Cold Hemagglutinin Associated with Leptospiral Hemolytic Anemia in the Sheep[1] (35165)

M. J. FREEMAN, R. L. MORTER, AND M. J. DECKER
(Introduced by E. V. Morse)
Department of Veterinary Microbiology, Pathology and Public Health, Purdue University, Lafayette, Indiana 47907

Leptospirosis is a zoonotic disease which affects many species of animals as well as man. In ruminants, *Leptospira pomona* is frequently the etiologic agent. The clinical signs of infection in sheep include fever, icterus, and a hemolytic anemia. Several investigations have failed to elucidate conclusively the mechanism of this anemia (1–4). The detection of a transiently occurring cold-hemagglutinin (CHAn) associated with the hemolytic crisis in the plasma of experimentally infected anemic sheep, has recently been reported (5). Some characteristics of this CHAn are reported here.

Materials and Methods. A total of 15 sheep free of lepotospiral agglutinins was used in three separate experiments. All sheep were housed in isolation units, provided with hay and a complete pelleted ration and water *ad libitum*. One noninfected control was maintained in contact with the infected sheep in each experiment. The sheep were infected with *Leptospira pomona* by subcutaneous inoculation with 2.5 ml of a 10% emulsion, in 0.85% NaCl solution, of liver and spleen obtained from leptospiremic, moribund hamsters.

The microscopic agglutination test employing live *L. pomona* as antigen, using 7- to 10-day cultures in Stuarts' fluid medium (Difco Laboratories, Detroit, Mich.) with 10% rabbit serum, was used for the detection of leptospiral agglutinins (6) in the serum of the experimental sheep. Rectal temperatures and packed RBC volumes and hemoglobin values were determined daily to define the development of the infection and the anemia which has been described in detail elsewhere (5).

For the isolation of CHAn, large volumes of blood were obtained from normal control sheep or experimentally infected sheep during the hemolytic crisis in either Alsever's (7), ACD (8) or citrate (1 vol of 0.5 *M* Na citrate for 5 vol of blood) anticoagulant solution. Plasma was separated at 25° and its globulin fraction concentrated by precipitation at 40% saturation with ammonium sulfate. The precipitated globulins were dissolved in a minimal volume of water and dialyzed against 0.85% NaCl solution until free of detectable SO_4^{-2}.

To absorb cold-reactive hemagglutinins, autologous, preinoculation erythrocytes (RBC) were added to the concentrated globulin solution and the suspension was incubated at 4°. The RBC were then sedimented and washed at 4° with 0.85% NaCl solution until the wash was as free as possible of hemoglobin or other proteins. The washed RBC were then resuspended in 0.85% NaCl solution and the temperature raised to and maintained at 25° to elute the CHAn. The final eluate from the RBC was concentrated with 40% ammonium sulfate as described above.

Cold hemagglutinin activity was demonstrated by the microtiter method (9) by reaction of twofold serial dilutions of 0.05 ml of serum or purified CHAn, in 0.85% NaCl solution, with an equal volume of a 0.25% suspension of autologous preinoculation RBC. Separate titration series were incubated at 25 and 4°. After several hours of settling, titers were recorded as the reciprocal of the last dilution for which a positive settling pat-

[1] Supported in part by U.S. Public Health Service Research Grant AI 04029-07. Submitted as journal paper 3763, Agricultural Experiment Station, Purdue University.

tern (an even mat of cells) was observed.

The antigenic properties of CHAn preparations were characterized using immunodiffusion (10) and immunoelectrophoretic (11) assays. Sucrose density gradient ultracentrifugation (12) was used to estimate the molecular size of the CHAn. The preparation of ovine immunoglobulins used for immunoprecipitin tests has been described (13). Antisera were prepared by repeated immunization of rabbits with ovine serum or partially purified IgM and CHAn preparations in conjunction with complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Developing antisera, as necessary, were absorbed by repeated stepwise additions of small amounts of chromatographically purified, ovine IgG_1 and IgG_2 or hemoglobin (Pentex Inc., Kankakee, Illinois) to remove antibody reactive with the antigenic determinants of those proteins.

The effects of heat (56 and 65° for 60 min), 2-mercaptoethanol (0.1 *M* for 16 hr at 25°), 10 cycles of freezing and thawing and storage at low temperature (8, −20, and −70°) for short and long terms on the CHAn activity were also determined.

Results and Discussion. Experimental infection was varified by the detection of leptospiral agglutinins in the sera of 11 of 15 exposed sheep. One sheep died and three sheep in the last experiment were exsanguinated before agglutinins appeared. The severity of the hemolytic anemia of the affected sheep was indicated by the fact that the packed cell volumes of 12 of the 15 febrile, experimental sheep decreased rapidly by a factor of one-fifth or more, relative to their preinoculation levels.

Initially, CHAn were detected in low titer (2 to 4) in the unconcentrated plasma of one sheep in the first experiment reported here. Although cold hemagglutinins were not subsequently detected in the unconcentrated plasma of the other anemic sheep, CHAn were detected in the plasma of all anemic sheep after RBC absorption and elution and ammonium sulfate concentration of the eluate. The titers of CHAn recovered ranged from 1:1 to 1:8 after a hundredfold concentration. The failure to detect CHAn in the one hundredfold concentrated globulin fractions of plasma from uninfected, nonanemic control sheep suggested a role for CHAn in the genesis of the anemia.

However, the relative amounts of CHAn activity isolated in individual experiments cannot be readily compared to the severity of the anemia or be used to estimate the total plasma level of CHAn or the efficiency of the isolation procedures themselves, since minor modifications in procedures were introduced in various experiments to determine the most efficient and convenient methods for purification. Although the amounts of CHAn appear to be low, these may nevertheless be of considerable biologic significance in view of the facts that only a single molecule of IgM antibody may be required to lyse a single RBC and that antibody may recycle and initiate the lysis of additional RBC (14).

The existence of three distinct components in the concentrated eluate from the RBC was indicated by immunoelectrophoretic analysis of CHAn preparations using a broad spectrum anti-bovine whole serum (Fig. 1). By analogy and positional characteristics, these lines were identified presumptively as IgG, IgM, and hemoglobin, Hb, (Fig. 1A, B, C). These tentative designations were confirmed by absorption of the developing antiserum with IgG_2 and Hb which abolished or diminished the intensity of the lines designated IgG and Hb, respectively. The third line, presumptively identified as IgM, was unaffected by absorption with either IgG_2 or Hb. Since insufficient ovine IgM was available to absorb the developing serum, an indirect method of identification was used. Development of CHAn with a specific anti-IgM serum (which developed only a single, IgM, line in whole ovine serum) resulted in the development of only a single short precipitin arc, characteristic of IgM, near the origin of migration (Fig. 1D2).

The tentative identification of the components of the CHAn preparation was substantiated by additional immunoelectrophoretic tests using an antiserum to CHAn which had been prepared by immunizing a rabbit with the original, partially purified CHAn preparation (Fig. 1D1). Absorption of this

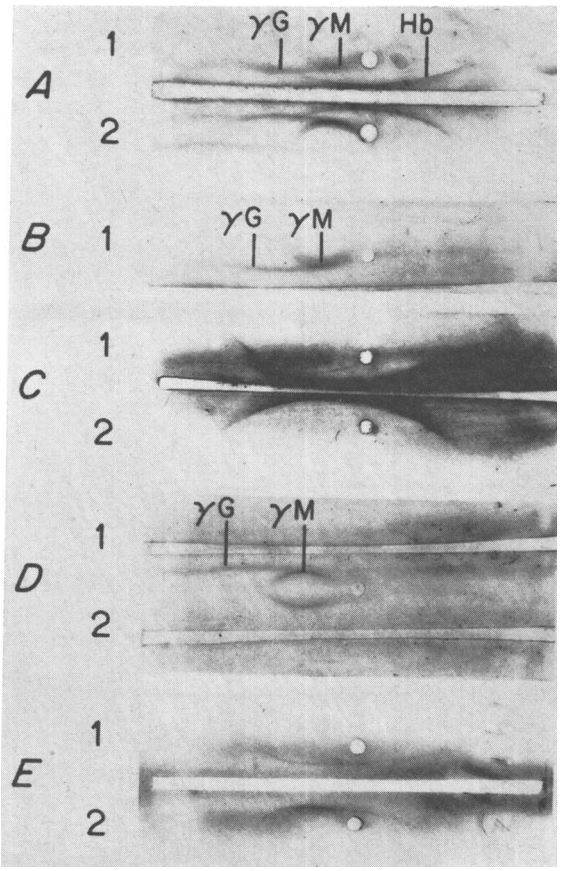

FIG. 1. Immunoelectrophoretic analysis of cold hemagglutinin (CHAn) preparations: (slides A and B) Three different CHAn preparations developed with a broad spectrum rabbit anti-ovine normal serum. Although differing in concentration of individual components, preparations (A1) and (A2) were similar in composition. Both contained γG and γM globulins and hemoglobin (Hb) precipitin arcs which were identified on the basis of their position and by diminution of the arcs after absorption with the appropriate purified protein. Preparation (B) was one in which less hemolysis occurred during purification of the CHAn than for (A1) and (A2) and only γG and γM were detected. (slide C1) Another CHAn preparation; and (2) purified ovine hemoglobin (Hb) developed with rabbit anti-ovine normal serum. A faint IgM line may be seen in (1) beneath the heavy Hb line. The amount of hemoglobin in this particular preparation was high. (slide D) Cold hemagglutinin (CHAn) preparation (center well) developed with (1) antiserum to CHAn and (2) an anti-γM serum. Arcs like γG and γM and γM are seen in (1) and (2), respectively. (slide E) Reaction of anti-CHAn serum with normal ovine serum (1) and a CHAn preparation (2) after absorption of the developing serum with γG and hemoglobin. The γG arc in both preparations is greatly diminished.

anti-CHAn serum with ovine IgG_2 and Hb inhibited the development of both the IgG and Hb arcs when either whole ovine serum or CHAn preparations were tested (Fig. 1E2). From these immunoelectrophoretic tests it was concluded that the remaining line was IgM. The failure of the anti-CHAn serum to develop any additional lines (other than IgG and IgM) in the several CHAn preparations which were tested or in ovine serum itself suggested a limited antigenic heterogeneity of the CHAn eluates themselves (Fig. 1A–1D). This assumption would, of course, depend upon whether any other components in the CHAn preparation used for preparation of the anti-CHAn serum were

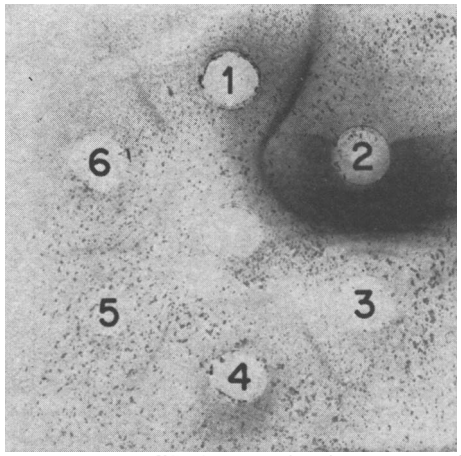

FIG. 2. Immunodiffusion reaction between a cold-hemagglutinin (CHAn) preparation (center well), purified ovine γM (well 1) and antiserum to normal ovine serum (well 2). Normal ovine serum (wells 3 and 5) and a monospecific anti-γM serum (wells 4 and 6) were used to fill the remaining wells.

functionally as immunogenic as the three proteins to which the immunized rabbit actually made antibodies.

Three components (or lines of precipitation) were also detected by immunodiffusion assay when the anti-CHAn serum described above was used to test several CHAn preparations. One of these lines coalesced with ovine IgG. Another coalesced completely with a purified preparation of ovine IgM (Fig. 2.1). A third precipitin line, developed only with anti-CHAn serum, could not be identified. The development of this third precipitin line was unaffected by absorption of the anti-CHAn serum with hemoglobin, sheep RBC lysate, intact sheep RBC, or a preparation of leptospiral antigens prepared by sonic disruption of whole leptospiral cells. Since antisera to whole ovine serum, ovine IgA, ovine IgM, and several antisera to whole ovine serum, ovine IgA, ovine IgM, and several antisera to various leptospiral antigens also failed to develop the third precipitin line, its identity has remained unestablished.

The significance of the multiple components detected in some CHAn preparations is not entirely clear since the CHAn preparation from one experiment in which collection of plasma in Na citrate inhibited the massive hemolysis experienced previously had only a single line when tested with anti-ovine whole sera. This suggested that some of the multiple components in the other CHAn preparations may have been merely products of the continued cell lysis which occurred during the washing process. Unfortunately, only small amounts of CHAn were recovered in the latter experiment, perhaps as a result of the inhibitory action of the citrate. However, the single precipitin arc which was observed coalesced with the line of precipitation formed by a purified preparation of ovine IgM and anti-ovine whole serum (Fig. 2). Although anti-IgM serum reacted with both the IgM and CHAn preparations, the concentrations of antigens and the strength of the developing serum were not sufficient to obtain readily visible coalescence patterns. Nevertheless, the bulk of the other evidence obtained strongly favors identification of the CHAn as an IgM antibody.

The effects of physical and chemical treatments on the serologic activity of CHAn were also consistent with the properties of many IgM proteins. For example, mercaptoethanol reduction or heating at 65° for 1 hr destroyed CHAn activity. Although storage at 8° or lower temperatures for 1 week or 10-times repeated freezing and thawing had no effect, the activity of the purified CHAn preparations was diminished upon storage at −20° over a period of 6 to 8 weeks.

The CHAn activity sedimented (Fig. 3) more rapidly than hemoglobin or a 7S marker (^{125}I human γ-globulin) by sucrose gradient ultracentrifugation, but did not sediment as rapidly as would have been expected for a 19S protein (15).

Despite the strongly suggestive immunochemical data and the supporting physical and chemical data, the identification of CHAn as an IgM protein can only be regarded as tentative in the presence of multiple proteins in some preparations of CHAn and the limited characterization so far possible. The presence of hemoglobin, is, of course, readily explicable and might be eliminated in subsequent experiments by the use of erythrocyte ghosts instead of intact RBC for absorption during the isolation of CHAn. The

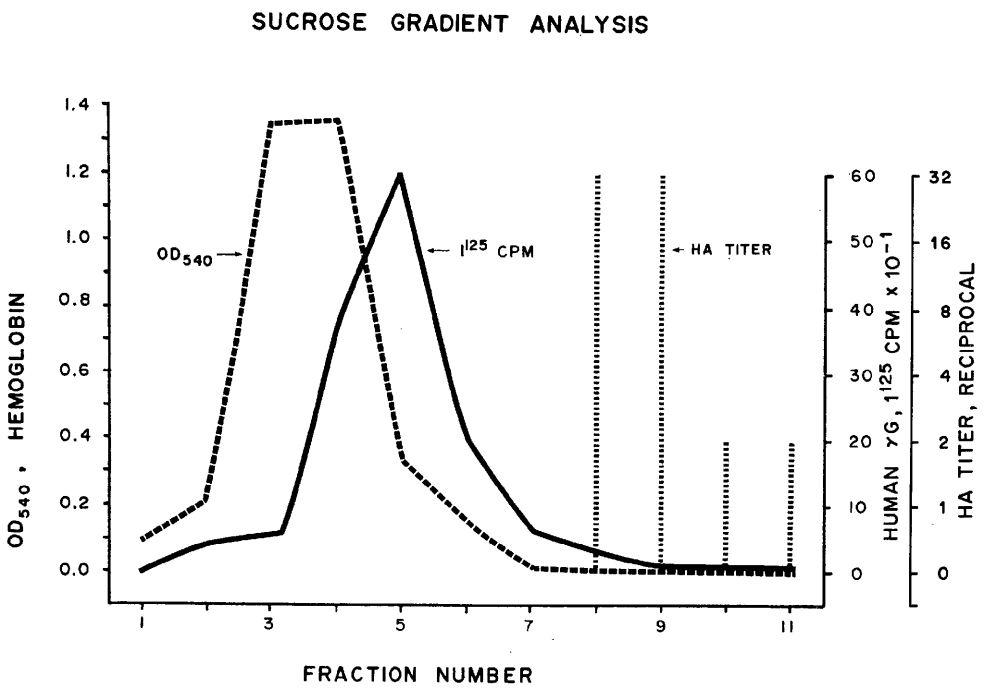

FIG. 3. Sucrose density gradient separation of cold hemagglutinin activity: the sedimentation position of the cold hemagglutinin (striped bar); contaminating hemoglobin (expressed as OD_{540}) (- - -); and a marker of ^{125}I labeled 7S human γ-globulin (—). The direction of sedimentation was to the right.

IgG detected may simply have represented nonspecific contamination. On the other hand, it may actually have represented the occurrence of hemagglutinin activity in the IgG as well as the IgM class of ovine immunoglobulins. However, the failure to observe CHAn activity in the 7S region of the gradient argues against this interpretation.

Since abundant precedent exists in human medicine for the role of a CHAn in immunologically-mediated, hemolytic disease (17), the discovery of CHAn as a consistent feature of leptospirosis suggested that CHAn may be involved in the pathogenesis of leptospiral hemolytic anemia. Although CHAn occur in several human diseases or syndromes, the most consistent association of CHAn with a specific infectious disease has been in primary atypical pneumonia. In one recent study (17), 70% of affected individuals had CHAn, which appeared during the febrile phase of the infection and diminished rapidly thereafter. A similar pattern has been observed following experimentally induced leptospiral infection (5). However, wide variations in the occurrence and persistence of CHAn associated with other hemolytic anemias have been recognized (16).

Since the optimal reaction between CHAn and RBC occurs at a temperature much lower than that of the body, a paradoxical situation may appear to be posed for any postulated role for a CHAn as a mechanism of any biologic significance in the genesis of anemia in the intact animal. However, although CHAn react optimally *in vitro* at low temperatures, CHAn may effect hemolysis or other erythrocyte alterations at *in vivo* temperatures (18). Unfortunately, the mechanism by which erythrocyte-reactive antibodies may effect erythrocyte destruction and clinical hemolytic anemia are complex and unclear.

If the CHAn recognized in the present investigation can be conclusively demonstrated to have a functional role in the genesis of the acquired hemolytic anemia, experimental leptospirosis would offer a useful and unique

system for the study of an interesting immunologically-mediated disease.

1. Bauer, D. C., Eames, L. N., Sleight, S. D., and Ferguson, L. C., J. Infec. Dis. **108**, 229 (1961).

2. Ferguson, L. C., Ramge, J. C., and Sanger, V. L., Amer. J. Vet. Res. **18**, 43 (1968).

3. Kanich, R. F., Hopps, H. C., and Stauch, J. E., Antibiot. News **1968**, 7.

4. Morter, R. L., Proc. Soc. Exp. Biol. Med. **106**, 600 (1961).

5. Decker, M. J., Freeman, M. J., and Morter, R. L., Amer. J. Vet. Res. **31**, 873 (1970).

6. Morse, E. V., Morter, R. L., Longham, R. F., Lundberg, A., and Ulney, D. E., J. Infec. Dis. **101**, 129 (1957).

7. Kabat, E. A., and Mayer, M. M., "Experimental Immunochemistry," 2 ed., p. 149. Thomas, Springfield, Ill. (1961).

8. "Pharmacopeia of the United States of America," 15 ed., p. 650 (1955).

9. Sever, J. L., J. Immunol. **88**, 32 (1962).

10. Ouchterlony, O., Progr. Allergy **6**, 30 (1962).

11. Scheidegger, J. J., Int. Arch. Allergy Appl. Immunol. **7**, 103 (1955).

12. Edelman, G. M., Kunkel, H. G., and Franklin, E. C., J. Exp. Med. **108**, 105 (1958).

13. Pan, I. C., Kaplan, A. M., Morter, R. L., and Freeman, M. J., Proc. Soc. Exp. Biol. Med. **129**, 867 (1968).

14. Borsos, T., and Rapp, H. J., Science **150**, 505 (1965).

15. Freeman, M. J., Immunology **15**, 481 (1968).

16. De Grauchy, G. C., "Clinical Hematology in Medical Practice," 2 ed., p. 254. Davis, Philadelphia (1964).

17. Krech, U., Pathol. Microbiol. **29**, 558 (1966).

18. Swisher, S. N., *in* "Mechanisms of Anemia" (I. M. Weinstein, ed.), p. 271. McGraw-Hill, New York (1962).

Received Oct. 24, 1969. P.S.E.B.M., 1970, Vol. 135.