THE ACCUMULATION OF IRON IN TUBERCULOUS AREAS

IV. THE EFFECT OF FERRIC CHLORIDE ON THE COURSE OF TUBERCULOSIS IN REINFECTED RABBITS*,†

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Previous studies by the writer have demonstrated that repeated intravenous injections of dilute ferric chloride solution are followed by an accumulation of iron in tubercles. Concomitantly with this accumulation the course of the disease in rabbits was found to be retarded, as evidenced by an increase in the survival time and by the less extensive involvement of the lungs of experimental animals as compared with controls that had had the disease for the same length of time (1-4). These results were obtained in two independent series of experiments. In the earlier investigations bovine tuberculosis was induced by intravenous inoculation of a virulent culture. When the intravenous ferric chloride treatment promptly followed subcutaneous injection of the bacilli, the dissemination of the latter from the site of inoculation was retarded (5). In a preliminary communication (6) it was shown that reinfected rabbits receiving repeated ferric chloride injections survived for more than 6 months after the death of the last control, which succumbed about 4 months following reinfection with virulent tubercle bacilli.

The present experiments give in greater detail some of the data on the combined effect of partial immunity and ferric chloride administration on the course of experimental tuberculosis. A comparison is

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made with the results of treatment of a non-vaccinated group of tuberculous animals. Some observations are brought forward indicating the mechanism possibly responsible for the favorable effect of ferric chloride in experimental tuberculosis.

TABLE I
Effect of Ferric Chloride Injections on Tuberculous Rabbits

	Control animals			Experimental animals			
	Rabbit No.	Weight at beginning of experiment	Survival time after inoculation with virulent tubercle bacilli	Rabbit No.	Weight at beginning of experiment	Amount of 0.25 per cent ferric chloride injected	Survival time after inoculation with virulent tubercle bacilli
		<i>gm.</i>	<i>days</i>		<i>gm.</i>	<i>cc.</i>	<i>days</i>
Group I, non-vaccinated	7-71	2230	91	7-60*	2015	201	112
	7-31	2195	102	7-51	1820	219	161
	7-80	1670	126	7-59	1860	214	287
	7-83	3010	162	7-30	2200	218	326
	7-81	2635	170	7-79	1695	202	344†
Average.....			130				246
Increase in survival time of experimental animals: 89 per cent							
Group II, preliminary vaccination with relatively avirulent strain	5-97	2900	47	6-07*	2450	232	81
	6-26	2060	55	5-41*	2500	278	110
	6-02	2250	67	6-16	2125	278	131
	6-24	2520	108	5-96	2200	278	334†
	6-28	2500	130	6-04	2955	278	334†
Average.....			81				198
Increase in survival time of experimental animals: 144 per cent							

* Superimposed upper respiratory infection at time of death.

† These animals were killed; all others died of their diseases.

EXPERIMENTAL

The technic followed was practically the same as that employed in the earlier experiments; for this reason it will not be taken up in great detail.

In brief, a series of 10 Flemish brindle rabbits was inoculated subcutaneously in the thigh each with 0.05 mg. of a virulent bovine Ravenel strain of tubercle bacilli. Intravenous injections of ferric chloride (0.25 per cent in distilled water) were started immediately in 5 of these rabbits and continued for a period of about

4 months. As a rule about three injections of 6 cc. each of the iron salt were made every week. At the beginning the injections were performed more frequently and the individual doses were larger (10 cc.). Subsequent observations indicated that 5 to 6 cc. of 0.25 per cent ferric chloride (lukewarm) was innocuous to rabbits when injected slowly into the circulation. The stock solution of ferric chloride was kept on ice when not in use.

A second group of 10 brindle rabbits was inoculated intravenously each with 0.005 mg. of a relatively avirulent strain of bovine tubercle bacilli (Cernay). About 6 weeks later each of these animals received subcutaneously in the thigh 0.05 mg. of a virulent bovine Ravenel strain of tubercle bacilli precisely as in the first series of rabbits. The injections of ferric chloride were started promptly in 5 of the group and were continued for about 4 months as described above. The purpose of this second series of experiments was to determine whether partial immunity induced by primary inoculation would enhance the retarding effect of ferric chloride.

Effects of Ferric Chloride on Survival Time

The results of the experiments are shown in Table I. Group I summarizes the observations on the non-vaccinated rabbits. Five controls lived from 91 to 170 days with an average survival time of 130 days. Four of the experimental rabbits survived from 112 to 326 days and one was still alive 344 days after subcutaneous inoculation with virulent tubercle bacilli. At that time this animal was sacrificed and a thorough postmortem examination was performed. When this last animal is included in the calculations, the average longevity of the experimental rabbits is found to be 246 days, an increase of 89 per cent over the survival time of the controls. The weight changes of the longest survivors of the control and experimental animals in this group are plotted in Chart 1. It is clear that death follows shortly after the controls begin to lose in weight (see control Rabbits 7-83, 7-81, and 7-80, Chart 1). On the other hand the weight curves of the experimental rabbits show a definite protracted course even when the animals start to show a loss in weight (see experimental Rabbits 7-59, 7-30, and 7-79, Chart 1). The controls began losing weight between the 12th and the 15th week of their disease; while with the exception of Rabbit 7-30, the experimental rabbits began to show a decline in their weight curves at the 22nd week of their disease; *i.e.*, about 6 weeks after discontinuing the ferric chloride injections. In brief, the data both on the increase in survival time and on weight

CHART 1. Weight changes of some of the non-vaccinated tuberculous rabbits (longest survivors) repeatedly injected with ferric chloride, and some of the non-vaccinated control tuberculous rabbits (longest survivors).

— control rabbits.

- - - - - experimental rabbits.

CHART 2. Weight changes of two vaccinated tuberculous rabbits (longest survivors, killed) repeatedly injected with ferric chloride, and of two vaccinated control tuberculous rabbits (longest survivors, died).
 — control rabbits.
 ----- experimental rabbits.

changes with administration of ferric chloride are in complete accord with the writer's previous observations (3, 4). The more marked increase in longevity obtained in this group of animals as compared with the previous series is presumably referable to the effect of ferric chloride on the milder type of disease induced by subcutaneous inoculation of tubercle bacilli. In the earlier experiments the disease had been induced by intravenous inoculation of the microorganisms.

Group II in Table I summarizes the results obtained in the reinfected series of rabbits. These animals were not inoculated with the virulent bacilli (Ravenel) simultaneously with the non-vaccinated rabbits of Group I; nor was the same suspension employed. Hence the two series can be compared only in regard to the respective differences between survival time of experimental and control rabbits. However, the actual values in survival time of Group I are not comparable with those of Group II. In the latter the 5 controls lived between 47 and 130 days with an average survival time of 81 days. Three of the experimental rabbits succumbed 81, 110, and 131 days after subcutaneous reinoculation with virulent tubercle bacilli. Two of the animals had superimposed upper respiratory infections at the time of death. The 2 remaining experimental rabbits (Nos. 6-04 and 5-96) progressively increased in weight, as indicated in Chart 2. They lived for over 6 months after the death of the last control animal. On the 334th day after reinoculation with virulent tubercle bacilli these 2 animals were killed and thorough postmortem examinations were performed. The average survival time of the experimental group is unknown, since 2 of the animals were finally sacrificed. However, prior to the autopsy of the 2 survivors amongst the experimental animals the average life span of this group of rabbits was 198 days as compared with 81 days in the control series, or an increase of 144 per cent over the average survival time of the controls.

In Chart 2 are plotted the fluctuations in weight of the longest survivors of both control and experimental reinfected rabbits. Here again control Rabbits 6-28 and 6-24 succumbed shortly after they began losing weight; whereas experimental Rabbits 6-04 and 5-96 maintained steady weights for over 200 days after the death of the last of the controls. Small temporary losses in weight, as indicated on the chart, were associated as a rule with summer heat waves.

With relief in weather conditions, the animals invariably regained their original weights. The effects obtained by repeated intravenous injections of ferric chloride in reinfected tuberculous rabbits are evidently even more striking than those in the non-vaccinated animals of Group I (Table I). This doubtless is referable to the partial immunity induced by primary inoculation which results in a less acute form of tuberculosis upon reinfection with a virulent strain. The degree of immunity conferred by primary inoculation is associated with considerable individual variation. The immunity may be of sufficient magnitude to induce a high degree of resistance in control reinfected animals; *i.e.*, those receiving no iron. In a recent study on the BCG and human strains of tubercle bacilli, the writer has found this to be the case. When, however, the Cernay strain of bacilli was employed for primary inoculation, as described in the foregoing experiments, only a relatively mild degree of immunity must have been induced, since all the controls succumbed with considerable tuberculous involvement.

Effect of Ferric Chloride on the Extent of Tuberculous Lesions

The morphology of the organs in tuberculous rabbits treated with ferric chloride has already been described (3-6) and will be touched upon but briefly.

As a rule, gross lesions were seen only in the lungs, the kidneys, and at the site of subcutaneous inoculation. In the latter, the lesion appeared in the form of a fairly well circumscribed sacculated abscess which frequently buried itself deep into the anterior muscles of the thigh. It was filled either with a yellowish, suppurating material, of a fairly fluid consistency; or else contained a more viscous substance which formed a partially inspissated caseous mass. This local subcutaneous lesion seemed (in some cases) to be slightly larger in controls than in experimental animals. The kidneys presented the usual picture of discrete caseous foci, situated primarily in the cortical region. The gastrointestinal tract was infrequently involved in the form of miliary tubercles located chiefly in the ileum and in the neighborhood of the cecum. Though the appearance of the lungs varied somewhat in both series, their constant involvement influenced their selection as the main object for study, in comparing lesions of control and experimental rabbits. In the control animals of the non-reinfected group (I), the lungs at post mortem revealed extensive confluent pulmonary tuberculosis only in the longest survivors (*e.g.*, Rabbits 7-83 and 7-81, Table I). The animals which succumbed earlier presented relatively fewer discrete lesions. The reason for this well known

variation in extent of involvement in regard to time of death is not clear. The lungs of experimental rabbits of this group were often characterized by an intense congestion of the parenchyma interspersed by caseous foci; a tendency to confluence was noted at the bases and margins of the organ.

The extent of involvement showed some degree of variation, but it is noteworthy that in the longest survivors of the experimental group the degree of tuberculosis was far less than in some of the longest surviving controls. (See Fig. 1 and Table I, Rabbit 7-83, control, and Rabbit 7-30, experimental.) Some of these experimental rabbits were still alive 5 to 6 months after the death of the last of the controls (Table I, Group I), and yet the extent of tuberculous involvement in the former was considerably less than in the latter.

There is another point of difference between experimental and control rabbits which may be mentioned here. In animals treated with ferric chloride, the presence of iron is demonstrated (by the Prussian blue reaction) not only in caseous areas of lung tissue, but also within mononuclear phagocytes. The iron appears in the form of yellowish brown pigment, indistinguishable from hemosiderin, and is practically always observed in abundance within the sinuses and reticular cords of the spleen; in the reticular cells of the bone marrow; and fairly frequently in the Kupffer cells of the liver. In the latter organ, however, yellow, iron-containing granules were also occasionally found within the parenchymatous cells of animals treated with the iron salt. While it is true that such pigmented material is occasionally seen in the spleen of a normal rabbit, it has not been encountered by the writer, at least in the present series of experiments, either in the bone marrow or Kupffer cells of the liver of untreated animals, although Rous and others have observed normal rabbit livers containing red cells in various stages of degradation together with hemosiderin. The present observations indicate that some degree of hemosiderosis was evidently induced in the experimental animals by repeated intravenous injections of ferric chloride. The injections had no evident effect on the hemoglobin percentage. A slight increase in the reticulocyte level when compared with that of controls was noted. The average reticulocyte count in the experimental animals studied was 1.2 per cent, while that of the controls was 0.8 per cent. This is doubtless due to some degree of hemolysis in the ear

vein occasioned by the sudden introduction of a relatively large volume of hypotonic ferric chloride (5 to 6 cc. of 0.25 per cent concentration). It is doubtful, however, whether this problematic, slight hemolysis is sufficient to account for the extensive hemosiderin deposition in the various organs. When a 0.25 per cent ferric chloride solution rendered isotonic with mammalian blood (by the addition of 680 mg. of sodium chloride to 100 cc. of solution) was employed instead of the hypotonic solution, the same picture of hemosiderosis was observed. Recent studies by the writer have shown that the addition of 0.25 per cent ferric chloride to tissue cultures of mesenteric lymph nodes is followed by the appearance in large mononuclear cells of iron-containing pigmented material indistinguishable from hemosiderin. The above observations which are being reported in greater detail in separate communications (7) indicate that hemosiderin is not derived solely from the product of hemoglobin degradation, but may result from the action of cells in general upon iron contained in body fluids. For this reason it has been suggested that, for the pigment in question, *cytosiderin* would represent a more accurate name than the more specific term *hemosiderin*. Studies are now in progress to determine whether this iron-containing material plays any rôle in protracting the course of the disease.

In regard to the degree of tuberculosis in the control and experimental reinfected rabbits of Group II, the general involvement was somewhat similar to that described above for the non-vaccinated series. The lungs of controls showed in practically all cases widespread confluent caseating tuberculosis, a picture differing markedly from that observed in animals treated with ferric chloride. Those members of the latter group that succumbed during the period of ferric chloride injection (Rabbits 6-07, 5-41, and 6-16) frequently exhibited extensive pulmonary congestion. In these congested lungs a moderate number of discrete caseous foci were scattered about. In the 2 rabbits (Nos. 5-96 and 6-04, Table I) which survived for more than 6 months after the death of the last control, the amount of vascular congestion was not appreciable. One may recall that at the time that these animals were sacrificed, the ferric chloride injections had been discontinued for about 32 weeks. Hence it is quite reasonable to suppose that the congestion in the lungs is probably associated with the accompanying

intravenous injections of the ferric salt. The postmortem findings in these 2 rabbits, which were killed 334 days after reinoculation with virulent tubercle bacilli, have been adequately described already in a short preliminary note (6). Suffice it to point out here that the scant tuberculous involvement in these 2 animals was noteworthy, especially when compared to the massive, confluent caseating tuberculosis in the lungs of the longest control survivors which had died more than 200 days previously (see Fig. 2). Connective tissue proliferation frequently occupied a more prominent position at the peripheries of caseous foci in experimental rabbits than in the controls. Medlar (8) has pointed out that the bovine tuberculous lesion in the rabbit is characterized by a slight degree of fibrosis. This is well exemplified in the lungs of the longest surviving control (No. 6-28, Fig. 4) where at the peripheries of large caseous areas, connective tissue cells are few in number. It is interesting to note, on the other hand, in experimental Rabbit 6-04, which was still alive 334 days after reinoculation with tubercle bacilli, that the few large caseous areas present in the lung are circumscribed by a fairly wide zone of connective tissue proliferation (Fig. 5). Fibrosis in the tubercle, as Opie recently pointed out, is doubtless an expression of resistance to the further spread of the disease (9).

The mechanism to account for the favorable effect of ferric chloride is now under investigation. For the present we shall only call attention to the frequent finding of intense congestion in the pulmonary tissue of animals repeatedly injected with the iron salt, particularly if these animals are killed within a relatively short time after the period of ferric chloride treatment. The reader is referred to Figs. 1 and 3 for illustrations of the degree of congestion in the lungs of experimental rabbits. The frequent occurrence in the lungs of treated animals of a zone of engorged capillaries about tubercles, at times associated with some fibroblastic proliferation, suggests the end-stage of a superimposed inflammatory reaction induced by the accumulation of iron in tubercles (Fig. 6). This possibility is supported by the fact that ferric chloride, in the concentration employed, is in itself an intense inflammatory irritant. This is quite evident when the salt is injected into the dermis of an animal. The lungs of animals surviving long after cessation of treatment were often characterized

by moderate connective tissue proliferation at the peripheries of tubercles (Fig. 5, Rabbit 6-04). This fibrous zone, in its location with respect to the tubercles, corresponded in a general way to the area of engorgement found at an earlier stage. The presence of areas of congestion in control tuberculous rabbits was observed in a relatively small percentage of cases. In support of the concept that ferric chloride is responsible for the reactive pulmonary changes the following histological data were collected. The lungs of 24 controls were studied for the presence of vascular congestion and connective tissue proliferation about caseous areas. Congestion was found in 10 cases or 42 per cent of the lungs studied; while the incidence of fibrous tissue was noted in 12 cases; *i.e.*, in 50 per cent of the organs examined. The lungs of 27 experimental rabbits that had received varying amounts of ferric chloride yielded the following information: 16 of the lungs or 60 per cent of cases revealed marked vascular congestion; while 24 lungs or 90 per cent of the total cases were characterized by varying degrees of fibrosis.

In connection with these facts, there are in the literature some interesting clinical and pathological observations which may be mentioned. About 90 years ago, Rokitsansky pointed out the rarity of pulmonary tuberculosis in lungs that were passively congested. He even asserted that cardiocirculatory diseases producing passive congestion of the lungs were a preventative of phthisis (10). Weiss (11) expressed the opinion that in mitral stenosis accompanied by pulmonary congestion tuberculosis of the lungs was infrequent. More recently Hyndman and Landt (12) in some experimental studies came to the conclusion that the number of tuberculous lesions in congested organs was smaller than in the corresponding non-congested organs of control animals.

DISCUSSION

The results of the present series of experiments show definitely that repeated intravenous injections of dilute ferric chloride solution retard markedly the course of development of tuberculosis in rabbits. Furthermore, the present observations indicate that the combined action of partial immunity induced by a first infection and of ferric chloride administration enhances the favorable effects of the iron salt in reinfected animals. The implications of these findings are obvious, especially when it is recalled that the tuberculosis of white adults

is generally regarded as a disease of reinfection following the primary infection of infancy or childhood.

The results of seven independent series of experiments representing a total of 100 rabbits are summarized in Table II. The studies on

TABLE II
Summary of All Studies on Effect of Ferric Chloride in Tuberculous Rabbits

Series	Results published in	Total No. of rabbits	Average survival time		Increase in survival time of experimental animals
			Control animals	Experimental animals	
			<i>days</i>	<i>days</i>	<i>per cent</i>
*1	1932	16	61	109	78
*2	1933	20	94	135	44
3	1934	10	130	246	89
†4	1934	10	81	198	144
Average.....			91.5	172	89
Total No. of rabbits.....		56			

* Series 5 (1933). 16 tuberculous rabbits sacrificed between 45th and 79th day of the disease. Extent of tuberculous involvement in lungs of experimental animals considerably less than in controls

Series 6 (1933). 19 tuberculous rabbits sacrificed between 35th and 108th day of the disease. Tubercle bacilli inoculated subcutaneously. Experimental rabbits showed definite retardation in spread of the bacilli as evidenced by extent of lesion, particularly in the lungs

* Series 7 (1931). 9 rabbits with bovine tuberculosis; 2 of these treated for about a month with ferric chloride intravenously prior to inoculating with tubercle bacilli. They survived considerably longer than controls: 124 and 115 days respectively. Average survival of 7 controls: 54 days

Total No. of rabbits in all series: 100

* Bovine tuberculosis induced by intravenous route; all others were inoculated subcutaneously.

† This group received a preliminary vaccination with a relatively avirulent strain of tubercle bacilli (Cernay).

survival time include 56 rabbits. The average survival time of all control rabbits is 91.5 days; that of the experimental animals is 172 days. The average increase in the survival time of experimental rabbits is 89 per cent. Series 5 and 6, comprising 35 animals, represent

a study of the extent of lesions in control and experimental animals that had the disease for the same length of time. These, as previously reported (4, 5), were in entire accord with the results obtained on survival time; a comparison of the pathological lesions substantiated the fact that the course of the disease following ferric chloride administration was protracted.

The explanation for this favorable action on the part of ferric chloride is still somewhat problematical owing to the multiplicity of factors concerned. It is possible that the retarding effect on the development of the disease may be the resultant of several factors. The frequent appearance of intense vascular congestion associated with some fibroblastic proliferation in tuberculous lesions of iron-treated animals suggests the end-stage of a superimposed inflammatory reaction induced by the accumulation of the iron in tubercles. The introduction of this additional synergistic element would enhance the various factors in the tubercle which naturally tend to dispose of the bacilli. Furthermore the frequent deposition within mononuclear phagocytes of an iron-containing pigment, indistinguishable from hemosiderin, may, by activating the cells of the reticulo-endothelial system, constitute a factor in the favorable effect of ferric chloride administration. The recent observation of Rous and Beard (13) that Kupffer cells loaded with ferromagnetic iron oxide can be dislodged with relative ease into the circulating blood from their attachment to the liver parenchyma renders it conceivable that cells of the reticulo-endothelial system loaded with the iron-containing pigment may be more readily detached and may thus be disseminated in greater numbers to tuberculous foci.

SUMMARY AND CONCLUSIONS

Repeated intravenous injections of dilute ferric chloride solution in tuberculous rabbits markedly retard the development of the disease as evidenced both by a prolongation in the survival time and by comparison of lesions in control and experimental animals.

Partial immunity induced by first infection combined with ferric chloride administration enhances in reinfected animals even more strikingly the favorable effects of the iron salt. Some of the experimental animals were still alive and apparently well about 6 months

after the death of the last of the controls which succumbed about 4 months after reinfection with virulent tubercle bacilli.

Factors that may perhaps account for the favorable effect of ferric chloride in experimental tuberculosis have been discussed.

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EXPLANATION OF PLATES

PLATE 25

FIG. 1. Comparison of lung of (a) Rabbit 7-83 (control, 162 days, non-vaccinated group) and that of (b) No. 7-30 (experimental, 326 days, non-vaccinated group). Both of these animals were allowed to die of the disease. Rabbit 7-30 had received by repeated intravenous injections a total of 218 cc. of 0.25 per cent ferric chloride. Even though the experimental animal survived over 5 months after the death of the control, the extent of tuberculous involvement in its lungs is distinctly less than in the control animal. The congestion in the lungs of the experimental rabbit is pronounced.

FIG. 2. Comparison of lungs of (a) Rabbit 6-28 and (b) Rabbit 6-24 (longest control survivors, 130 and 108 days respectively, vaccinated group) and those of (c) Rabbit 5-96 and (d) No. 6-04 (longest experimental survivors, killed on 334th day after reinfection). Each experimental rabbit had received a total of 278 cc. of 0.25 per cent ferric chloride intravenously. They were killed more than 6 months after the death of the last of the controls. The amount of tuberculous involvement is scant in comparison with the extensive confluence of lesions in the controls.

FIG. 3. Comparison of lung of (a) Rabbit 8-21 (control, killed on 63rd day of disease) and that of (b) No. 8-15 (experimental, killed on 63rd day of disease). This

rabbit had received a total of 57 cc. of ferric chloride. The disease was induced by intravenous inoculation of the bacilli. The involvement in the control lung is extensive as compared to that of the experimental animal. The intense congestion in the lung of the iron-treated rabbit is striking. About 100 more viable bacilli were cultured and recovered as individual colonies from the control than from the experimental lung.

PLATE 26

FIG. 4. Photomicrograph of lung section of control Rabbit 6-28 (died 130 days after reinfection) showing extensive caseation with diffuse cellular infiltration at periphery. There is practically no evidence of any fibrosis. $\times 162$.

FIG. 5. Photomicrograph of lung section of experimental Rabbit 6-04 (killed 334 days after reinfection) showing at periphery of large area of caseation a moderately dense zone of connective tissue proliferation. Compare with Fig. 4 (control Rabbit 6-28). $\times 85$.

PLATE 27

FIG. 6. Drawing of microscopic section through lung of an experimental rabbit that had received intravenously a total of 226 cc. of 0.25 per cent ferric chloride administered over a period of about 10 weeks. Note the dilatation, moderate engorgement, and large number of small vessels at the periphery of a caseous area. There is a slight connective tissue reaction. $\times$ about 500.

(Menkin: Iron in tuberculous areas. IV)

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