THE ACCUMULATION OF IRON IN TUBERCULOUS AREAS

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PLATES 37 AND 38

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In previous communications (1, 2, 3) it has been shown that a vital dye, trypan blue, or iron in the form of its salt, ferric chloride, when injected into the circulating blood stream rapidly accumulates in an area of inflammation, where the substance is fixed and fails to drain to the tributary lymph nodes.

Bowman, Winternitz, and Evans by microscopic studies (4) found that, in experimental tuberculosis, trypan blue injected intravenously stains tubercles in the liver. They pointed out the great affinity of giant and epithelioid cells for this vital dye, which was always found as granules within these cells. Lewis (5) showed that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. If the animal is given an intravenous injection of trypan red 24 hours or more after such inoculation, the fluid in the anterior chamber of the inoculated eye always becomes colored. We have been able to confirm these earlier studies by gross examination. Trypan blue injected in the circulating blood stream of rabbits rapidly accumulates in tuberculous areas of the lung.

In view of the observation that iron, intravenously injected, accumulates in inflamed areas, experiments were set up to determine whether this metal when injected into the circulating blood stream would accumulate and be detectable in tubercles. In a preliminary report by one of us from The Henry Phipps Institute (6) it was shown in a few experiments that after several intravenous injections of ferric chloride an accumulation of iron within the caseous areas of tubercles in lungs was readily demonstrable by a qualitative test (Prussian blue reaction). These studies have been continued and considerably extended so as to include quantitative determinations on the accumulation of this metal in tuberculous areas.

EXPERIMENTAL

Bovine tuberculosis was induced in rabbits by the intravenous injection of either 0.01 mg. or 0.001 mg. of a saline suspension of Ravenel strain. Several weeks later a large number of these animals were killed by ether anesthesia. The lungs which showed, as a rule, extensive tuberculosis were carefully removed and dipped for about an hour in acidified potassium ferrocyanide (3 parts of 1 per cent HCl to one part of 2 per cent $K_4Fe(CN)_6$).

TABLE I

Presence of Iron in Tubercles of Non-Injected Animals

Rabbit No.	Interval between injection of tubercle bacilli and death of animal	Prussian blue reaction in tubercles of lung
	days	
1*	14	0
2*	19	0
3*	19	0
4*	20	0
5	22	0
6**	25	0
7**	28	0
8*	35	Trace
9	36	Trace in 2 or 3 tubercles
10***	40	0
11	52	0
12	52	0
13	52	0
14	53	0
15	53	0

* These rabbits received 0.01 mg. of bovine Ravenel strain. All the others received 0.001 mg.

** These two rabbits each received a single injection of 20 cc. 0.25 per cent ferric chloride.

*** This rabbit was allowed to die; the lungs were tested for iron on the day of its death.

The results of the tests are shown in Table I. It is evident that in the vast majority of these animals the tubercles in the lungs exhibited a negative Prussian blue reaction. In this connection it is interesting to note that many years ago Gierke (7) pointed out that calcified tubercles in the lungs failed to react to the microchemical test for iron. It should, however, be mentioned that frequently when a

rabbit was allowed to die spontaneously of tuberculosis and then placed on ice for a day or two before removing the lungs, the latter would reveal a distinct positive Prussian blue reaction within the caseous areas of tubercles. Evidently such areas contain a considerable quantity of iron bound up in a form that does not give the Prussian blue reaction prior to some degree of postmortem change. For this reason the qualitative test was always made on the lungs of an animal immediately after killing it, or, when the animal died spontaneously, on the day of death (Rabbit 10, Table I).

The experimental rabbits received an intravenous injection of either 0.01 or 0.001 mg. of saline suspension of bovine tubercle bacilli (Ravenel strain). From 3 to 5 weeks later these animals were started on a course of daily intravenous injections each of 5 cc. of a 0.25 per cent solution of ferric chloride crystals. The solution was usually heated slightly and injected very slowly. The number of injections varied a great deal. This material proved injurious when it escaped into the tissue about the marginal vein of the ear. After numerous daily injections, the development of thrombi in vessels and of an extensive local inflammatory reaction often prevented further administration of the iron salt. The rabbits as a rule were killed after several injections of the ferric salt solution. The lungs which showed extensive pulmonary tuberculosis were carefully removed and placed for about 1 hour in acidified potassium ferrocyanide.

Within a very short time, in some cases amounting to only a few minutes, the tubercles exhibited a marked Prussian blue reaction on gross examination. The blue color was intense in the caseous or central part of the tubercle (See Fig. 1 and Table II, Rabbit 20). The result was very different from that in ordinary inflamed areas. Dye or iron which penetrates into the periphery of these by way of the blood stream fails to enter the central part in which the circulation is relatively inactive (1, 2). It is to be noted in this connection that various dyes that cannot penetrate living cells are able to stain dead or dying cells (8).

The results of all the experiments are shown in Table II. Both Rabbits 25 and 26 lived for 13 days after the injections of ferric chloride had been discontinued. These two animals died of their tuberculosis and the lungs were tested on the day of death. In both of these rabbits an intense blue reaction in the caseous areas was obtained a few minutes after dipping the lungs into acidified potassium ferrocyanide solution. It was found that a single injection of 20 cc. of 0.25

per cent solution of ferric chloride produced no demonstrable Prussian blue reaction in the tubercles (Rabbits 6 and 7, Table I). Two injections of the ferric salt likewise produced essentially a negative Prussian blue reaction in Rabbit 18 (Table II). Several intravenous injections of the ferric salt at least are necessary before a positive Prussian blue reaction can be elicited in the tubercles of the lung.

The Prussian blue reaction involves the tubercles on the surface of the lung since these are the only ones in direct contact with the potassium

TABLE II

Presence of Iron in Tubercles of Animals after a Number of Intravenous Injections of Ferric Chloride

Rabbit No.	No. of 5 cc. injections of 0.25 per cent ferric chloride	Interval between injection of tubercle bacilli and death of animal	Prussian blue reaction in tubercles of lung
		days	
18	2	22	Trace in few tubercles
19	3	24	+
20*	16	37	+
21	9	38	+
22	4	39	+
23	2	42	+
24	7	43	+
25**	22	84	+
26**	19	87	+

* Received 0.01 mg. bovine Ravenel strain. All other rabbits received 0.001 mg.

** These rabbits were allowed to die of their disease; the lungs were tested for iron on the day of death.

ferrocyanide solution. The deeper layers of caseating tissue consequently do not reveal the presence of the metal by the qualitative test.

Histological studies of lung tissue of animals injected intravenously several times with ferric chloride and tested for the presence of the iron by the Prussian blue reaction show the metal to exist chiefly within the caseous centers of the tubercles (See Fig. 2 and Table II, Rabbit 26). Fig. 2 shows that the intense blue reaction (shown on the figure in black) is not distributed homogeneously throughout the caseous area. The figure is a low power reproduction; careful studies under higher magnification show that the granules sur-

rounding the caseous area are not iron deposits but nuclei. There is no evidence that iron particles have been previously taken up by cells which have then migrated into the growing tubercle and have broken down there. On this assumption one would still expect to find at the periphery of the caseous area a great number of mononuclear phagocytes loaded with particles giving the test for iron. These were not found. Only rarely, here and there, were blue granules observed within the cytoplasm of mononuclear phagocytic cells. The iron seems therefore to be deposited primarily within the areas of caseation. There is no indication that proliferation of fibrous tissue in the lungs is greater in injected than in uninjected animals.

In several animals the injections of ferric chloride were started on the day following inoculation with 0.01 mg. of bovine tubercle bacilli. Daily injections of the salt were performed for about 2 weeks. The lungs of these animals were then studied and found studded with miliary tubercles. On gross examination these showed either no areas of caseation or at most very few of them. When testing for iron in such tubercles the Prussian blue reaction was negative. This constitutes another evidence that iron is apparently deposited only where caseous areas are present.

Quantitative Studies on the Accumulation of Iron in Tuberculous Areas

In a previous communication (2) it has been shown that in uninjected animals an old inflammatory lesion of at least 70 hours duration reveals the presence of iron by the qualitative test. This is probably due to the degradation, in the late stages of inflammation, of hemoglobin from red corpuscles phagocytosed by tissue macrophages. It has been pointed out above that when the lungs of an uninjected tuberculous animal were tested for the presence of iron a positive Prussian blue reaction in the caseous areas was frequently obtained if the test was made some time after death. Presumably tubercles as such contain a larger quantity of iron than normal lung parenchyma. For this reason it was thought advisable to substantiate the qualitative findings on the accumulation of iron in tuberculous areas by quantitative determinations.

Portions of the lungs of rabbits that had received daily intravenous injections of 5 cc. of 0.25 per cent ferric chloride solution were placed in a dry oven for about

16 hours at a temperature ranging from 120°C. to 130°C. The lungs of tuberculous animals that had received no iron were treated in the same manner, to serve as controls. Both sets of animals had received the same dose of tubercle bacilli intravenously (0.001 mg. of a bovine Ravenel strain, with the exceptions of control Rabbit 8 and experimental Rabbit 20, each of which received 0.01 mg.).

Quantitative determinations of iron in tissue were performed according to the method of Kennedy (9) with only slight modifications. 20 cc. of concentrated sulfuric acid and 17 cc. of 60 per cent solution of perchloric acid were added to a carefully weighed portion of dried lung in a Kjeldahl flask. The mixture was heated over a low flame for about 10 minutes until complete digestion took place

TABLE III

Iron Content of Tuberculous Lungs in Animals Injected Daily with Ferric Chloride and in Non-Injected Animals*

Non-injected animals			Injected animals			
Rabbit No.	Interval between injection of tubercle bacilli and death of animal	Iron content*	Rabbit No.	No. of 5 cc. injections of 0.25 per cent ferric chloride	Interval between injection of tubercle bacilli and death of animal	Iron content*
	days				*days*	
8**	35	55.5	20**	16	37	165.3
9	36	120.0	21	9	38	185.2
14	53	118.3	24	7	43	289.5
16**	66	163.6	25***	22	84	876.1
17***	67	123.6	26***	19	87	686.9

* Figures are expressed in milligrams of iron per 100 gm. of dry tissue.

** Received 0.01 mg. of bovine strain of tubercle bacilli.

*** These animals were not killed but allowed to die of tuberculosis.

and the solution appeared almost colorless. While the mixture was still hot, 0.8 cc. of 30 per cent hydrogen peroxide was added and the liquid was then cooled and diluted to 100 cc. with distilled water. 10 cc. of this solution was pipetted into a 50 cc. stoppered cylinder to which were added 10 cc. of amylic alcohol and 5 cc. of 20 per cent sodium thiocyanate. The mixture was immediately shaken and the iron was extracted by the amylic alcohol layer. This layer was pipetted off into a colorimeter cup and compared with a standard treated in exactly the same manner.

The results of the analyses are shown in Table III. It is clear that the lungs of tuberculous animals that had received repeated intravenous injections of ferric chloride contain more iron than do the lungs of non-injected animals. Although the marked increase in iron con-

tent of lungs of injected animals is consistent, the variation in individual rabbits of this group is evident and is not entirely accounted for by differences in the number of injections of ferric chloride. The amounts of iron in the lungs of the control animals, although definitely less than in the injected animals, show also marked individual variations. The varying intervals between the injection of tubercle bacilli and the death of the animal had no evident relation to the individual differences in iron content (*cf.* Rabbits 8, 9, and 17).

Analyses were also performed to determine the iron content of normal lung tissue in two non-tuberculous animals receiving no iron. The values obtained were 70.2 and 78.8 mg. of iron per 100 gm. of dry tissue respectively. When these two values are compared with those obtained in tuberculous animals receiving no iron (Table III) it is seen that with the exception of one case (Rabbit 8) there is distinctly more iron in tuberculous than in normal lung tissue. This is not surprising when it is recalled that in previous work (2) a greater amount of iron was likewise recovered from inflamed than from normal cutaneous areas of non-injected animals. These results with non-injected animals show that lung tissue normally contains an appreciable quantity of iron, which is evidently bound up in a form that does not give the Prussian blue reaction. It is also conceivable that in caseous areas of non-injected animals iron exists in a loosely bound form which a certain degree of postmortem change renders capable of reacting to potassium ferrocyanide.

Determinations were made on the lung tissue of two non-tuberculous animals that previously had received 12 and 20 daily intravenous injections of 5 cc. of 0.25 per cent solution of ferric chloride crystals respectively. The purpose of these additional controls was to ascertain whether iron accumulates to the same extent in normal as in tuberculous lung tissue. The values obtained were 93.6 and 119.0 mg. iron per 100 gm. of dry tissue respectively. These results definitely show that the accumulation of iron in normal lungs after repeated intravenous injections of the ferric salt is slight when compared to its accumulation in tuberculous lungs of injected animals (Table III). In this connection it is interesting to note the recent demonstration of Fowweather and Polson (10) that in normal rabbits there is a lack of an adequate reservoir of iron in the lungs. They point out that the stor-

age of iron after its injection takes place principally in the liver. This is in agreement with earlier work. Boycott and Price-Jones (11) showed that in rabbits infected with *Trypanosoma brucei* an anemia developed owing to the destruction of red cells. The iron of the destroyed hemoglobin was found stored in the liver and in the spleen. Muir and Dunn (12) demonstrated that in acute hemolytic anemia nearly all the iron from the destroyed hemoglobin was deposited in the liver, spleen, and kidneys.

The quantitative results obtained further corroborate the qualitative tests and show that an accumulation of iron in tuberculous areas follows repeated intravenous injections of ferric chloride.

These experiments suggest that the accumulation of iron in tuberculous areas may have clinical application. It is conceivable that iron or iron-containing substances by their accumulation in tuberculous areas may alter the character or course of development of the disease. Further experiments are being conducted to investigate this question.

CONCLUSIONS

Repeated daily intravenous injections of ferric chloride solution are followed by an accumulation of iron in tuberculous areas of lungs. The iron accumulates in the caseous areas of tubercles and is demonstrable by the Prussian blue reaction.

Quantitative determinations corroborate these results and show that the iron content of lung tissue in tuberculous animals injected with ferric chloride exceeds that in normal animals injected with this salt, as well as that in non-injected tuberculous animals.

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EXPLANATION OF PLATES

PLATE 37

FIG. 1. Lung of Rabbit 20 (see Table II). This animal received 16 intravenous injections of ferric chloride solution. The animal was killed and the lung placed in acidified potassium ferrocyanide. The Prussian blue reaction is limited to the caseous areas of tubercles. About actual size.

PLATE 38

FIG. 2. Camera lucida drawing of microscopic section of lung (Rabbit 26, Table II). This animal received 19 intravenous injections of ferric chloride solution. Iron, as shown by the Prussian blue reaction, is deposited in the caseous area. All dots at the periphery of the area of caseation are nuclei. (A hematoxylin-eosin preparation; magnified about 85 times.)

FIG. 1

(Menkin and Menkin: Iron in tuberculous areas)

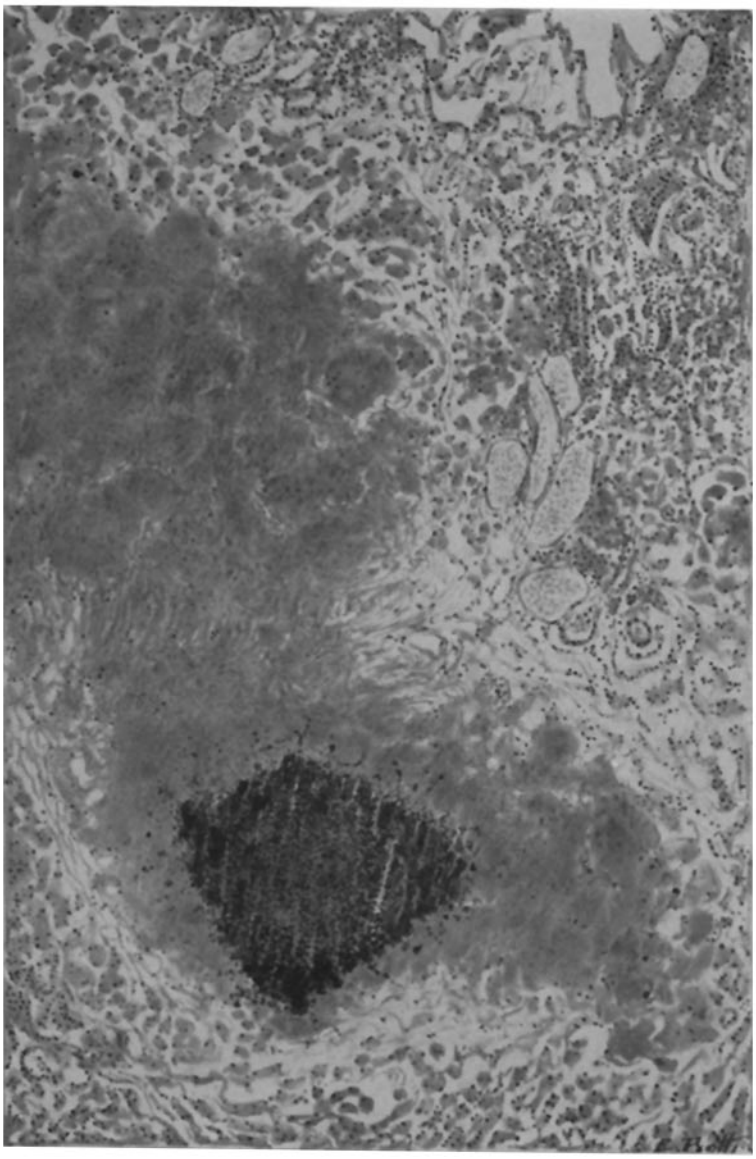

FIG. 2

(Menkin and Menkin: Iron in tuberculous areas)