STUDIES ON BARTONELLA MURIS ANEMIA

VII. The Protective Action of Copper and Iron against Bartonella muris Anemia

By DAVID PERLA, M.D., and J. MARMORSTON-GOTTESMAN, M.D.

(*From the Laboratory Division of Montefiore Hospital, New York*)

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The extensive experiments of Hart, Steenboch, and Elvehjem and their coworkers (1–5), Beard and Myers and their associates (6–11), and others have established the importance of copper in hemoglobin formation. Young rats fed on a diet of milk deficient in copper develop an anemia within 6 to 8 weeks which can readily be prevented by the administration of small quantities of copper in the diet. A minimal requisite of 0.025 mg. of elemental copper per rat per day was determined by these investigators. Since albino rats are subject to *Bartonella muris* infection spontaneously during the early weeks following weaning, it was thought that the milk anemia of the rat may be complicated by infection with *Bartonella muris*. The prophylactic effect of copper in the milk anemia of rats suggested its trial in *Bartonella muris* anemia of splenectomized adult rats.

The rats used in these experiments were all carriers of *Bartonella muris*. They were of stock raised in our laboratory for many years and maintained under constant environmental and dietary conditions. The diet for the past 10 years has consisted of 15 gm. per rat per day of a mixture composed of hominy 100 parts, rolled oats 25 parts, fine meat and bone 25 parts, dried skim-milk 16 parts, and salt 1½ parts. Twice a week the rats received whole milk and bread *ad lib.* and greens (lettuce leaves). The exact copper content of this diet was difficult to estimate but the food mixture was found to contain about 0.025 mg. of elemental copper per 15 gm. of food. In the experiments reported the copper was added in the form of copper sulfate in doses equivalent to 0.1 mg. of elemental copper per day. Lactose was used as a vehicle. The iron was added in the form of iron ammonium citrate in doses equivalent to 1 mg. of elemental iron per day.

Following splenectomy, daily hemoglobin estimations and smears were made on all the rats and the red cell counts were made every other day. In determining

the protective action of copper and iron supplements to the normal diet, against *Bartonella muris* anemia in splenectomized rats, it was necessary that the animals should be observed for a period of 1 month to exclude a delayed appearance of anemia following splenectomy. If the hemoglobin percentage and the red cell count did not fluctuate more than is usual in the normal rats and *Bartonella* bodies were not present or only sparsely found, it was considered that complete protection against the infection and the anemia was obtained.

In the experiments reported both immature and mature rats were used. The experiments were divided into three groups. In the first group of experiments, the copper and iron supplements to the diet were given for a period of 2 days prior to and 1 month subsequent to splenectomy. In the second group, the copper and iron supplements to the diet were given during a period of 9 to 12 days prior to and 1 month subsequent to splenectomy. In the third group, copper and iron supplements to the diet were given for a period of 2 months prior to and 1 month subsequent to splenectomy.

The Effect on Bartonella muris Anemia of Copper and Iron Supplements to an Adequate Diet

In the first group of experiments, 18 rats, 3 to 4 months old, were used. Four received daily supplements of iron in the form of iron ammonium citrate in amounts equivalent to 1 mg. of elemental iron per day, 8 received additions of copper as copper sulfate in amounts equivalent to 0.1 mg. of elemental copper per day, and 6 received both copper and iron additions daily. The supplements were commenced 2 days prior to splenectomy and continued thereafter.

All the animals developed severe *Bartonella muris* anemia. No protective action of the copper and iron was observed in this group. (See Table I.)

In the second group of experiments, 46 rats were fed diets supplemented with copper, iron, or copper and iron during a period of 9 to 12 days prior to and 1 month subsequent to splenectomy. Thirty untreated splenectomized adult rats were used as controls during the same period.

Of the treated rats, 20 received supplements of copper alone in amounts equivalent to 0.1 mg. of copper per day.[1] Of these, 8 were immature rats, and 12 were 4 months of age. Of the group of immature rats 3, or 37 per cent, were completely protected against *Bartonella muris* infection and failed to develop any evidence of anemia following splenectomy. Of the mature animals 9, or 75 per cent, were protected against the anemia. Fourteen rats received supplements of iron in the form of iron ammonium citrate in amounts of 1 mg. of iron per rat per day. Of

[1] It was found that intraperitoneal injections of copper as copper acetate in physiological salt solution are as effective as feeding the copper.

these, 4 were immature rats and 10 mature rats. Of the young rats only 1 was protected. Of the mature rats 5 were protected. (See Table I.)

Twelve rats received both iron and copper supplements in the same amounts as used in the previous experiments (0.1 mg. copper and 1 mg. iron per rat per day) during 9 to 12 days prior to and 1 month subsequent to splenectomy. Of 4 young

TABLE I

Effect on the Incidence of Bartonella muris Anemia Following Splenectomy of Additions of Copper and Iron to an Adequate Diet
Summary of Experiments

No. of rats	Age	Addition to diet	Feeding commenced prior to splenectomy	Protected	Unprotected	Protected
			days			*per cent*
4	3–4 mos.	Fe	2	0	4	0
8	3–4 mos.	Cu	2	0	8	0
6	3–4 mos.	Cu and Fe	2	0	6	0
8	9 wks.	Cu	9–12	3	5	37
12	4 mos.	Cu	9–12	9	3	75
4	9 wks.	Fe	9–12	1	3	25
10	4 mos.	Fe	9–12	5	5	50
4	9 wks.	Cu and Fe	9–12	4	0	100
8	4 mos.	Cu and Fe	9–12	6	2	75
10	9 wks.	Control	—	0	10	0
20	3–5 mos.	Control	—	0	20	0
11	4 mos. (at time of operation)	Cu	60–80	7	4	63
8	4 mos. (at time of operation)	Fe	60–80	2	6	25
8	4 mos. (at time of operation)	Cu and Fe	60–80	3	5	36

rats treated, all were protected. Of the 8 mature rats, 6, or 75 per cent, were completely protected against the *Bartonella* infection and did not develop any anemia.

Of the 30 control splenectomized adult rats observed in the same period, all developed a severe anemia.

From these experiments (see Table I) it is evident that copper and iron protect a large percentage of rats against the *Bartonella* infection following splenectomy if the copper and iron supplements are given during a period of approximately 10 days prior to splenectomy and continued thereafter. Copper is definitely more effective than

iron, and copper and iron slightly more effective than copper alone. In the groups fed diets supplemented by copper alone or iron alone, the adult rats were protected in twice as many instances as were the immature rats, doubtless because of the fact that the severity of the infection with *Bartonella muris* (without anemia) is much greater in the immature rat with intact spleen than in the adult rat in which the infection is entirely latent. This is manifested by the reaction of the spleen. The percentage weight of the spleen to the body weight of immature rats of carrier stock is much greater than that of mature rats. The spleen in the young rat of *Bartonella muris* carrier stock shows the histologic changes of congestion of the pulp and hyperplasia of the follicles.

Copper protects 75 per cent of adult rats against *Bartonella* anemia following splenectomy if it is added as a supplement to an adequate diet for a period of 10 days prior to splenectomy.

In the third group of experiments, 27 rats received supplements of copper and iron to the normal diet during a period of 2 months prior to and 1 month subsequent to splenectomy. Six untreated rats fed on the normal diet were used as controls. All the rats were 7 to 8 weeks of age at the time the supplemented feedings were commenced and about 4 months of age when splenectomized. All the controls developed a severe infection following splenectomy. Of the treated rats, 11 received supplements of copper alone in amounts equivalent to 0.1 mg. of elemental copper per rat per day. Of these, 7, or 63 per cent, were completely protected against the infection and the anemia following splenectomy. Eight rats received supplements of iron in amounts equivalent to 1 mg. of elemental iron per rat per day. Of these, 2 were protected. Eight received both copper and iron supplements in amounts equivalent to 0.1 mg. and 1 mg. respectively of the elemental metal per rat per day. Of these, 3 were protected.

From these experiments (see Table I), it is evident that supplements of copper to the diet given during a long period of time protected a considerable number of rats against *Bartonella muris* anemia following splenectomy. The addition of iron resulted in protection in only a few instances and the addition of both copper and iron resulted in less protection than the addition of copper alone. The protective action of supplements of copper and iron to the diet, when these are added during a period of 2 months preceding splenectomy, is not as great as that of copper and iron when added for a period of 10 days prior to splenectomy.

It is of considerable interest that in two instances the rats fed copper alone for 2 months developed a slight anemia several days prior to splenectomy. At operation the percentage weight of the spleen to body weight of the rat (0.9 per cent) was considerably greater than the average for this age period (0.27 per cent). Following removal of the spleen the anemia cleared up and the rat remained free of infection during the period of observation of 1 month. The large amount of copper may have resulted in injury to the pulp cells of the spleen. This was manifested by a recurrence of the *Bartonella muris* infection. That the removal of the spleen in these cases resulted in a lessening of the infection and disappearance of the anemia is a unique experience in our observations of *Bartonella muris* anemia. The fact that rats fed diets supplemented with iron, or copper and iron for a period of 2 months prior to splenectomy were not protected was probably the result of cellular injury resulting from excess storage of the metals—primarily the iron. The liver and spleen were found heavily laden with iron pigment. The total quantity of copper salt received by the treated rats to the day of splenectomy was equivalent to 6 to 8 mg. of elemental copper per rat and of iron salt, 60 to 80 mg. of elemental iron per rat.

Cannon and McClelland (13), Haendel and Haagen (14), Friedberg (15), Rosenthal and Zohmann (16), and Judenik (17) observed that repeated injections of suspensions of India ink or other inert colloidal substances may be followed either by spontaneous *Bartonella muris* anemia in the rat with intact spleen or by an increased susceptibility to the injection of blood of an anemic splenectomized rat (superinfection). They attribute this depression in the acquired resistance to *Bartonella muris* infection to the cellular injury resulting from blocking of the reticulo-endothelial system. The effect obtained is similar to that which follows splenectomy.

DISCUSSION

The effect of the addition of small amounts of copper to an adequate normal diet during a period of a little more than a week to prevent *Bartonella muris* anemia in albino rats of carrier stock is of importance in relation to the physiologic utilization of copper in the body. The need for small amounts of copper in the production of hemoglobin has been demonstrated by work already referred to. Copper is essential as a catalytic oxidative agent in the formation of hemoglobin. From the present work it would seem to be an important substance in the maintenance of resistance to *Bartonella muris* anemia. The *Bartonella* infection occurs spontaneously in rats of carrier stock between the 5th and 7th weeks.[2] It remains latent but the acquired resistance

[2] McCarrison and Singh (18) recently observed *Bartonella muris* bodies on the red cells in new-born rats of normal mothers during the first 4 days of life in about 20

established on the first invasion of the animal can be broken down by splenectomy. The anemia that follows is severe and striking, and results in a mortality of at least 30 per cent. Protection against this infectious anemia may be due either to a direct toxic action of copper and iron on the *Bartonella muris*, or to an indirect action intimately related to splenic function. In several instances we have observed, some weeks after copper had been discontinued, a recurrence of *Bartonella* infection with anemia in rats splenectomized and protected for 1 month by copper. It seems probable that the copper exerts an indirect protective effect rather than a direct toxic effect on the bacterium.

Feldt and Schott (19) have maintained that the chemotherapeutic action of salvarsan against spirochetal infections is due to an indirect action on the reticulo-endothelial cells. They found that when these cells were blocked with metallic colloids and splenectomy had been done in mice infected with *Spirocheta febris recurrentis*, the animals could not be cured by chemotherapeutic agents otherwise specific in their action. This is in accord with our observations in experiments in which an excess of copper and iron was fed to rats over a period of 60 to 80 days. The excess storage of the metallic substances may be thought to have injured the reticulo-endothelial cells and thus to have prevented the action of the copper and iron against the *Bartonella muris* anemia. The difference between the toxic dose of copper and the physiological requirement of this metal is, however, considerable.

In a previous study we have been able to procure from the spleen a substance (20) which when injected into adult albino rats, beginning on the day of splenectomy, protects in a large percentage of instances against *Bartonella muris* anemia. The extract was prepared in the manner of Hartman's suprarenal cortical extract. The specific relation of the *Bartonella muris* anemia in the adult rat to splenic function is well established. That an extract can replace the spleen in protecting the animal against this anemia strongly suggests that the specific protective function of the spleen is due to a substance secreted by the pulp cells which in some manner influences cellular response to infection. This extract contains no protein material, and no trace of either copper or iron. We have suggested that it contains a specific hormonal substance elaborated by the spleen.

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per cent of instances. Apparently in some cases infection with *Bartonella muris* may occur at birth.

It is possible that copper exerts its effect by influencing the oxidative processes involved in cellular activity[3] and in this way affects the resistance of the cell to toxic substances.

The literature on the relation of the spleen to iron metabolism is exhaustively and critically reviewed by Lauda (22). He states that the experimental evidence reported, is inconclusive in demonstrating any relationship between the physiology of the spleen, and either the storage or utilization of iron. The relation of copper to the spleen may be somewhat analogous to the relation of calcium to the parathyroid gland. The small amount of copper in the normal diet of the rat is insufficient for the needs of the rat in the absence of the splenic hormone. An excess of the metallic element in the diet may compensate for a deficiency of the hormone. Further, the copper, administered as an inorganic salt cannot be utilized at once but must be converted into a form that is utilizable by the body in the mechanism of resistance to *Bartonella muris* anemia.

Cunningham (23), in a careful study of the relative amounts of copper in various animal and plant tissues, found that the rat has less copper in its organs than any other animal studied. The copper ingested is stored primarily in the liver (21). Corper (24) found very little copper stored in any other organ than the liver, and only traces were recovered from the spleen. The liver of the rat contains 1/20th to 1/30th the percentage weight of copper found in the liver of the rabbit, the guinea pig, the sheep, or the ox (21). This is probably a result of great differences in the copper content of the food of these animals. The fodder of the guinea pig and the rabbit contains large amounts of green vegetables very rich in copper (22). It may be that the resistance of the adult guinea pig and the rabbit to *Bartonella muris* infection, which exists whether the spleen is present or not (25), is dependent on the high copper content of the diet. This would explain the susceptibility of the suckling young of these species, in which copper is as yet not stored in significant amounts (25).

These studies suggest a possible importance of dietary copper and of iron in relation to resistance to infectious anemias in human beings.

[3] The influence of copper on oxidative processes has been demonstrated by Voegtlin (21) in the case of glutathione. Glutathione combined with copper oxidized 1000 per cent more rapidly than glutathione not prepared in this way.

We plan to investigate the relation of dietary copper to various types of infection.

There is considerable literature on the administration of copper salts in the treatment of infectious diseases, but most of it is difficult to evaluate, and the reports are contradictory or the experiments poorly controlled. Good results have been reported from its use in oidiomycosis, actinomycosis, and sporotrichosis by Bevan (26). Its use in tuberculosis therapeutically, both experimentally and in patients, receives some support from the work of von Linden (27), Meissen (28), and Straus (29) but as Corper points out, their work is inadequately controlled. Corper could find no evidence of a therapeutic action of copper in experimental tuberculosis in guinea pigs, but he was not essentially concerned with the *physiological* rôle of copper in the mechanism of resistance. The animals employed in his experiments (guinea pigs and rabbits) have large stores of copper as result of a diet naturally rich in copper. An excess of copper added to their diet might well have a detrimental effect on cellular physiology. A relative paucity of copper in the diet, as normally occurs in the care of the rat, may, in the absence of a splenic hormone influencing infection, result in a diminution of resistance to certain types thereof. Additions of copper to the diet of these rats may raise their resistance. It does not follow, however, that an excess of copper added to a diet containing foods naturally rich in copper would necessarily increase the resistance of the host.

SUMMARY

The effect on *Bartonella muris* anemia of adding copper or iron or both to an adequate diet was studied.

The addition to the diet of copper (0.1 mg. per rat per day), or iron (1 mg. per day), or both during a period of 2 days prior to splenectomy and 1 month subsequent thereto failed to protect adult albino rats against *Bartonella muris* anemia.

The addition of copper to an adequate diet for a period of 10 days prior to splenectomy and 1 month subsequent thereto protected 75 per cent of the rats against the anemia.

The addition of iron (1 mg. per rat per day) for a period of 10 days prior to and 1 month subsequent to splenectomy protected 50 per cent of the rats against this anemia.

The addition of both copper and iron for a period of 10 days prior to and 1 month subsequent to splenectomy protected 75 per cent of these rats against *Bartonella muris* anemia.

The addition of copper alone for a period of 2 months prior to and 1 month subsequent to splenectomy protected 63 per cent of the rats against *Bartonella muris* anemia.

The addition of iron, or of both copper and iron during a period of 2 months prior to splenectomy and 1 month subsequent thereto protected about one-third of the rats against *Bartonella muris* anemia.

CONCLUSIONS

Copper plays a rôle in the mechanism of resistance to *Bartonella muris* anemia in the rat. The small amount of the element in the ordinary diet of the rat is insufficient to protect the animal after splenectomy. An excess of copper, however, may give protection in the absence of the spleen. Its utilization would seem to be intimately associated with splenic function.

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