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STUDIES ON BARTONELLA MURIS ANEMIA

VI. A Lipoid Extract of the Spleen That Prevents Bartonella muris Anemia in Splenectomized Albino Rats

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In previous work (1), the authors have demonstrated that minute splenic autoplastic transplants made 7 weeks prior to splenectomy protect a large percentage of splenectomized rats against *Bartonella muris* anemia. A comparative histological study of the transplants of protected and unprotected rats revealed a regeneration of the pulp cells in the protected rats, and an exhaustion destruction of the pulp in the unprotected rats. This supported the hypothesis that the reticular and endothelial cells of the pulp of the spleen elaborate some internal secretory substance having an influence to prevent *Bartonella* infection. Lauda and Flaum (2) found that rats joined by parabiosis are protected against *Bartonella muris* anemia if the spleen of only one animal is removed. From their experiments these investigators concluded that the protective action is due to a hormonal substance produced in the spleen.

Unsuccessful attempts have been made by numerous investigators to demonstrate some substance in the spleen which would replace this organ in protecting adult splenectomized rats against *Bartonella muris* anemia. During the past 3 years we have made many attempts to obtain such an extract. Feeding raw or cooked spleen of ox, calf, pig, or rat had no effect on the course of the disease. Recently, however, lipoid extracts of the spleen have been prepared which possess the property of protecting splenectomized adult albino rats against *Bartonella muris* anemia in a large percentage of instances.

Since the anemia is more severe in the male, only male rats were used in testing the extracts. In no instance among 440 male rats of carrier stock used for studies of *Bartonella muris* anemia during the

past 3 years, has the anemia failed to develop following splenectomy. Similar observations on the prevalence of the disease in the male have been made by Ederle and Kriech (3). It is plain that the spleen plays a specific rôle in the protective mechanism of the body to the infection. Rats of carrier stock are infected early in life, they recover, and remain carriers of the infection.[1] Subsequent splenectomy is followed by a recurrence of the infection with the development of a severe anemia.

Preparation of the Extract

The method of extraction is the same as that used by Hartman (10) in the preparation of the cortical suprarenal hormone. Ox spleens were obtained from freshly killed animals, packed in ice, and immediately transported to the laboratory. 10 pounds of spleen were extracted at a time.

The fat and as much of the capsule as possible is stripped off, and the pulp is ground in a meat grinder. The trimming and grinding is done by artificial light and the material kept cold. The ground material is placed in large round bottom flasks and 1.5 liters of peroxide-free ether is added for each kilo of spleen. The ether extraction is done in the dark in an atmosphere of CO_2, and the extraction is assisted by a rotary motion of 15 to 20 rotations per minute. More rapid agitation may result in emulsification. If possible the temperature of the room should be kept below 10–12°C. The flasks are shaken for a period of 3 hours, and allowed to stand overnight. The ether is decanted, and rapidly filtered through coarse filter paper. The ether extraction is repeated for a total of 3 extractions. The ether removed is evaporated at a temperature of 15°C. *in vacuo*. All the ether fractions are added together. It is best to evaporate the ether immediately after decanting and to add the new fractions to the residue. The residue of the ether extraction is now extracted with 95 per cent alcohol. 100 cc. of 95 per cent alcohol is used for each kilo of spleen extracted. The alcohol is warmed to 60°C. for a period of 30 minutes, the flask being shaken every 10 or 15 minutes. The flask is slowly cooled to ice temperature and the alcohol extract decanted. The same quantity of alcohol is again added, and extraction repeated 4 times, to insure thorough extraction.

The alcohol fractions are pooled, and chilled to −10°C. for 2 hours in chopped ice to which $CaCl_2$ has been added. This process causes the precipitation of large amounts of cerebrosides. The chilled material is then rapidly filtered in the ice box, and the filtrate is now a clear brownish yellow liquid. It is essential that filtration be done rapidly and in the cold. If the filtrate is not clear the chilling process should be repeated before proceeding. The spleen contains large amounts of inert lipoids and their removal is attended with difficulty. The alcohol filtrate

[1] See previous publications of the authors on this subject (4–8). For a review of the literature to 1930 see Lauda (9).

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is now evaporated to one-third its volume *in vacuo* at a temperature of 45–50°C. The alcohol fractions will keep for several weeks. The reduced volume of alcohol is again chilled, and filtered in the manner outlined above. The filtrate is now evaporated to dryness *in vacuo* at 45–50°C. The residue is repeatedly extracted with a small amount of ether. More complete extraction is obtained by agitation and scraping with a rubber "policeman" during the process. This procedure is repeated 4 times with at least 10 cc. of ether and is carried out in the dark room by red light, the flask being kept on ice during the procedure. The ether extractions are filtered through coarse and then fine filter paper, and evaporated to dryness *in vacuo* at a low temperature. Great difficulty will be encountered in this last step if there is a large amount of alcohol residue. In that case, in order to eliminate inert material, it is advisable to do another alcohol extraction with subsequent chilling and filtering.

The final residue of the last ether extraction is a small amount of brownish oily material. If the extractions have been carefully carried out not more than a few drops of this material remain. It is thoroughly emulsified with distilled water (50 cc. for 5000 gm.) in the required amount, and filtered through a Seitz filter. The resultant aqueous extract has a yellowish tinge. It is brought up to isotonicity, and preserved with 0.1 per cent of benzoic acid. Physiological salt solution may be used for the emulsification in place of distilled water. 1 cc. of the extract is equivalent to 100 gm. of spleen. The aqueous extract will keep for several weeks if a preservative is added.

Numerous attempts to obtain larger yields were made by suspending the oily residue of the final ether extraction in olive oil. The resultant emulsion produced severe peritoneal irritation when injected intraperitoneally into rats. An attempt to fractionate the final ether-soluble material into acetone-soluble and insoluble fractions was made in the following manner: An excess of acetone was added to the final ether extract until no further precipitation occurred. The precipitate was filtered off and dissolved in benzine. The filtrate was evaporated and dried *in vacuo*, taken up in water, and passed through a Seitz filter. The benzine extract of the acetone-insoluble fraction was evaporated to dryness *in vacuo* and taken up in water. When tested neither the acetone-soluble nor the acetone-insoluble fraction showed evidence of potency.

Extracts of fresh spleen, made by extraction either with acetone or with HCl, proved unsuccessful.

On analysis, the final aqueous lipoid extract gave no reactions for either protein or carbohydrate. Traces of nitrogen were detectable (Kjeldahl). No copper or iron could be detected by qualitative tests.

EXPERIMENTAL DATA

Twenty-nine splenectomized male albino rats of carrier stock were tested with the lipoid extract of spleen. Of these, 8 were 6 to 8 weeks of age and 21 were 3 to 5 months old. The extract was administered twice daily intraperitoneally in

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amounts of 0.5 cc. The injections were started 24 hours prior to splenectomy. Hemoglobin estimations with the Dare hemoglobinometer, red blood cell counts, and smears were made daily. A rat was considered completely protected if the blood count and hemoglobin did not show greater variation than is observed in normal rats, and *Bartonella* bodies were not found on the red cells. The animals were under observation for at least 1 month following splenectomy, to eliminate the possibility of a delayed appearance of the anemia.[2] All the rats were of *Bartonella muris* carrier stock, raised in the laboratory, and used for studies of this anemia during a period of several years.

TABLE I

The Protective Action of an Aqueous Lipoid Extract of Spleen against Bartonella muris Anemia in Male Splenectomized Rats of Carrier Stock*

No. of rats	Age	Completely protected†	Unprotected	Protected
				per cent
Treated				
8	6–8 wks.	3	5	37
21	3–5 mos.	14	7	66
Controls				
0.5 cc. saline twice daily				
40	3–5 mos.	0	40	0
Theelin (ovarian follicular hormone) 10 units per day				
12	3–5 mos.	0	12	0

* The extract was administered twice daily intraperitoneally in amounts of 0.5 cc. (1 cc. of the extract is equivalent to 100 gm. of spleen).

† A rat was considered completely protected if the blood count and hemoglobin did not show greater daily variations than is found in normal rats, and if *Bartonella* bodies were not found in the red cells.

In the first group of immature rats, complete protection against *Bartonella muris* infection and anemia was obtained in 3 out of 8 rats. In the second group of mature rats, 14 out of 21 rats were completely protected. Of 40 male splenectomized rats injected daily with physiological salt solution, all developed *Bartonella muris* anemia. As a

[2] In the control splenectomized untreated rats the anemia develops in most instances during the 1st week.

further control, the ovarian follicular hormone, Theelin (Parke, Davis and Co.) an aqueous extract of the crystalline hormone, was administered to 12 adult male splenectomized rats in amounts of 10 rat units per day injected intraperitoneally. No protection against *Bartonella muris* anemia was observed.

Rats of carrier stock between the ages of 6 and 8 weeks, with the spleen intact, suffer from a severe infection of *Bartonella muris* with little or no anemia. This is manifested by the occasional appearance of *Bartonella muris* bodies on the blood cells and the marked hyperplasia and congestion of the spleen. Protection in such animals against anemia following splenectomy by an extract of the spleen is, therefore, less effective. In the adult, the infection is latent and the spleen shows little evidence of hyperplasia and congestion. In these rats protection was obtained in a large percentage of instances.

DISCUSSION

Numerous investigators have found that liver extracts potent in the treatment of pernicious anemia have no effect on the course of *Bartonella muris* anemia. Recently Ederle and Kriech (3) have studied the effects of injections of a commercial extract of liver, administered subcutaneously in amounts equivalent to 2 gm. of liver a day. They observed that the anemia in the treated rats was less severe, and the mortality was less than in the control group. On the other hand, Vedder (11) obtained entirely negative results with liver therapy.

The literature contains numerous references to the use of spleen extracts in various diseases, but interpretation of such results is difficult. There are many evidences of splenic function, but their significance is not plain. The body of most animals contains accessory splenoid tissue in varying amounts. The rat possesses less hemolymph tissue than other mammals (12) and a much larger spleen, and the removal of the spleen in this animal seems to have an unusually marked effect on resistance. The specific relation between *Bartonella muris* anemia in the rat and the function of the spleen offers a possible index of function of the pulp tissue of this organ.

The protective action of a splenic extract against *Bartonella muris* anemia in the splenectomized adult male rat upholds the hypothesis that such an extract contains a specific internal secretory substance.

SUMMARY

An aqueous lipoid extract of ox spleen was prepared which protects adult male albino rats of carrier stock in a large percentage of instances against *Bartonella muris* anemia following splenectomy. It is suggested that the extract contains a specific hormonal substance.

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