activity was classified as 1 (no symptoms but active) had titers ranging from 0 to 1 : 64 with bacillary extract-sensitized cells, from 0 to 1 : 32 with O.T.-sensitized cells and from 0 to 1 : 32 using sensitized collodion particles. Thus the range of titers of sera from active cases with no symptoms was wide.

Collodion particles and red cells sensitized with the aqueous bacillary extract were not always agglutinated by the same dilution of the same serum. Differences in titer of two or more doubled dilutions were noted with approximately 30% of sera tested. Bacillary extract-sensitized cells were in general agglutinated by higher dilutions of serum than were O.T.-sensitized cells.

Hemolytic modification of the hemagglutination test. The apparent lack of specificity of the hemagglutination reaction as herein employed led us to explore Middlebrook's hemolytic modification of the test (12). Both tests were run simultaneously on identical blood samples using the same lot of

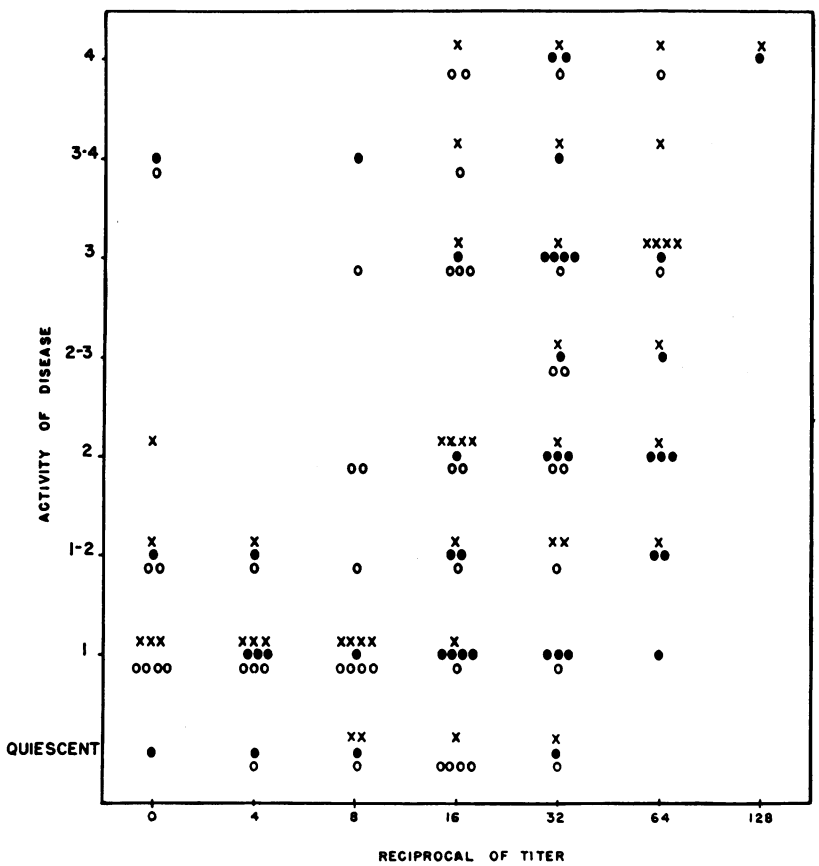

FIG. 1. TITERS OF TUBERCULOUS SERA VERSUS ACTIVITY OF DISEASE
● Hemagglutination: aqueous extract-sensitized sheep cells
○ Hemagglutination: O.T.-sensitized sheep cells
× Collodion particles: aqueous extract-sensitized

TABLE III

Hemagglutination and hemolytic modification titers of non-tuberculous sera

Antigen: Lederle's four-times concentrated O.T.

	Hemolytic modification		Hemagglutination	
	Non-TB sera	TB sera	Non-TB sera	TB sera
No. of individuals	13	21	13	21
Median titer reciprocal	0	128	8	16
Modal titer reciprocal	0	256	8, 16, 32*	16, 32†
Range of titers	0 to 32	0 to 256	0 to 32	0 to 64
Titers 1 : 8 and over	4 or 30%	18 or 85.7%	9 or 69%	19 or 90%
Titers 1 : 16 and over	3 or 23%	18 or 85.7%	6 or 46%	18 or 85%

* Three sera each.
† Eight sera each.

cells sensitized with Lederle's four-times concentrated O.T. Results are presented in Table III. It is evident that titers with the two types of tests do not correspond. In the non-tuberculous group (both tuberculin positive and tuberculin negative individuals), the distribution of hemagglutination titers is quite uniform in the range of 0 to 1 : 32, but distribution of the hemolytic titers tends to be weighted toward low values, which would indicate some improvement in specificity. The percentage of non-tuberculous sera with "significantly" high titers by the hemolytic modification, however, was still too high for the test to be of diagnostic value. The percentage of "significant" hemagglutination titers of both non-tuberculous and tuberculous groups was of the same order as in the first group (Table I), although somewhat higher in this comparatively short series of cases. Hemolytic titers of sera from the tuberculous group were in general higher than those of the hemagglutination test. The tuberculous group was chosen to contain patients with varying stages of disease activity. Symptoms of patients exhibiting high titers were as likely to be quiescent as severe; thus as with the hemagglutination test, no correlation was observed between the degree of activity of the disease and hemolytic titer.

DISCUSSION

Middlebrook (13) repeatedly speaks of the specificity of the hemagglutination reaction if the proper antigen is used for sensitizing red cells. In the hands of Scott and Smith (2) and Rothbard, Dooneief and Hite (3), Lederle's four-times concentrated O.T. appeared to constitute such an antigen. In later work with a larger number of sera, however, Smith and Scott (4) modified their views considerably, stating that the possibility of "biologically false positive or false negative reactions" had not been eliminated. The latter authors also found that sera from some tuberculin negative individuals agglutinated O.T.-sensitized sheep cells. They concluded after this study that the hemagglutination test does not determine the clinical activity of a tuberculous lesion. Results of the test as recorded in the present report support their later view.

Repeated intracutaneous injection of tuberculin in five Mantoux negative reactors failed to raise the agglutinating titer of sera for O.T-treated sheep red blood cells. Failure of correlation of skin reactivity to tuberculin and presence of agglutinins in non-tuberculous individuals is illustrated in Table II. Correlation of skin response to tuberculin and serological titer of antibody has, of course, never been previously observed (14).

Lack of correlation of results of hemagglutination and collodion particle agglutination tests with approximately 30% of sera tested, using the same antigen for sensitizing particles as herein reported, indicates that the two carriers were adsorbing different substances from the complex bacillary extract. Figure 1 suggests that the material adsorbed by collodion particles might have had greater specificity in detecting moderate or severe activity of the tuberculous process than did material adsorbed on sheep erythrocytes. The surface of a collodion particle is chemically more homogeneous than that of a red blood cell. It might therefore be expected that material adsorbed from a complex mixture would be more uniform in nature. However, high collodion particle agglutination titers of sera from quiescent cases and from cases with few symptoms, as well as with sera from healthy individuals, dampens enthusiasm for the test, at least with this antigen, for diagnostic purposes. Results with the hemolytic modification of the hemagglutination test perhaps indicate a trend toward greater specificity but fall far short of results desired in a diagnostic test. Middlebrook (12) has stressed the importance of a suitable antigen for specificity of the hemagglutination test and its hemolytic modification. It would appear that the antigens used in the work herein reported are not sufficiently specific for diagnostic purposes.

Differences between hemagglutination and hemolytic titers of the same sera were also noted by

Middlebrook (12), who discussed, as a reason for failure of correlation, the possibility that the antibodies involved in the two reactions were different in certain sera. Precise 50% hemolysis end-points were not employed for complement dosage in either Middlebrook's work, as he points out, or in the present studies. It is possible that more rigorous control of the dose of complement and other variables might yield somewhat different and possibly more satisfactory results.

Lack of a suitable antigen may be mainly responsible for failure of these tests to differentiate between tuberculous and non-tuberculous sera. Irregularities of titers with tuberculous sera could, for example, be explained by infection with different types of human tubercle bacilli containing different antigens, analogous to types of pneumococci. Keogh, North and Warburton (15) have indicated the polysaccharide nature of antigens from organisms such as *H. influenzae* and certain other bacteria which sensitize red cells for agglutination with specific antisera. Furthermore, Middlebrook's first observation of the hemagglutination reaction with tuberculous sera was obtained with red cells sensitized with a polysaccharide fraction of the tubercle bacillus. Although specific carbohydrates conferring type specificity on tubercle bacilli have not yet been recovered, they may nevertheless exist. It is known that immunization of guinea pigs with one strain confers a degree of immunity for other strains; this fact does not argue against the possible occurrence of type specific carbohydrates, however, inasmuch as the immunizing fraction of the tubercle bacillus is likewise unidentified.

In spite of the questionable feasibility for diagnosis of these reactions as presently employed, the fact that high titered sera (1 : 16 and over) were obtained from tuberculous individuals in significantly higher percentages than from non-tuberculous individuals should probably not be ignored. The natural inference is that the proper antigen has not been used. Two other possible explanations for irregularities present themselves. In the first place, assuming that positive tests detect specific antibody, failure of these as well as other serological tests in the diagnosis of tuberculosis could be accounted for by either qualitative or quantitative peculiarities of antibody for the tubercle bacillus or by its irregular occurrence in a free state in the fluid portion of the blood.

It is also possible that, instead of measuring specific antibody for the tubercle bacillus, these tests reflect rather a general non-specific host response to an irritant. Two abnormal constituents of serum, presumably results of tissue destructive processes, have already been demonstrated, that is, (*a*) the pneumococcus C-reactive protein found in sera from persons in the acute stages of a variety of diseases (16–20) and (*b*) a substance associated with the $alpha_2$ globulin of blood serum which appears to increase in tuberculosis, cancer and other diseases involving tissue destruction (21–23). McCarty points out (17) that C-reactive protein may be only one of several new substances, unrecognized simply for lack of suitable reagents for testing for their presence, to appear in the blood in the course of pathological processes. Serological results herein reported might reflect the occurrence of such a substance in blood serum rather than specific antibody.

SUMMARY AND CONCLUSIONS

Hemagglutination tests using sheep erythrocytes adsorbed with either four-times concentrated O.T. or an aqueous extract of tubercle bacilli, and collodion particle agglutination tests using the latter antigen, were performed on groups of non-tuberculous and tuberculous sera. Using O.T.-sensitized erythrocytes, titers of 1 : 16 and higher were obtained with 28% of 21 non-tuberculous sera and with 54% of 46 tuberculous sera. Using aqueous extract-sensitized red cells, supposedly significant titers were obtained with 39% of 28 non-tuberculous sera and 74% of 43 tuberculous sera and using aqueous extract-sensitized collodion particles, with 35% of the 28 non-tuberculous sera and 65% of the 43 tuberculous sera. There was no correlation between presence or absence of agglutinins in non-tuberculous sera and skin-reactivity to tuberculin.

The hemolytic modification of the hemagglutination test also appeared to be unsatisfactory for diagnosis using four-times concentrated O.T. as antigen. Twenty-three per cent of 13 non-tuberculous sera and 85.7% of tuberculous sera had titers considered significant by other observers.

Moderate and severe symptoms of tuberculosis usually were correlated with high collodion particle agglutination test titers; the correlation of little or no disease activity with low titers was less

consistent. Correlation of disease activity with hemagglutination titers (either antigen) was even less constant than with collodion particles.

Repeated intracutaneous tests of five tuberculin negative supposedly healthy individuals with increasing concentrations of O.T. failed to raise either hemagglutination (O.T.) or collodion particle agglutination (aqueous extract) titers.

Agglutination of aqueous extract-sensitized erythrocytes and collodion particles by the same sera failed to yield the same end point with approximately 30% of the sera tested, indicating adsorption of different fractions of this complex antigen by the two kinds of carriers. Hemagglutination and hemolytic titers, using O.T.-sensitized red cells also differed.

In our hands, none of these tests was sufficiently specific with the antigens employed to be useful in the diagnosis of tuberculosis. The occurrence of high titers in a far higher percentage of tuberculous than non-tuberculous sera, however, is perhaps too significant to be ignored. Whether observed irregularities are due to the use of unsuitable antigens, or to peculiarities of antibody for the tubercle bacillus, or to non-specific constituents of serum as yet unidentified, remains to be determined.

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