

**Anticryptococal Serum Factors in Experimental Liver Disease**

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Lysozyme and ß-lysin activity in rat serum have been studied biochemically and microbiologically after induction of hepatic dysfunction. Under the conditions of these experiments ß-lysin activity was adversely affected by liver necrosis or cirrhosis, while both factors (ß-lysin ** and lysozyme) were affected by hyperbilirubinemia. Lysozyme and ß-lysin activity were unaffected in serum from rats afflicted with (a) malnutrition, (b) fatty liver in the absence of significant necrosis, (c) disturbance in porphyrin metabolism as represented by hexachlorobenzene intoxication, and (d) extent of glycogen deposition in the liver.

The potential role of these and other pertinent factors in host resistance to cryptococcosis has been discussed.

A recent study has shown 1 that serum from certain patients with Hodgkin's granuloma, reticulum cell sarcoma, or leukemia exhibited anti-cryptococcal activity which deviated from the normal. Exacerbation and subsequent restabilization of the neoplastic process could be charted by plotting the inhibitory activity of the serum. In addition, it was shown that some patients with hepatomegaly in the absence of obvious reticuloendothelial disease have yielded serums with reduced activity. It was thus the aim of the present study to define the role of the liver in decreased serum inhibition of cryptococci by measuring this activity in rats with experimentally induced hepatic dysfunction.

**Materials and methods**

*Animals* — Male, Holtzman rats (Rowley Farms, Plymouth, Michigan) ranging in body weight from 160 to 180 gm., were used throughout this study. Each ex-

Donaldson, were unable to establish serological identity between serum anticryptococcal inhibitor and Donaldson's ß-lysin. For sake of convenience the term ß-lysin is used in this paper to indicate similarity to but not identity with the thermostable bactericidin.

*Cryptococcus neoformans* ist der Erreger der sogenannten Cryptococcose (Europäische Blastomykose Busse-Busdike, Torulose). Die Cryptococcose der Ratte wie beim Menschen nimmt ihren Ausgang von einer Inhalationsinfektion der Lungen. Metastasierung mit Befall von Nieren, Leber, Milz und Knochenmark ist häufig zu sehen. Öfters ist die Infektion bemerkbar bei Reticulosen, H o d g k i n - scher- oder Zuckcr-Krankheit oder bei Krankheiten, wobei die Wirtsresistenz vermindert ist.

1 H. H. Gadebusch, Antimicrobial Agents and Chemotherapy — p. 640 [1964].
perimental group included at least five animals per drug and an equal number of suitable placebo-treated controls.

**Drugs and Drug Protocols** — Ethionine (ETH), a-methyl, ot-methionine (METH), thioacetamide (TAA), [Nutritional Biochemical Corp., Cleveland, Ohio]; cortisone acetate (CORT) [the Upjohn Co., Kalamazoo, Michigan]; and carbon tetrachloride (CCl₄) [Mallinckrodt Chem. Co., St. Louis, Mo.] were administered by injection. Dimethylthrosamine (DMN) [Eastman Kodak Co., Rochester, N. Y.] and alphaphosphyl-isothiocyanate (ANIT) [K and K Laboratories, Jamaica, N. Y.] were suspended in peanut oil [Eastman Kodak Co., Rochester, N. Y.] and alpha-lipoic acid [Eastman Kodak Co., Rochester, N. Y.] were offered to individually caged animals and given with fifty rats (160—180 gms) which were weighed ad libitum for a period of twelve weeks.

At various intervals after administration of the drugs, animals were exsanguinated to obtain serum and the livers rapidly excised for fixation in 10% buffered formalin or sectioned directly in freezing microtome for oil red O staining. In addition, 24-hour urine samples were collected from HCB-fed rats before sacrifice.

**Protein Depletion** — Protein depletion was started with fifty rats (160—180 gms) which were weighed and caged separately. Twenty of these animals were given Normal Protein Test Diet (General Biochem. Corp., Chagrin Falls, Ohio) and the remainder placed on Protein Deficient Test Diet for 12 days. At the end of that time rats of the latter group weighing more than 90% of their original weight were discarded and the remainder placed on Protein Deficient Test Diet for 10 days. All diets were supplied in pellet form ad libitum. Body weights of all animals were recorded daily. Twenty-two days after initiation of the experiment all surviving animals in both groups were exsanguinated and the livers handled as described previously for drug-treated animals.

**Biochemical Determinations** — When indicated, total protein was determined by the biuret reaction and total hirurbin by the diazosulfanilic acid reaction of van den Bergh as modified by Malloy and Evelyn and adapted for microdetermination by Hogg and Meites. Alkaline phosphatase (AP) and glutamic pyruvic transaminase (GPT) activity in serum were measured by the method of Powell and Smith and Reitman and Franks respectively with the aid of commercial reagents (Sigma Chemical Co., St. Louis, Mo.). Serum iron and total iron binding capacity were determined by the method of Kittes et al. and Ramsay respectively. Uroporphyrin in 24-hour urine samples from HCB-fed animals was estimated spectrophotometrically. Liver glycogen was determined by the method of Kemp and Kits van Heijningen.

Electrophoresis of serum by the polyacrylamide gel method was carried out on each sample. Quantitation of the electrophoregrams was accomplished by a densitometric scan at 640 mλ using a modified Beckman DU spectrophotometer followed by estimation of the areas under the curves from Gaussian equations.

**Histologic Studies** — Paraffin sections of formalin-fixed tissue were stained with hematoxylin and eosin. Histologic changes were graded essentially according to Popper et al.

**In Vitro Antifungal Activity** — The presence of known anti-cryptococcal factors in freshly collected rat serum was measured by first assaying the serum for lysozyme (muramidase EC 3.2.1.17) activity. Subsequently, the serum was absorbed with bentonite to remove the lysozyme and assayed for β-lysin activity as described previously.

Briefly, this system consisted of a duplicate series of tubes each containing medium and final concentration of 20% of the serum under test. Suitable controls were included. Each tube then received an inoculum of the assay organism (Cryptococcus neoformans BR1) and was incubated at 37°C.

At intervals all tubes were mixed well on a Vortex mixer and the optical density (OD) at 650 mλ was recorded with an un inoculated-medium tube as a blank to adjust the instrument (Bausch and Lomb Spectronic 20 colorimeter) to 100% transmission. The assay was terminated as soon as growth in the control tubes (containing no serum) had reached an OD of approximately 1.00 and the arithmetic mean values were plotted on graph paper.

The resulting growth curves were compared by dropping a perpendicular to the abscissa from the point where the control curve crossed the 0.800 OD line. The OD values of the test sera and normal control
serums thus derived were numerically scored by applying the following formula:

\[ \text{absorbancy of test serum} - \text{absorbancy of control serum} \times 100 \]

\[ \text{absorbancy of test serum} \]

Under these circumstances a value of less than 10 signified that inhibition of the assay organism by the test serum was within normal limits. Abnormal inhibition was denoted by values between 10 and 60. Above 60, it must be assumed that the serum provided a substance stimulatory to growth and/or reproduction of the organism.

**Results**

*Acute Studies* — The administration of large single doses of various known hepatotoxic agents to rats has defined the biochemical and histological alterations which occur during the course of their intoxication (Table 1). The most significant changes in serum of treated animals included increases in (a) alkaline phosphatase (ANIT, TAA), (b) glutamicoxylic transaminase (ANIT, TAA, DMN, CCl₄) and (c) total bilirubin (ANIT, DMN, TAA). While serum protein values varied only slightly among the groups, electrophoretic analyses demonstrated consistent increases in the albumin fraction (TAA, CCl₄). These observations were well correlated with concomitant decreases in alpha and gamma globulins as well as severe centrolobular necrosis. DMN-treated animals, in contrast, demonstrated considerable variation in serum proteins which has been ascribed to the hemorrhagic nature of the liver lesions.

Acute ethionine intoxication, which was reported to cause an increase in quinine oxidase in rat serum evoked no change in alkaline phosphatase (AP), glutamic-pyruvic transaminase (GPT) or bilirubin. Fat vacuole formation was noted, however, upon histological examination of the liver sections. Another methionine analog, α-methylmethionine, was essentially non-toxic to rats in spite of its reputed greater effect on apparent free methionine in the liver of female rats.

Alterations in serum anticryptococcal factors were mixed and varied. Activity due to β-lysine was reduced in most cases in the early stages of intoxication (TAA, DMN, ANIT, CCl₄) but never dropped more than ca. 30% even in the face of severe centrolobular liver necrosis (CCl₄). When hyperbilirubinemia was present in the later stages, β-lysine activity was further reduced or completely abolished (ANIT). In contrast, significant increases in lysozyme were noted only in animals treated with TAA. Since liver lysosomes are devoid of lysozyme, it was reasoned that the increased amounts of this enzyme probably could be traced to leukocytes which had infiltrated the area. A modest reduction in lysozyme occurred as soon as hyperbilirubinemia became evident. Alpha-methylmethionine and ethionine treated animals exhibited no changes in β-lysine or lysozyme activity when compared to placebo-treated animals.

*Chronic Studies* — Long term administration of carbon tetrachloride or ethyl alcohol resulted in hepatic cirrhosis in rats as confirmed by gross and histopathological examination. The serum changes in CCl₄-treated animals paralleled those reported for acute studies. Ethanol-treated rats showed slight increases in GPT, lysozyme and in β-lysine activity.

The induction of experimental porphyria in rats by means of hexachlorobenzene ingestion showed serum changes not unlike those seen in ethanol-treated animals at a time when large amounts of porphyrins (ca. 63 mcg/24 hours) were excreted in the urine.

Hypoproteinemia, whether the result of malnutrition (reduced protein intake) or induced by increased protein catabolism (cortisone), did not significantly alter serum enzymes or anti-cryptococcal activity. It should be pointed out that glycogen stores were increased during cortisone administration and decreased (histological evidence) when protein intake was restricted.

**Effect of bilirubin upon anti-cryptococcal factors in rat serum** — In view of the marked reductions in anti-cryptococcal activity noted during hyperbilirubinemia it was of interest to determine the reason(s) for this effect. Bilirubin (crystalline; \( E_{453}^{1%} = 1.02 \times 10^3 \) in CHCl₃) was taken up in a minimum of ammonium hydroxide to affect solution and added to several pools of normal rat serum. The serum pools were adjusted for pH (7.4) and bilirubin concentration. Lysozyme assays were followed by bentonite absorption and subsequent determination of β-lysine activity as outlined previously. Representative assays as depicted in Table 2


17 M. S. Dunn and E. A. Murphy, Cancer Res. 18, 569 [1958].
Table 1. Correlative Summary: Acute Hepatotoxicity

**Abbreviation: AP = alkaline phosphase; GTP = glutamic-pyruvic**

Table 2. Effect of bilirubin in rat serum upon lysozyme and β-lysin activity. * For procedure, c.f. text. ** Values above 10 indicate altered inhibition of the test organism by serum.

established that both lysozyme and β-lysin activity were adversely affected by the presence of bilirubin in serum at concentrations above 2 mg-per cent.

**Effect of iron upon lysozyme and β-lysin activity in rat serum** – Iron has been incriminated in a number of experimental situations with the inability of an animal host to contain a given pathogen. Since an increase in serum iron or change in iron-binding capacity might have occurred following procedures designed to derange liver function, it was deemed necessary to determine the potential effect of this metal ion on anti-cryptococcal activity.

Serum samples from each experimental group (minimum of 5 animals) were pooled in iron-free glassware and the iron content determined on two separate occasions. Under these conditions the serum iron content of none of the groups of rats exceeded 174 mg/100 ml. (normal range 134 – 148 mcg/100 ml.). Total iron-binding capacity under the same circumstances remained within normal limits, and none of the rats yielded serums with reduced lysozyme or β-lysin activity. The calculated per cent saturation increased only ca. 10 per cent.

**Discussion**

With the aid of a number of experimental drugs whose adverse effects on liver metabolism were generally well documented, it has been possible to advance our knowledge in the area of host resistance mechanisms vis-a-vis Cryptococcus neoformans. Thus, rats subjected to various chemical or physiological insults, yielded serums which possessed less anticryptococcal activity largely due to apparent reductions in lysozyme and β-lysin. Bilirubin in vitro and hyperbilirubinemia in vivo were capable of interfering with the activity of lysozyme and β-lysin. An excess of porphyrins in the urine following hexachlorobenzene intoxication, on the other hand, was unable to alter serum inhibition of this fungus.

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20 H. Ungar, E. Moran, M. Einser, and M. Eliaikim, Arch. Pathol. 73, 427 [1962].
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<tr>
<td>β-Lysin activity***</td>
<td>Total Protein [gm. %]</td>
<td>Electrophoresis** [% Protein Fraction]</td>
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<td>Pre-Albumin</td>
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<tr>
<td>3.2 ± 1.7</td>
<td>6.3 ± 0.2</td>
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<tr>
<td>8.5 ± 2.7</td>
<td>6.4 ± 0.1</td>
<td>8.17</td>
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<tr>
<td>5.3 ± 2.1</td>
<td>5.8 ± 0.2</td>
<td>0.82</td>
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<tr>
<td>7.2 ± 1.5</td>
<td>6.1 ± 0.1</td>
<td>Variable</td>
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<tr>
<td>2.1 ± 2.2</td>
<td>6.7 ± 0.2</td>
<td>0.85</td>
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<tr>
<td>5.8 ± 2.1</td>
<td>6.9 ± 0.2</td>
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<td>0.1 ± 1.0</td>
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<td>7.9 ± 1.2</td>
<td>6.7 ± 0.9</td>
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<td>5.6 ± 0.4</td>
<td>6.1 ± 0.2</td>
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<tr>
<td>6.9 ± 0.3</td>
<td>6.5 ± 0.3</td>
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\[\text{asaminase, Routes = s.c. = subcusaneous; p.o. = per os.} \]
\(\text{** Standard errors of the mean for these data vary between }\)
\(\text{10 signify impaired inhibition.} \)

In spite of extensive liver necrosis or hemolysis in some instances, significant increases in serum iron could not be demonstrated. For chronic processes it may well be that the excess iron is preferentially sequestered in hepatic parenchymal cell lysosomes as indicated for CCl\textsubscript{4} \textsuperscript{25} or iron-dextran loading \textsuperscript{26}. These findings, however, do not rule out the possibility that serum iron increases in other in vivo situations could occur (massive intravascular hemolysis of erythrocytes, excessive absorption of iron, ineffective hemapoieis) and that these events could alter the ability of serum to inhibit C. neoformans. In addition, the oxidation state of the iron as well as the pH or Eh may be even more important. An instance of in vitro loss of inhibitory activity has been reported \textsuperscript{27}. These authors dialyzed human serum against phosphate buffer and attributed the decrease in inhibition to the presence of phosphate ion. A more likely explanation stems from the work of Rogers \textsuperscript{28} who found that horse serum dialyzed in a similar manner lost antibacterial activity which he attributed to iron contamination of the phosphate buffer used.

The role of iron in the establishment of an infectious process appears to take many forms depending upon the pathogen and the physiological circumstances which obtain. Thus, Candida and bacteria such as Pasteurella, Staphylococcus, and Salmonella (but noh Escherichia and Shigella \textsuperscript{18}) have been reported to grow in human serum only in the presence of excess iron. From the evidence presented it is not clear whether the effect shown is due to inhibition of the antimicrobial activity of transferrin, whether indeed large amounts of iron are required by these organisms for continued growth and reproduction, or both. In the case of Cryptococcus neoformans the Fe\textsuperscript{2+} requirement is extremely low \textsuperscript{29} so that this metal ion would probably not become a growth-limiting factor in vivo. The complexity of iron in host resistance phenomena is further complicated by the recent reports of Rogers et al. \textsuperscript{28}. These investigators have defined a bacteriostatic system for Clostridium perfringens type A in vitro which consists of transferrin together with β\textsubscript{2} and γ-globulin.

From a clinical standpoint of view, one might reasonably deduce from these experiments that patients with advanced Hodgkin’s disease or other acute liver disorder would be less likely to contain the spread of cryptococci via the hematogenous route. Jaundice when present would further compromise the activity of the host resistance factors in question.

The able technical assistance of Judy Henry and Douglas Bundy is acknowledged.

\textsuperscript{25} R. J. Stenger, Amer. J. Pathol. 43, 867 [1963].
\textsuperscript{26} G. Kent, O. T. Minick, F. I. Volini, E. Orfeli, and J. de la Huerga, Lab. Invest. 12, 1102 [1963].
\textsuperscript{28} H. J. Rogers, Immunology 12, 285 [1967].
\textsuperscript{29} H. H. Gadebusch, Unpublished experiments [1965].