Enhancement of Canine Isohemagglutinin A by a Heat Labile Serum Factor, Probably Complement.* (20180)

FREDERICK STOHLMAN, JR.† (Introduced by T. Koppanyi.)

From the Hematology Laboratory and the Department of Medicine, Georgetown University Hospital and the University School of Medicine, Washington, D.C.

The demonstration by Young and his colleagues(1) of a variety of isohemagglutinins in the dog, which in many respects mimic those observed in humans, has facilitated *in vivo* investigation of immune hemolysis, heretofore limited by the inability to study adequately such reactions in human subjects. Sporadic reports of canine isoantibodies have appeared in the literature in the past and are adequately reviewed by Hamilton(2) and Young(1). Young and his co-workers(3) have reported 6 canine agglutinogens A, B, C, D, E, and F. These do not correspond to human agglutinogens of similar designation. The incidence of these agglutinogens and their corresponding agglutinins are summarized by Christian(4) and Young(3). Hemolytic reactions following the transfusions of incompatible blood or plasma and hemolytic disease of the newborn have been produced readily with anti-A but have not been reported with other canine isoantibodies(5).

The A agglutinogen or its variant A′ has been found in 63% of a large group of random dogs and has been demonstrated to be strongly antigenic, in most instances producing high titre anti A within 8 to 21 days(3,4). *In vitro* anti-A acts as a specific hemolysin as well as an agglutinin; is thermostable; most active at 37°C; fixes complement; and sensitizes red cells for the antiglobulin reaction(4,14). An apparently unique characteristic of canine anti-A is the enhancement of its agglutinative capacity by a heat labile factor in normal dog serum. Moreover, fixation of complement in normal fresh canine sera by an antigen-antibody precipitate destroys the capacity of such sera to enhance the antibody. The addition of guinea pig complement, however, does not restore this activity(4). Likewise human and bovine albumin have been ineffective(4). Initial studies on the nature of this heat labile factor in normal dog serum are the subject of this report.

Methods and material. Of the anti-A sera used throughout this study, 5, (6, 16, 22, 65 and 75) were produced in this laboratory by multiple small transfusions (5 cc) of A cells into A negative dogs. A sixth sera (49A) was kindly supplied by Drs. L. E. Young and W. A. O'Brien of the University of Rochester. Anti-dog serum rabbit serum for use in the antiglobulin test (Coombs) was prepared as described by Christian *et al.*(4). Pooled human, guinea pig sera (from this laboratory) and lyophilized commercial guinea pig serum (lyocomplement, Sharp and Dohme) were absorbed with pooled canine cells prior to use. Fresh bovine sera‡ were absorbed 3 times at 4°C with pooled A cells prior to use. Sera from 12 steer as well as a pool of bovine sera (from 6 steer) were prepared. Thirty per cent commercial albumin and 25% human albumin (National Red Cross) were used as indicated. Complement and its component parts were inactivated as follows: C′1, 2, by heating at 56°C for 30′; C′3 by an insoluble, carbohydrate fraction of yeast, zymosan, as described by Pillemer(6); C′4 was inactivated by incubation at 25°C with .15 N NH_3, as cited by Kabat and Mayer(7). Mid-piece (C′1 and 3) and end-piece (C′2 and 4) were separated by dilution with 10 volumes of M/200 KH_2PO_4 at 4°C. The precipitate thus formed was redissolved in 5 volumes of 0.9% NaCl and the pH adjusted to 7.3 with .1 N $NaHCO_3$ the supernatant was made isotonic with 10% NaCl and pH adjusted to 7.3 with N $NaHCO_3$. The final dilution of mid-piece was 1:5 and end-piece was 1:12. Alternatively,

* Supported in part by a grant-in-aid from the National Institutes of Health of the Public Health Service.

† Postdoctorate Research Fellow of the National Institutes of Health.

‡ Obtained through the generosity of William Schluderberg and Joseph Lochte of Schluderberg and Kurdle Co. (Esskay), Baltimore, Md.

separation was produced by dialysis against 4 liters of distilled H_2O at 4°C, or phosphate buffer of pH 5.4 and ionic strength .02(7). Dialysis was continued over an 8-hour period with 2 changes of the bath. An increase in dialyzing surface was obtained by the insertion of a pyrex tube within the cellophane dialysis bag as suggested by Bier(8). Following the dialysis the precipitate was washed twice with phosphate buffer and restitution of pH and tonicity was effected as in the dilution method. Final dilutions were 1:10 for end-piece and 1:5 for mid-piece. Since results obtained with both methods were identical, no distinction is made in the text. Complement fixation was obtained by the use of an antigen-antibody precipitate using anti-dog-rabbit serum and dog serum. Alternatively, fixation was produced with anti-bovine dog sera from an immunized animal and bovine cells. Complement fixation was carried out at 4°C. Sera were checked for residual C′ with a sheep cell indicator prior to use. All antisera were inactivated at 56° for 30′ prior to use and dilutions were made with saline§ throughout. Preliminary studies indicated that there were no significant differences between titres obtained with heated antisera diluted with saline and those using heated antisera and fresh normal dog serum as a diluent, provided that fresh serum is subsequently added to the system. Christian *et al.* reported a similar experience (4). Immune sera were stored at –20°C when not used the day of collection as was absorbed human, guinea pig and bovine sera. Canine A blood was collected without anticoagulant, the serum separated and the cells washed from the clot. Prior to use all cells were washed three times in 0.9% NaCl and resuspended in sufficient buffered saline to make approximately a 4% suspension. Titrations were performed using a total volume of 0.2 cc consisting of .05 cc of antisera; .05 cc of saline suspended A cells; .05 cc of each of two substances to be tested. When only one substance was tested the additional .05 cc consisted of buffered saline. All titrations were incubated at 37°C for 15′ and centrifuged at 300 G for 30″. Antiglobulin tests (Coombs) were performed as described by Christian(4). The degree of hemolysis is indicated by numerals 0-4; where 4 is complete hemolysis, 3 marked hemolysis, 2 moderate hemolysis and 1 slight hemolysis. Agglutination is indicated by +'s with ++++, a solid clump of cells; +++, large clumps of cells; ++ medium clumps of cells; + small clumps of cells giving a granular appearance; ± microscopic agglutination without visible macroscopic agglutination.

Results. Preliminary studies of the characteristics of anti-A, including observations on thermal range, hemolytic activity, complement fixation, sensitization for antiglobulin reaction, and serum enhancement, were in agreement with those reported by Christian(4). It was noted that high titre anti-A sera were capable of producing agglutination in the first dilutions in the absence of the serum factor. Maximal agglutination and hemolysis were lacking in such systems. Weak anti-A sera were incapable of agglutinating A cells in the absence of the enhancing factor. It also was demonstrated that there was a wide individual variation amongst A cells both in agglutinative capacity and susceptibility to hemolysis. Consequently, Christian(4) has subdivided the A agglutinogens into two groups, A and a weak variant A′. We have observed, as did Christian, a broad spectrum of activity within each group. These variants are due to differences in the agglutinogens rather than in content of serum enhancing factor as is shown by similar hemolytic and agglutinating titres obtained with homologous as well as autogenous sera, an example of which appears in Table I.

The most unique characteristic of canine anti-A is the enhancement by fresh canine sera. Table I demonstrates that this effect differs from the usual incomplete or univalent isohemagglutinin. The latter generally manifest a similar degree of activity in fresh or heated serum, albumin and albumin-serum mixtures. There is, however, a marked reduction in the activity of canine anti-A when heated serum or saline replaces fresh serum in the reaction. The addition of human or bovine

§ Following demonstration of the importance of divalent cation in the reaction, studies were repeated using a veronal-bicarbonate buffered saline with optimal Mg^{++} and Ca^{++} added(9). Unless otherwise indicated, saline in this paper refers to buffered saline with added Mg^{++} and Ca^{++}.

TABLE I. Comparison of Titres of Anti A Serum against A Cells in Presence of Fresh Serum with Those Obtained in Saline, Heated Serum, Albumin and Serum-Albumin Mixtures.

Reagent 1	Reagent 2	Final dilution of anti A serum (49A) 4	8	16	32	64	128	256	512
U. A. S.*	Saline	4	4	3+	2+++	++++	++	±	—
U. H. S.	"	4	4	3+	2+++	+++	++	±	—
U. A. S.	"	++	+	±	—	—	—	—	—
B. Al.	"	++	++	+	—	—	—	—	—
Hu. Al.	"	++	++	±	—	—	—	—	—
U. H. S.	B. Al.	4	3++	1++++	++++	+++	+	—	—
H. A. S.	"	+++	++	+	±	—	—	—	—
Saline	Saline	++	+	—	—	—	—	—	—

* U. = unheated; A. = autogenous; S. = serum; H. = heated; B. Al. = bovine albumin; Hu. Al. = human albumin.

Degree of hemolysis is expressed numerically (4–); agglutination by +'s.

albumin-serum mixtures to such reactions has failed to produce significant enhancement in our experience.

The observations, that inactivation of fresh sera by heating at 56° for 30′ or fixation of complement destroys the enhancing effect of fresh serum, suggest that complement is involved. Accordingly, the effect of inactivation of specific fractions of complement, separation of mid-piece and end-piece, and complement fixation were studied. A typical protocol is shown in Table II. It may be noted that such procedures resulted in complete loss of enhancing effect. It is difficult to establish with certainty from these preliminary data whether C′ or one or more of its fractions is required for this reaction, since the method of inactivation while specifically destroying one fraction may also significantly reduce other components(7). It seems likely however, that the entire C′ rather than one or more of its components is necessary for the manifestation of maximum canine anti-A activity.

TABLE II. Effect of Inactivation of Complement (C′), Its Components and the Binding of MG^{++} on the Reaction of Anti A and A Cells.

Reagent 1	Reagent 2	Final anti A (#22) titre†
Unheated S.*	Saline	256
Heated S.	"	16
NH_3 inactivated S.	"	8
Yeast inactivated S.	"	8
Mid-piece	"	4
End-piece	"	8
C′ fixed S.	"	8
Unheated S.	.15M citrate	16

* S = serum.

† Inactivated sera—1-2 + agglutination at 1:2; unheated serum, complete hemolysis at 1:8; partial hemolysis at 1:32; ++++ agglutination at 1:64.

Further evidence of the role of complement in this reaction is provided by the effect of Mg^{++}, a divalent cation essential for optimum complement activity(10). Binding Mg^{++} by a .15 Molar concentration of citrate effectively reduce the activity of anti-A sera (Table II). Restoration of free Mg^{++} by the addition of excess Mg^{++} as $MgCl_2$ restores this activity. It has also been shown that removal of Mg^{++} by dialysis, binding with phosphate or the sodium salt of ethylene diamine tetracetic acid, a chelating agent, at pH 7.1 will influence the anti-A reaction in a similar fashion. Moreover, if an excess of Mg^{++} is introduced into fresh serum there will be a reduction in activity, although not of the magnitude noted with binding of Mg^{++}. These observations are in keeping with those of Mayer *et al.* on the effect of the magnesium ion in the complement reaction(10).

A variety of animal sera have been studied to observe whether fresh heterospecific sera are capable of producing maximum anti-A activity. Extensive studies have been carried out on absorbed guinea pig complement and human sera. These sera, while capable of producing hemolysis in the lower dilutions of anti-A, do not demonstrate the agglutinative activity noted with fresh canine sera. Limited observations on rabbit, horse and swine sera have thus far been negative. In bovine sera, which lack lytic activity with most antigen-antibody systems(11,12) but have been shown to have varying degrees of conglutinating complement as well as conglutinin(13), the agglutinative capacity of anti-A is equivalent to that observed in fresh canine sera.

TABLE III. Effect of Bovine Serum on the Enhancement of Canine Anti A.

Serum added*	Final anti (#6) titre
Unheated canine	256§
Unheated B.†	512‖
Saline	4¶
B. serum‡	256‖
Heated B.	8¶
NH_3 inactivated B.	8¶
Yeast inactivated B.	8¶

* Each tube contained .05 cc anti A dilution; .05 cc saline, and .05 cc serum.

† B = bovine serum.

‡ Unheated bovine serum added to washed cells from saline titration in above row.

§ Complete hemolysis at 1:8; partial hemolysis at 1:64; 4 + agglutination at 1:64.

‖ 4 + agglutination — at 1:128, hemolysis absent in all dilutions.

¶ Maximum agglutination was 1 — 2 + at 1:2.

TABLE IV. Effect of Fresh Canine, Bovine and Antiglobulin Sera on the Reaction of Anti A and A′ Cells.

Serum added	Final titre anti A (#6)
Unheated canine	4*
Antiglobulin	128†
Unheated bovine	4*
Antiglobulin	128†

Antiglobulin serum was added to washed cells from row immediately above it.

* 1+ agglutination — no hemolysis.

† 4+ agglutination at 1:8; 3+ at 1:32.

Pooled absorbed sera and ten individual samples have yielded similar results when tested against six different anti-A sera. A typical protocol is presented in Table III. One further sample of fresh bovine serum was ineffective when added to the reaction alone, but when end-piece or heat inactivated dog sera was added, typical enhancement was noted. Another serum failed to demonstrate the enhancing effect even in the presence of heated canine serum. The agglutination of A′ canine cells in bovine sera is similar to that observed with fresh dog sera and weaker than with antiglobulin serum (Table IV). Inactivation of bovine sera by heat, zymosan or NH_3 or binding of Mg^{++} results in loss of enhancement activity (Table III). It could be demonstrated, with these two exceptions, that bovine sera contained all the factors necessary for enhancement by incubating inactivated sera and A cells in a saline titration, washing 3 times and resuspending in bovine sera with results as depicted in Table III. This indicated that the heat labile enhancing substance is not involved in the union of antigen-antibody but rather in a physical manifestation of this union. This concept is supported by the effect of antiglobulin(14). If anti-A titrations are made in saline or inactivated sera, weak agglutination results. When the cells from the above are washed and absorbed anti-dog serum rabbit serum (Coombs) is introduced, agglutination equivalent to or greater than that observed with fresh serum occurs (Table V) indicating that the cells were sensitized in the absence of complement.

Discussion. Enhancement of activity by a heat labile component of serum is apparently unique to canine anti-A amongst erythrocyte isoantibodies. Data have been presented in an effort to clarify this phenomenon. It has been demonstrated that fixation of complement or inactivation of its component parts results in a decrease in anti-A activity. Moreover, the concentration of magnesium, a cation essential for complement activity, influences this reaction. Guinea pig, human, and in limited studies, rabbit, equine and swine sera, did not manifest the enhancing activity noted in canine sera, although, with the exception of equine sera, hemolysis was increased in an otherwise inactivated system. Bovine serum, although hemolysis is not present, does contain a heat labile component capable of restoring maximal agglutination. This would suggest that the canine anti-A reaction requires complement and may be analogous to the conglutinin phenomenon recently reviewed by

TABLE V. Demonstration by Antiglobulin Technic of Sensitization of A Cells by Anti A Sera in Presence of Inactivated Complement.

Treatment of reagent sera	Titre of anti A sera (dog #6 GT) Initial	Antiglobulin
Fresh sera	256	1024
Saline	16	512
Heated	16	512
NH_3 treated	8	512
Yeast treated	8	512
Mid-piece	16	512
End-piece	16	512

Cells from initial titration washed 3 times in saline and subsequently incubated with antiglobulin serum.

Hole and Coombs(15). This reaction differs from the albumin-serum effect on incomplete Rh antibodies erroneously designated conglutination by Wiener(16), which does not require fresh serum or complement. It also differs from the Donath-Landsteiner reaction where C'4 is necessary for union of antibody and antigen(17). In the canine anti-A system sensitization of cells occurs in the presence of inactivated sera as may be demonstrated by the subsequent addition of antiglobulin (Coombs) sera or fresh sera to the sensitized cells.

The possibility that this serum effect is conglutination raises many questions. Streng (18) was unable to demonstrate conglutinin in canine sera using a conventional sheep cell system. Many investigators have failed to detect conglutinating as well as hemolytic complement in bovine sera. Most of these observations were carried out using a sheep cell-anti-sheep cell indicator. Rice(19) however, has recently demonstrated that complements and conglutinins are not mutually interchangeable and will vary in reactivity with the type of indicator used. Moreover, she has detected both hemolytic and conglutinative complement in bovine sera if anti-rabbit cell sheep serum is used as antibody and rabbit cells as antigen in the indicator system(19). Although deficiencies might exist in individual animals, it would certainly seem unreasonable to assume that one or more species lack such a ubiquitous protein complex as complement. It seems more likely that with the use of other antigen-antibody complexes, complement and conglutinin may be demonstrated in species, which heretofore, have been considered devoid of these substances. Accordingly, quantitative studies are being conducted on reactivation of dog complement and the interchangeability of complement fractions and conglutinin of various species utilizing the canine anti-A system.

Summary. Data have been presented suggesting that the enhancing effect of a heat labile component of canine serum on the agglutinative capacity of the canine isohemagglutinin A is due to complement. Although guinea pig and human serum are ineffective, bovine serum, in most instances, will restore maximum activity to a previously inactivated system. Further data suggest that this enhancement, which appears to be unique so far as erythrocyte isoantibodies are concerned, is related to conglutination.

The author gratefully acknowledges the advice and encouragement of Drs. Lawrence E. Young and William A. O'Brien of the University of Rochester, who also supplied us with various antisera, Dr. John F. Kent, of the Army Medical Graduate School, who reviewed in part the manuscript, and Dr. Charles E. Rath of Georgetown University whose cooperation made this study possible.

1. Young, L. E., Ervin, D. M., and Yuile, C. L., *Blood,* 1949, v4, 1218.
2. Hamilton, A. S., *J. Physiol.,* 1949, v154, 525.
3. Young, L. E., O'Brien, W. A., Miller, G., Swisher, S. N., Ervin, D. M., Christian, R. M., and Yuile, C. L., *Trans. N. Y. Acad. Sc.,* 1951, v13, 201.
4. Christian, R. M., Ervin, D. M., and Young, L. E., *J. Immunol.,* 1951, v63, 37.
5. Young, L. E., Christian, R. M., Ervin, D. M., Swisher, S. N., O'Brien, W. A., Stewart, W. B., and Yuile, C. L., *Proc. Intern. Soc. Hemat.,* 1950, 226.
6. Pillemer, L., and Ecker, E. E., *J. Biol. Chem.,* 1941, v137, 139.
7. Kabat, E. A., and Mayer, M. M., *Exp. Immunochemistry,* 1948, Charles C. Thomas and Co.
8. Bier, O. G., Leyton, G., Mayer, M. M., and Heidelberger, M., *J. Exp. Med.,* 1945, v81, 499.
9. Mayer, M. M., Croft, C. C., Gray, M. M., *J. Exp. Med.,* 1948, v88, 427.
10. Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.,* 1946, v84, 535.
11. Rice, C. E., *Canad. Comp. Med.,* 1950, v14, 369.
12. Rice, C. E., and Crowson, C. N., *J. Immunol.,* 1950, v65, 201.
13. Rice, C. E., *J. Immunol.,* 1950, v65, 499.
14. Coombs, R. R. A., Mourant, A. E., and Race, R. R., *Brit. J. Exp. Path.,* 1945, v26, 255.
15. Hole, N. H., and Coombs, R. R. A., *J. Hygiene,* 1947, v45, 480.
16. Wiener, A. S., *J. Lab. Clin. Med.,* 1945, v30, 662.
17. Jordan, W. S., Jr., Pillemer, L., and Dingle, J. H., *J. Clin. Invest.,* 1951, v30, 11.
18. Streng, O., *Z. f. Immunitatsf. u Exp. Therap.,* 1909, v2, 415.
19. Rice, C. E., and Boulanger, P., *J. Immun.,* 1952, v68, 197.

Received February 11, 1953. P.S.E.B.M., 1953, v82.