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Ammonium Sulfate Coprecipitation Antibody Determination with Purified Staphylococcal Enterotoxins

JACK GRUBER AND GEORGE G. WRIGHT
Medical Sciences Laboratory, Fort Detrick, Frederick, Maryland 21701

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The ammonium sulfate coprecipitation technique of Farr was applied in a study of the purified enterotoxins of *Staphylococcus aureus*. Ammonium sulfate coprecipitation of iodine-131-labeled enterotoxins A, B, and C, with the use of a 1.6 M concentration of $(NH_4)_2SO_4$, revealed differences in the antigen-binding capacity of normal and immune rabbit sera for the enterotoxins. The coprecipitation technique provided a quantitative test for detecting antibody to enterotoxin that was more sensitive than agar-gel diffusion methods. Antigen-binding tests suggested the presence of similar antigenic determinant groups in all three toxins. Measurable antigen-binding capacities for enterotoxins A, B, and C were detected in sera of normal human subjects and became elevated in several subjects accidently exposed to enterotoxin.

The ammonium sulfate coprecipitation technique of Farr (5) provides a quantitative and sensitive measurement of the antigen-binding capacity of antisera. Addition of ammonium sulfate to mixtures of iodine-131-labeled antigen and antiserum precipitates the globulin, and with it the antigen that is bound to antibody; unbound antigen remains in the supernatant fluid. The proportion of labeled antigen precipitated is a function of the antibody activity of the serum. This technique has been used with bovine serum albumin and other serum proteins in the investigation of a variety of immunological phenomena (4, 17, 21, 23; M. M. Wei and A. B. Stavitsky, Bacteriol. Proc., p. 52, 1965).

The Farr procedure is now considered more sensitive than other established techniques for measuring antibody to bovine albumin (14). Despite its sensitivity, the use of this technique with microbial antigens has been limited (7, 8, 11; J. Gruber and G. G. Wright, Fed. Proc., p. 308, 1966) and largely neglected in the study of disease and resistance to infection.

The sensitivity and precision of the ammonium sulfate coprecipitation procedure seemed to warrant its application to staphylococcal enterotoxins. These highly purified antigens involved in the pathogenesis of disease were viewed as excellent model proteins for extending the scope of the Farr technique, and for developing a sensitive, quantitative, and specific method for determination of antibody against enterotoxin.

MATERIALS AND METHODS

Antigens. Staphylococcal enterotoxin B, obtained from *Staphylococcus aureus* strain S6, was kindly supplied by E. J. Schantz (18). This material was purified by chromatography on Amberlite CG-50 resin and carboxymethyl cellulose. It has been characterized as a simple protein with a molecular weight of 35,000. It is a homogeneous material of purity greater than 99%, as indicated by ultracentrifugation, free electrophoresis, and agar-gel diffusion tests. Purified staphylococcal enterotoxins A and C were kindly provided by M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison. The enterotoxin A was obtained from strain 100 and the enterotoxin C from strain 137. These enterotoxins were also estimated to be at least 99% pure (1).

Antisera. The antienterotoxin B reference rabbit antiserum employed (MI-1) was a pool of several sera obtained from five immunized New Zealand rabbits. The antienterotoxoid B rabbit antsera (MCR) were obtained from five rabbits injected with type B enterotoxoids. A pool of four of these sera (MCR-2) was also employed. The specific antienterotoxin B serum (JG-1), used to study both the reproducibility of the test procedure and combination with heterologous enterotoxins, was a pool of 15 sera obtained from immunized rabbits. This serum had a 50% coprecipitation titer of 864 against 1 μg of homologous enterotoxin B per ml. Rabbit antisera to enterotoxins A and C were kindly provided by M. S. Bergdoll.

Gel diffusion precipitation tests. Double-diffusion agar-gel precipitation tests were performed by the method of Ouchterlony (15). Test or reference sera were added to the plates 18 to 24 hr prior to the addi-

tion of soluble antigen, and then all plates were incubated at 5 C for at least 72 hr. Conventional Ouchterlony block titration of MI-1 serum versus purified enterotoxin B indicated that the maximal sensitivity for detection of antibody was obtained when the concentration of enterotoxin was 10 μg/ml. Ouchterlony titers cited are the reciprocals of the highest serum dilutions reacting with the standard enterotoxin concentration. Single-diffusion agar-gel precipitation tests were performed by the technique of Oudin (16), as described by Weirether et al. (24). In the Oudin procedure, test sera were evaluated by comparing the migration rate of enterotoxin B in various serum dilutions to migration in similarly diluted MI-1 reference serum. To avoid negative values, the MI-1 serum was arbitrarily given the value of 100. Oudin block titration of MI-1 serum versus purified enterotoxin B indicated that the maximal sensitivity for detection of antibody was obtained with a toxin concentration of 25 μg/ml. Oudin titers represent the antibody content of sera as compared with the value of 100 for the reference MI-1 serum.

Labeling. Carrier-free iodine-131 was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn. Enterotoxins A, B, and C were iodinated by a microdiffusion procedure previously described (12). Adequate specific activity was obtained by using 1.0 ml of a solution containing 1 or 2 mg of protein per ml, 0.2 ml of 0.002 M KI carrier, and 50 to 100 μc of ^{131}I (as Na ^{131}I). Free iodine was generated by the addition of 0.2 ml of an acidified-dichromate solution (0.014 M $Na_2Cr_2O_7$ in 1.8 N H_2SO_4).

Antibody determination. Antisera for testing were prepared by an initial 1:2.5 dilution of serum, followed by twofold dilutions. All dilutions were made in 0.85% NaCl (saline). To each 0.5-ml sample of diluted serum, 0.5 ml of iodine-labeled antigen was added. Similar control tubes were prepared by use of the same solutions of labeled antigen and saline. After incubation of antigen-antiserum solutions for 30 min, 1 ml of 3.2 M $(NH_4)_2SO_4$ was added to each 1-ml antigen-antiserum or antigen-saline solution and immediately mixed. After 18 hr of incubation at 5 C, the tubes were centrifuged for 30 min at 2,300 rev/min. The nonprecipitated soluble antigen was determined by counts on an accurately measured portion of the supernatant fluid. In most cases, the dilution of serum giving 50% precipitation of the radioactive antigen was determined by graphic interpolation and was taken as the titer. Where indicated, the reciprocal of the highest serum dilution employed giving 50% precipitation of antigen was taken as the titer.

Radioactivity measurements. Radioactivity was measured with a Nuclear-Chicago model 132B analyzer-computer in conjunction with a model DS-302 scintillation well detector containing a 7.6-cm (diameter) sodium iodide crystal. Background radioactivity usually measured less than 50 counts/min.

RESULTS

Studies with enterotoxins A, B, and C. Microdiffusion iodination (12) was employed to label each toxin (1 mg/ml concentration), yielding labeling ratios of 1.7, 1.0, and 1.9 atoms of iodine bound per molecule of enterotoxin A, B, and C, respectively. Gel diffusion precipitation tests indicated full retention of the precipitating activity of the enterotoxins after iodination and dialysis.

Labeled enterotoxin preparations were stable at 5 C; repeated coprecipitation tests with toxin stored for 2 weeks gave data identical to those obtained with freshly prepared material. Furthermore, even after storage for 30 days at 5 C, all of the iodine precipitated with trichloracetic acid. Chromatography by linear gradient elution from carboxymethyl cellulose indicated that enterotoxin B remained homogeneous after iodination.

The solubility of the labeled enterotoxins was determined in various concentrations of ammonium sulfate. The toxins were only slightly soluble in half-saturated (2 M) ammonium sulfate. However, no detectable precipitation occurred in solutions containing 1 μg of toxin per ml and 1.6 M ammonium sulfate.

The effects of incubation time and temperature on the interaction between enterotoxin B and antibody were investigated at intervals ranging from 10 min to 24 hr and at 5 and 25 C. Dilutions of an antiserum pool (MCR-2) were selected to provide approximately 50 and 100% precipitation of enterotoxin. After the appropriate incubation periods, an equal volume of 3.2 M ammonium sulfate was added. The binding of labeled enterotoxin B by antiserum was rapid, resulting in maximal precipitation after 10 min, the shortest interval tested; results were similar at both incubation temperatures.

The effect of antigen concentration on precipitation was determined in serological tests performed with labeled enterotoxin B at concentrations of 10, 2, and 1 μg/ml. At the lower concentrations of antigen, essentially 100% of the toxin was precipitated by the immune serum (Fig. 1). Serum titer was taken as the reciprocal of the serum dilution causing precipitation of 50% of the labeled toxin antigen. As Fig. 1 indicates, serum titers of 16, 60, and 104 were obtained with antigen concentrations of 10, 2, and 1 μg/ml, respectively. As reported previously for the anthrax system (Gruber and Wright, Federation Proc. **25**:308, 1966), the titer of immune serum appeared inversely related to the concentration of enterotoxin B employed.

To study the reproducibility of the test system, a pooled rabbit antiserum (JG-1) prepared against enterotoxin B was assayed on three occasions with two independent assays on each occasion. The serum was tested at 10 dilutions, 1:10 through 1:5,120, and the labeled toxin was used at a concentration of 1 μg/ml. Coprecipitation in all tests was performed with ammonium sulfate at a final

concentration of 1.6 M. The data obtained for the six trials were essentially identical. The 50% precipitation titers ranged from 736 to 992, with a mean value of 864 and a standard deviation of 108.

The capacity of rabbit antisera to combine with radiolabeled enterotoxins A, B, and C was ascertained. The toxins were employed at a final concentration of 1 μg/ml, and coprecipitation was performed with 1.6 M ammonium sulfate. Normal and immune sera differed in their combining capacities for the toxins. The amount of the toxins precipitated by normal sera diluted 1:10 was 25% or less, whereas similarly diluted immune sera precipitated more than 90% of their homologous toxins (Fig. 1–3). With all systems, over a considerable range of dilutions, the proportion of antigen precipitated by immune sera remained markedly greater. The serum-combining capacities for the toxin antigens are presented in Table 1. The antisera to the enterotoxins combined with their homologous toxins to a considerable extent. Both normal and bovine serum albumin immune sera failed to combine with the labeled enterotoxins. The reactions of the anti-B serum (JG-1) with the A and C enterotoxins were appreciable.

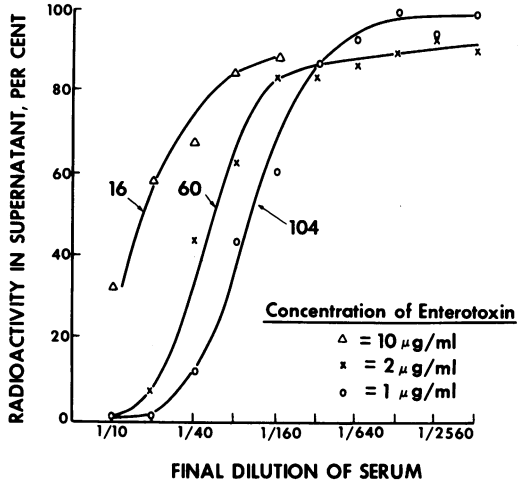

FIG. 1. *Effect of enterotoxin B concentration on coprecipitation with immune serum.*

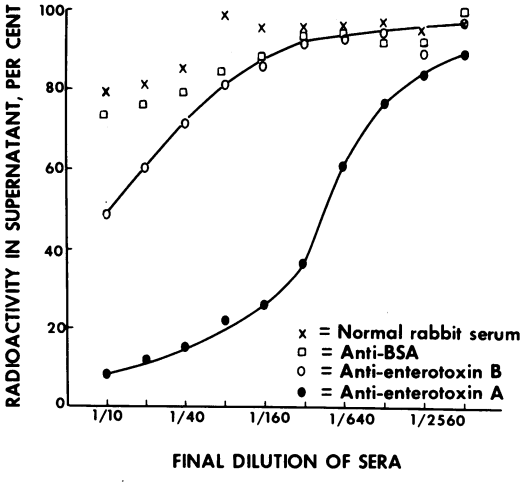

FIG. 2. *Coprecipitation of labeled enterotoxin A and rabbit sera with 1.6 M ammonium sulfate.*

Comparison with the quantitative precipitin test. Precipitin test data and three pools of rabbit antisera to enterotoxin B were kindly provided by our colleague S. J. Silverman. Ammonium sulfate coprecipitation tests were performed with purified enterotoxin B at a final concentration of 1 μg/ml. The results of the coprecipitation tests (Table 2) are suggestive of a correlation between coprecipitin test titer and antibody nitrogen content.

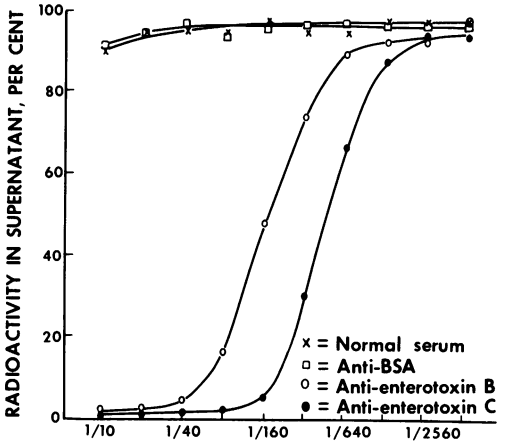

FIG. 3. *Coprecipitation of labeled enterotoxin C and rabbit sera with 1.6 M ammonium sulfate.*

TABLE 1. *Coprecipitating antibody to staphylococcal enterotoxins in rabbit sera*

Serum	50% titer		
	A toxin	B toxin	C toxin
Normal IV	0[a]	0	—[b]
Normal VI	0	0	0
Anti-bovine albumin	0	0	0
Anti-A toxin	480	—	—
Anti-B toxin	10	864	160
Anti-C toxin	—	—	480

[a] Sera diluted 1:10 failed to precipitate 50% of the toxin.

[b] Not done.

Comparison with gel diffusion tests. The sensitivity of coprecipitation serology relative to conventional agar-gel diffusion procedures was determined with antibody to Formalin-treated enterotoxin B. Sera obtained from five immunized rabbits at seven time periods were assayed for coprecipitation antibody to type B toxin with the use of 1.6 M ammonium sulfate for precipitation. The results (Table 3) indicate that the coprecipitation technique was capable of measuring antibody produced in the rabbit after a single injection of Formalin-treated enterotoxin B. Booster injections elicited a secondary response, producing antibody in higher titer which persisted for a longer time.

Ouchterlony and Oudin tests of these sera detected precipitating antibody only in the sera obtained shortly after booster injections. Both of these techniques failed to detect response to the initial injection of antigen, or of the antibody persisting after booster injection. Figure 4 illustrates the relationship between coprecipitation determinations and the gel diffusion results. No serum that was positive by a gel diffusion method was negative by coprecipitation. Of the 16 sera that contained antibody by coprecipitation, six (37.5%) were negative by the gel methods.

TABLE 2. *Quantitative precipitin and ammonium sulfate coprecipitation assays of antienterotoxin B rabbit sera*

Pool of serum	Coprecipitation 50% end point	Antibody N	Ratio
		μg/ml	
I	448	230	1.95
II	480	204	2.35
III	384	196	1.96

TABLE 3. *Detection of staphylococcal enterotoxin B antibody by coprecipitation with labeled toxin*[a]

Status	Antibody titers[b] with rabbit no.				
	3	5	7	10	15
Preinjection	0	0	0	0	0
Postinjection, 1 month	10	20	0	20	0
Postinjection, 3 months	0	0	0	0	0
Postinjection, 5 months	0	0	0	0	0
Postbooster 1, 5 days	80	320	160	160	160
Postbooster 1, 3 months	10	10	0	10	0
Postbooster 2, 9 days	40	320	160	80	160

[a] Iodinated enterotoxin antigen used at a final concentration of 1 μg/ml.

[b] Titer is the reciprocal of the highest serum dilution used causing precipitation of 50% of the radioactive antigen. Zero indicates that a 1:10 dilution of serum failed to precipitate 50% of the antigen.

Studies with human sera. The sera from 53 normal, healthy, human subjects were tested by the coprecipitation technique for antibody to enterotoxin B. All of the sera precipitated measurable amounts of labeled toxin (1 μg/ml concentration). Because the combining power of these sera was low, the titers were expressed as the percentage of antigen precipitated with serum diluted 1:10. The range and distribution of the precipitation values was from a low of 2.8% to a high of 98.8%, with a mean of 33.2% (Fig. 5). More than half of the sera precipitated antigen in the 11 to 20% and 21 to 30% ranges. Of the 53 sera, 20 precipitated more than the mean value, and 8 of these precipitated more than 50% of the toxin.

Four of the normal sera were also tested for antibody to enterotoxins A and C. All precipitated measurable amounts of both the A and C toxins (Table 4). There was no evident relationship among the combining power of the three toxins.

A group of five individuals studying isolation of

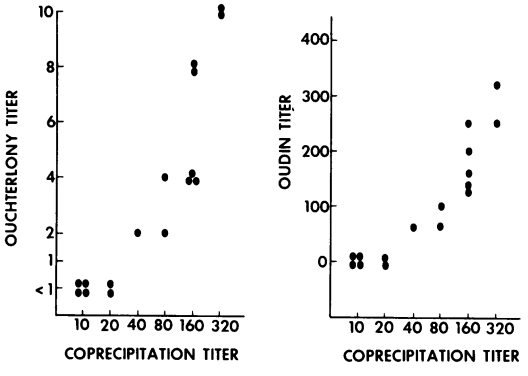

FIG. 4. *Comparison between coprecipitation and gel diffusion titers.*

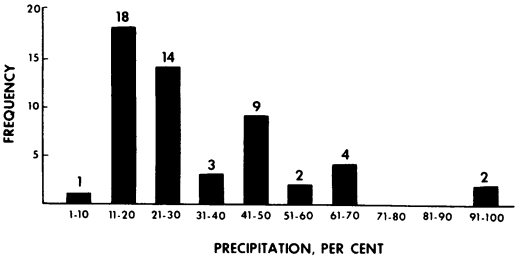

FIG. 5. *Distribution of coprecipitation values obtained with normal human sera.*

enterotoxin B by column chromatography were considered as having been exposed to enterotoxin during normal laboratory activities. The exposure was apparently minimal because the symptoms were limited to lachrymation, conjunctivitis, and periorbital distress. This accident afforded an opportunity to determine the antibody response of human subjects following exposure to enterotoxin B. The exposed individuals were bled at the time of the incident and again 2 weeks later. These sera were designated "acute" and "postexposure," respectively. From an existing serum bank, a serum specimen from each individual (taken at least 2 months prior to the incident) was designated "pre-exposure." Serum antibody to enterotoxin B was measured by coprecipitation serology by use of toxin at a concentration of 1 μg/ml (Table 5). All of the sera precipitated the test antigen to some extent. The sera from three of the subjects (E. L. C., L. L. D., D. P. H.) showed a rise in antigen-binding capacity between the acute and the postexposure samples. Two of these three (L. L. D., D. P. H.), who had the highest pre-exposure antibody, also had the highest postexposure levels. It is noteworthy that these two individuals with high pre-exposure antibody levels exhibited the mildest clinical symptoms with no eyelid or periorbital edema.

TABLE 4. *Antibody to staphylococcal enterotoxins in normal human subjects*

Subject	Precipitation (%)[a]		
	A toxin	B toxin	C toxin
MIK-1	49.7	66.0	21.6
MIK-3	30.2	16.1	10.7
MIK-7	46.3	45.1	14.0
MIK-32	67.1	13.8	—

[a] Sera at 1:10 dilution.

TABLE 5. *Precipitation of labeled staphylococcal enterotoxin B by sera of exposed human subjects*

Subject	Precipitation (%)[a]		
	Pre-exposure sera	Acute sera	Post-exposure sera
E. L. C.	14.1	19.0	28.8
L. L. D.	28.6	23.8	36.6
D. P. H.	24.9	34.6	43.9
R. H.	16.4	14.8	13.5
W. T.	19.7	19.2	16.3

[a] Sera at 1:10 dilution.

DISCUSSION

Application of Farr's procedure to microbial antigens requires careful selection of an appropriate concentration of ammonium sulfate. Farr used solubility in half-saturated ammonium sulfate to separate free from bound antigen (5). Freter (6) and Grey (11), by altering the concentration of ammonium sulfate employed in the procedure, also obtained systems in which unbound test antigen did not precipitate with globulin. Freter used 37% saturated ammonium sulfate with vibrio antigen; Grey used 40% saturation with streptococcal M protein. In the present work, the purified staphylococcal enterotoxins were relatively insoluble at half-saturation, but satisfactory and consistent results were obtained with 1.6 M ammonium sulfate (40% saturation).

Not all globulins are precipitated at this concentration of ammonium sulfate. It is possible that under the conditions employed there were still some antigen-antibody complexes that remained in solution, in which case some portion of the total antibody of sera may not have been detected. Farr demonstrated that 80% of an anti-bovine serum albumin antibody eluate was precipitated by 40% saturated ammonium sulfate (5), and has indicated that the procedure is applicable to antigens soluble at 40% saturation (13). At 40% saturation, a test system was obtained that detected anti-M protein antibody not revealed by the spontaneous precipitin reaction (11). Even at 37% saturated ammonium sulfate, all agglutinin and mouse-protective antibody to vibrio antigen was precipitated (6). It thus appears that any antibody missed by the modified Farr technique would be of a type that is also missed by conventional serological methods.

Few reports have appeared on the in vitro characterization of normal or immune sera with the use of purified enterotoxin as test antigen. In vivo neutralizing antibodies have been demonstrated in the sera of immunized animals (20). These antibodies also have been associated with in vivo precipitation of enterotoxin in agar (2). However, gel diffusion and other serological procedures have been employed primarily for assay of enterotoxin (9, 10, 19).

Silverman (19) assayed culture filtrates for enterotoxin B, and found excellent correlation between the results of gel diffusion precipitation and quantitative precipitin tests. In the present work, a similar relationship was suggested between the results of the quantitative precipitin test and ammonium sulfate coprecipitation. A relationship was also evident between coprecipitation serology and the agar gel diffusion proce-

dures, but the coprecipitation procedure proved to be markedly more sensitive.

This sensitivity of the coprecipitation procedure has been demonstrated previously in a study employing bovine serum albumin (14). The coprecipitation technique was more sensitive than gel diffusion and other serological procedures tested. The present report indicates that the coprecipitation technique was also more sensitive than gel diffusion methods in the detection of antibody to staphylococcal enterotoxins. Low levels of antibody to enterotoxin B could be detected by ammonium sulfate coprecipitation, whereas both Ouchterlony and Oudin techniques failed to do so. Furthermore, with antisera obtained after a booster injection of toxoid, 50% coprecipitation titers could be determined with sera diluted as much as 1:320; detection of antibody in the same serum by the Ouchterlony technique was limited to a dilution of no more than 1:10. Additionally, whereas the titration of enterotoxin B antibody by Ouchterlony and Oudin tests required toxin concentrations of 10 and 25 μg/ml, respectively, the ammonium sulfate coprecipitation test required an antigen concentration no higher than a 1 μg/ml.

Antigenic specificity is the basis for establishing the enterotoxin types (3). The coprecipitation antigen-binding tests with the three enterotoxins provide additional evidence regarding the specificity of the antigenic types. Specific hyperimmune anti-enterotoxin B serum was not as effective in binding either enterotoxin A or C as it was in binding its homologous toxin. However, it is evident that heterologous interactions may also be significant. Thus, the type B antiserum had considerable combining power for type C toxin, and the reaction involved all of the iodinated protein present in the test antigen and not some heterologous toxin contaminating the preparation. The data appear consistent with the concept that similar antigenic determinant groups are present on the three toxins. Similar heterologous combining reactions have been described in other systems (11, 22).

The sera of all of the normal human subjects tested contained measurable antigen-binding capacity for purified enterotoxin B. That this coprecipitating antibody is specific in nature for enterotoxin B is indicated by the markedly different precipitation values obtained when several of these sera were also tested with enterotoxins A and C. The presence of these antibodies may indicate some natural exposure to all three enterotoxins.

The fact that the coprecipitation procedure detected antibody in all of the human subjects surveyed indicates that this serological technique can be useful in following humoral response to enterotoxin stimulation. The data obtained with the accidently exposed human subjects further support this view. At every test period, each of the subjects' sera demonstrated some antigen-binding capacity. Three of the five subjects examined presented clear evidence of a rise in coprecipitating antibody following exposure. Additionally, the relationship between clinical symptoms and titer of pre-exposure antibody is the sort that would be expected if the antigen-binding capacity of serum was involved in host protection.

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