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Mechanism of Tuberculostasis in Mammalian Serum

II. Induction of Serum Tuberculostasis in Guinea Pigs[1]

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The growth of tubercle bacilli in serum samples of untreated animals depends upon the availability of ionic iron which serves as a growth factor in supporting bacillary multiplication. The amount of available iron in serum is determined by the ratio between iron-saturated and iron-free transferrin; a low value for the ratio is associated with tuberculostasis (e.g., human serum, 0.4), whereas a high value is associated with the growth-supporting quality (e.g., guinea pig serum, 5.6). The treatment of guinea pigs with lipopolysaccharide of *Escherichia coli* or tuberculous cell wall material consistently and significantly reduced serum iron levels; a similar but less striking effect was observed in BCG-vaccinated animals. Pronounced differences were observed in the time of appearance and duration of serum hypoferremia; in lipopolysaccharide-treated animals, it appeared in 1 day and lasted for several days, whereas in BCG-vaccinated animals it appeared in about 2 weeks and lasted for much longer time periods. The induced hypoferremia was always associated with the concomitant development of serum tuberculostasis which could be neutralized by the addition of iron. These results indicate, therefore, that the mechanism of induced serum tuberculostasis in lipopolysaccharide- or tuberculous cell wall-treated and BCG-vaccinated guinea pigs is the same as that present in tuberculostatic sera of untreated animals.

The inhibitory effect of human serum upon the growth of tubercle bacilli has been attributed to the unavailability of ionic iron (10). Tubercle bacilli can grow in human serum only if sufficient iron is provided to give an excess of the metal over the unsaturated capacity of transferrin. The role of transferrin in the mechanism of serum tuberculostasis is limited to the property of this protein to bind ionic iron. Results suggest that iron alleviates serum tuberculostasis either by serving as an essential metal for bacillary growth or by exerting a neutralizing effect upon an iron-sensitive tuberculostatic factor.

In contrast to tuberculostatic human, bovine, sheep, rabbit, and mouse sera, guinea pig serum supports bacillary multiplication (11). However, after BCG vaccination or infection of guinea pigs with tubercle bacilli or staphylococci, their sera become tuberculostatic (13). The appearance of an antituberculous response in the serum of *Staphylococcus*-infected animals suggests the nonspecific nature of the phenomenon.

It has been reported that the treatment of animals with lipopolysaccharide (LPS) of gram-negative bacteria induces hypoferremia (1, 9; G. C. Chandlee and G. M. Fukui, Bacteriol. Proc., p. 45, 1965) and nonspecific immunity (6, 19, 22). Whether the hypoferremia is a contributing factor to the mechanism of nonspecific resistance is not known. However, results obtained in several laboratories suggest that the treatment of infected animals with iron increases their susceptibility to several bacterial parasites (24), and, therefore, iron may be an important factor in host-parasite interactions.

Results presented in this report show that serum tuberculostasis can be induced in guinea pigs by treating them with LPS, with tuberculous cell wall preparation (TCW), or with live cells of tubercle bacilli. The tuberculostasis in the sera of treated animals results from a lack of ionic iron which serves as an essential growth factor in the multiplication of tubercle bacilli.

MATERIALS AND METHODS

Treatment of guinea pigs. In this study, we used guinea pigs weighing between 500 and 700 g. Animals were obtained from the Camm Research Institute, Inc., and local dealers.

Guinea pigs were vaccinated with a subcutaneous injection, into the groin area, of 5 mg (wet weight) of

[1] A preliminary report of these findings was presented at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4-9 May 1969.

tubercle bacilli (BCG) which had been grown in Dubos Tween-albumin-dextrose medium for 10 days. Other guinea pigs were injected intraperitoneally with LPS or TCW. The LPS used in this study was prepared from *Escherichia coli* (Difco), and the TCW material was prepared from BCG (generously supplied by E. Ribi). Both lyophilized preparations were weighed and suspended in saline at the proper concentrations, and the desired doses per 100 g of body weight were injected immediately after their preparation.

Sera. Blood samples were obtained from untreated, BCG-vaccinated, LPS- or TCW-treated guinea pigs. Guinea pigs were anesthetized by ether and bled only once by cardiac puncture. Occasionally, other mammalian blood samples were collected for a comparative study. Sera were separated from clots by centrifugation, and they were sterilized by filtration through sintered-glass filters. Sera were stored in a refrigerator in screw-cap tubes and were used within a few days for determinations of tuberculostasis, serum iron (SI) content, and total iron-binding capacity (TIBC). Serum samples which showed evidence of hemolysis were not used in these tests.

Tests for tuberculostasis. The detailed description of techniques employed in determining serum tuberculostasis has been described in previous communications (10–12). Bacillary inhibition or growth in 2-ml serum samples was determined after a 2-week incubation period at 37 C by a routine plating method. Procedures used in investigating the mechanism of tuberculostasis in serum samples of BCG-vaccinated, LPS- or TCW-treated guinea pigs are described in appropriate contexts.

Tests for SI and TIBC. SI levels were determined by the method of Landers and Zak (15). The TIBC of serum samples was determined by the saturation of serum with iron (5 μg/ml) and the subsequent precipitation of unbound iron with magnesium carbonate (26). Iron in the saturated serum was determined by the same technique as the SI level in untreated serum. Since all or nearly all iron in a serum sample is bound to transferrin (Tr), the SI and TIBC values were used to calculate the percentage of iron-saturated Tr.

RESULTS

Results presented in our previous communication (10) suggested two explanations for the neutralization of serum tuberculostasis by iron. According to the first explanation, iron served as a growth factor, and tuberculostasis would therefore be attributed to the absence of ionic iron in the serum. The second explanation proposed the existence in tuberculostatic serum of an iron-sensitive antimycobacterial factor which, in the absence of ionic iron, inhibits bacillary multiplication.

To decide which of the above explanations is correct, experiments were performed in which the interplay between Tr or Desferal (desferrioxamine B methane sulfonate; Ciba Pharmaceutical Products, Inc., Summit, N.J.) and iron was investigated in Dubos Tween-albumin-dextrose medium. Both substances, which are specific iron-chelating agents, were dissolved in Hanks solution and added in various concentrations to Dubos liquid medium. Iron determinations showed that this medium contained 4 μg of iron per ml at a 1:4 dilution. The mixtures of Dubos medium and solutions of chelating agents were inoculated with BCG, and after a 2-week incubation period the extent of bacillary growth was determined by plating samples on Dubos solid medium. Both Tr and Desferal inhibited bacillary growth in concentrations at which they were able to bind all ionic iron (Table 1). It is reasonable to conclude, therefore, that serum tuberculostasis is not caused by the presence of antimycobacterial factor, but it is due to the lack of ionic iron which serves as an essential growth factor in bacillary multiplication.

It is interesting to note that tubercle bacilli could not utilize iron which was bound not only to Tr, as reported previously (10), but also to Desferal. Since Desferal binds iron very firmly and effectively eliminates it from the body, this, chelator should be evaluated as an adjunct in tuberculosis therapy.

It was reported previously that guinea pig serum, in distinction to other mammalian sera, supports good growth of tubercle bacilli (11). We found that the tuberculostasis exerted by human serum could be neutralized by the addition of iron and that the tuberculostasis in iron-neutralized human serum could be reconstituted by the addition of Tr (10). This Tr-iron interplay in the mechanism of serum tuberculostasis suggested that human and guinea pig serum samples should be investigated in terms of SI and TIBC. Results of this study are presented in Table 2.

TABLE 1. *Antimycobacterial activity of Tr and Desferal in Dubos medium diluted 1:4*[a]

Substance tested	Dose	No. of generations
	mg/ml	
None	None	12.7
Tr	4.0	0.0
	2.0	8.1
	1.0	12.5
	0.50	11.1
Desferal	0.40	0.0
	0.20	0.0
	0.10	11.3
	0.05	10.7

[a] Dubos medium diluted 1:4 in Hanks solution contained 4 μg of iron per ml of the solution. The *p*H during the 2-week growth period was maintained at 7.5.

TABLE 2. *Correlation between levels of iron-saturated Tr and degrees of bacillary growth in mammalian sera*

Source of serum	No. of tests	Amt of iron (μg)[a]		Tr saturation[d]	Tuberculostasis in serum[e]
		TIBC[b]	SI[c]		
				%	
Man	10	327	97	30.0	Present
Cow	4	490	191	39.0	Present
Rabbit	8	317	204	64.3	Limited
Mouse	10	382	230	60.2	Limited
Guinea pig	20	323	273	84.4	Absent

[a] Individual determinations of TIBC and SI fell within 10% variation of the mean value shown in the table.

[b] TIBC value shows the mean of the amount of iron present in 100 ml of iron-saturated serum.

[c] SI value shows the mean of the amount of iron present in 100 ml of untreated serum.

[d] Percentage of iron-saturated Tr in serum sample equals (SI/TIBC) × 100.

[e] Tuberculostasis was scored as "present" when bacillary growth was less than 1 generation, "limited" when it was less than 5 generations, and "absent" when it varied between 5 and 14 generations during a 2-week incubation period.

Most mammalian sera, and especially human and guinea pig sera, do not differ significantly in TIBC values. Since a TIBC value mirrors the level of Tr in serum, we concluded that these sera, with the possible exception of bovine serum, possess similar amounts of Tr. There are pronounced differences, however, in the values for SI. In comparison to SI in human serum, rabbit and mouse sera contained two times and guinea pig serum three times as much SI. Consequently, the saturation percentage of Tr in the latter sera was much higher than in human and bovine sera. The last entry in Table 2 shows that the fate of tubercle bacilli in mammalian serum depends upon the degree of saturation of Tr with iron; human and bovine sera with around 30% saturated Tr were tuberculostatic, whereas guinea pig serum with 84.4% saturated Tr supported bacillary multiplication. Limited bacillary multiplication (from one to five generations) was present in rabbit and mouse sera which contained 60% saturated Tr. These results correlate well with our previous findings which were obtained in a study of tuberculostatic activity of mammalian sera (11).

Tuberculostasis in serum samples of normal animals could be neutralized with the addition of iron (Fig. 1). The quantities of iron required for the neutralization of human and rabbit serum samples were proportional to the amounts of iron-free Tr in respective sera (refer to Table 2). The addition of iron to guinea pig serum did not alter bacillary growth significantly.

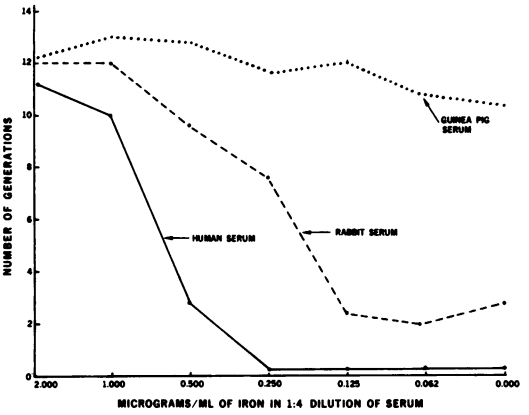

FIG. 1. *Fourteen-day growth of tubercle bacilli in serum samples with various quantities of added iron.*

Experiments described above show conclusively that the growth of tubercle bacilli depends upon the availability of ionic iron. It seems that this bacillary need for iron cannot be supplied by iron-Tr or iron-Desferal complexes. Since tubercle bacilli did not grow in human serum with 30% iron-saturated Tr, but multiplied in guinea pig serum with 85% iron-saturated Tr, it is suggested that serum with a high percentage of saturated Tr possesses a small but sufficient amount of ionic iron for bacillary growth. This suggestion is supported by observations recorded in the literature which indicate that there is a dissociation balance between Tr·Fe, Tr, Fe^{3+}, and HCO_3^- in plasma (16). For this reason, the ratio between iron-saturated and iron-free Tr may be used as a rough indication of the amount of ionic iron in the serum. The value for this ratio in human serum is low (0.4), and, therefore, only traces of ionic iron are present in this tuberculostatic serum. In guinea pig serum, the value is high (5.6), and, therefore, the amount of ionic iron is sufficient to support good bacillary multiplication.

It has been shown repeatedly that treatment of animals with LPS of gram-negative bacteria induces a nonspecific or rather broad immunity to a variety of parasites. Since such broad immunity could be due to the presence of humoral antibacterial factors, we performed experiments to determine whether LPS- and TCW-treated guinea pigs develop hypoferremia and tuberculostasis in their sera.

The experiment presented in Table 3 shows that, 24 hr after the treatment of guinea pigs with LPS or TCW, their sera became hypoferremic (see SI) and tuberculostatic. The results indicate

that the degree of hypoferremia and tuberculostasis is dependent upon the amounts of LPS or TCW used for treatment. Since the TIBC values in sera of LPS- or TCW-treated guinea pigs remained the same as in untreated animals, the decrease in the percentage of iron-saturated Tr was not caused by the increase in the amount of the protein but by the decrease in serum iron.

Hypoferremia induced with LPS lasted for about 5 days, whereas that induced with TCW was longer in duration, lasting about 14 days (Table 4). This prolonged hypoferremia in TCW-treated guinea pigs could be attributed to the large dose of TCW material (20 times that of LPS) from which LPS is released over a prolonged time period. This suggestion is supported by observations of the toxic effects of LPS and TCW.

TABLE 3. *Iron-saturation levels of Tr and tuberculostasis in sera 1 day after the treatment of guinea pigs with LPS or TCW preparations*[a]

Animals injected with	Treatment dose	Amt of iron (μg)		Tr saturation	Tuberculostasis in serum
		TIBC	SI		
	mg/100g			%	
Saline		360	300	83.4	Absent
LPS	0.500	320	80	25.0	Present
	0.100	340	80	23.5	Present
	0.050	330	130	39.4	Present
	0.010	330	200	60.6	Present
	0.005	360	220	61.1	Limited
TCW	1.500	340	90	26.5	Present
	1.000	330	120	37.0	Present
	0.500	340	160	47.0	Present
	0.100	350	140	40.0	Present
	0.010	320	230	73.0	Absent

[a] For an explanation of symbols used, see the footnotes of Table 2.

Although the former material, in doses comparable to TCW, is quite toxic and causes a high mortality rate, the latter material only induces mild toxemia. Sera reported in Table 4 to have less than 70% iron-saturated Tr were tuberculostatic; limited serum tuberculostasis was observed in serum samples containing up to 75% iron-saturated Tr.

To determine whether the serum tuberculostasis in LPS- and TCW-treated animals is the consequence of an induced hypoferremic state, attempts were made to neutralize the acquired tuberculostasis with the addition of iron. The tuberculostatic sera, obtained from guinea pigs on days 1, 2, and 3 after treatment, were diluted 1:4 in Hanks solution containing various quantities of iron (added as ferric chloride). The LPS- and TCW-induced tuberculostasis could be effectively neutralized with the addition of sufficient iron (Table 5).

Results in Table 5 also show that a limited degree of hypoferremia and tuberculostasis developed in BCG-vaccinated guinea pigs in about 2 weeks after the vaccination. Since the BCG-induced hypoferremia was not pronounced and its significance could only be assessed by using a larger number of samples, 15 sera of 3-week BCG-vaccinated and 15 sera of untreated animals were tested for hypoferremia and tuberculostasis. This survey showed that, in contrast to bacillary growth-supporting normal guinea pig serum, with 83.0% saturated Tr, sera of vaccinated animals were weakly tuberculostatic and their mean percentage of saturated Tr was 74.4. In all experiments, the BCG-induced serum tuberculostasis was eliminated by the addition of minute amounts of iron. The effectiveness of this iron neutralization indicates that the mechanism of tuberculostasis in sera of BCG-vaccinated

TABLE 4. *Percentage of iron-saturated Tr in guinea pig serum on various days after LPS and TCW treatments*

Treatment dose	Iron tests[a]	Amt (μg) of iron on day					
		1	2	3	5	10	14
LPS (0.05 mg/100 g)	TIBC	368	360	340	348	320	360
	SI	110	204	200	260	300	294
	Saturation (%)[b]	29.9	56.7	60.0	74.7	93.8	82.8
Cell wall (1.0 mg/100 g)	TIBC	400	380	340	360	360	292
	SI	120	160	220	240	246	230
	Saturation (%)	30.0	42.1	64.7	66.3	68.3	78.8
Saline Controls	TIBC	352	340	330	360	360	330
	SI	312	280	260	294	300	320
	Saturation (%)	88.6	82.3	78.8	81.6	83.4	93.9

[a] On each day, iron determinations were performed on a sample of serum of an individual guinea pig in each experimental group. Animals were bled only once.

[b] All sera which possessed below 70% iron-saturated Tr exhibited tuberculostatic activity.

TABLE 5. *Iron-neutralization of tuberculostasis in sera of LPS- or TCW-treated and BCG-vaccinated guinea pigs*[a]

Days after treatments[b]	Tr saturation	Generations in serum with iron (μg/ml)				
		8	4	2	1	0
	%					
LPS						
1	17.5	9.2	8.0	0.4	0.0	0.0
2	42.1	12.2	10.7	11.3	10.6	0.0
3	60.2	11.1	9.5	11.9	11.8	0.0
TCW						
1	26.5	9.5	9.5	9.9	5.1	0.0
2	42.1	10.6	10.0	10.3	10.2	0.5
3	59.3	11.0	10.7	9.3	9.7	1.2
BCG						
3	86.2	11.2	11.0	11.9	11.5	10.5
7	79.4	11.7	12.1	11.3	10.6	9.1
14	75.0	11.1	12.3	10.2	9.5	4.3
21	75.3	10.7	9.9	10.3	9.1	0.4
28	68.6	11.3	10.9	10.2	9.7	0.0
Untreated	85.6	12.1	11.5	11.6	11.8	11.5

[a] Tests for the presence and the neutralization of tuberculostasis were performed in a 1:4 dilution of serum samples.

[b] On day "0," animals were injected intraperitoneally with 0.05 mg of LPS, 1 mg of TCW preparation, or 1 mg of BCG cells per 100 g of body weight.

guinea pigs is the same as that induced in LPS- and TCW-treated animals.

DISCUSSION

Results of this study show that serum tuberculostasis, whether present naturally or induced in animals experimentally, can be neutralized with the addition of iron, which serves as an essential growth factor in bacillary multiplication. The amount of iron needed for the neutralization of the antibacterial activity of serum was thought to be of such a quantity as to provide an excess of the cation over the unbound iron-binding capacity of Tr (24). Our study shows that tubercle bacilli can multiply in the presence of small amounts of iron-free Tr (as in guinea pig serum) and questions, therefore, the need for oversaturation of serum with iron as a means of promoting bacillary growth in serum. Results suggest that the dissociation balance between iron-Tr, Tr, and iron in serum determines the amounts of ionic iron whose availability defines the growth-supporting quality of a serum sample. According to this, the level of ionic iron in serum is determined by the ratio between iron-saturated and iron-free Tr, and, therefore, this ratio is of crucial importance in the study of serum tuberculostasis. A similar conclusion has been reached by Schade, who studied the dependence of staphylococcal growth rate in human serum on the percentage iron saturation of Tr (21).

It is of considerable interest that the substances which induce serum tuberculostasis are those which quite frequently have been implicated in the induction of nonspecific immunity to facultative intracellular parasites. In view of this, this discussion deals more with the possible significance of our findings as they pertain to native and acquired immunity in tuberculosis than with a comparative analysis of results in this and similar studies. References were made in our previous report (10) to findings which stress the importance of iron in the development of diseases to a variety of bacterial species.

It is the predominant but not unanimous conclusion that immunity in tuberculosis is of a cellular nature. This acquired cellular resistance may be defined as a state in which macrophages develop an increased capacity to destroy tubercle bacilli. This cellular immunity is thought to be an atypical immunological phenomenon, because it is effective not only against the pathogen which induced it but against other facultative intracellular parasites as well (18). According to the concept of cellular immunity, delayed hypersensitivity is its inseparable part, because it has been observed that immunizing procedures which do not induce delayed hypersensitivity also do not provide the host with cellular resistance. The mechanism by which activated, hypersensitive cells are able to destroy intracellular parasites is not known. It was suggested that the destruction of tubercle bacilli in these cells could be attributed to their high content of lysosomes and associated enzymes (5).

Some studies conducted in tissue cultures of infected normal and immune macrophages challenged the concept of cellular immunity (7; I. Kochan, Ph.D. Thesis, Stanford Univ., Palo Alto, Calif., 1958). These studies, as well as a more recent one by Patterson and Youmans (Bacteriol. Proc., p. 70, 1969), showed that tubercle bacilli multiply at the same rate in normal and immune macrophages. However, when normal and immune macrophages are maintained in the presence of tuberculostatic serum or streptomycin, intracellular bacillary multiplication is inhibited much more in immune than in normal cells (14; R. J. Patterson and G. P. Youmans, Bacteriol. Proc., p. 70, 1969). This finding suggests that immune cells respond to the intracellular bacilli in such a way as to enable antimyco-

bacterial substances of the tissue culture medium to reach and to inhibit the parasites. The importance of medium in tissue culture studies was appreciated by Wheeler and Hanks (25), who found that the intracellular growth of tubercle bacilli is not so much influenced by the components, metabolism, or immunological properties of host cells as by compounds and conditions provided by the extracellular environment. It has been observed that serum of BCG-vaccinated animals protects immune cells from degenerative effects of infection with tubercle bacilli but not against damage with Old Tuberculin (8). These findings attributed the significant protective role in tuberculosis to humoral rather than cellular factors.

It is a well-documented and accepted fact that the serum of BCG-vaccinated animals fails to protect the recipient against infection with tubercle bacilli (I. Kochan, Ph.D. Thesis, Stanford Univ., 1958). The lack of protection in immune serum-treated animals led some investigators to conclude that humoral factors play no role in the resistance to infection with tubercle bacilli (18). The possible fallacy of this conclusion becomes evident when one considers the mechanism of serum tuberculostasis based upon the Tr-iron interplay. The transfer of a small quantity of tuberculostatic serum of a BCG-vaccinated animal to a normal animal would not appreciably change the ratio between iron-saturated and iron-free Tr, which determines the level of ionic iron and consequently the fate of tubercle bacilli in serum.

The nonspecific nature of cellular immunity makes it identical or similar to nonspecific immune states induced in LPS-treated animals. Dubos and Schaedler (6) observed that bacterial products with endotoxin activity produce, in treated animals, a resistant state to a variety of pathogens. The spectrum of resistance induced by LPS of gram-negative bacteria has been shown to be broad (22). Recently, Youmans and his associates (4, 27, 28) showed that tubercle bacillus possesses two immunizing moieties. The first was associated with the bacterial cell wall and produced a nonspecific immunity, and the second was found in the ribosomal fraction and induced a specific immunity in animals. Since the latter fraction was found to produce an effective immunity rather than a delayed hypersensitivity, the authors concluded that acquired cellular immunity is not mediated by the hypersensitive state. These results, as well as findings which indicate that infected tissue cultures of normal and immune macrophages, when maintained in the absence of streptomycin, do not differ in their growth-supporting qualities for bacillary multiplication (14; R. J. Patterson and G. P. Youmans, Bacteriol. Proc., p. 70, 1969), question the foundation on which the concept of cellular immunity was built and developed to exaggerated proportions.

It is quite possible that Rich's suggestion, that a reproduction-inhibiting substance should be sought as a major factor of immunity in tuberculosis (20), is pertinent to the Tr-iron interplay in serum tuberculostasis described in our work. The chronic nature of the disease does not suggest the destruction of the parasites by an antibody or cellular activities but implies a fluctuating bacteriostasis. Such fluctuation in tuberculostasis at the focus of infection could be visualized by considering variations in *p*H, accumulation of iron-binding materials, etc. It is known that these and similar factors disturb the tuberculostasis-determining ratio between iron-saturated and iron-free Tr.

LPS treatment and BCG vaccination effectively induce serum tuberculostasis in guinea pigs. Recently, we found that these treatments increase serum tuberculostasis in rabbits (*unpublished data*). This suggests a mechanism by which one can explain the native and acquired resistance to several bacterial parasites. In fact, endotoxin-induced immunity to *Klebsiella pneumoniae* and *Salmonella typhimurium* has been attributed to the ability of endotoxin to cause hypoferremia in experimental animals (G. C. Chandlee and G. M. Fukui, Bacteriol. Proc., p. 45, 1965). Bullen and his associates (2, 3) reported that the protective effect of antisera to *Clostridium welchii* and *Pasteurella septica* can be abolished by treatment of passively protected animals with iron. Sword (23) reported that the treatment of mice with iron compounds predisposes them to infection with *Listeria monocytogenes* and that plasma iron decreases during progressive infection. Sword found, however, that iron-treatment of immunized animals fails to suppress the acquired resistance. Since the decrease in SI is associated with the increase in iron-free Tr (as it is shown in our report), more iron should be used to abolish resistance in immune animals than to induce susceptibility in normal animals. In view of this, one would have more confidence in Sword's results pertaining to the inability of iron to decrease acquired immunity if more iron were used to accomplish this.

On theoretical grounds, this discussion suggests that the mechanisms of native and acquired resistance to tubercle bacilli are the same. Depending upon differences in SI levels among animal species, there are various susceptibilities to infection; guinea pigs with high SI are much more susceptible than man with low SI. Similarly, the

development of a broad immunity in BCG-vaccinated or LPS-treated animals can be attributed to the decrease in SI levels and associated inhibition of bacillary multiplication. In view of this reasoning, the suggestion made by Lurie (17), that acquired resistance to tuberculosis is determined by and superimposed on native immunity, is interesting and may even be prophetic.

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