Equine Infectious Anemia: Preparation of a Liquid Antigen Extract for the Agar-gel Immunodiffusion and Complement-fixation Tests

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SUMMARY

An agar-gel immunodiffusion test recommended for the diagnosis of equine infectious anemia was evaluated. Our preliminary observations confirmed those of Coggins concerning the mechanism of the test and the results obtained. Furthermore, emphasis was put on the difficulties encountered in the production of spleen antigens with an optimum amount of reactivity. Acetone-ether extraction procedures for the preparation of a liquid antigen extract are described. This type of antigen was reactive in the complement-fixation test in 1:8 or greater dilution and it is proposed to use the complement-fixation test in assessing and standardizing the liquid antigen extract activity to be used in the immunodiffusion test. This antigen can also be concentrated or diluted, if required, to meet the reactivity of a standard antigen used in the test.

RÉSUMÉ

Les auteurs ont évalué l'épreuve de l'immunodiffusion dans l'agar, tel que recommandé pour le diagnostic de l'anémie infectieuse des chevaux. Leurs observations ont confirmé celles de Coggins en ce qui concerne le méchanisme de l'épreuve et les résultats obtenus. De plus, il ont étudié plus particulièrement les difficultés rencontrées dans la production, à partir de rates infectées, d'antigènes possédant une réactivité optimum. Ils décrivent des méthodes d'extraction à base d'acétone-éther pour la préparation d'un antigène liquide. Ce type d'antigène a réagi dans l'épreuve de la fixation du complément lorsque dilué 1:8 ou plus. Ils se proposent d'employer l'épreuve de la fixation du complément pour titrer et standardiser la réactivité de l'antigène liquide destiné à être employé dans l'épreuve de l'immunodiffusion. Cet antigène peut être concentré ou dilué, lorsque requis, pour obtenir une réactivité comparable à l'antigène standard employé dans l'épreuve.

INTRODUCTION

The infectious nature of equine infectious anemia (EIA) was demonstrated in France in 1859 by Anginiard and cited in the monograph by Goret *et al* (5). In 1902, in Canada, Torrance (6) studied a disease which appeared similar to the one observed in Europe. In 1904, Carré and Vallée (3) were successful in transmitting the infection with blood and tissue fluid filtrates passed through porcelain filters; consequently it was concluded that a virus was the cause of the infection.

Until recently, no single *in vitro* test for the diagnosis has been generally recognized as proof for this infection. The final diagnosis was based on the inoculation of suspected material into susceptible horses. In 1970, Coggins and Norcross (4) recommended an agar-gel immunodiffusion (AGI) test which detected antibodies in experimentally infected ponies as early as 18 days and for more than three years after inoculation. In their test the antigen consisted of finely minced spleen collected

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from ponies acutely infected with equine infectious anemia virus.

The present report will summarize our preliminary evaluation of the AGI test and will describe the preparation of a reactive liquid antigen extract which can be standardized for use in the AGI test. Its activity can also be titrated by the complement-fixation (CF) tests.

MATERIALS AND METHODS

Source of Virus

The Wyoming EIA virus in the form of infective serum (YO-157) obtained from Dr. L. Coggins, Cornell University, Ithaca, New York, U.S.A., was used in the inoculation of the horses for the production of infected spleens. In addition blood or serum from three proven Canadian field cases of EIA, one chronic (D-9187) and two acute (E-3169; F-8696), were also used for experimental transmission studies.

Experimental Animals

Horses and ponies were used in our experimental studies. They were bought from individual owners or from commercial horse dealers. Before being used in the experiment they were kept in isolation under observation. Their temperature was taken daily and they were proven normal, at first, by animal cross-inoculation with blood from another animal in the same group. Eventually, after the development of the AGI test, this test was used to assess their health status instead of animal inoculation.

Agar-Gel Immunodiffusion Test

The method described by Coggins and Norcross (4) was adopted and in summary was applied as follows: The test was performed in plastic Petri dishes of 100 mm diameter. On the day of the test, a first layer of 5 ml of 2% Noble's special agar[1] in a borate buffer, pH 8.6, was poured to seal the bottom of the plastic dish. After solidification of the agar, a second layer of 15 ml of 1% similar agar was added as diffusion media. Four sets of seven wells were punched in the top layer of the agar using a specially designed agar gel punch. Each set consisted of seven holes, 7 mm in diameter, placed at 3 mm from each other and from the central well. The antigen was placed in the central well and the positive reference serum in wells 1 and 4. Wells 2, 3, 5 and 6 contained the sera under test (Fig. 1). The tests were incubated at 25°C for 48 hours in a humid chamber. The reading was performed while holding the agar plate over a beam of light. Special attention was paid to the characteristics of the precipitation lines as to whether they were curved, diffused, dissolved or non-specific.

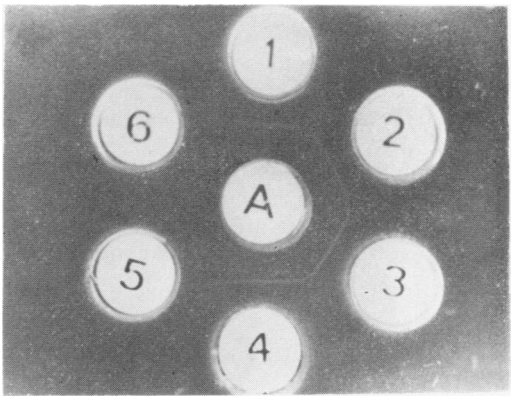

Fig. 1. Wells 1 and 4. Positive reference sera giving sharp precipitation lines.
Wells 2 and 3. Positive test sera giving sharp precipitation lines forming identity with the reference antiserum lines.
Wells 5 and 6. Negative test sera giving no precipitation.
Well A. Contains the spleen antigen.

The radial immunodiffusion test was performed as above with the exception that the reference antiserum was incorporated in the 1% agar layer in an appropriate final dilution (1:10 final dilution for serum 980). The diameter of the wells containing the antigens under test and their distance from each other were the same as described above. The incubation of the test and the reading of the reactions were also performed in a similar way.

Comflement-Fixation Test

The standard direct type of complement-fixation test was used in the present studies. Except for the antigen which will be described later, the technique employed has been summarized in a previous publication (2).

[1]Noble's special agar, Difco Laboratories Inc., Detroit 1, Michigan, U.S.A.

PRODUCTION OF SPLEEN ANTIGEN

Twenty-four ponies and/or horses were inoculated with the Wyoming virus in the form of infective serum or subpassages as indicated in Tables I and IA. The sources and amount of inoculum are indicated in the Table together with the results. The reactivity of the various antigens was compared with a reference spleen antigen obtained from Dr. L. Coggins.

EXTRACTION OF THE ANTIGEN IN A LIQUID STATE

Long extraction procedure — The method used was based on a procedure described before (1) and applied as follows: 10 grams of spleen pulp were blended in a Virtis homogenizer with approximately 200 ml of chilled acetone. After two hrs of shaking in a mechanical shaker placed in a refrigerator at a temperature of approximately 9°C, the ground tissue was centrifuged at 1000 x g for 10 min and resuspended in the same volume of chilled acetone. This procedure was repeated once with acetone, then with an equal mixture of acetone and anhydrous ether, then twice with anhydrous ether only. All extractions were made, as much as possible, in the cold room. For convenience the acetone-ether mixture extraction was done for an overnight period instead of the usual two

TABLE I. Inoculation of Horses for Production of Spleen Antigens

Recipients						Donors	
Horse No.	Temp. Rise >103° at D. P. I.	No. of Days T. >104°	Peak Temp. at D. P. I.[c]	Killed at D. P. I.	Reaction[a]	Horse No. and Inoculum	Days of Temp. >103°
H- 6-70	7	9	106.1°-14	died-16	−	WYO 157 5 ml. ser.	?
H- 7-70	3	3	107.0°-8	9	++	H-6-70 100 ml.bl.[b]	1st.
H- 9-70	10	9	107.0°-11	died-22	−	H-6-70 30 ml.bl.	1st.
H-10-70	3	6	105.6°-19	died-22	−	H-6-70 30 ml. bl.	1st.
H-11-70	7	4	106.2°-8	10	+++	H-6-70 30 ml. bl.	1st.
H-12-70	8	4	105.6°-8	12	++	H-6-70 30 ml.bl.	1st.
H-13-70	7	6	106.0°-8	12	−	H-6-70 25 ml.ser.	2nd.
H-14-70	9	6	105.6°-10	14	−	H-6-70 30 ml.ser.	1st., 7th.
H-15-70	7	2	104.8°-9	10	+	H-14-70 100 ml. + 50 ml.bl.[b]	1st., 2nd.
H-16-70	5	3	105.4°-9	10	++	H-14-70 100 ml. + 50 ml.bl.[b]	1st., 2nd.
H- 1-71	5	2	105.0°-6	8	+	H-16-70 100 ml.bl.[b]	5th.
H- 3-71	6	3	106.8°-9	10	++	H-11-70 100 ml. ser.	4th.

[a] ++ or +++ = Spleens containing enough antigenic reactivity to be usable in the test
+ or − = Spleens not usable in the test; because of too little antigenic content
[b] = The donor was infected by direct blood transfusion
[c] = D.P.I. means day post-infection; bl., means blood and ser., stands serum

hrs at 9°C. After the final extraction, the material was centrifuged, the supernatant discarded and the residual ether evaporated by agitation with a glass rod until the sticky splenic material appeared as a dry powder. The dry powder was re-suspended in 20 ml of physiological saline and agitated overnight at approximately 9°C in a mechanical shaker. The following morning the material was frozen and thawed three to five times and again shaken overnight in the cold room. The next morning the suspension was centrifuged at approximately 8000 x g to remove the tissue particles. Approximately 10 to 15 ml of liquid antigen was obtained from 10 grams of original splenic tissue extracted. The liquid extracted antigen was stored frozen or lyophilized.

Short extraction procedure — In addition to this long extraction procedure we have investigated a short extraction method. This simplified procedure consisted in homogenizing 10 grams of spleen pulp in 200 ml of chilled acetone-ether mixture. After two to four hrs shaking in a mechanical shaker placed in the refrigerator at a temperature of approximately 9°C the ground tissue was centrifuged at 1000 x g for 10 min, the supernatant discarded and the residual acetone-ether mixture evaporated by agitation with a glass rod until the sticky splenic material appeared as a dry powder. The dry powder was

TABLE IA. Inoculation of Horses for Production of Spleen Antigens

	Recipients					Donors
Horse No.	Temp. Rise >103° at D. P. I.	No. of Days T. >104°	Peak Temp. at D. P. I.[c]	Killed at D. P. I.	Reaction[a]	Horse No. and Inoculum	Days of Temp. >103°
H- 4-71	7	2	105.0°-7	10	−	H-11-70 100 ml. ser. 4th.	
H- 5-71	9	11	106.7°-10	22	−	H-11-70 100 ml. ser. 4th.	
H- 6-71	6	3	106.0°-8	10	+	H-11-70 100 ml. ser. 4th.	
H- 7-71	6	5	105.9°-9	10	+	H-11-70 100 ml. ser. 4th.	
H- 8-71	7	2	105.7°-8	9	++	H-4-71 100 ml. ser. 1st.	
H- 9-71	4	3	105.0°-7	9	+	H-5-71 100 ml.bl.[b] 2nd.	
H-10-71	5	3	105.5°-8	9	±	H-5-71 100 ml.bl.[b] 2nd.	
H-12-71	7	2	104.8°-12	died-13	++	H-9-71 100 ml.bl.[b] 1st.	
H-13-71	5	3	105.4°-7	9	−	H-8-71 100 ml.bl.[b] 1st.	
H-15-71	?	?	?	10	+	H-13-71 100 ml.bl.[b] 2nd.	
H-16-71	5	4	105.8°-7	9	++	H-11-70 15 ml.ser. 4th.	
H-17-71	3	6	106.4°-5	8	++	H-11-70 15 ml. ser. 4th.	

[a] ++ or +++ = Spleens containing enough antigenic reactivity to be usable in the test
+ or − = Spleens not usable in the test because of too little antigenic content
[b] = The donor was infected by direct blood transfusion
[c] = D.P.I. means days post-infection; bl., means blood and ser., stands for serum

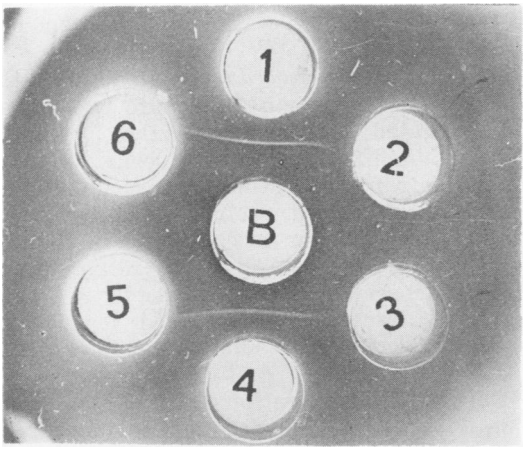

Fig. 2. Wells 1 and 4. Positive reference sera giving sharp precipitation lines.
Wells 2 and 3. Positive test sera with a low antibody content causing a curve at the corresponding end of the reference antiserum lines.
Wells 5 and 6. Negative test sera giving no precipitation.
Well B. Contains the spleen antigen.

resuspended in 20 ml of physiological saline and agitated overnight at approximately 9°C in a mechanical shaker. The following morning the material was frozen and thawed twice, shaken for one hour at 9°C and centrifuged at approximately 8000 x g to remove the tissue particles. The liquid antigen extract was stored frozen or lyophilized.

Source of Serum Antibodies

The reference serum antibodies (311-Sept. 70) needed in the test to evaluate the identity of the precipitation line was at first obtained from Dr. L. Coggins. Eventually the serum from a field case of EIA (980) giving a typical, sharp identity precipitation line, comparable with the reference serum of Coggins, was used as a control in the test.

In order to evaluate the sensitivity and specificity of the test the sera collected by serial bleeding from ten known negative horses which were infected experimentally in 1966-67 as indicated in Table II, were tested for the presence of antibodies.

RESULTS

Agar-Gel Immunodiffusion Test

Our preliminary observations confirmed those of Coggins concerning the mechanism of the test and the results obtained. When we incorporated in the test an optimally adjusted antigen-reference-antiserum combination, the reaction of the test sera appeared as sharp precipitation lines forming an identity with the lines of other reference antisera (Fig. 1). When the amount of antibodies in the serum under test was low, the reaction appeared as a deviation of the end of the reference serum precipitation line toward the central well containing the antigen (Fig. 2). However, when the antigen-reference-antiserum combination was not in optimum proportion i.e. when the reaction occurred in a

TABLE II. Results of the Coggins' Agar-gel Immunodiffusion Test on Sera of Serial Bleeding from Ten Experimentally Infected Horses

Identification of Horses	Inoculum	Negative Reactions		Positive Reactions	
		No. of Bleeding	Time of Bleeding	No. of Bleeding	Time of Bleeding
			(days)		(days)
H-3-66	10 ml bl.[a] I.V. from D-9137	1	Pre.	2	27 to 54
H-4-66	90 ml bl. I.V. from E-3169	2	4, 5	3	37 to 96
H-2-67	20 ml bl. I.V. from D-9137	3	Pre. to 11	35	18 to 296
H-3-67	20 ml ser. I.V. from H-4-66	5	Pre. to 11	12	16 to 60
H-10-67	10 ml bl. S.Q. from E-3169	2	Pre. 16	3	23 to 43
H-11-67	10 ml bl. S.Q. from E-3169	1	Pre.	9	39 to 105
H-12-67	10 ml ser.[a] S.Q. from E-3169	2	Pre. 11	5	18 to 45
H-17-67	135 ml ser.[b] S.Q. from H-4-66	3	6 to 17	3	25 to 39
H-21-67	10 ml bl. S.Q. from E-3169	2	Pre. 11	4	18 to 56
H-22-67	10 ml spl.[a] S.Q. from F-8696	1	15	44	20 to 288
		22	Pre. to 17	120	16 to 296

[a] = Bl.: blood; ser.: serum; spl.: spleen emulsion 1:10 dilution
[b] = This serum was treated with TTE (1, 1, 2- Trichlorotrifluorethane)

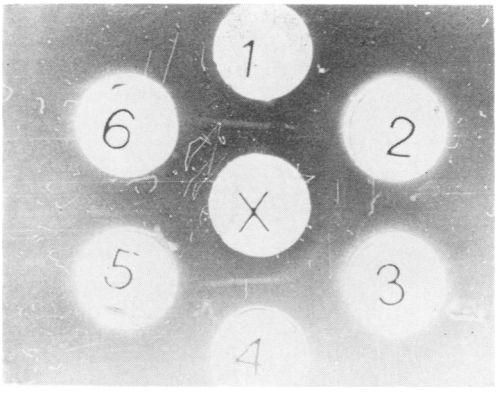

Fig. 3. Wells 1 and 4. Positive reference sera giving sharp precipitation lines.
Wells 2 and 3. Positive test sera with a high antibody content giving diffused or dissolved precipitation lines forming identity with the reference antiserum lines.
Wells 5 and 6. Negative test sera giving no precipitation.
Well X. Contains the spleen antigen.

zone of moderate antibody excess, the reaction appeared as a broad, diffused precipitation line (Fig. 3). In certain cases, this diffuse precipitation line appeared to dissolve and the corresponding reference precipitation line was shorter by one third and even one half of its normal length. The broad diffuse precipitation reaction had to be differentiated from the non specific reactions, i.e., precipitation reactions due to antigens other than EIA agent. Such reactions might be due to other infectious agents or to iso-antibodies. In this latter case, they could be removed by absorption of the test serum with normal splenic tissue powder. However from a practical point of view it was important to recognize and differentiate the non-EIA reactions. They did not give a line of identity with the reference precipitation line. They either crossed the reference control line or failed to join the control line smoothly as they continued on into the test serum well to form a spur (Fig. 4).

Production of Spleen Antigen

Coggins and Norcross in their publication (4) mentioned, that for antigen preparation it is important to select animals that reacted severely within the first week after infection and to harvest the spleen after three to five days of high temperature. Out of 24 horses or ponies inoculated as indicated in Tables I and IA, the spleen of nine animals contained enough antigenic activity to be used in the AGI test. However no single criterion was reliable in predicting the antigenic activity of the spleen. In fact even though the reactive spleens were obtained from animals having a temperature above 104°F for three to six days and which were killed on the ninth or tenth day after infection, the spleen of animals (H-6-71; H-7-71; H-9-71; H-10-71; H-13-71) with this type of clinical manifestations were of low activity.

Standardization of Spleen Antigens

Various degrees of antigenic activity were displayed by the nine spleens which met the minimum acceptable in the test. A general assessment of the activity of various antigens was obtained (Fig. 5) by placing groups of them around a central well containing a reference serum. The length of the precipitation line and its position between the serum well and the opposite well containing the antigen, were indications of the activity of the various antigens. A strong antigen formed a precipitation line located closer to the central well containing the reference serum, than a weaker antigen. For example, in Fig 5, well 5 which contained spleen H-8-71 exhibited less activity than well 6 which contained the acetone-ether liquid extract of

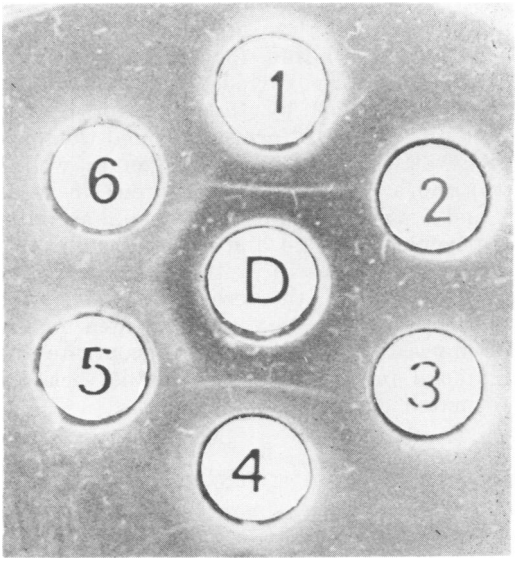

Fig. 4. Wells 1 and 4. Positive reference sera giving sharp precipitation lines.
Wells 2 and 3. Negative test sera giving no precipitation.
Wells 5 and 6. Negative test sera giving diffuse non-specific precipitation lines forming spurs with the reference precipitation ilnes.
Well D. Contains the spleen antigen.

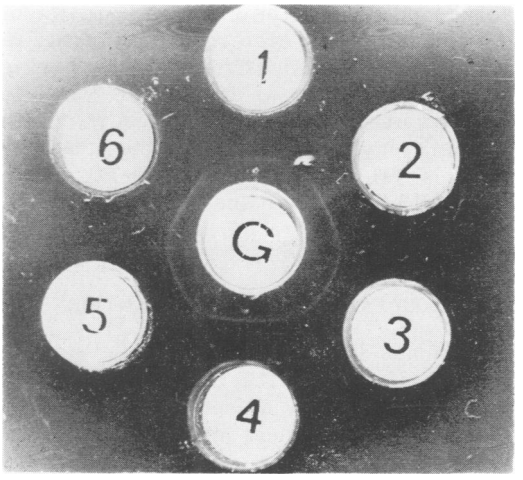

Fig. 5. Comparison of the reactivity of various spleen antigens. The central well, G. contains the reference positive serum and the peripheral wells contain the antigens being compared as follows: Well 1: H-11-70; Well 2: H-12-70; Well 3: H-16-70; Well 4: Coggins' antigen; Well 5: H-8-71; Well 6: H-8-71 extract liquid antigen.

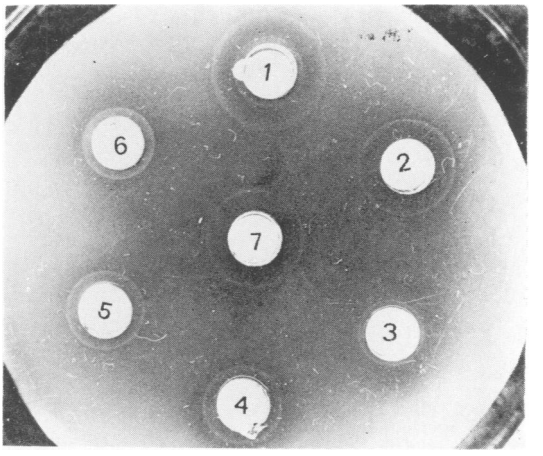

Fig. 6. Comparison of the reactivity of various spleen antigen by radial immunodiffusion. The immune serum was incorporated into the agar and the seven wells contained the spleen antigens being compared as follows: Well 1: H-11-70; Well 2: H-12-70; Well 3: H-16-70; Well 4: H-8-71; Well 5: H-8-71 extract liquid antigen; Well 6: H-7-70; Well 7: Coggins' antigen.

the same spleen. Also in the same figure, antigens in wells 1, 2 and 4 contained more activity than that in well 3 which was the least active antigen in this group.

The activity of the spleen antigens was also assessed by radial immunodiffusion (Fig. 6). In this instance, the reference positive serum was incorporated in the diffusion agar in appropriate dilution (1:10 final dilution for serum 980), the activity of the various spleen antigens could be judged by the diameter of the precipitation ring forming around the antigen well. For example, antigen in well 1 was more reactive than antigens in wells 2 and 7 which were of about equal activity. Antigens in wells 4 and 5 were comparable whereas antigens in wells 3 and 6 were the least active of the group. However the nature of the spleen pulp antigen makes it impossible to dilute or concentrate the material in view of adjusting its activity to match a standard antigen.

Reactivity of Antigen in Liquid State

The acetone-ether extraction procedure yielded from 1 to 1.5 ml of antigen for each gram of spleen extracted. Comparison of the activity of the liquid antigen extracted by the long method with various spleen pulp antigens can be seen in Fig. 5 (wells 6 and 5) and in Fig. 6 (wells 5 and 4). In the AGI test, the liquid extracted antigen was more active, even before concentration, than the pulp antigen from which it originated. Also, reactive liquid antigen was obtained by the long extraction method from infected spleens such as H-13-71 (Table IA) which were non-reactive or showed only faint reaction when used as pulp in the AGI test. However, the short extraction procedure, yielded good antigen only from reactive spleens and could not be used with faintly reactive ones. No reactions were demonstrable in the liquid antigen extracted from non-infected horse spleen.

In the CF test, the extracted liquid antigen (long procedure) from spleen H-8-71 was active in 1:8 dilution with our standard serum 980. It even reacted partly in 1:16 and 1:32 dilutions with other sera with a higher antibody content. The results indicated that the CF test would serve as an indicator of the activity of a liquid antigen extract intended for use in the AGI test. Also the liquid antigen extract can be concentrated by evaporation or dessication, and can be diluted as required to adjust its activity to that of a reference control antigen. The activity of liquid antigen extract could not be removed by centrifugation at 155,000 x g for one hour. The activity remained in the supernatant fluid. The antigen can be stored for weeks either frozen or lyophilized.

RELIABILITY OF THE AGAR GEL IMMUNODIFFUSION TEST

In Table II are listed the results of the AGI test sera collected by serial bleeding from ten horses experimentally inoculated with known virulent blood. Before inoculation, these ten animals were proven negative by cross inoculation. Twenty-two sera collected before inoculation and up to 17 days after, were negative in the test. One hundred and twenty sera collected from the 16th to 196th days post inoculation reacted in the test. In summary, all the sera collected at or after the 18th day post inoculation reacted positively. In one instance, (H-3-67) the serum collected 16 days after inoculation was also reactive.

DISCUSSION

The present studies support the report of Coggins and Norcross (4) on the behaviour of the AGI test in the diagnosis of equine infectious anemia. The main difficulty encountered was in regularly obtaining reactive spleens from infected horses. Also amongst the reactive spleens, various amount of activity was observed. Consequently a serum containing an adequate amount of antibodies had to be selected for each antigen in order to obtain the optimum antigen-reference-antiserum combination giving a sharp reference precipitation line. The development of a liquid antigen extract which could be diluted or concentrated as required should facilitate the calibration of the test. The liquid antigen extract was also active in the CF test and this test could be used in its preliminary standardization. However in this case it is important to select an antiserum with good CF and AGI titers against the EIA viral antigen. In general a liquid antigen extract which was reactive in a 1:8 dilution in the type of CF test used gave a sharp precipitation reaction in the AGI test with the known positive reference serum.

As reported (2) we attempted to prepare a tissue extract antigen to be used in the CF test. In this study the antigen was extracted from the tissues of EIA dying equine, from normal equine and bovine, with physiological saline. In the CF test this type of antigen was detecting transient antibodies which formed in the serum of animals after primary exposure to the infection. This physiological saline extracted antigen was labile, being destroyed by extraction with ether and also during storage in the frozen state at —20°C. The acetone-ether extracted liquid antigen in use in the present study was prepared from spleen collected from horses during the first height of pyrexia, that is from nine to ten days after infection. It is stable upon storage, is not destroyed by ether treatment, does not detect tissue antibodies and is specific for EIA viral antibodies.

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