SOME ASPECTS OF INTRACELLULAR PARASITISM OF PATHOGENIC MICROORGANISMS
A REVIEW

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The phagocytic system, distributed throughout the blood and tissue compartments of the body, acts as an intermediary between the host and pathogenic microorganisms. This is not a specific function, it being one of the physiological properties of phagocytes to engulf particulate matter and even larger molecules which have gained access to blood or tissues of an organism. Metchnikoff (15), recognizing clearly this function, overestimated the destructive effect exerted by phagocytes upon pathogenic microorganisms. His interpretation of the role of the phagocytic system in infections applies almost entirely to pathogens causing acute infections (27), but should not be extended indiscriminately to chronic infections. In chronic infections the relation between the causative agent and the cells, and especially the phagocytes, may last for a long period of time, often for the lifespan of the host. It is in these chronic infections that intracellular parasitism has been recognized as one form of host-parasite relationship.

This form of host-parasite relationship is of special interest to the student of leprosy, because of the almost purely intracellular habits of the two types of leprosy bacilli. This attribute, at least in the case of *Mycobacterium leprae murium,* is perhaps explained in part by evidence that the bacilli are extremely sensitive to components of serum (4). These properties caused almost insurmountable difficulties to an efficient experimental approach to problems of leprosy. Hanks and Gray (6), in a forthcoming paper, present and discuss evidence obtained by metabolic analysis of the biology of the leprosy bacillus in relation to its intracellular parasitism. Because of these shortcomings in experimentation with leprosy bacilli, it seems justified to review in the following pages results obtained with other agents causing chronic infections, as brucellosis and tuberculosis.

During certain phases of the infective process these agents are able to survive or even to multiply intracellularly or extracellularly (7, 9). For this reason they might be classified as facultative intracellular parasites. The discussion will deal in the first part with problems of chemotherapy of intracellular parasites; the second part will present recent findings on the intracellular parasitism of tubercle bacilli.

CHEMOTHERAPY OF INTRACELLULAR INFECTIONS

Since the experiments of Rous and Jones (18) on the protection of intracellular typhoid bacilli against the bactericidal activity of antisera, it is known that microorganisms which have been phagocytized may escape the influence of antibacterial agents to which they are susceptible when not within cells. This is of no concern for the chemotherapy of acute bacterial infections, but it can be a great obstacle to the efficient treatment of chronic infections. Although the underlying mechanism had not been realized as such, it was soon apparent that streptomycin could not rid the body entirely of infective agents like brucella, salmonella and tubercle bacilli, in spite of the fact that these microorganisms are extremely sensitive to the antibiotic *in vitro*. Reviewing the literature on chemotherapy of tuberculosis (21) I emphasized the fact that even after prolonged treatment with streptomycin, viable tubercle bacilli can be recovered from tissues of infected experimental animals. Similarly, in human tuberculosis treated with streptomycin the subsequent course of the infection often indicates the survival of the tubercle bacilli in the lesions.

The first indicative observation was made by Barski (2), who exposed tissue cultures of lungs and spleens infected with tubercle bacilli to as much as 50μgm./ml streptomycin for a period of 12 days and thereafter continued cultivation of the infected tissues in a medium free of streptomycin. The tubercle bacilli which had remained confined to the cells during the presence of streptomycin overgrew the tissue culture soon after removal of the antibiotic. This was taken as clear evidence of the protective property of the cells against streptomycin.

Jensen (8) obtained similar results when studying the effect of treatment with streptomycin on the multiplication of tubercle bacilli in lungs and spleens of normal and BCG-vaccinated guinea-pigs. The animals were exposed to an aerosol of tubercle bacilli and received injections of 5,000 μgm. streptomycin every 12 hours. Tubercle bacilli were cultivated from lungs and spleens of animals killed at increasing intervals of time after infection. A typical experiment is presented in Text-fig. 1, and the results can be summarized as follows:
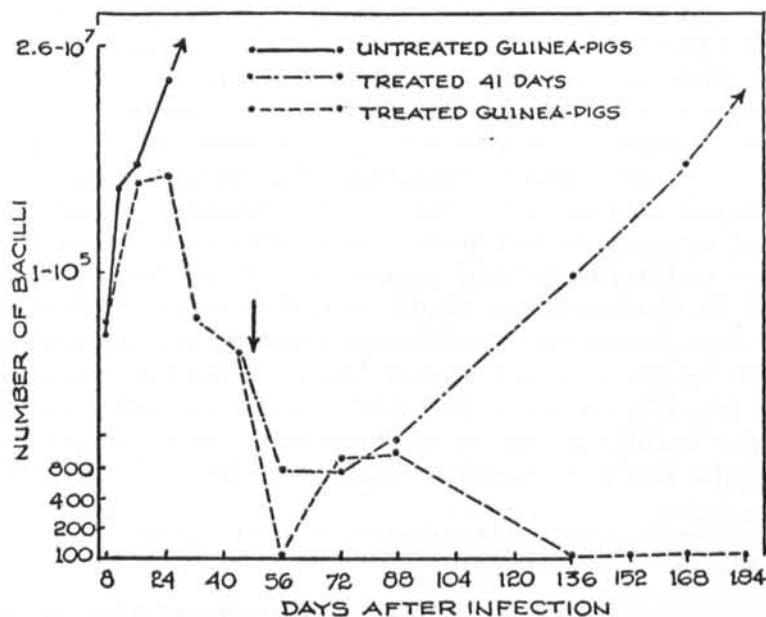
TEXT-FIG. 1. Recovery of tubercle bacilli from the lungs of guinea pigs with and without streptomycin treatment. (Fig. 1 in reference 8.) The guinea pigs were infected by the inhalation technique and killed at increasing intervals of time thereafter.

Tubercle bacilli multiply in the lungs of both treated and untreated animals at the same rate, for a time. An effect of streptomycin becomes apparent only 12 to 15 days after infection. When treatment is discontinued, whether on the 41st or the 184th day after infection, bacilli begin to increase in numbers after a latency period of about 30 days, and the infection progresses until death of the animal. Vaccination with BCG prior to the infection results in a more prompt and more pronounced response to the treatment with streptomycin. Jensen concluded that streptomycin had no effect upon the course of the infection until some immune reaction had appeared which would intensify the activity of the drug. He states, "Streptomycin and the immunity together have a much stronger effect on the tubercle bacilli than has either factor by itself."

In the light of Barski's findings a more rational explanation of Jensen's results is possible. During the first phase of the infection with tubercle bacilli, from the 1st until about the 15th day, the bacilli multiply within phagocytes and are not affected by streptomycin. When hypersensitivity has developed, the cells containing large numbers of bacilli may be destroyed and thus release their bacillary content. These organisms, having become extracellular, are then susceptible to streptomycin. The combined effect of streptomycin and immunity, emphasized by Jensen, can be ascribed to the property of the phagocytes in the immune animal to retard multiplication of intracellular tubercle bacilli. Strepto-

mycin, then, would restrict extracellular multiplication, and the acquired state of the phagocytes would limit intracellular multiplication.

When studying multiplication of tubercle bacilli within monocytes cultured *in vitro*, it was found that much higher concentrations of streptomycin were required to prevent their growth inside the phagocytes than was sufficient for complete inhibition in a liquid medium containing Tween 80 and albumin (22). By contrast, isoniazid (isonicotinic acid hydrazide) retained its full activity against tubercle bacilli even when they were multiplying within phagocytes. These findings have been confirmed by Mackaness and Smith (11), the former of whom also had studied comparatively the antituberculosis activity of a number of chemotherapeutic agents in a cell system and in a cell-free culture medium (10). He found that most of the agents tested were much less active on intracellular bacilli than on extracellular ones. Streptomycin and para-aminosalicylic acid were especially conspicuous in this respect (Table 1).

TABLE 1.—*Minimal inhibitory concentrations of various drugs on tubercle bacilli cultivated in Tween-albumin medium and in macrophage cultures.*[a]

Drug	Minimal concentration of drug, /μgm./ml., causing complete inhibition	
	Tween-albumin medium	Macrophage culture
Streptomycin	0.5	10 100
Isoniazid	0.05	0.05
Neomycin B	1.56	25.0
P. A. S.	1.56	100.0

a The data are taken from references 10 and 22.

In a more recent study the bactericidal effect of streptomycin, isoniazid, and combinations of both against intracellular tubercle bacilli was investigated (12). Mackaness and Smith cultivated infected macrophages in the presence of varying concentrations of the therapeutic agents. After some days of cultivation the medium was replaced by one without antituberculosis drug, and 24 hours later the culture was air-dried to kill the macrophages. This procedure did not harm the tubercle bacilli, which began to produce microcolonies after the culture had been embedded in an oleic acid-albumin medium with agar. Judging the bactericidal effect of the drug according to the number of microcolonies developing from the infected and killed phagocytes under such conditions, it was found (Text-fig. 2) that streptomycin alone had almost no bactericidal activity, but it had an additive effect in combination with isoniazid.

Experimentation with brucella under similar conditions has led to almost identical results. When studying comparatively the effect of strepto-

mycin on intracellular brucella and on brucella in cell-free media, Ma-
goffin and Spink (13) had found that organisms phagocytized by poly-
morphonuclear leucocytes survived for days in the presence of strepto-
mycin in concentrations as high as 50 μgm./ml., whereas they were killed

## % INFECTED MACROPHAGE RESIDUES
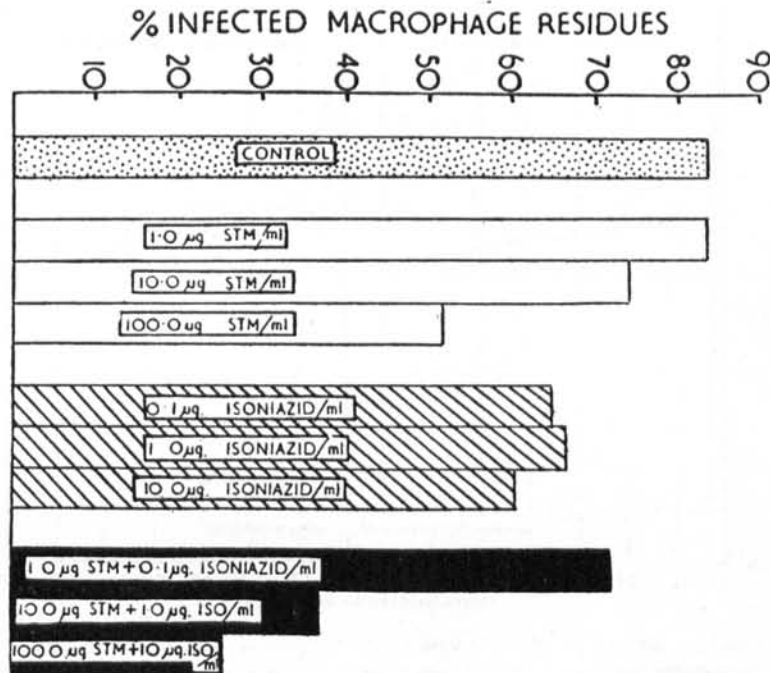
TEXT-FIG. 2. Percentage of macrophages containing viable tubercle bacilli in cul-
tures which had been exposed for four days to varying concentrations of streptomycin
and isoniazid, singly and in combination. (Fig. 13 in reference 12.)

within hours in a medium without cells (Text-fig. 3). Mouse phagocytes
protected brucella organisms not only against streptomycin but also
against the bactericidal effect of rabbit antiserum (19). In more recent
experiments mice were infected with brucella and treated thereafter
with different therapeutic agents for three weeks. Recovery of the bru-
cella in cultures from the spleens of mice killed at the end of the treat-
ment period and four weeks later showed that aureomycin and terramycin
not only caused a reduction of the pathogens by the end of the treatment
but that the reduction continued for four more weeks without treatment.
This was not the case with either streptomycin or sulfadiazine. Combina-
tions of the various agents were always more effective than any one
alone (20).

All these results obtained with tubercle bacilli or brucella are indica-
tive of the complexities with which one is confronted when analyzing the
efficacy of chemotherapy of chronic infections. Brucellosis might be
compared superficially with leprosy. In either infection the pathogen
finds a hostile environment in the body fluids but manages to survive

within phagocytic cells. In the case of brucella the active factor has been identified as an antibody; in leprosy it is probably natural inhibition (6). This, however, should be no hindrance to the application of some of the
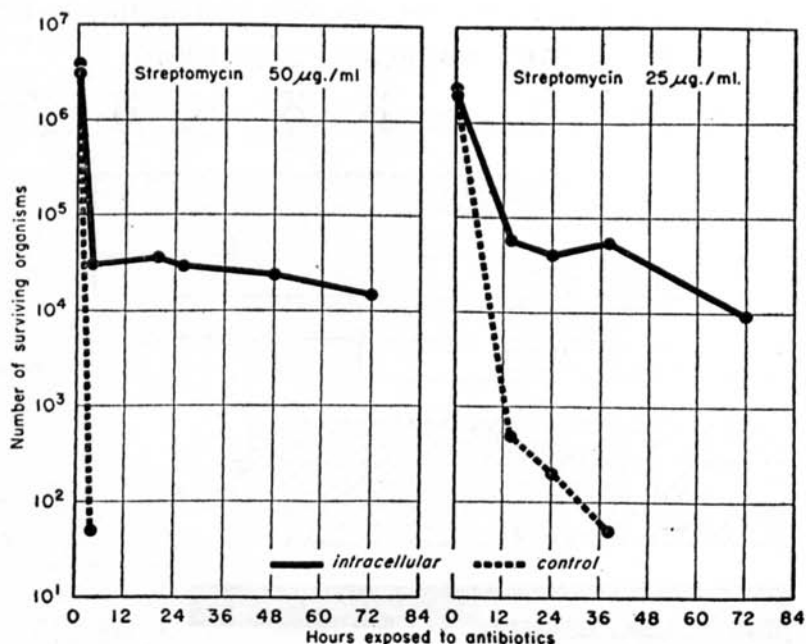
TEXT-FIG. 3. Effect of streptomycin on intracellular and extracellular *Brucella suis*. The leucocytes were obtained from human buffy coat. (Fig. 1 in reference 13.)

knowledge gained with these organisms to problems in leprosy. The most important lesson seems to be that combinations of chemotherapeutic agents are more potent and effective than any agent alone, even if the partners show very little activity themselves. This is true for brucella as well as for tubercle bacilli. It is not yet clear how much of the combination effect, especially of sulfadiazine and streptomycin, may be due to a toxic effect of sulfadiazine rendering the phagocytes more permeable to the other drug, thus favoring streptomycin activity.

INTRACELLULAR PARASITISM OF TUBERCLE BACILLI

In a discussion of chemotherapy, as shown above, tuberculosis can be compared with other chronic infections. But tuberculosis seems to occupy a unique position when the mechanism of immunity is considered. The agents of brucellosis, typhoid fever and probably leprosy are characterized by the survival of the organisms within phagocytes even after some form of immunity has been established. Recent studies on the fate of tubercle bacilli within macrophages cultivated *in vitro* revealed that phagocytes from vaccinated animals possess inhibitory power against the bacilli, whereas the normal macrophages allow tubercle bacilli to multiply within their cytoplasm. Since the results obtained so far might be im-

portant for the consideration of immunity in leprosy, the principles of the technique as well as the results will be summarized in the following.

To study *in vitro* the interaction between macrophages and tubercle bacilli, some technical conditions had to be fulfilled. First, the cells had to be cultivated in such a way as to be accessible to the oil-immersion objective of the microscope after fixation and staining. This was achieved by cultivating macrophages derived from peritoneal exudates in a single-cell layer on coverslips which could easily be handled. Secondly, the cells had to be infected uniformly with a very small number of tubercle bacilli. This was made possible by the addition of a diluted suspension of finely dispersed bacilli to the suspension of phagocytes used for the preparation of the cell cultures. Thirdly, the infection of the cultures had to be restricted to an intracellular condition. The property of streptomycin not to influence intracellular microorganisms while active against extracellular ones was essential for these experiments. Streptomycin in concentrations from 2 to 5 μgm./ml, added to the liquid culture medium for the macrophages, prevented extracellular proliferation of the bacilli without interfering with those which were intracellular. Thus, with the help of the antibiotic the tubercle bacilli were confined artificially to the intracellular phase. These experimental conditions resemble the natural conditions in infections with brucella and leprosy bacilli.

Using this technique, intracellular multiplication of different strains of tubercle bacilli of varying degrees of virulence was measured quantitatively by counting the number of bacilli present in the macrophages after increasing periods of cultivation (23). The strains were classified as virulent, attenuated and avirulent according to their ability to multiply in normal animals or to cause lesions and progressive disease (17, 25). As summarized in Table 2, it was found that both virulent and

TABLE 2.—*Multiplication of various strains of tubercle bacilli within normal macrophages cultivated* in vitro, *and effect of bacilli upon the cells.*[a]

Strains of bacilli	Multiplication of bacilli	Extent of damage to cells
Virulent: H37Rv	++++	++++
Attenuated: RiRv	++++	++
Attenuated: BCG	++++	+
Avirulent: H37Ra	0	0

a Compiled from reference 23.

attenuated tubercle bacilli could multiply within phagocytes from normal animals, whereas the avirulent variant R37Ra did not multiply. In addition to their ability to multiply, the tubercle bacilli exerted a direct

effect upon the cells in which they were harbored. This could result in destruction of the cells depending on the degree of virulence of the bacilli; the more virulent the strain the more cells were found destroyed. Destruction of the phagocytes led to the spread of the bacilli. Table 2 gives in column 2 an arbitrary estimation of the degree of destruction caused by the different strains.

From these results it was concluded that virulence of tubercle bacilli would depend on at least two properties: (a) the ability to multiply intracellularly, and (b) the capacity of destroying the host cells (23). The destructive effect of virulent tubercle bacilli on macrophages may be related to the inhibition of migration of polymorphonuclear leucocytes by virulent tubercle bacilli described by Allgöwer and Bloch (1). These authors had found that leucocytes from blood buffy coat which had phagocytised virulent tubercle bacilli (H37 Rv) and which were then explanted into a suitable plasma containing medium would no longer migrate out of the explant. The avirulent variant H37 Ra did not have any effect of this nature. A certain correlation between degree of virulence or attenuation and inhibition of migration has been established (14) which is duplicated in macrophage cultures.

In contrast to the free multiplication of tubercle bacilli within macrophages from normal animals, bacillary proliferation was almost completely suppressed when phagocytes from previously vaccinated animals were used for the experiments. This growth-inhibitory effect of macrophages from immunized animals gives these cells a key position in acquired immunity in tuberculosis (9). This view is further supported by the results which were obtained when cultivating monocytes from normal and vaccinated animals in a medium containing normal serum or serum taken from vaccinated animals. As summarized in Table 3, it

TABLE 3.—*Multiplication of tubercle bacilli (strain BCG) in cultures of macrophages from normal and immunized animals in the presence of normal serum and serum from an immunized animal.*[a]

Source of cell culture	Multiplication of bacilli	
	Normal serum	Immune serum
Normal animal	++++	++++
Immunized animal	0	0

a Compiled from data in reference 24.

was consistently found in those experiments that cells from immunized animals did not support bacillary growth, whether they were cultivated in presence of normal or "immune" serum. Likewise, serum of either kind—i.e., normal or immune—did not influence the multiplication of the tubercle bacilli within normal macrophages (24).

The fact that macrophages from immunized animals can suppress bacillary proliferation is of importance for an understanding of the pathogenesis and immunity of tuberculosis. The question arises by what means the infection progresses when the phagocytes no longer allow multiplication. If the bacilli do remain confined within the phagocytes permanently, multiplication cannot continue after immunity has been established. This probably is the case in infections with an attenuated strain of tubercle bacilli such as the Calmette-Guérin bacillus (BCG). In a first phase the bacilli multiply, and then their number remains stable, and later it gradually declines. Virulent bacilli, on the other hand, can escape the phagocytes by means of their destructive capacity and establish extracellular foci in which they are no longer exposed to the inhibitory effect of the macrophages. Multiplication thus will continue more or less unhindered. This interpretation of the results obtained in tissue-culture experiments are compatible with the findings by Pierce and Dubos (3, 17) in mice. In their experiments virulent tubercle bacilli continued to increase in lungs and spleens beyond the time during which immunity would develop, whereas multiplication of attenuated bacilli was brought to a standstill after an initial increase of the number of bacilli.

The nature of the inhibitory property of the cells is unknown. The fact that the inhibitory property of macrophages and skin hypersensitivity to tuberculin appear simultaneously after infection is no proof for the identity of the two phenomena (24).

There is no reason to doubt that leprosy bacilli multiply within phagocytic cells, thereby inducing the formation of the lepra cells (16). How immunity manifests itself in leprosy is unknown. Based on the results obtained with tubercle bacilli, two possibilities might be discussed briefly. (*a*) Either the phagocytes acquire the power to inhibit and to eliminate leprosy bacilli, or (*b*) a high degree of cellular sensitivity is established which brings about the destruction of the phagocytes loaded with bacilli which in turn become exposed to an unfavorable extracellular environment. No facts support the first hypothesis except for the analogy to tuberculosis. The second possibility, however, is based on the experience that the benign form of leprosy, the tuberculoid type, is characterized by reactivity towards lepromin and usually negativity for bacilli, whereas negativity to lepromin and abundance of bacilli are typical findings in the so-called malign form of leprosy, the lepromatous type (26). Unfortunately, there are no facts available which would offer a safer basis for rationalization. But it is hoped that some of the knowledge gained with tubercle bacilli will help eventually to solve practical or theoretical problems of leprosy.

SUMMARY

Some new knowledge gained in the field of chemotherapy of chronic infections is discussed in the first part of this paper. It is emphasized

that pathogenic agents causing chronic infections may survive or even multiply within phagocytic cells, and that many therapeutic agents are inactive against intracellular microorganisms.

In the second part, recent experimental observations on the interaction between tubercle bacilli and macrophages are reviewed. Evidence is presented which supports the view that the macrophages play a dominant role in immunity against tuberculosis.

The possible application of these facts to an understanding of problems of the infection with leprosy bacilli is stressed.

RESÚMEN

El autor hace un repaso de la relación parásito-celular. Se dá enfasis al hecho que ciertos agentes patogénicos causantes de infecciones crónicas pueden vivir y aún multiplicarse dentro células fagocíticas, y que muchos agentes terapéuticos son inactivos contra microorganismos intracelulares. Se presenta evidencia de la interrelación entre los fagocitos y los bacilos de la tuberculosis, la que demuestra que los macrófagos juegan un papel de importancia en la inmunidad contra la tuberculosis. Se discute la posible aplicacion de estos principios a los problemas de la lepra.

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