Adaptation of the Zymosan Assay of Properdin to Animal Sera. (23575)

JOHN F. KENT,§ ANDRE J. TOUSSAINT,* AND WILLIAM A. HOOK
Department of Serology, Walter Reed Army Institute of Research, Washington, D.C.

The reaction of zymosan with properdin of certain animal species was found to be inhibited in the presence of properdin-free human complement (RP), an essential reagent in the standard assay(1). The present report describes methods for resolving the problem, and provides examples of their applicability.

Reagent standardization and technic. The reagents (RP, R3, and zymosan) were prepared according to standard methods(1). The following technical modifications were introduced, however, to simplify procedure and allow the use of reagents standardized for other purposes; comparative tests showed that they did not affect the outcome of the assay: 1. The diluent(2) for all reagents was a triethanolamine buffered saline solution (TBS) containing divalent cation in concentrations optimal for immune hemolysis. One liter of stock aqueous solution (conc., 10 ×) contained 75.0 g NaCl, 1.0 g $MgCl_2 \cdot 6H_2O$, 0.2 g $CaCl_2 \cdot 2H_2O$, 28.0 ml $N(CH_2CH_2OH)_3$, and 180 ml HCl (1 N). Before use, one volume of stock solution was diluted with nine volumes of distilled water; the molarity of the diluted solution was 0.15, the pH 7.3-7.4 at 20°C. 2. The erythrocyte suspension was prepared from sheep's blood collected at least 5 days earlier in modified Alsever's solution (3). The cells were washed three times with 10-20 volumes of TBS, and the suspension adjusted with TBS to contain 5×10^8 erythrocytes/ml. The initial suspension from a given lot of blood was adjusted on the basis of hemocytometer counts. Lysates of this suspension, however, were subjected to optical density measurements as a basis for standardizing later suspensions from the same lot. Triplicate measurements were made in 12 × 75 mm cuvettes, 0.3 ml aliquants of the cells being lysed each with 1.7 ml distilled water. The optical densities† of the lysates were read at 550 mμ against a water blank, and their mean calculated. Successive suspensions were adjusted with TBS to yield lysates with mean optical densities within plus or minus 1% of that of the initial suspension. Erythrocytes so standardized were sensitized at least 10 min. before use with an equal volume of optimally-diluted(4) hemolytic antiserum. 3. The reaction volume was 0.75 ml, as in the standard assay, but zymosan and test serum were used in the same aliquot volume (0.25 ml) as RP. This was accomplished by standardizing the zymosan suspension to contain the optimal quantity(1) in 0.25 ml, and by using serial 2-fold dilutions rather than decreasing quantities of test serum. The practice had advantages in simplicity and accuracy, and avoided supplementary, volume equalizing additions of TBS. Moreover, it was possible under the conditions to express properdin titer (units/ml) directly in terms of the highest dilution of serum yielding an endpoint reaction.

Adaptation of the assay to animal sera. Method Ia. Precipitation of properdin as euglobulin and reconstitution in human R3. The euglobulin was prepared as in reference (5), the details of preparation being as follows. Measure one volume (2.0 ml) of test serum into a 40 ml centrifuge tube, and immerse in a beaker containing ice and water. While rotating tube add slowly nine volumes (18.0 ml) of 0.0027 N HCl. Stopper with a rubber stopper and let stand at least 30 minutes in the iced-water bath. Sediment the euglobulin by spinning at least 10 minutes at 2000 G in the refrigerated centrifuge (0-2°C). Decant supernate and invert tube on filter paper for draining. Redissolve euglobulin in human R3, introducing initially about 0.5 ml, and using a glass rod to facilitate resolution. Transfer euglobulin solution quantitatively to

* Vet. Admin. Central Lab., Walter Reed Army Inst.

§ Present address: Division of Laboratories and Research, State Dept. of Health, Albany, New York

† The Coleman Jr. Spectrophotometer, Model 6 A, was used with adapter for 12 mm cuvettes.

a 15 ml graduated centrifuge tube, washing the 40 ml tube with successive small volumes (0.2-0.4 ml) of R3 and adding the washings to the 15 ml tube until the volume is brought to 2.0 ml. Remove any undissolved residue by spinning 10 minutes at 1000 G, and decant supernate into a 13 × 100 mm tube. Prepare serial two-fold dilutions in TBS, and add 0.25 ml to a series of 13 × 100 mm tubes containing 0.25 ml each of zymosan suspension and RP. Stopper with rubber stoppers, mix contents, and incubate 1 hour at 37°C in the water bath; shake tubes at 10 minute intervals. Centrifuge for 10 minutes at 1000 G, and decant supernates into 13 × 100 mm tubes. Test for residual C'3 using the simplified standard technic(1). *Method Ib.* Identical with method Ia except that the euglobulin was reconstituted in endpiece(6) of human complement. This alternative was suggested by Dr. L. Pillemer. Human pseudoglobulin is essentially properdin-free and contains only a residuum of C'3. Its components are more nearly complementary to euglobulin than are those of R3, but its use requires the preparation of an additional reagent. Attempts were made at first to reconstitute the euglobulins in media simpler than human R3 or endpiece. Guinea-pig, swine, canine, and chicken euglobulins dissolved in TBS reduced the hemolytic activity of human C' when incubated with the latter (1 hour at 37°C) in the absence of zymosan. Swine and canine euglobulins were anticomplementary also when reconstituted in bovine serum albumin (7%) or heat-inactivated human C'. Human euglobulin was satisfactory in all these media. *Method II. Deferred addition of RP.* Into a series of 13 × 100 mm tubes containing 0.25 ml of zymosan suspension pipette 0.25 ml of serial 2-fold dilutions of test serum. Stopper with rubber stoppers, mix contents, and incubate 1 hour at 37°C in the water bath; shake tubes at 10 minute intervals. After this primary incubation period add 0.25 ml RP to each tube, replace stoppers, mix contents and incubate an additional hour at 37°C, shaking the tubes at 10 minute intervals. Centrifuge 10 minutes at 100 G, and decant supernates into clean 13 × 100 mm tubes. Test for residual C'3 using the simplified standard technic(1).

Results. Results obtained in concurrent properdin assays using the standard (Std) and adapted methods appear in Table I; the examples are representative of the serum species. In tests of human sera, methods Ia and Ib yielded results closely comparable to those of the standard assay. Reproducibility was good, as indicated by duplicate titrations of the same sera. Method II yielded somewhat higher titers than the others, an event which was consistent with the longer period allowed for the properdin-zymosan reaction. In our experience, the standard method failed to yield clear-cut endpoints in titrations of guinea-pig, swine, canine, or chicken sera. Methods Ia, Ib, and II, however, yielded satisfactory results with guinea-pig and swine sera, and method II was applicable in the case of canine sera. None of the adaptations proved effective in titrating chicken sera, and it appeared that properdin of this species might not be demonstrable, at least by zymosan assay.‡ It should be noted in this connection that successful application of the adapted methods required some preselection of reagents as in the standard assay(1); combinations of RP and R3 found satisfactory for one species of animal serum did not necessarily yield acceptable results with another. In evaluating the adaptations, method II might be considered the procedure of choice on the basis of sensitivity and general applicability. However, it is somewhat more time consuming than the others, and it should be noted that C'3 contributed by test serum can be a source of error as in the standard assay; the influence of this component is considerably reduced in methods Ia and Ib.

Summary. The reaction of zymosan with properdin of certain animal species was found to be inhibited in the presence of properdin-free human complement (RP), an essential reagent in the standard assay. The problem was resolved by 1) precipitating the properdin as euglobulin and reconstituting for the assay in properdin-free residues (R3 or endpiece)

‡ The presence of properdin was nevertheless demonstrable in tests(7) for virucidal activity performed by Drs. J. L. Barlow, and H. Van Vunakis of the N. Y. State Health Dept.

TABLE I. Applicability of the Standard (Std) and Adapted (Ia, Ib, II) Methods in Zymosan Assay of Animal Properdin.

Serum		Assay		Hemolysis (%) in the simplified assay with serum diluted 1:						Properdin titer*
Species	Number	Date	Method	1	2	4	8	16	32	(units/ml)
Human	21457–2	7/13/57	Std		0	0	30	50+		4
			Ia		0	0	40	50+		4
			" †		0	0	50	50+		4
			Ib		0	0	45	50+		4
			II		0	0	0	50+		8
"	21457–3	8/ 7	Std		0	0	10	50		6
			Ia		0	0	40	50+		4
			Ib		0	0	15	50+		6
			" †		0	0	15	50		6
			II		0	0	0	40	50+	8
Guinea pig	52357‡	8/ 2	Std		50+	30	15	50+		
			Ia		0	0	0	50+		8
			Ib		0	0	0	50+		8
			II			0	0	10	50+	12
Swine	12557–7	8/ 6	Std		20	10	25	50+		
			Ia		0	0	10	50		6
			Ib		0	0	0	45		8
			II		10	0	0	45		8
			" †		10	0	0	50		8
Canine	73157–10	8/15	Std		50	45	35	15	20	
			Ia		50+	20	5	5	20	
			Ib		50+	50+	5	5	25	
			II			0	0	20	50	12
Chicken	80557‡	8/ 6	Std		50+ at all dilutions (1:1 through 1:32)					
			Ia							
			Ib							
			II							

* The reciprocal of the highest serum dilution causing complete inhibition of hemolysis, or if no more than 20% hemolysis was observed at the next dilution, the mean of their reciprocals.

† Duplicate assay, using a separate euglobulin preparation in the cases of methods Ia and Ib.

‡ Pooled sera of several animals.

of human complement, or 2) deferring the addition of RP until properdin-zymosan complex had formed. The methods were applied successfully in the assay of guinea-pig, swine, and canine properdin, but were ineffective in demonstrating properdin in chicken serum.

We take this occasion to thank Dr. Louis Pillemer and his associates of the Institute of Pathology, Western Reserve University, for the generous contributions in personnel training, material, and advice made since these studies were initiated. Special thanks go also to Drs. H. Van Vunakis and J. L. Barlow of the N. Y. State Health Dept. who performed the tests for virucidal activity on chicken sera, and to Mrs. Patricia Wells and Mr. Earl H. Fife, Jr., of our own department, who provided the standardized erythrocyte suspensions and reagent diluent.

1. Pillemer, L., Blum, L., Lepow, I. H., Wurz, L., and Todd, E. W., *J. Exp. Med.*, 1956, v103, 1.

2. Kent, J. F., Otero, A. G., and Harrigan, R. E., *Am. J. Clin. Path.*, 1957, v27, 539.

3. Bukantz, S. C., Rein, C. R., and Kent, J. F., *J. Lab. and Clin. Med.*, 1946, v31, 394.

4. Kent, J. F., *Science*, 1947, v105, 316.

5. Ericson, J. O., Volkin, E., Craig, H. W., Cooper, G. R., and Neurath, H., *Am. J. Syph. Gonor. and Ven. Dis.*, 1947, v31, 374.

6. Ecker, E. E., Pillemer, L., and Seifter, S., *J. Immunol.*, 1943, v47, 181.

7. Van Vunakis, H., Barlow, J. L., and Levine, L., *Proc. Nat. Acad. Sci.*, 1956, v42, 391.

Received September 9, 1957. P.S.E.B.M., 1957, v96.