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Heterophile antibodies in pathologic human sera resembling antibodies stimulated by foreign species sera

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SUMMARY

Studies were performed on heterophile antibodies originally described by Hanganutziu and Deicher and referred to as H–D antibodies. It was confirmed that these antibodies appear as a result of injections of foreign species sera. They differ from Forssman antibodies by combining with bovine erythrocytes and from Paul–Bunnell antibodies by reacting with guinea-pig kidney.

It was demonstrated that H–D antibodies react in double diffusion gel precipitation tests with: (1) crude extract of bovine erythrocyte stromata; (2) purified fraction of this extract devoid of Paul–Bunnell antigen; (3) whole bovine serum and sera of several other species; and (4) thermostable ethanol-insoluble fractions of serum and organs of oxen and several other species. These various antigenic preparations gave usually reactions of complete or partial identity with each other. In several instances, two or even three precipitation lines could be detected. H–D-negative human erythrocytes became coated with H–D antigen upon simple incubation with H–D-positive sera.

H–D antibodies were also detected in some pathological human sera without any indication that the patients had ever received injections of foreign species sera. Such antibodies were undistinguishable from H–D antibodies engendered by injections of foreign sera.

INTRODUCTION

In our previous studies, heterophile antibodies of infectious mononucleosis (IM) sera (Paul–Bunnell (P–B) antibodies) have been extensively investigated by means of a variety of serological procedures (Kano & Milgrom, 1964; Milgrom & Loza, 1966; Milgrom & Loza, 1967; Malave, Kano & Milgrom, 1973; Malave & Milgrom, 1973; Milgrom, Loza & Kano, 1975).

In the present study, sera of patients suffering from diseases other than IM were examined for the presence of heterophile antibodies. Several sera of these patients were found to contain antibodies agglutinating sheep erythrocytes and producing complement-mediated lysis of sheep and bovine erythrocytes. The reaction with bovine erythrocytes precluded the Forssman (Forssman, 1911) nature of these antibodies. They were also different from P–B antibodies of IM (Paul & Bunnell, 1932) since they were absorbed by guinea-pig kidney sediment. Accordingly, it appeared to us that we are dealing with a type of antibodies known as 'serum sickness antibodies' which had been described by Hanganutziu (1924) and Deicher (1926).

Both Hanganutziu and Deicher observed that sera of patients who had received therapeutic injections of horse antitoxins contained agglutinins for sheep erythrocytes and both authors correctly recognized that these antibodies are different from Forssman antibodies. Appearance of these antibodies could not be related to any clinical symptoms of serum sickness or to the volume of horse serum injected. Deicher

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(1926) also reported that the antibodies elicited by injections of horse sera would combine with erythrocytes of other species origin, e.g. rabbit, horse, guinea-pig, and pig in addition to those of sheep. He also noted that immunization with sera of other than horse origin, e.g. bovine serum, may stimulate formation of very similar antibodies.

Antibodies described by Hanganutziu and Deicher (to be referred to as H–D antibodies) elicited rather little interest. However, with the development of serodiagnosis of IM based on detection of P–B antibodies, all diagnostic tests have to demonstrate that the positive results with IM sera are indeed due to P–B antibodies and not to H–D or Forssman antibodies (Davidsohn & Walker, 1935).

Recently, Pirofsky, Ramirez-Mateos & August (1973) studied sera of forty-six patients who received γ globulin fractions of goat antisera to human thymocytes for therapeutic trial. The vast majority of these patients formed antibodies which agglutinated sheep erythrocytes and were absorbable by both guinea-pig kidney and boiled bovine erythrocytes. The authors justifiably recognized these antibodies as H–D antibodies.

In the study to be reported, H–D antibodies were studied by a variety of serological procedures. The major purpose was to obtain more information on the nature of these antibodies and their corresponding antigen and to compare the reaction produced by H–D antibodies stimulated by injection of foreign species sera with those that appeared spontaneously in the course of various diseases.

MATERIALS AND METHODS

Human sera. Sera from patients with various renal diseases were sent to our laboratory from the affiliated hospitals of the State University of New York at Buffalo for tests for histocompatibility allo-antibodies. Sera from patients with liver diseases and with infectious bowel diseases were kindly supplied by Dr L. A. Katz of the Department of Medicine and sera of patients suffering from multiple sclerosis by Dr W. A. Olszewski of the Department of Neurology of this University. Sera of patients with leukemias or lymphomas and with fever of unknown origin (FUO) were collected from patients of one of us (M.L.B.). Sera of patients with infectious mononucleosis (IM) were kindly supplied by Dr P. F. Hoffman of the Student Health Service of the University. Sera of patients with other diseases were obtained at the Buffalo General Hospital, Buffalo, New York.

One FUO patient (J.B.) whose serum was extensively studied in this communication is a 25-year-old male university student who has been suffering from recurrent episodes of fever and lymphadenopathy but has never been injected with any animal serum. He has been hospitalized several times with diagnosis of IM because of a high titre of agglutinins to sheep erythrocytes.

One serum sample of patient injected with horse antiserum (whole serum) to tetanus toxin (H-ATT) was a gift of Dr C. E. Arbesman of the Department of Medicine of this University. Sera from patients who received γ globulin fractions of goat antisera to human thymocytes (G-ATG) were kindly supplied by Dr B. Pirofsky of the Division of Immunology and Allergy, University of Oregon, Portland, Oregon, and sera of subjects injected with γ-globulin fractions of horse antisera to human lymphocytes (H-ALG) were generously donated by Dr R. M. Condie of the Department of Surgery, University of Minnesota, Minneapolis, Minnesota. Normal human sera were obtained from healthy staff members of this department. All sera were heated at 56°C for 30 min before they were tested.

Animal sera. These were obtained from animals exsanguinated at our laboratory or local slaughterhouse.

Human tissues. These were obtained at autopsy in the local hospitals.

Animal tissues. Bovine tissues were obtained from animals slaughtered at a local abattoir and tissues of rabbits and guinea pigs were obtained from animals exsanguinated in our laboratory. Erythrocytes of oxen and sheep were obtained from animals raised at a local farm.

Bovine stroma extracts. Extract of bovine stromata was obtained as previously described (Milgrom *et al.*, 1975). Briefly, stromata obtained by lysing erythrocytes in distilled water at 4°C were washed repeatedly with distilled water and saline. They were exposed to supersonic vibration for 90 sec at 20,000 cycles/sec and centrifuged at 64,000 *g* for 30 min. The clear supernatant recovered was referred to as 'crude extract'.

The procedures used for the preparation of various fractions of bovine stroma extracts will be published separately by Dr J. M. Merrick of this department. They were all based on extraction of aqueous suspension of bovine stromata with chloroform-methanol mixture. In some preparations, aqueous layer contained only P–B antigen at a concentration demonstrable in a precipitation test. One such preparation used in this study was referred to as 'P–B fraction'. In most other instances, the aqueous layer contained H–D antigen in addition to P–B antigen. H–D antigen could be separated by DEAE or thin layer column chromotography. Such preparations were referred to as 'H–D fractions'.

BE preparations. Resistant to boiling and ethanol-insoluble preparations (BE) of sera and tissues were obtained as previously described (Milgrom & Witebsky, 1962). Briefly, 1:2 diluted serum or 20% (w/v) tissue suspension in saline was heated for 60 min at 100°C. The extract separated after centrifugation at 12,000 *g* for 10 min was autoclaved for 45 min at 121°C and

centrifuged again in a similar way. The supernatant was mixed with 3 volumes of 95% ethanol and left overnight at room temperature. The precipitate was separated after centrifugation, dissolved in saline and reprecipitated with ethanol. The precipitate was dried *in vacuo* and preserved as grayish powder at room temperature. Before use, it was dissolved at a desired concentration in phosphate-buffered saline, pH 7·2 (PBS).

Cytolysis in agar gel. The test was performed as previously described (Milgrom & Loza, 1966; Fuji *et al.*, 1971; Juji, *et al.*, 1971: Zaleski & Milgrom, 1971). Bovine or sheep erythrocytes were suspended in medium 199 at a concentration of 1·25%. Fifty microlitres of the cell suspension were added to 0·4 ml of 0·7% agar solution and the mixture was spread immediately on a prewarmed microscope slide. After the agar layer solidified, 5 μl droplets of serum dilutions were placed on its surface. The slides were incubated in a moist chamber for 60 min at 37°C, washed with saline and incubated again for 60 min at 37°C with a 1:10 diluted rabbit serum as a source of complement. The slides were then washed, air dried, and fixed with ethanol. Antibody titres were expressed as reciprocals of the highest serum dilution at which definite cytolysis was noted.

Double diffusion gel precipitation. The test was performed using 1% agarose (Seakem, Marine Colloids, Incorporated, Springfield, New Jersey) in saline. Convenient patterns were used with wells 4 mm or 5 mm in diameter and diffusion distance of 3 mm. All human sera tested for antibodies were used undiluted. Whole sera tested for the antigen were also used undiluted. BE preparations and crude stroma extract were used at concentrations of 5–10 mg of dry weight per millilitre. P–B and H–D fractions of bovine stromata were employed at concentrations of 0·5–1 mg/ml. The plates were left for 3–4 days at 4°C before readings and pictures were taken.

Immunoelectrophoresis. This was performed following the procedure of Scheidegger (1955) using 1% agarose solution in barbital buffer of pH 8·6 and ionic strength of 0·075. Concentration of reagents was similar to the one used in double diffusion gel precipitation.

Sheep cell haemagglutination. Serial dilutions of serum in PBS in 0·1 ml volumes were mixed with 0·1 ml of 1% suspension of sheep erythrocytes in PBS. The test was left at room temperature for 2 hr, the tubes were centrifuged at 1500 rev/min for 2 min, and then the agglutination was estimated after shaking the tubes gently.

Inhibition of sheep cell haemagglutination. 0·1 ml of human serum containing six agglutinating units was mixed with 0·1 ml of serial dilutions of inhibiting animal serum. The mixture was left at room temperature for 60 min. Then 0·1 ml of 1% suspension of sheep erythrocytes was added and the test was continued as described above.

Simple passive haemagglutination. Human group O erythrocytes were washed three times with PBS. The packed cells were mixed with three volumes of undiluted horse or human serum which were heated at 56°C for 30 min. Because of strong natural agglutinins to human erythrocytes present in bovine serum, 10% bovine serum albumin fraction (w/v) was employed instead of whole serum. The mixtures were incubated at 37°C overnight. The erythrocytes were washed three times with PBS and resuspended at a concentration of 1%. One-tenth of a millilitre of this cell suspension was mixed with 0·1 ml of tested serum at various dilutions. The tubes were kept at room temperature for 2 hr and the pattern agglutination was recorded.

Tanned cell haemagglutination. The test was performed as described by Witebsky & Rose (1956). The concentration of tannic acid used for treatment of human group O erythrocytes was 1:20,000. The final concentration of coated erythrocytes was 1%. Egg albumin at a concentration of 0·2% in PBS was used to prepare the dilutions of tested sera and the erythrocyte suspension.

Absorption experiments. Washed sediments of homogenized guinea pig kidneys or bovine erythrocyte stromata were used. The sediment was mixed with an equal volume of undiluted serum for the double diffusion tests or with 1:5 diluted serum for the cytolysis and hemagglutination tests. The mixtures were incubated for 60 min at room temperature and centrifuged at 3000 *g* for 3 min. Then the supernatant was recovered. The procedure was repeated two or three times.

2-Mercaptoethanol (ME) treatment. Equal volumes of 0·2 M ME and of 1:5 diluted serum were mixed and incubated at 37°C for 60 min. Without dialysis, the serum dilutions were immediately made and tested.

RESULTS

As shown in Table 1, 417 various human sera were examined for lysis of bovine erythrocytes. No IM sera were included into this study. Among tested sera, thirty had lytic antibody titres of 80 or higher. None of twenty control sera from healthy staff members of this department had such titres. The thirty positive sera were also tested for lysis of sheep erythrocytes, and in all instances, titres of 80 or more were observed. Absorption of all thirty positive sera with guinea-pig kidney sediment reduced their titres against bovine as well as sheep erythrocytes to 20 or less. This would indicate that the heterophile antibodies under investigation belonged to the type of H–D antibodies. The appearance of the H–D antibodies seemed to have no direct relationship to any disease except that, surprisingly, four out of five patients with fever of unknown origin (FUO) had increased titres of these antibodies. It may be noted that six of seventy (8·6%) renal disease sera, nine of 126 (7·1%) sera of patients with liver diseases and four of forty-nine (8·2%) of lymphoma-leukaemia sera showed increased antibody titres.

We studied only one serum of a patient injected with whole horse serum (H-ATT) and found a titre

TABLE 1. Lysis of bovine erythrocytes by various human sera

	No. of sera which lysed bovine erythrocytes at a titre of:								
	< 80	80	160	320	640	1280	2560	5120	Total
Renal diseases	64	5	1						70
Liver diseases	117	1	4	3	1				126
Infectious bowel diseases	23	3							26
Fever of unknown origin (FUO)	1	1	1	1			1		5
Lymphomas, leukaemias	45	2	1	1					49
Multiple myeloma	2		1						3
Drug hypersensitivity	1			1					2
Collagen disease	26		1						27
Multiple sclerosis	58	1							59
Carcinoma of various organs	10								10
Viral diseases	11								11
Burns	9								9
Healthy subjects	20								20
Total	387	13	9	6	1		1		417
Patient who received H-ATT					1				1
Patients who received G-ATG						1	2	1	4
Patients who received H-ALG	30	1		2					33
Total	30	1		2	1	1	2	1	38

of 640. Sera of all four patients who received goat γ-globulin (G-ATG) showed titres of 1280 or higher whereas only three of thirty-three sera from patients injected with horse γ-globulin (H-ALG) had increased titres and even these were relatively low.

It was of some interest to study the effect of ME treatment on the H–D antibodies. As seen in Table 2, antibody activity of the serum J.B. was only slightly influenced by the ME treatment. In a similar way ME treatment left quite high antibody titres in sera of patients C.R. and V.A. who were injected with H-ATT and G-ATG, respectively. In contrast, all three sera of patients with liver diseases lost their activities after such treatment. This would indicate that at least a great part of H–D antibodies in sera of J.B., C.R. and V.A. belonged to the IgG class while apparently all antibodies of the liver disease sera were of the IgM class.

The H–D antibodies were studied by means of double diffusion gel precipitation tests. For the initial experiments we selected sera of patient C.R. who received injection of H-ATT and of patient J.B. who suffered from FUO and who received no injection of a foreign species serum. These two sera were tested against extracts of bovine erythrocyte stromata along with two IM sera. As shown in Fig. 1a, all four sera formed distinct precipitation lines with the crude stroma extract. The lines formed by sera of patients C.R. and J.B. which contained H–D antibodies merged into a reaction of identity with each other whereas they formed spurs over the lines produced by the IM sera. When subsequently these four sera were tested against P–B and H–D fractions of the bovine stromata, the P–B fraction reacted only with the IM sera (Fig 1b); and the H–D fraction combined only with H–D antibody-containing sera (Fig. 1c). These two fractions were also tested against the H–D antibody-containing serum of FUO patient P.N. Here, again, reaction was noted with the H–D but not with the P–B fraction.

The serum J.B. was tested against sera of ox, horse, sheep, rabbit, guinea-pig and rat origin. As seen in Fig. 2, serum J.B. formed precipitation lines with all these animal sera. The precipitation lines seemed to merge into reactions of complete or partial identity. In the reaction with bovine serum, the precipitation was clearly separated into two lines. The sharp line closer to the antigen well was uninfluenced by the adjacent reaction with horse serum but it did not extend over the line formed by sheep serum. Apparently, this line was produced by a component present in bovine and sheep but not in horse serum,

TABLE 2. Sensitivity to 2-mercaptoethanol (ME) of bovine haemolysins (B.H.L.) as well as sheep haemolysins (S.H.L.) and haemagglutinins (S.H.A.)

			Titre of antibodies in serum	
			Untreated	Treated with ME
FUO	J.B.	B.H.L.	2560	1280
		S.H.L.	5120	2560
		S.H.A.	320	160
	P.N.	B.H.L.	320	80
		S.H.L.	1280	80
		S.H.A.	80	< 20
Liver diseases	297	B.H.L.	320	< 20
		S.H.L.	640	< 20
		S.H.A.	< 20	< 20
	388	B.H.L.	160	< 20
		S.H.L.	640	< 20
		S.H.L.	< 20	< 20
	488	B.H.L.	640	< 20
		S.H.L.	1280	< 20
		S.H.A.	20	< 20
H-ATT injection	C.R.	B.H.L.	640	160
		S.H.L.	640	320
		S.H.A.	320	320
G-ATG injection	V.A.	B.H.L.	5120	320
		S.H.L.	> 5120	640
		S.H.A.	640	320
	R.S.	B.H.L.	2560	80
		S.H.L.	1280	80
		S.H.A.	160	80
	P.K.	B.H.L.	2560	20
		S.H.L.	1280	> 20
		S.H.A.	320	< 20
	D.A.	B.H.L.	1280	160
		S.H.L.	2560	20
		S H.A.	640	20
I.M.	A.T.	B.H.L.	2560	< 20
		S.H.L.	> 5120	< 20
		S.H.A.	640	< 20

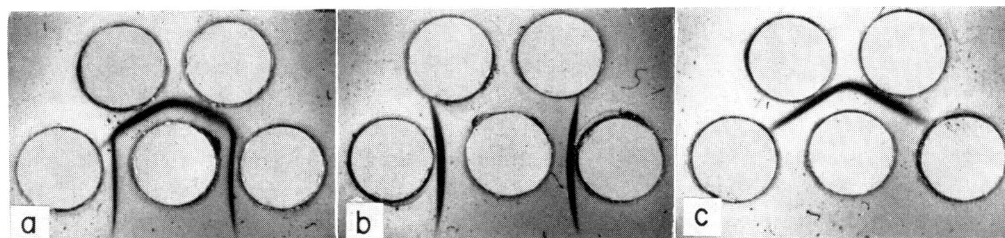

FIG. 1. Central well: (a) crude extract; (b) P–B fraction; and (c) H–D fraction of bovine stromata. Peripheral wells in all three plates: lower left and right, IM sera; upper left, H-ATT serum C.R.; upper right, FUO serum J.B.

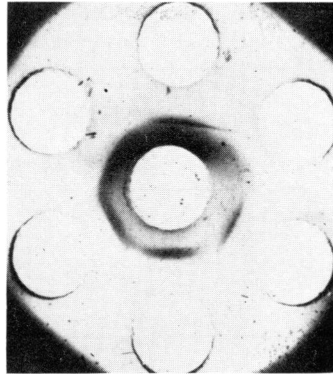

Fig. 2. Central well: FUO serum J.B. peripheral wells, clockwise from the uppermost well: sera of ox, horse, rabbit, guinea-pig, rat and sheep.

FUO serum J.B. and G-ATG serum V.A. were then tested against H–D fraction of bovine stromata, untreated bovine serum and BE preparation of this serum. Major precipitation lines formed by serum J.B. with the bovine serum and its BE preparation merged into a reaction of complete identity with the line formed with the H–D fraction (Fig. 3a). The line formed by serum V.A. with bovine serum BE merged into a complete identity reaction with the line formed with the whole bovine serum. The latter line, however, gave a partial identity reaction with the H–D fraction in that the reaction with the H–D fraction appeared richer than that with the bovine serum (Fig. 3b).

Presence of H–D antigen in sera of various species origin was further studied by means of inhibition of sheep cell haemagglutination (Table 3). In this experiment, three sera with H–D antibodies at a dilution corresponding to six agglutinating units were mixed with increasing dilutions of sera of various species. The agglutination of sheep erythrocytes was inhibited by sera of ox, horse, sheep and rabbit but not by human serum. Significantly, none of these sera inhibited agglutination by P–B antibodies. In this connection, it appeared interesting to find out if the H–D antigen would 'coat' H–D-negative erythrocytes upon simple incubation of such erythrocytes with H–D-positive serum. To this end, experiments were performed, the results of which are presented in Table 4. It may be seen that human erythrocytes were 'modified' by albumin fraction of bovine serum or whole horse serum in that they became agglutinable by H–D antibody-containing sera. As expected, human serum had no effect on the erythrocytes.

As shown in Fig. 3, sera with H–D antibodies precipitated BE fraction of bovine serum. This would mean that the H–D antigen may be characterized as one of thermostable and ethanol-precipitable antigens of mammalian tissues (Milgrom & Witebsky, 1962; Milgrom *et al.*, 1964; Milgrom, Tuggac & Witebsky, 1965). As seen in Fig. 4, the precipitation lines formed with the BE preparation of bovine serum by sera

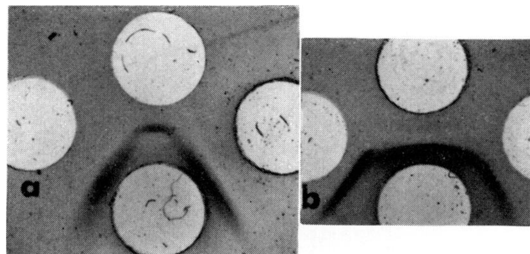

Fig. 3. (a) Lower well: FUO serum J.B. Left well: bovine serum BE. Upper well: H–D fraction of bovine stromata. Right well: bovine serum. (b) Lower well: G-ATG serum V.A. Left well: H–D fraction of bovine stromata. Upper well: bovine serum. Right well: bovine serum BE.

TABLE 3. Inhibition of agglutination of sheep erythrocytes by six agglutinating units of sera with H–D or P–B antibodies

Serum	Inhibiting titre of sera of:				
	Ox	Horse	Sheep	Rabbit	Man
J.B. (FUO) H–D	32	8	8	16	< 4
C.R. (H-ATT) H–D	32	4	8	8	< 4
V.A. (G-ATG) H–D	16	4	8	16	< 4
A.T. (IM) P–B	< 4	< 4	< 4	< 4	< 4

TABLE 4. Simple passive haemagglutination test. Reactions of human O erythrocytes incubated with bovine serum albumin, horse serum or human serum

Serum	Titre of agglutination of human erythrocytes coated with:		
	Bovine serum albumin	Horse serum	Human serum
J.B. (FUO) H–D	320	640	< 20
C.R. (H-ATT) H–D	320	640	< 20
V.A. (G-ATG) H–D	Not done	1280	< 20
A.T. (IM) P–B	< 20	< 20	< 20

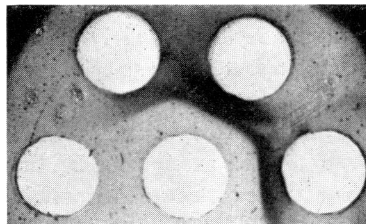

FIG. 4. Central well: bovine serum BE. Lower left well: IM serum A.T. Upper left well: H-ATT serum C.R. Upper right well: FUO serum J.B. Lower right well: G-ATG serum V.A.

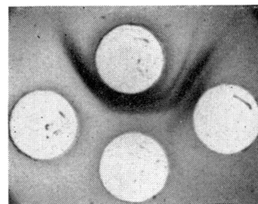

FIG. 5. Lower well: bovine serum BE. Remaining wells: FUO serum J.B., absorbed with guinea-pig kidney (left), unabsorbed (top), and absorbed with bovine stromata (right).

J.B., V.A. and C.R. merged into reactions of identity. The additional line formed by the serum J.B., however, did not have its counterpart in the reactions of sera C.R. and V.A. with the BE preparation. It should be noted that IM serum failed to react with bovine serum BE.

Both reaction lines formed with the bovine serum BE by the serum J.B. could be abolished completely by absorption of the serum J.B. with either guinea-pig kidney sediment or bovine erythrocyte stromata (Fig. 5). Thus, apparently both antigens detectable in the BE preparation by the serum J.B. belonged to the H-D 'complex.' Reaction lines that appear between unabsorbed and absorbed serum J.B. samples in Fig. 5 indicate that guinea-pig kidney as well as bovine stroma sediments released an excess of soluble H-D antigenic material into the absorbed serum. Interestingly enough, bovine stromata released a component which produced a precipitation line very close to the stroma-absorbed serum specimen and which was absent from the serum BE.

BE fractions of bovine brain, kidney, spleen and adrenal were also tested against H-D antibody-containing sera. As seen in Fig. 6, the patterns of the reactions given by three different sera were very similar even though the reactions with the serum C.R. were weaker than those with the other two sera. The reaction lines with the H-D fraction of bovine stromata were stronger than the lines with bovine BE preparations. The lines formed with BE preparations of organs merged into reactions of identity with the serum BE line. BE lines seemed to merge into reactions of identity with the H-D lines. (The spurs formed by H-D lines merged into reactions of identity with faint lines formed by BE preparations, which were separated from major lines. This is seen in Fig. 6b,c. Besides bovine tissues, BE preparations of rabbit and guinea-pig tissues also reacted with serum J.B. Six precipitation lines formed by BE preparations of various tissues of these three animals appeared to merge into reactions of identity as seen in Fig. 7.

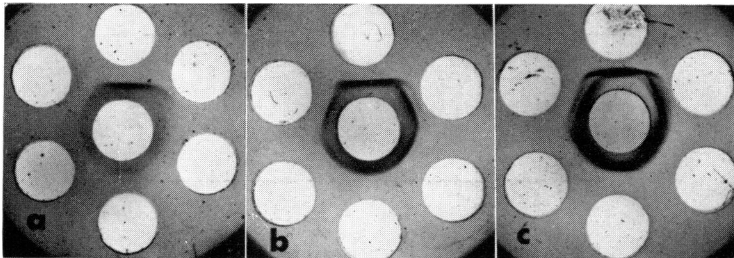

Fig. 6. Central well: (a) H-ATT serum C.R.; (b) G-ATG serum V.A.; and (c) FUO serum J.B. Peripheral wells in all three plates: uppermost well, H-D fraction of bovine stromata; remaining wells (clockwise), BE preparations of bovine brain, kidney, spleen, adrenal and serum.

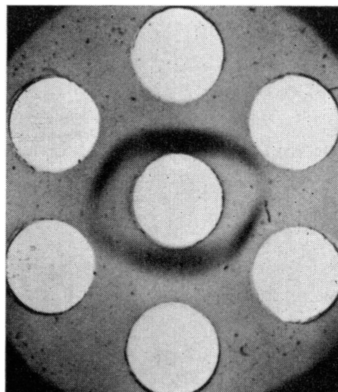

Fig. 7. Central well: FUO serum J.B. Peripheral wells, clockwise from the uppermost well: BE preparations of bovine spleen, rabbit brain, rabbit kidney, rabbit lung, guinea-pig liver and guinea-pig lung.

Tanned cell haemagglutination tests were also employed to demonstrate the reactions of BE antigens with sera containing H–D antibodies. Representative results of these experiments are shown in Table 5. Tanned human erythrocytes coated with bovine serum or adrenal BE preparations were agglutinated by all three tested sera with H–D antibodies but not by IM serum.

Subsequently, H–D fraction of bovine stromata and bovine serum BE were studied by immunoelectrophoresis against serum J.B. As seen in Fig. 8a, the H–D fraction formed a precipitation arc in the albumin region while the serum BE formed one strong and one faint arc in the similar region. In contrast, the line(s) formed with whole bovine serum extended from the albumin region to the γ globulin region (Fig. 8b). When the H–D antibody-containing sera, J.B. and V.A., were electrophoresed, precipitation lines with the bovine serum BE were observed in the position characteristic for IgG class of immunoglobulins.

Finally, BE preparations of human tissues were tested against H–D antibodies. In very preliminary experiments, brain, thymus and spleen BE preparations gave positive results in double diffusion gel precipitation. This is exemplified by results presented in Fig. 9.

TABLE 5. Tanned cell haemagglutination. Reactions of human O erythrocytes coated by bovine BE preparations

Serum	Titre of reaction with erythrocytes coated by 1 mg/ml concentration of BE preparation of:	
	Bovine serum	Bovine adrenal
J.B. (FUO) H–D	320	640
C.R. (H-ATT) H–D	20	40
V.A. (G-ATG) H–D	640	640
A.T. (IM) P–B	< 10	< 10

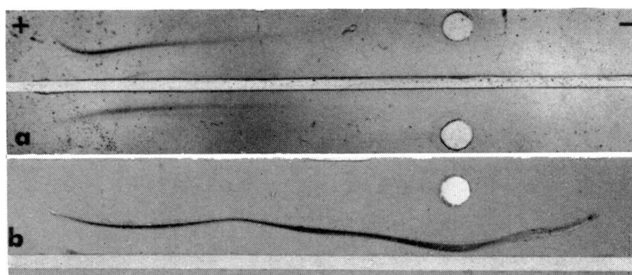

FIG. 8. Troughs in both plates: FUO serum J.B. (a) Upper well: H–D fraction of bovine stromata. Lower well: bovine serum BE. (b) Upper well: bovine serum.

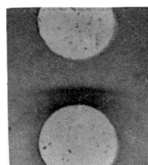

FIG. 9. Upper well: human spleen BE. Lower well: FUO serum J.B.

DISCUSSION

The term 'H-D antibodies' has been employed in this paper to denote what used to be called 'serum-sickness antibodies.' We believe that this is completely justified by the fact that these antibodies are not necessarily related to serum sickness or even to stimulation by a serum of a foreign species. We had at our disposal only one serum (C.R.) from a patient who received injections of whole horse serum. Serum C.R. gave typical reactions for H-D antibodies in all tests employed. Furthermore, we confirmed observation of Pirofsky *et al.* (1973) that sera of patients injected with G-ATG contained H-D antibodies. Similarly to Pirofsky *et al.* (1973), we have all reason to believe that H-D antibodies were obviously not formed in response to goat γ-globulin but to the H-D antigen 'contaminating' this fraction. Significantly, of thirty-three subjects injected with purified γ-globulins of H-ALG, only three formed H-D antibodies and these had rather low titres. Apparently, the horse γ-globulin used in these clinical trials was to a great extent free of H-D antigen.

It was rather unexpected finding that sera of several patients who never received injections of any foreign species serum had H-D antibodies. We could not identify any disease entity in which these antibodies would appear with high frequency but we found them in several patients suffering from various diseases.

Analysis of H-D antibodies was mostly performed by means of double diffusion gel precipitation tests. We obtained positive results with crude extract of bovine erythrocyte stromata and with whole bovine serum. Extracts of bovine stromata could be partitioned into P-B and H-D fractions. Further studies on preparation of these antigens in purified form are in progress and will be reported. It may be mentioned now that separation of pure H-D antigen is a much easier task than separation of pure P-B antigen. Bovine serum contained abundantly H-D antigen but it did not contain P-B antigen in readily detectable amounts.

Upon immunoelectrophoresis of whole bovine serum, the H-D antigen(s) was broadly distributed through all electrophoretic field. Therefore, it is not surprising that some γ-globulin preparations may contain this antigen. It appeared to us that blood plasma may well be the primary location of H-D antigen and that it is absorbed by erythrocytes in a similar fashion as some blood group antigens, first of all, the J antigen of cattle (Stromont, 1949) and R antigen of sheep (Rendel, Sorensen & Irwin, 1954). It was, therefore, interesting to find out that H-D-negative human erythrocytes became H-D-positive upon incubation in H-D-positive horse serum or bovine serum albumin.

The thermostability of the H-D antigen demonstrated by previous authors in horse serum and bovine erythrocytes (Stuart *et al.*, 1936; Tomcsik & Schwarzweis, 1948) was confirmed in these studies. Furthermore, we found that this antigen can be obtained following the procedure described for preparation of various tissue antigens, which involves extraction at boiling temperature followed by precipitation at 70% concentration of ethanol (Milgrom & Witebsky, 1962; Milgrom *et al.*, 1964; Milgrom *et al.*, 1965). We called tissue fractions obtained by this procedure BE fractions. In addition to BE of bovine serum, we found H-D antigen in BE preparations of many bovine organs and organs of other species origin. Interestingly, H-D antigen(s) in BE preparations as well as this antigen in preparation of bovine erythrocyte stromata were shown to be fast-moving components in immunoelectrophoresis.

In several instances the reaction between H-D antibodies and the antigenic preparations was composed of two or even three precipitation lines. Therefore, it seems justified to speak about H-D antigenic complex.

Significantly, reactions produced by H-D antibodies elicited by injection with a foreign species serum were undistinguishable from reactions produced by H-D antibodies occurring in various patients who did not receive such injections.

The antigenic stimulus responsible for the formation of the H-D antibodies by the patients who had never received injections of foreign sera still remains to be determined. However, we would like to propose that the antigen may be released from the patient's own tissues during the pathologic processes. This is supported by demonstrating the H-D antigen in BE preparations from some human organs.

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