Studies on the Production of the Complement Fixing Factor in Reaction to Autologous Liver Tissue

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INTRODUCTION

The presence of an organ- and species- non specific complement fixing factor has been demonstrated in the sera of patients having various liver diseases.[1-3] A higher incidence of positive results and higher serum titers have been observed in active chronic hepatitis and primary biliary cirrhosis.[4-6] The clinical significance of the complement fixing factor in liver diseases has been investigated from a number of view points,[7][8] but further studies into this problem are necessary.

The present study aims to provide additional informations on which has resulted from a series of experiments in which the complement fixing factor appeared following liver damage caused by the injection of thioacetamide.

MATERIALS AND METHODS

Hepatic damage was produced by intraperitoneal injection of thioacetamide. A total of 20 male Wistar rats which showed no anticomplementary effect to a 1 : 16 serum dilution were used. The rats weighted from 130 to 160 gr.. These rats were divided into two groups of 10 rats each. The first group received a single injection of thioacetamide and following the injection, sera were taken at intervals of 2 hours, 12 hours, 36 hours, and then, after 36 hours, once each day for 12 days. After the taking of sera at 12 hours, 36 hours, 48 hours, 72 hours, 96 hours, one week, and one month, rat was sacrificed on each of those occasions. The second group of 10 rats received a weekly injection of thioacetamide for five weeks. After the fifth weekly injection of thioacetamide sera were taken at the same interval as for the first group. After the taking of sera at 12, 36, 48, 72, 96 hours, one rat was sacrificed for histological examination. At one week, one month and two months after the fifth weekly injection one additional rat was sacrificed too. No sera were taken on these last three occasion. All injections were intraperitoneal injection of thioacetamide at 30 mg per 100 gr. of body weight.

In addition, the livers of 5 more male Wistar rats were used for making various liver fractions.

Preparations of various liver fractions

Normal rat liver was perfused in situ with cold normal saline solution. The liver tissue was cut into small pieces and minced, washed throughly by several changes of saline solution and homogenized in a glass homogenizer. A twenty per cent suspension solution was then prepared in normal saline solution. This suspension solution was centrifuged at 3000 rpm for 10 minutes. The supernatant solution thus obtained was used as a saline extract of liver.

Fractionation of liver homogenate was performed using the modified method of Schneider & Hogeboom.[9] Perfused rat liver was minced and washed with ice cold 0.25 M sucrose buffer solution. The liver tissue was homogenized in the same buffer solution and differential centrifugation was performed. Sediments at 7, 000 G (mitochondria) and 105, 000 G (microsome) were suspended with normal saline solution. The supernatant solution was dialyzed for 3 days with normal saline solution to remove sucrose and was used as a cell sap fraction. Similarily mitochondrial and microsomal fractions were washed several times with a normal saline solution, and were suspended with normal saline solution after hemogenizing by a closed teflon pestle. All procedures were carried out at 4°C.

Fig. 1. demonstrates the method used in the present studies for preparation of liver fractionation and the electron micrographs of mitochondria and microsome.

Immunization of rabbits

Three adult rabbits were used for immunization. Rabbits received subcutaneous injection of 1 ml of liver cell sap solution containing 10 mg of protein with an equal volume of complete Freund's ajuvant at weekly intervals for 2 months. All rabbits showed high titer 7 days after the last immunization.

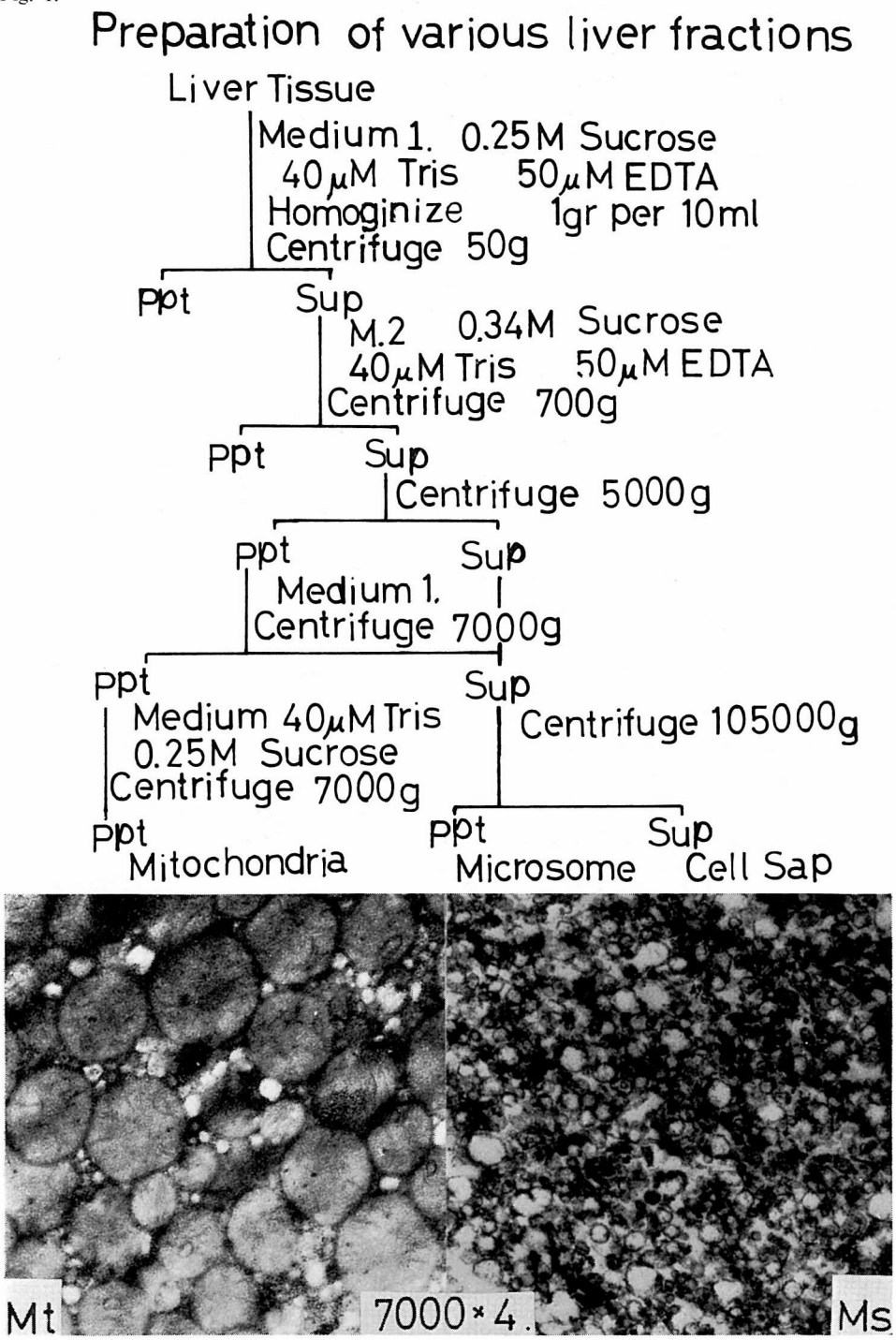
Detection of hepatic protein in serum

The hepatic proteins in the serum of the rats were detected by the diffusion technique using the rabbit anti-rat liver cell sap serum absorbed with normal rat serum. The agar plate was incubated at 4°C for 5 days, thereafter the precipitin line was examined.

Complement fixation test

Qualitative complement fixation test was performed by the method described by Kolmor.[10] The serum was inactivated at 56°C for 30 minutes and diluted serially with a phosphate buffered solution containing Mg^{++} (20 μg/ml). A quarter ml of saline extract of liver and 2 units of complement were incubated overnight at 4°C. The following day, 0.25 ml of a sensitized 2 per cent suspension solution of sheep red blood cells were added and incubated in a water bath at 37°C for 30 minutes and read optically for hemolysis. Complement fixation test was expressed as negative if there was a complete hemolysis at 1 : 16 serum dilution.

Fig. 1.


Preparation of various liver fractions
Liver Tissue
Medium 1. 0.25M Sucrose
40μM Tris 50μM EDTA
Homoginize 1gr per 10ml
Centrifuge 50g
Ppt
Sup
M.2 0.34M Sucrose
40μM Tris 50μM EDTA
Centrifuge 700g
Ppt
Sup
Centrifuge 5000g
Ppt
Sup
Medium 1.
Centrifuge 7000g
Ppt
Sup
Medium 40μM Tris
0.25M Sucrose
Centrifuge 7000g
Centrifuge 105000g
Ppt
Mitochondria
Ppt
Microsome
Sup
Cell Sap
Mt
7000×4.
Ms

Treatment with mercaptoethanol

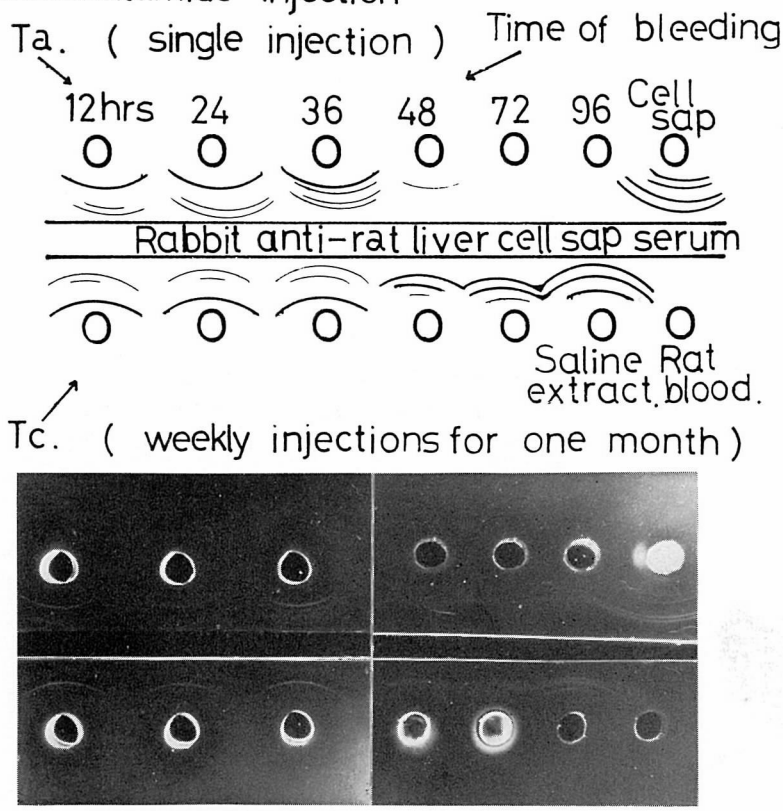
Treatment with 2-mercaptoethanol was carried out by a modification of the method described by Deutsch and Morton.[11] Equal volumes of sera diluted 1 : 10 and 1 M mercaptoethanol were mixed and incubated at room temperature for 24 hours. After incubation, the serum was dialysed for 24 hours against buffered saline containing 0.02 M iodoacetamide.

RESULTS

Detection of circulating hepatic protein

The rabbit anti-liver cell sap serum absorbed with normal rat serum revealed four precipitating lines against liver cell sap. The sera taken at 2, 12, 36, 48, 72 and 96 hours after thioacetamide injection were examined against rabbit anti-liver cell sap serum. The amount of hepatic protein in the rat serum was studied by examining the highest dilution showing precipitin lines. As shown in Fig. 2,

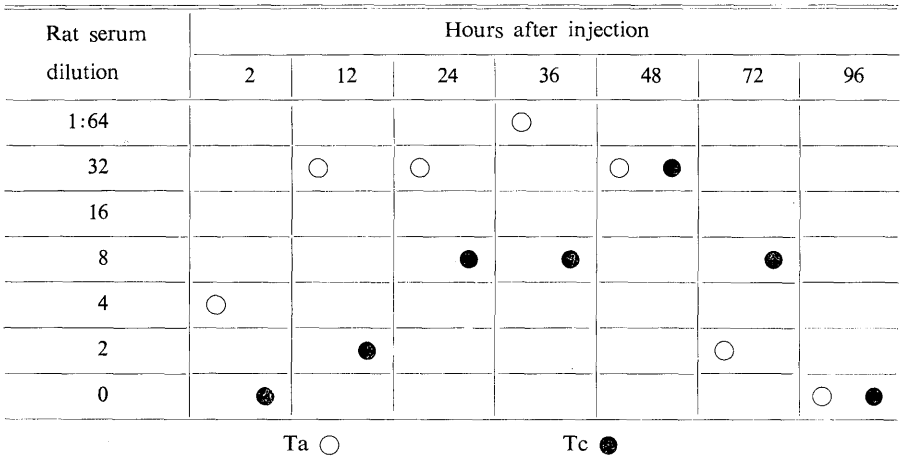
Fig. 2. Detection of circulating liver protein after Thioacetamide injection



circulating hepatic proteins began to be detected within a few hours after the thioacetamide injection and had disappeared by 72 hours after administration of injection. In the sera taken at 12 to 36 hours after injection, four precipitin lines were obtained and the highest level of hepatic proteins was found. The sera obtained at 2, 48, 72 and 96 hours showed only two precipitin lines against anti-liver cell sap serum.

Similar results were demonstrated in the sera of rats after the fifth weekly injection of thioacetamide. However, the hepatic proteins in this group were slow in appearing after the fifth injection and reached a peak at 36 to 48 hours after injection and the hepatic proteins titers were lower. Fig. 3 shows the hepatic protein titers in the sera of rats treated with thioacetamide.

Fig. 3. Liver antigen titers after thioacetamide injection



Incidence of complement fixing factor

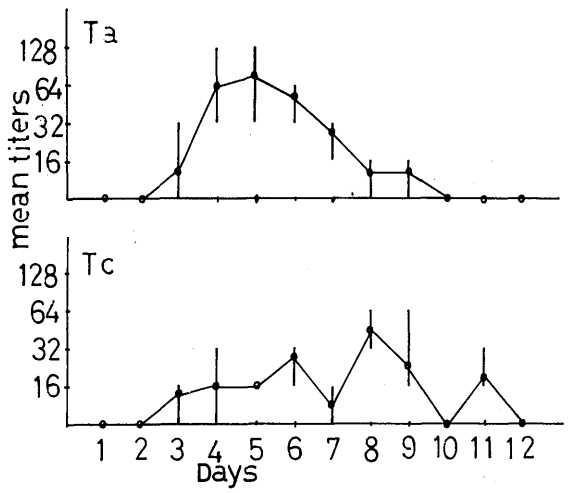
Complement fixation test was performed on sera taken each days for 12 days after the thioacetamide injection. The sera from rats given injection of thioacetamide fixed complement with the saline extracts of both autologous and homologous rat livers. Generally, the incidence of a complement fixation factor against homologous liver was much higher than against autologous liver (Fig. 4). After a single thioacetamide injection the complement fixing factor began to appear on the third day and it disappeared on the tenth day. The highest complement fixation titer was at a 1 : 128 serum dilution on the 5th day (Fig. 5). In the rats given five weekly injections of thioacetamide, complement fixing factor was found sporadically in low titers. (Fig. 5) The sera which fixed complement with saline extract of rat liver were used to react with various liver fractions. Antigenic activity in the sera from thioacetamide treated rats was found in microsome fraction. A negative result was obtained with both mitochondrial and cell sap

fraction (Fig. 6). The complement fixing factor was inactivated by mercaptoethanol treatment. This activation seemed to be related to IgM.

Fig. 4. CFT with auto- and Homologous liver antigen

Serum No	Antigen \ Day	1	2	3	4	5	6	7	8	9	10	11
Ta No. 2	auto-	−	−	−	+	++	+	−	−	−	−	−
	Homo-	−	−	−	+	++	+	+	−	−	−	−
Ta No. 4	auto-	−	−	+	+	+	−	−	−	−	−	−
	Homo-	−	−	+	+	++	+	+	+	+	−	−
Tc No. 2	auto-	−	−	−	−	+	−	−	−	+	−	−
	Homo-	−	−	+	−	+	+	−	++	+	−	+
Tc No. 4	auto-	−	−	−	−	+	−	−	+	−	+	−
	Homo-	−	−	−	+	+	+	+	++	+	−	+

Fig. 5.



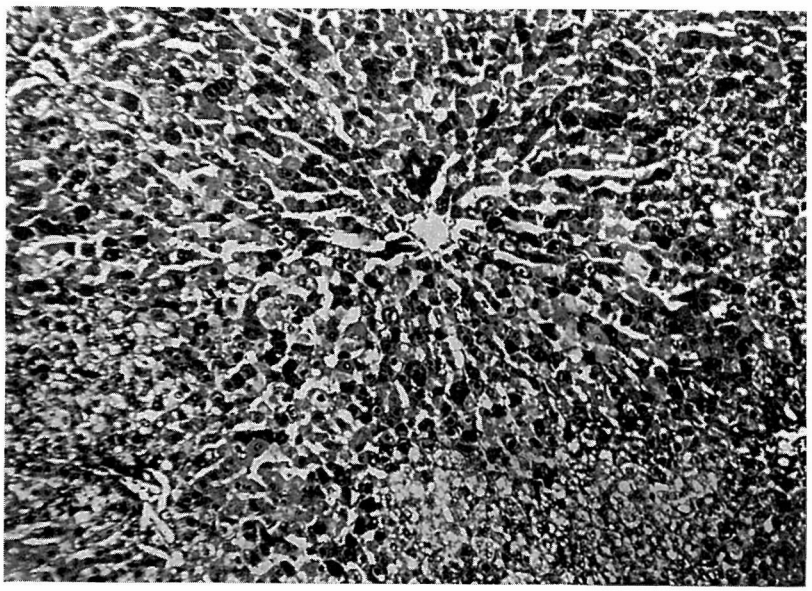
Histological findings

At 12 hours after a single injection of thioacetamide the hepatic cells were eosinophilic and sometimes fragmented. After 24 to 48 hours, hydropic degeneration and ballon cells were found in the centrolobular zone and cellular infiltration appeared among these cells. The necrosis reached a maximum at 24 to 48 hours (Fig. 7). Thereafter, centrolobular necrosis diminished and the livers began to have a normal appearance within 2 weeks.

Fig. 6. CFT with various liver fractions

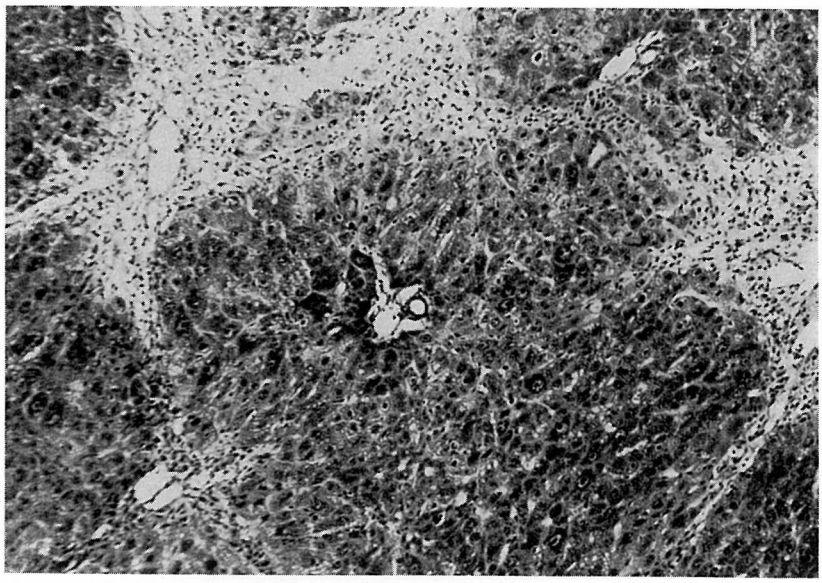
No of Serum	Days of Bleeding	Liver fraction			Saline extract
		CS	MS	MT	
Ta No. 2	4 Days	−	++	−	−
	5	−	+++	−	−
	6	−	+	−	−
Ta No. 4	4	−	+++	−	−
	5	−	+	−	−
	6	−	+	−	−

Fig. 7.



Severe hepatic necrosis and fibrosis were found in the rats given five weekly injections of thioacetamide (Fig. 8). Although the relationship between the degree of hepatic damages and lapse of time was not clear, liver damage was completely repaired within 2 months except for a slight fatty infiltration.

Fig. 8.



DISCUSSION

Four hepatic proteins were detected in the serum of the rats given thioacetamide injection. These proteins were of hepatic origin as shown in Fig. 1. In maximum titer sera, four precipitin lines were found by the use of anti-rat liver cell sap rabbit serum using Ouchterlony's method. Although the nature of hepatic protein in the blood was not examined, it may be tissue break-down products and a type of hepatic enzymes previously reported by Rees et al..[12)]

The hepatic proteins appeared 2 hours after a single injection of thioacetamide, and had disappeared by 72 hours after the injection, with the maximum level in the blood being at 36 hours. In the rats receiving five weekly injections of thioacetamide, hepatic proteins appeared more slowly in the blood, and had disappeared by 96 hours. The serum level of hepatic proteins in rats given five weekly injections was lower than in the rats given the single injection, which suggests that the amount of hepatic protein in the liver decreases with repeated injections of thioacetamide. Histologically, severe hepatic necrosis and fibrosis were observed after the repeated injections.

Similar clearance curves of hepatic proteins were obtained for all rats whether they received a single or repeated injections of thioacetamide. There are indications, as yet unproven, that the leakage of hepatic protein is related to acute hepatic injury. Histological examination of rat livers after a single thioacetamide injection indicated that there may be a correlation between the serum level of hepatic protein

and the degree of necrosis.

At such times, when hepatic proteins had disappeared from the blood, the sera from the rats became to fix complement with the saline extracts of both autologous and homologous rat livers. The complement fixing titers reached a peak on the fifth day after the thioacetamide injection, and had disappeared by the tenth day after the injection.

Similar complement fixing factors against the rat liver homogenate were found in the rats which received injections of carbon tetracloride.[13][14] It has been well known that the heated aggregated gamma globulins are anticomplementary.[15] Recently, Beall reported that complement fixing reaction is due to aggregation of serum gamma-globulin with the normal tissue protein containing protein with some similarities to serum gamma-globulin.[16] However, as reported by Weir, the appearance of the complement fixing factor was prevented by irradiation and splenectomy and it's activity present in the macroglobulin fraction of the serum. Wier[14] indicated that the complement fixing factor was autoantibody in nature but he did not actually demonstrate the hepatic protein in the blood after carbon tetracloride injection. The experimental results presented here show that hepatic proteins are released into the blood by the thioacetamide injection, and that subsequently autoantibodies against autologous liver homogenate appear in the blood.

Although the sera of rats whose liver had been damaged by carbon tetracloride fixed especially complement with mitochondrial fraction of the liver, the activities of the complement fixing factor which was detected in the sera of the thioacetamide damaged rats was found in the microsome fractions. This may due to the qualitative difference of hepatic proteins released by hepatotoxin.

In the rats given repeated injections of thioacetamide, the complement fixing factor was detected transiently following the 5th injection. This suggests that the complement fixing factor is mainly a relation to acute hepatic injury. It is especially noticeable that these serum factors appeared sporadically in lower titers. These rats may become tolerant to the hepatic proteins which are released continuously by repeated injections of thioacetamide. A selective loss of tolerance in rats to hepatic antigen was induced by immunizing them with autologous liver emulsified in complete Freund's adjvant and such antisera reacted with autologous rat liver as determined by haemagglutination.[17] These antibodies were considered to as being formed by the break in normal immunological homeostasis as reported by Burnet.[18] The complement fixing factor caused by acute hepatic damage in our experiments, may differ from the autoantibodies caused by the loss of selective tolerance to autologous tissue proteins and formed in response to specific hepatic antigen.

The results of histological examination presented here showed that severe hepatic necrosis was repaired completely within 2 months, as demonstrated in the rats given repeated thioacetamide injection. On the basis of this observation, there

was no evidence that complement fixing auto-antibodies play a pathologic role in liver damage. The complement fixing autoantibodies related to IgM may be an immunological response to the results of hepatic break down which is caused by acute hepatic damage.

SUMMARY

Hepatic proteins were demonstrated in the sera of rats given thioacetamide. These hepatic proteins remained in the blood for 3 to 4 days. At such times, when hepatic proteins had disappeared from the blood, complement fixing factors reacting with the saline extracts of both autologous and homologous rat livers were found in the sera from the rats, and disappeared by the tenth day. In rats which had been given weekly injections of thioacetamide for a month and this followed by a maintenance injection, it was found that complement fixing factor appeared sporadically in low titer. Complement fixing factor may be related to IgM.

After the thioacetamide injection severe hepatic necrosis and fibrosis were found in all rats, but this damage was completely repaired within 2 months except for a slight fatty infiltration.

Complement fixing autoantibodies may be an immunological response to the hepatic proteins which were released following acute hepatic damage.

Acknowledgment

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REFERENCES

1) Gajdusek, D. C.: *Nature* (London), **179**: 666, 1957.
2) Hackett, E., Beech, M. and Forbes, I.: *Brit. Med. J.*, **2**: 17, 1960.
3) Deicher, H. R., Holman, H. R. and Kunkel, H. G.: *Arthr. and Rheum.*, **3**: 1, 1960.
4) Gajdusek, D. C.: *Arch. Int. Med.*, **101**: 9, 1958.
5) Willcox, R. C. and Isselbacker, K. J.: *Am. J. Med.*, **30**; 185, 1961.
6) Göken, M.: *J. Lab. Clin. Med.*, **59**: 533, 1962.
7) Mackay, I. R. and Larkin, L.: *Australian Ann. Med.*, **7**: 251, 1958.
8) Pasnik, L. J., Beall, G. N. and Van Arsdel, P. D.: *Am. J. Med.*, **33**: 774, 1962.
9) Hogeboom, G. H. and Schneider, W. C.: *The Nucleic Acids*, Vol. 1, 1955. Academic Press. P. 105.
10) Yamamura, Y. and Ishizaka, K.: *Immunochemistry*, 1963, Asakura, Tokyo. P. 36.
11) Deutsch, H. F. and Morton, J. I.: *Science*, **125**: 600, 1957.
12) Rees, K. R. and Sinha, K. P.: *J. Pathol, Bacteriol.*, **80**: 297, 1960.
13) Weir, D. M.: *Lancet*, **1**: 1147, 1961.
14) Weir, D. M.: *Immunolgy*, **6**: 581, 1963.
15) Ishizaka, T. and Ishizaka, K. and Borsis, T.: *J. Immunol.*, **87**: 433, 1961.
16) Beall, G. N. and Ohanian, S. H.: *J. Lab. Clin. Med.*, **61**: 67, 1963.
17) Richter, M., Sargent, A. U., Myers, J. and Rose, B.,: *Immunology*, **10**: 211, 1966.
18) Burnet, F. M.: *Brit. Med. Bull.*, **19**: 245, 1963.