EXTRACELLULAR INHIBITORS IN LEPROTIC INFECTIONS AND THEIR ROLE AS BARRIERS TO EXPERIMENTAL TRANSMISSION[1]

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Previous work has shown the adverse influence of serum, and the favorable influence of purified serum albumin, on the infectiousness of *Mycobacterium leprae murium* incubated in tissue homogenates (9). It has also shown the corresponding effects of these protein solutions on respiration (5) and hydrogen transfer capacity (11) of washed suspensions of murine leprosy bacilli. Finally, there has been observed a series of positive correlations between infectiousness and hydrogen transfer capacity (HTC) following refrigeration in various solutions (12). Bacilli which had been refrigerated in albumin for three months possessed much greater infectiousness than was predicted by their hydrogen transfer capacity. It was evident, therefore, that prolonged soaking in albumin had conferred upon them a special property which afforded protection against some adverse condition encountered in the animal body after inoculation.

When the bacilli are transferred experimentally to a new animal they lie for some hours in a serous exudate along the needle track before they are all ingested by phagocytic cells. It was, therefore, regarded as of interest to examine the time required for inhibition of metabolic activity in serum. It was hoped on this basis to judge whether the damage to the endogenous metabolism of bacilli in the usual inocula (unprotected by albumin) might provide an explanation of the foregoing observations.

Although experimental transmission of murine leprosy is accomplished readily, this report presents evidence that the success of this transmission is determined in part by factors other than the classical intracellular relationships between such mycobacteria and their host cells. Similar concepts with respect to the role of extracellular inhibitors in tuberculosis (15) and virus infections (1) have been reached independently by other workers. An evaluation of these concepts will be undertaken in the discussion.

METHODS

Purified suspensions of *M. leprae murium* were obtained from infected rat testicle tissue and their concentration was standardized by methods previously described (7). The test dose of 0.1 ml of suspension corresponding to nephelometer No. 40 was added to 0.4 ml of balanced salt solution (BSS), serum or other modifying solution, and rotated at 37° for 24 hours in the presence of air containing 30 mm. CO_2 to provide the optimal pH, 7.5. The residual hydrogen transfer capacity (HTC) of the bacilli

[1] With the assistance of Miss Tobey Backerman and Miss Rachel Barrett.

was then tested by the addition of 0.1 ml of 1 per cent tetrazolium violet and by anaerobic incubation for 96 hours. The micrograms of formazan then extractable in acetone was regarded as a measure of the HTC of the incubated suspensions. The original paper ([7]) failed to state that the evacuated anaerobic bottles are refilled with 600 mm. partial pressure of nitrogen. Otherwise, there is excessive distillation of water vapor from the tests to the walls of the anaerobic vessels.

It has been shown that the HTC after 24 hours incubation in the control BSS is usually about 40 per cent of the original values ([11]). For purposes of the present comparisons, the residual HTC after 24 hours in BSS was regarded as the base line and assigned the value of 100 per cent. Sources of albumin and yeast autolysate have been stated ([12]).

Oxygen consumption at 37°C was measured by conventional techniques ([17]) in a sensitive modification of the Warburg apparatus ([14]), with 0.1 ml of 10 per cent KOH in the center well to absorb CO_2. Small reaction vessels and manometer capillaries were used to provide a system two or three times more sensitive than standard equipment ($KO_2 = 0.5$). Each flask received 0.5 ml of washed bacilli of nephelometer No. 80 in M/60 phosphate buffer at pH 7.5; water or test substance in water was added to make a final volume of 0.9 ml. These methods are described in greater detail elsewhere ([5]).

EXPERIMENTAL RESULTS

The average degree of inhibition by 50 per cent native rat serum during incubation for 24 hours, and the lessening of this toxicity by added albumin and yeast supplement, are shown in Table 1. The two levels of albumin are equivalent to 20 per cent and to 100 per cent of the total protein in 50 per cent serum, while the concentration of yeast supplement is much lower than those used hitherto. It is evident that 0.7 per cent albumin decreased toxicity of serum by four times, and 3.5 per cent albumin by 7 times, while 3.5 per cent albumin with yeast supplement

TABLE 1.—*Effect of rat serum and of albumin and yeast supplement on the HTC of murine leprosy bacilli during incubation for 24 hours.*

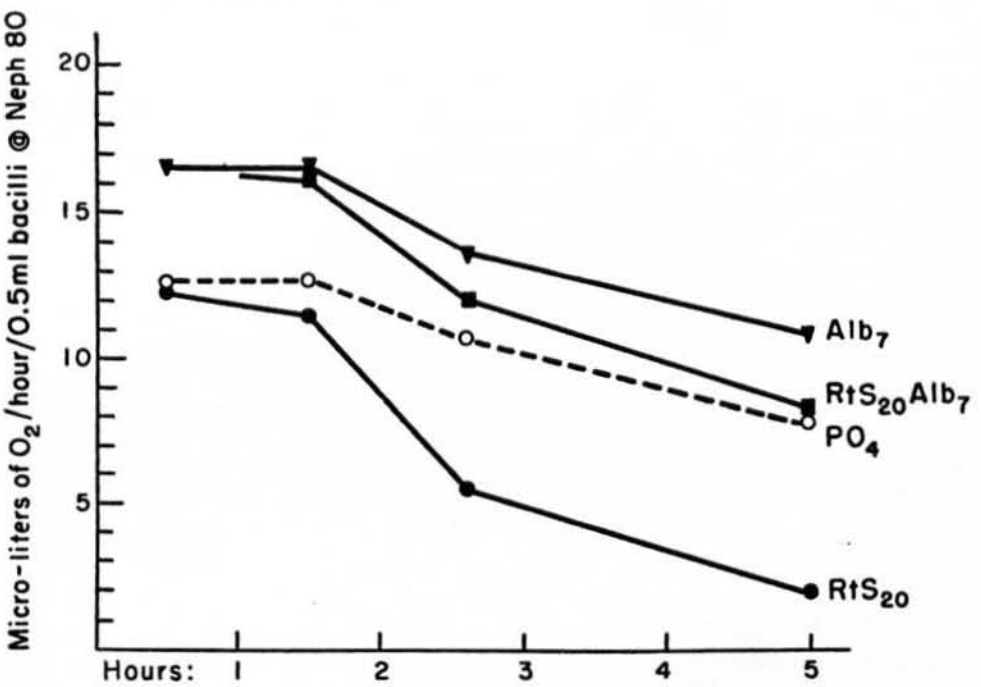
Incubation in the presence of:[a]	HTC		Values compared with 50% rat serum
	μgm.	%	
Balanced salt solution	51	100	10
Alb 3.5%, YS 4%[b]	182	357	36
Alb 3.5%	131	256	26
Rat serum 50%	5	10	1
Rat serum 50% with Alb 0.7%	21	41	4
Rat serum 50% with alb 3.5%	35	69	7
Rat serum 50% with Alb 3.5%, YS 4%	52	102	10

a Incubation for 24 hours, aerobic, 30 mm. CO_2, pH 7.5, prior to testing residual HTC by adding TzV and incubating anaerobically for 96 hours.

b Alb = purified bovine albumin; YS = yeast supplement 1:25. All components were diluted in the control balanced salt solution.

1:25 abolished the toxicity. Other body fluids are less inhibitory, in accordance with their lower content of lipo and muco-proteins. The benefits of adding albumin and yeast supplement to such fluids are relatively greater.

Since inhibition of respiration in the presence of serum diluted to 20 per cent becomes measurable after 1.5 hours (Text-fig. 1), it is evident that brief exposure of the bacilli to serous and inflammatory exudates must exert promptly an adverse effect on the endogenous metabolism. It has already been shown that this result must be interpreted as a decrease



TEXT-FIG. 1. Respiration rates of *M. leprae murium* in the presence of serum and serum albumin. Reagents: PO_4, M/60 phosphate buffer as inorganic background for endogenous metabolism; RtS_{20}, rat serum 20%; Alb_7, serum albumin 7%; $RtS_{20}Alb_7$, mixture in which albumin exceeds rat serum protein by 5 times.

in both viability and infectiousness (8, 12). The loss of 50 per cent of the respiratory rate in three hours and the fall to an almost negligible rate after six hours indicate, furthermore, that the damage is progressive and very serious. It will also be noted that when albumin was added in concentrations equal to 5 times the total serum proteins, but without prior soaking into the bacilli, it caused an initial enhancement of rate but did not eliminate the toxic effect of serum. More actively respiring (i.e., less inhibited) suspensions show less enhancement by albumin and greater percentage inhibition by serum.

Two experiments were conducted to explore the influence of time, and of the order of exposure, to both serum and albumin on the degree

of inhibition. The results given in Table 2 show that when the bacilli were incubated aerobically in the presence of serum for only five hours, the relative inhibition of HTC was comparable to that induced by 24-hour exposure. This inhibition was reduced by adding albumin, or albumin with yeast supplement, after the lapse of one hour. Exposure to these solutions for one hour prior to adding serum, however, gave only slightly higher HTC values. The loss of HTC was spared to about the same extent as when these three solutions were combined simultaneously (Table 1).

TABLE 2.—*The neutralizing effect of albumin and yeast supplement on serum toxicity; influence of time and order of exposure.*

Incubation[a]			Results	
A. First hour	B. Next four hours			
Substance[b]	Addition:[c]	Final mixture	μgm.	%
BSS	BSS	BSS	48	100
Alb_8	BSS	$Alb_{2.2}$	70	146
Alb_8 YS_8	BSS	$Alb_{2.2}$ $YS_{2.2}$	141	294
BSS	RtS_{100}	RtS_{40}	5	10
Alb_8	RtS_{100}	$Alb_{2.2}$ RtS_{40}	29	60
Alb_8 YS_8	RtS_{100}	$Alb_{2.2}$ $YS_{2.2}$ RtS_{40}	51	107
RtS_{100}	BSS	RtS_{40}	4	8
RtS_{100}	Alb_8	$Alb_{2.2}$ RtS_{40}	27	56
RtS_{100}	Alb_8 YS_8	$Alb_{2.2}$ $YS_{2.2}$ RtS_{40}	42	88

a Incubation for five hours before addition of TzV, in two periods. All subscript figures indicate percentage of the substances when added; e.g., Alb_8 = albumin 8%, Rts_{100} = rat serum 100%.

b To 0.1 ml of bacillus suspension, added 0.2 ml of the substance indicated. Thus, for example, an 8% concentration becomes 5.33%.

c Addition of 0.2 ml of the substance indicated, giving the final mixtures shown in the next column.

Similar results were obtained when bacilli were refrigerated in the solutions shown in Column A of Table 2 for 24 hours, prior to completing the combinations and incubating for four hours. From these results it is evident that brief exposure of the bacilli to albumin does not provide the unusual degrees of protection that were observed when bacilli had been refrigerated in albumin for a period of three months.

Certain properties of the bacilli, in fact, are altered after prolonged storage in albumin, although not shorter intervals. When bacilli are rigorously washed after refrigeration in albumin for three months and examined after Ziehl-Neelsen staining, some 40 per cent of them occur as bright blue bacterial cells containing deeply-stained red granules. Experi-

mental staining of albumin and other protein films on glass slides reveals that albumin has a remarkable capacity for dye-binding. Since the bacilli described had been rigorously washed, and since the property described is not acquired during refrigeration for several days, it was concluded that these bacilli had become permeated with albumin during prolonged refrigeration.

DISCUSSION

The data assembled here indicate that suspensions of *M. leprae murium* in tissue homogenates, simple solutions, or recently suspended in albumin or yeast supplement, suffer a serious loss in metabolic activity and capacity very soon after inoculation into animals. It is only after long periods of refrigeration that albumin permeates the bacilli sufficiently to afford significant protection. Neither albumin nor yeast supplement provides this degree of protection after brief incubation, or when combined simultaneously with serum. These observations are consistent with the earlier finding that the increased infectiousness of bacilli in these reagents is roughly proportional to the enhancement of HTC (12). The failure of greater protection is probably explained by the superficial situation of the two beneficial reagents in early periods, and also by the extracellular neutralization which occurs between serum and these reagents. Preliminary information on the nature and physical state of the inhibitors in native serum has been recorded (10); further details are to be published elsewhere.

In an earlier summary of metabolic relationships among the mycobacteria, reasons for anticipating more serious inhibition in *M. leprae* were stated (14). Since human leprosy seems never to have been transmitted experimentally from one human to another, it is of interest to note two reasons why inhibitions of the type described for *M. leprae murium* may occur during the procedures which have been employed in attempts to transmit human leprosy. The preparation of homogenates inevitably exposes the bacilli to inhibitory action by serum and body fluids contained in the leproma. Evidence that *M. leprae murium* is inhibited in various degrees when recovered from such homogenates has been presented (11). After variable intervals in this unfavorable environment, the bacilli are inoculated into sites where they are exposed to serous exudate until taken up by cells. It is only when *M. leprae murium* is protected against this hazard that its full infectiousness can be appreciated (12). Following such exposure it is probable that the human type bacilli are no longer capable of diverting, or competing with, the metabolism of the host cells. The reasons for this view have been discussed in an earlier paper (12). It seems necessary, therefore, to include both the preparation of homogenates and the further action of exudate and body fluids among the factors unfavorable to experimental transmission which have already been discussed (13).

A provisional summary has been made of the probable manner in which natural extracellular inhibitors prevent proliferation of human and murine leprosy bacilli in cell cultures, and of the uncertainty whether these inhibitors play a decisive role in limiting pathogenesis during the more quiescent phase of leprosy (14). Other investigators have been more sanguine in emphasizing the limitation of disease by extracellular, nonimmunologic mechanisms. The evidence, however, is confined to experimental observations such as those here described. The data with respect to viruses[2] are from two general sources: (*a*) the demonstration of reduced infectiousness after exposure of viruses to serum or serum fractions (1); and (*b*) the observations that components of ground substance limit virus production without damage to cultivated cells (3), and that high serum content in cell-culture media, while increasing the cell crop, may decrease virus synthesis (4). The presumed important role of tissue inhibitors in tuberculosis (15) is dependent upon the liberation or activation of such components because of pathological changes in that disease. Although there are already many indications that natural extracellular inhibitors play a determining role in certain instances of host-parasite specificity, the exact importance of these inhibitors during insidious phases of diseases caused by intracellular agents remains to be evaluated.

In an earlier publication it was suggested that the inhibitors present in serum and body fluids may play a role during the treatment of leprosy. The present work provides additional insight into the reasons why brief exposure to extracellular inhibitors may decrease infectiousness. Differences between the definitely fatal action of these inhibitors and the mode of action of two antimetabolic drugs have been emphasized (14). The failure of INH (16) and of streptomycin (2) to diminish the infectiousness of *M. leprae murium* after incubation of tissue homogenates in the presence of these drugs is in complete accord with the conclusion that such drugs do not depress the endogenous metabolism of mycobacteria (6). Furthermore, it is known that the viability of mycobacteria can be supported for considerable periods by endogenous materials and without utilization of extraneous substrate.

In view of the contrast between this limitation in the action of these drugs and the remarkable way in which the natural inhibitors handicap the endogenous metabolism and the infectiousness of organisms such as *M. leprae murium*, these natural body components are of more than passing interest in problems of chemotherapy.

SUMMARY AND CONCLUSIONS

1. The unfavorable effect of serum from rats and other animals on the infectiousness of *M. leprae murium* was first observed in very dilute

[2] The literature on this subject is abundant. The references given serve only for illustration.

solutions. The adverse effect is here shown to occur within the time during which it is known that inoculated mycobacteria lie in serous exudate prior to phagocytosis. Because of the progressive, serious damage to the endogenous metabolism of the bacilli, it is concluded that this exposure to natural extracellular inhibitors lowers significantly both their viability and their infectiousness when inoculated into animals.

2. Similar inhibitory action, both during the preparation of tissue homogenates and following inoculation, is postulated to be an impediment to the experimental transmission of human leprosy.

3. In the case of *M. leprae murium*, protection against such damage is demonstrable only after prolonged refrigeration in albumin solutions. This information may be applicable to other mycobacterial diseases in which transmission is difficult.

4. The possible importance of normal extracellular inhibitors in the natural transmission and pathogenesis of other diseases caused by intracellular agents has been discussed.

RESÚMEN Y CONCLUSIONES

1. El efecto desfavorable del suero de ratas y otros animales en la infecciosidad del *M. leprae murium* fué observada primero en soluciones muy diluidas. Se ha demostrado que en estos casos el efecto contrario ocurre durante el tiempo en el cual se sabe que los micobacterios inoculados estan contenidos en un exudado seroso anterior a la fagocitósis. Por este daño progresivo y considerable al metabolismo endógeno, se llega a la conclusión que éste riesgo a los inhibidores naturales estracelulares disminuye notablemente tanto la vitalidad como la infecciosidad de éste organismo después de la inoculación animal.

2. Una acción inhibidora similar, ya sea durante la preparación de tejidos emulsionados o como consecuencia de la inoculación, parece ser un obstáculo para la transmissión esperimental de la lepra humana.

3. Tratándose del *M. leprae murium*, protección contra tal daño se logra solamente, después de una prolongada refrigeración en soluciones de albúmina. Este conocimiento puede aplicarse a otras enfermedades micobacteriales de difícil transmisión.

4. Se ha discutido la probable importancia de los inhibidores estracelulares normales en la transmisión natural y en la patogénesis de otras enfermedades causadas por agentes intracelulares.

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