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NATURALLY OCCURRING ANTI-TISSUE ANTIBODIES IN RAT SERA

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SUMMARY

Seventy per cent of normal rat sera have been shown to contain heat labile serum component(s) active against various rat organ homogenates as demonstrated by haemolytic complement fixation and passive haemagglutination tests. The main antigenic activity in rat liver has been found in the mitochondrial fractions.

It was also demonstrated by the indirect fluorescent antibody technique that both guinea-pig complement and high molecular weight rat globulins were fixed to rat organ sections.

Chemotactic activity has also been observed with rat serum and rat liver mitochondria and it is suggested that these naturally occurring antibodies may be implicated in the removal of tissue breakdown products.

In a previous report, describing the stimulation of anti-liver autoantibodies following carbon tetrachloride induced liver damage, it was noted that some 15% of 'normal rat' sera contained a heat-stable anti-liver autoantibody (Weir, 1963). A similar autoantibody, directed against kidney, has been found in normal rat sera (Digby & Loewi, 1965). Rats appeared not to be tolerant to rat liver antigen as far as IgM antibody was concerned (Pinckard & Weir, 1966). Moreover, the response described following the injections of carbon tetrachloride had the features of a secondary response. These observations suggested that some anti-liver autoantibody should be detectable in all normal sera, provided a sufficiently sensitive test was used, as a response to the continual release of intracellular breakdown products from effete liver cells. This report describes the finding of a heat-labile serum factor with the sedimentation and gel filtration characteristics of a high molecular weight immunoglobulin reacting with various tissues detectable by: (1) complement fixation in the haemolytic complement fixation test; (2) passive haemagglutination; (3) the localization of bound complement by the fluorescent antibody technique; and (4) the indirect fluorescent antibody technique.

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MATERIALS AND METHODS

Animals

Albino rats of both sexes were used, obtained from the University Animal Breeding Station. The animals were bled under light ether anaesthesia by heart puncture or from the tail vein.

Complement fixation test

The test was carried out as previously described using a four drop system and 1·25 MHD_{100} of guinea-pig complement (Pinckard & Weir, 1966), except that the rat sera were not inactivated by heating at 56°C.

Liver fractionation procedure

The method used was modified from that of Schneider & Hogeboom (1950). Seven fractions were prepared from whole liver homogenate by differential centrifugation in 0·25 M-sucrose (Pinckard & Weir, 1966).

Antigen dilution test

The method used was that previously described (Pinckard & Weir, 1966) where, in the complement fixation test, antigen dilutions were set up with a constant antiserum concentration.

Passive haemagglutination test

This was a modification of Boyden's (1951) technique (Cruickshank, 1965) in which the sheep cells were tanned with 0·32 mg tannic acid/ml packed cells and coated with various rat organ homogenates. The concentrations of antigen and of horse serum in phosphate buffer, pH 7·0, which gave stable cell suspensions were determined by checkerboard titrations for each antigen. This varied between 140 and 200 mg wet weight of tissue/ml of packed sheep cells.

Serum fractionation procedures

Sucrose density gradients were prepared by the method of Kunkel (1960) in which layers of 40, 30, 20 and 10% sucrose in phosphate buffered saline, pH 7·0, are allowed to equilibrate for 24 hr at 4°C, and serum diluted 1:2 in buffer layered on top. An M.S.E. Superspeed 40 centifuge was used with a three-place swing out rotor, the tubes containing 1 ml of each sucrose concentration and 0·5 ml of the diluted serum. The tubes were centrifuged for 16 hr at 35 000 rev/min at 4°C. Nine fractions of 0·5 ml were taken from the top using a 1-ml syringe and stored at −20°C until required for use. Using this technique to fractionate early primary rabbit anti-bovine serum albumin (BSA) serum, two peaks of antibody activity were found when the fractions were tested by passive haemagglutination and the Farr test (Farr, 1958; Minden & Farr, 1966) using [^{131}I]BSA. The more slowly sedimenting peak (7S globulins) was found in fractions 3–4 and the more rapidly sedimenting high molecular weight peak was found in fractions 7–8 (the 19S globulins). Chicken anti-BSA serum taken 6 days after primary injection, which is known to contain its main haemagglutinating

activity in the 19S class globulins (Dreesman *et al.*, 1965), was also found to have its main haemagglutinating activity in fractions 7–8.

Fluorescent antibody methods

An antiserum reactive against guinea-pig complement was prepared in two rabbits which were immunized with sodium sulphate precipitated guinea-pig globulin emulsified with an equal volume of Freund's complete adjuvant followed 3 weeks later by a precipitate of rabbit antiserum to BSA and BSA made at equivalence in the presence of fresh guinea-pig serum. The washed precipitate with its adsorbed complement was injected subcutaneously. The antisera were tested against guinea-pig globulin in gel diffusion tests and after two further injections of guinea-pig globulin (1 and 3 mg subcutaneously without adjuvant) an antiserum was obtained which gave clear-cut precipitin bands appearing within 24 hr with guinea-pig globulin. The ammonium sulphate precipitated globulins were conjugated with fluorescein isothiocyanate according to the method of Marshall, Eveland & Smith (1958). The anti-rat serum was prepared similarly by injecting rabbits subcutaneously with rat serum emulsified with Freund's complete adjuvant (1 ml of serum plus an equal volume of adjuvant). Injections were continued and test bleeds made until an antiserum was obtained which gave good precipitin bands with rat serum in gel diffusion tests.

Tissue sections cut from snap frozen blocks of liver and kidney were cut at 5 μ in a cryostat. The sections were air dried, fixed in acetone at 37°C for 1 min and layered with a mixture of rat serum and fresh guinea-pig serum in equal parts. After 1 hr incubation at 37°C the excess serum was washed off in phosphate buffered saline, pH 7·0, and the sections stained for 30 min at 37°C with the fluorescein conjugated anti-guinea-pig globulin. Both the guinea-pig serum and the conjugated anti-guinea-pig globulin were absorbed prior to use with rat liver powder. After washing, the sections were mounted in buffered glycerine and examined using a Leitz fluorescence microscope. Similar tests were carried out with sera from which the complement had been absorbed with precipitates prepared at equivalence from rabbit anti-BSA serum and BSA. The sera usually required to be absorbed 2× (overnight at 4°C) and were tested against sensitized sheep red cells to ensure that no complement activity remained.

The tests using fluorescein conjugated anti-rat serum were carried out in the same way except that no guinea-pig serum was added in the first stage.

Sephadex chromatography

Sera were chromatographed on a column of Sephadex G-200 using 0·1 M-tris, 0·2 M-NaCl buffer adjusted to pH 8·0 as recommended by Fahey & Wirtz (1966). The proteins were eluted at a flow rate of 35 ml/hr. The eluate was passed through an L.K.B. Uvicord automatic scanning device and a tracing of the protein pattern obtained. Fractions of 8 ml volume were collected on an L.K.B. Radirac fraction collector and stored at −20°C until required for testing.

RESULTS

It was not possible to obtain sufficient serum from a single rat to allow it to be used in all the tests. Some sera were pooled for use in fractionation procedures and others were set aside for use in the various serological tests.

Complement fixation tests with 'normal' rat sera against organ homogenates:

One hundred unheated rat sera were tested against whole liver homogenate. Seventy of these sera gave positive complement fixation tests, twenty-two were negative and eight were anticomplementary.

Twelve of the positive sera were used in complement fixation tests against various rat organs homogenates. The results (Table 1) showed that the titres could vary independently

TABLE 1. Activity against rat organ homogenates (complement fixation titres)

Sera		Liver	Heart	Kidney	Brain	Muscle	Spleen	Lung
1	U	512	128	256	512	64	512	512
	H	4	0	128	8	0	16	0
2	U	256	64	256	512	32	512	512
	H	0	0	64	4	0	0	0
3	U	256	256	128	512	8	128	512
	H	16	0	16	0	0	32	512
4	U	128	64	64	512	8	64	128
	H	4	0	0	4	0	0	4
5	U	64	16	16	64	16	32	128
	H	8	0	16	0	0	4	16
6	U	256	8	32	256	0	256	128
7	H	0	0	0	0	0	0	0
7	U	32	8	16	512	0	16	32
	H	0	0	0	4	0	16	0
8	U	32	0	32	16	0	32	32
	H	0	0	0	0	0	0	0
9	U	512	512	512	512	512	512	512
	H	512	128	512	512	4	64	512
10	U	32	0	64	0	0	64	256
	H	0	0	16	0	0	0	0
11	U	128	32	64	64	32	128	128
	H	0	0	16	0	0	0	0
12	U	32	0	32	16	0	64	256
	H	0	0	32	0	0	8	16

U = Unheated sera; H = sera heated at 56°C for 30 min.
These results are the reciprocals of the titres obtained.

in different animals. On average the highest titres recorded were against lung and brain. The activity was reduced or removed by heating at 56°C for 30 min before testing. The activity against kidney was least affected by this procedure.

Fractionation of sera

(1) Five normal rat sera which had been shown to be complement fixation test positive against whole liver homogenate were fractionated on sucrose density gradients. The nine

fractions obtained were tested against whole liver homogenate and other rat organ homogenates with the results as shown in Table 2, indicating that the activity was present in the faster sedimenting portions of the sera.

(2) Five rat sera which were positive in the complement fixation test against whole liver

TABLE 2. Complement fixation and passive haemagglutination activity in sucrose gradient fractions against rat organ homogenate

Organ	No. of sera tested	Complement fixation activity in fraction
Liver	3	5, 6, 7
Lung	2	5, 6, 7
Spleen	2	5, 6, 7
Heart	2	5, 6, 7
Brain	2	5, 6
Muscle	2	5, 6, 7
Kidney	2	5, 6, 7

Organ	No. of sera tested	Passive haemagglutination activity in fraction
Lung	1	5, 6, 7, 8, 9
Brain	1	5, 6, 7, 8, 9
Spleen	1	5, 6, 7, 8, 9

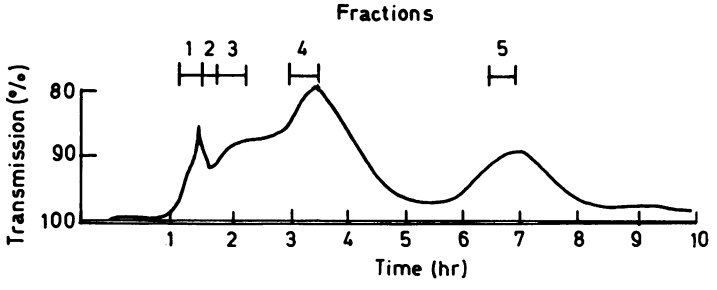

FIG. 1. Sephadex G-200 separation of rat serum indicating fractions taken. Buffer: 0·1 M-Tris, 0·2 M-NaCl, pH 8·0; flow rate, 35 ml/hr; sample, 1 ml rat serum.

homogenate were fractionated as described above by chromatography on a column of Sephadex G-200. The protein distribution pattern obtained is shown in Fig. 1. Fractions were taken from the ascending portions of the four peaks and the descending portion of the first as shown in Fig. 1.

The fractions when tested by complement fixation with whole liver homogenate were

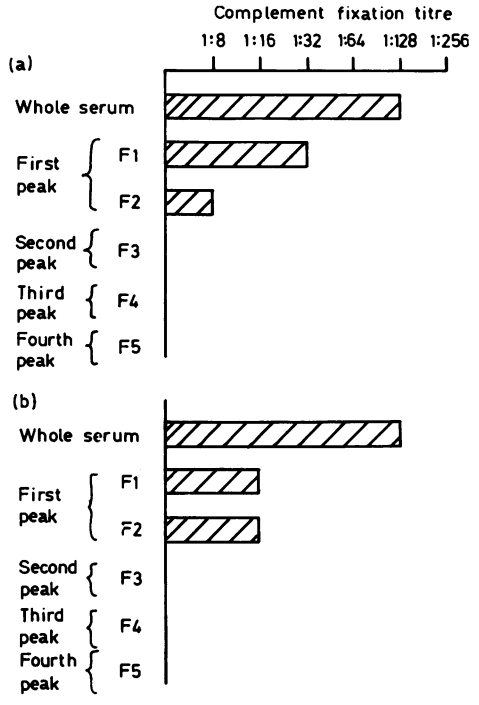

FIG. 2. Complement fixation titres of rat serum fractions (Fig. 1) tested against rat kidney homogenate. (a) Serum 1, (b) Serum 2.

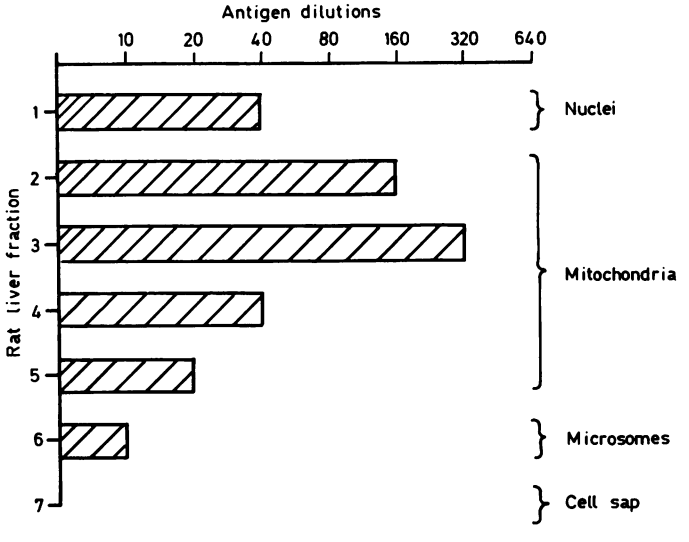

FIG. 3. Antigen dilution test with standard serum pool and liver fractions. Blocks represent positive complement fixation test at respective antigen dilution.

negative. Complement fixing activity was detected in tests with rat kidney antigen as shown in Fig. 2. This is consistent with the results shown in Table 1 in which the anti-kidney activity was relatively heat stable. However, even this activity was lost on concentration of the fractions by dialysis against carboxy-methyl cellulose.

The first proteins eluted through Sephadex G-200 are the high molecular weight immunoglobulins (IgM)+the α_2 macroglobulins (Flodin & Killander, 1962). The later peaks are dominated by the IgG and albumen respectively and Fahey & Wirtz (1966) have shown that the IgA appear just before the IgG. Immunoelectrophoretic patterns of the high molecular weight fractions obtained by both methods showed that they contained no contaminating IgG.

TABLE 3. Complement fixation (CF) and passive haemagglutination (PHA) antibody activity against various rat organ homogenates

Serum	Liver		Brain		Kidney		Lung		Spleen	
	PHA	CF	PHA	CF	PHA	CF	PHA	CF	PHA	CF
1	8	16	16	0	16	256	4	64	4	4
2	8	32	16	256	16	512	8	512	8	32
3	8	16	8	0	8	512	8	128	4	0
4	256	8	8	0	16	512	8	512	4	0
5	16	4	0	0	16	256	8	64	0	0
6	8	8	8	4	8	512	4	256	4	0
7	8	0	4	0	4	256	0	0	0	0
8	8	0	0	0	8	512	4	0	0	0
9	128	8	4	0	8	512	8	128	4	0
10	16	8	0	4	8	64	8	32	0	4
11	4	32	0	64	4	256	4	512	0	—
12	32	128	256	—	—	—	—	—	32	—

The results are shown as the reciprocals of the titres obtained. The above sera were unheated and from a different group of animals to that shown on Table 1.

Complement fixation test of normal sera against liver fractions

The liver fractions were prepared by differential centrifugation and tested as previously described (Pinckard & Weir, 1966). The fractions were made up to correspond to their concentration in the whole liver homogenate. They were doubly diluted in series and tested against a constant dilution of a pool of normal, unheated sera. Fig. 3 shows the results with each of the seven fractions. All but fraction 7, the cell sap, showed some activity, with the main peak of activity in fraction 3, the 'light mitochondria'.

Passive haemagglutination

Twelve sera were tested against various organ homogenates in the passive haemagglutination and complement fixation tests and the results are shown in Table 3. No correlations

between the titres obtained in the two tests is evident although the high molecular weight immunoglobulins appear to be involved in both instances (Table 2).

Detection of bound complement by fluorescent antibody

The uptake of guinea-pig complement when normal rat serum was layered on sections of rat organs was tested for by the use of a fluorescein conjugated anti-guinea-pig globulin. Eight sera were tested of which seven were found to show the uptake of guinea-pig globulin. The activity was removed by heating the sera at 56°C for 30 min and by the adsorption of complement from the added guinea-pig serum by immune precipitates.

Two rat sera were fractionated by zone ultracentrifugation in sucrose density gradients. Fractions 5, 7, 8 and 9 of these sera gave positive results. Controls with guinea-pig serum from which the complement had been absorbed with an immune precipitate were negative.

Indirect fluorescent antibody technique

The reaction between normal rat sera and rat kidney sections was detected by means of a fluorescein conjugated anti-rat serum. The tubules of the kidney have a high mitochondrial content (Walker *et al.*, 1965) and, as has been noted, this is the main sub-cellular antigen concerned in the reaction. Five unheated rat sera were tested and all were found to give faint staining. One rat serum was fractionated by density gradient centrifugation. Only fraction 7 was found to show activity in this procedure.

DISCUSSION

These results show, using a variety of immunological techniques, that most 'normal' rats have heat-labile complement fixing antibodies in their sera directed against a large number of different tissues and organs. The activity is associated with the high molecular weight serum components sedimenting with the IgM on sucrose density gradient fractionation and eluting with the α_2 and IgM globulins from Sephadex G-200. No activity was found in the fractions containing IgG although some was found in the sucrose fractions taken between the IgG and IgM peaks. It is uncertain yet whether this is due to traces of high molecular weight material in these fractions. However, on the basis of the tests carried out in the standardization of the centrifugation procedure which showed a clear separation between the IgG and IgM globulins, it is perhaps more likely that the activity in fractions 5 and 6 is due to 'IgA type' immunoglobulin. Such a possibility would be consistent with the relative heat lability of the serum activity.

The previous finding that rats could not be made tolerant to their particulate liver antigens and can form IgM antibody to such antigens as early as 21 days after birth (Pinckard & Weir, 1966) suggests that these animals should be quite capable of responding to cellular breakdown products released from effete cells. The results reported here support this possibility.

The anti-tissue antibodies found in rats after toxic liver damage (Weir, 1961, 1963, 1964) are stable to heating at 56°C for 30 min, unlike the present antibody activity, but both are associated in the complement fixation test with the high molecular weight immunoglobulin directed against the mitochondrial fraction obtained from liver by differential centrifugation. Preliminary results in the tanned cell test show that the mitochondrial fraction is a suitable antigen in this test. The fact that antibodies of this type can be induced in germ-free animals

following carbon tetrachloride injection (Arnason, Salomon & Grabar, 1964) renders it unlikely that the initial stimulus to the production of these antibodies is micro-organismal as suggested by Asherson & Rose (1963) with respect to the natural autoantibodies to liver and kidney in rabbits and autoantibody production to the gut in rabbits after injection of Freund's complete adjuvant (Asherson & Holborow, 1966). The finding that the antibody directed against the various organ and tissue homogenates can vary independently in different animals indicates the antibody must, to some extent, be tissue specific. This result can readily be explained if the antibody arises as the result of stimulation by cellular breakdown products. The immunoglobulins with the ability to take part in complement fixation and passive haemagglutination tests can also be seen to exist in varying proportions in different sera (Table 3).

The observations reported here may explain the common finding of anti-tissue antibodies in human disease states in conditions where cellular breakdown occurs, e.g. anti-heart antibodies in patients with myocardial infaction (Ehrenfeld, Gery & Davies, 1961), various forms of liver disease (Mackay & Gajdusek, 1958; Fraga, Toledo & Lima, 1964; Walker *et al.*, 1965), kidney disease (Kramer *et al.*, 1961) and following burn injury (Feodorov & Skurkovich), 1962; Pavkova, 1962). However, on the basis of their detailed observations of liver disease Doniach *et al.* (1966) conclude that not all types of cell injury in man are able to evoke anti-tissue antibody formation and that other factors must also be involved.

Serum antibodies have been implicated at various stages of the phagocytic processes, e.g. chemotaxis (Boyden, 1962; Hurley, 1964; Ward, Cochrane & Muller-Eberhard, 1965; Keller & Sorkin, 1965), phagocytosis (Stiffel *et al.*, 1964) and cytopepsis (Harris, 1961; Thorpe & Marcus, 1964). Preliminary evidence, using the two compartment chamber technique (Boyden, 1962) indicates that serum containing the immunological activity described here has a marked chemotactic effect on guinea-pig polymorphs when mixed with liver homogenate or isolated mitochondria. The reagents alone induce no chemotaxis or have only a slight effect. It therefore seems likely that the antibodies reported in this study may be implicated in the removal of tissue breakdown products and thus provide an experimental foundation to a physiological role of the immunological system as proposed by Grabar (1964).

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