Serum Enzymatic Changes Following Infection of Mice with *Mycobacterium lepraemurium*[1]

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For years we have been studying *Mycobacterium lepraemurium* infections of mice as a model of a chronic infectious disease whose outcome ultimately depends upon macrophage-parasite relationships. In our system, a rather large inoculum of bacilli (1.5×10^8) is administered per mouse to overcome the potentially protective effect of the cell-mediated immune response and to ensure the progression of the disease. This large dose of bacilli induces a relatively early state of cellular activity that peaks between 1.6 and 2.5 months after infection and that is reflected in the metabolic state of the peritoneal macrophages. This cellular immune response then vanishes as the infection progresses. Peritoneal macrophages from *M. lepraemurium*-infected animals show an increase in their content of several lysosomal enzymes ([8]) and in their capability of killing *Listeria monocytogenes,* another intracellular microorganism. Despite the early state of cellular immunity, the bacilli continue to grow, slowly but insidiously, until there is systemic involvement of the host. In the advanced stages of the disease (eight months or more), most organs are massively infiltrated with *M. lepraemurium*-containing macrophages. The spleen, lymph nodes, liver, genitalia, and skin are most affected. The kidney, heart, and lungs are much less affected and, sometimes, even spared.

Since immunobiochemical studies are being conducted in our laboratory during the course of the infection with *M. lepraemurium,* we needed a sensitive indicator of infection that was useful long before the clinical appearance of signs and symptoms of the disease. The liver being one of the most affected organs, we thought of those enzyme activities whose increase over normal values could somehow implicate liver damage: serum glutamate-oxalacetate and glutamate-pyruvate transaminases (GOT and GPT). Changes in the levels of serum lactate dehydrogenase (LDH) and alkaline phosphatase (AlkP) were also examined.

MATERIALS AND METHODS

Animals. Albino male NIH mice, 1.5 months old, were inoculated intraperitoneally (i.p.) with 1.5×10^8 *M. lepraemurium,* Hawaiian strain, or they were kept uninoculated as a control group. Every two weeks, 3 infected and 3 uninfected animals were individually sacrificed by chloroform inhalation. Each animal was then pin-fixed on a waxed tray, the skin on its thorax removed, and the blood withdrawn by cardiac puncture through a 21-gauge needle. This procedure allowed us to collect an average of 1.0 ml of blood per animal with little or no hemolysis. Sera were separated and stored at −76°C until used.

Biochemical assays. Enzyme determinations in serum were based on the methods of Wroblewski and LaDue ([9]) for LDH; Karmen ([5]) for GOT; Reitman and Frankel ([7]) for GOT and GPT; and Bessey, *et al.* ([3]) for AlkP. Assays were carried out in the Abbott automated clinical chemistry analyzer, ABA 100, using commercial Beckman kits. For GPT, GOT, and AlkP, direct determinations were done in undiluted 5 μl samples; while for LDH, because of the high activity present, the kinetic method in 5 μl of 1:5 or 1:10 diluted serum was required. Calibration of the analyzer was performed with reagents intended for the analysis of human samples.

Statistical analysis. Statistical analysis of the results was done by using a *t* test for small samples. ([2]).

RESULTS

The Figure shows the levels of GOT, GPT, LDH, and AlkP found during the infection

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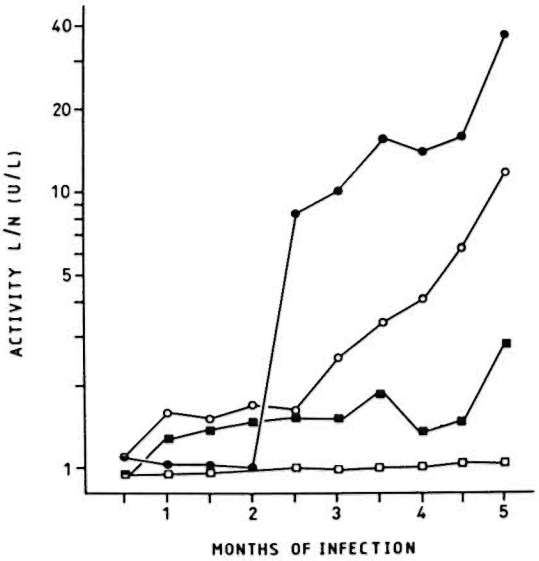
up to month 5. Results are given, for each particular time, as the ratio of average units per L in the infected group/average units per L in the control group.

It is observed that although differences between infected and control animals are apparent as early as 30 days after inoculation in regard to the levels of GOT and GPT, these differences are consistently significant only after 90 days in the case of GOT ($p < 0.01$) and after 150 days in the case of GPT ($p = 0.05$).

Changes in the activity of LDH seem to appear later, two months after infection, but the differences are much greater than those found with GOT and GPT. Two and one-half months after inoculation, LDH activity in the infected animals is 8 times greater than that found in their controls ($p < 0.01$), and by the fifth month it is 40 times higher ($p < 0.001$). This increase is not merely dependent on the physical presence of bacterial cells in the animals, since mice injected six months previously with heat-killed autoclaved *M. lepraemurium* (1.8×10^8 per mouse, i.p.) showed, as expected, no disease and LDH levels comparable to those found in the uninjected controls. The average LDH activity in the injected group ($n = 10$) was 0.262 ± 0.3; average activity in the control group ($n = 10$), 0.285 ± 0.02; for a ratio inoculated (L)/control (N) = 0.91. In this case, activity is given as the average optical density (OD) (530 nm) reading of the bluish-violet color developed after incubation (30 min/37°C) of duplicate 10 μl aliquots of undiluted fresh test serum with a mixture containing 6.0 mM sodium lactate, 0.07 mM NAD, 0.041 mM NBT, 0.11 mM phenazine methosulfate, and 2.55 ml (2.6 ml in blanks without enzyme) of 0.03 M Tris-HCl buffer, pH 9.0 ([1]).

For our more recent experiments, we adapted this colorimetric method to have a more versatile means to measure LDH activity in mice sera. The color developed in this assay remains stable at least for 60 min after stopping the reaction with 0.2 ml of 5% acetic acid. Regardless of these changes, the levels of AlkP in the control and infected groups are similar and do not show modifications with the time of infection. The Table shows the results after five months of infection in other similar groups of *M. lepraemurium*-infected and control animals.



THE FIGURE. Effect of the infection of NIH mice with *Mycobacterium lepraemurium* (1.5×10^8 bacilli per mouse) on the serum levels of lactate dehydrogenase (●——●), glutamate-oxalacetate transaminase (○——○), glutamate-pyruvate transaminase (■——■), and alkaline phosphatase (□——□). Each point is the ratio of average activity in infected animals/average activity in controls. Average activity (in units per liter) was calculated from three infected or control serum samples assayed individually.

Here again, while levels of serum LDH, GOT, and GPT are elevated, in that order, in the infected animals, the AlkP activity is the same in both control and infected groups. Although differences in the levels of LDH, GOT, and GPT are not as large as in the previous experiment, they are clearly evident, with LDH the best indicator of infection. Because enzyme activities in the normal animals from the first and second experimental groups are about the same (all of them fall within the average normal values, see below), it is possible that the lower figures observed in the second experiment for LDH and GOT, as deduced from the N/L ratios, relative to the five-month values, are a real consequence of the more advanced disease.

On the average, normal values (mean ± standard deviation) of serum LDH, GPT, GOT, and AlkP in mice (48 animals from the first and second experiments) are: 909 ± 112 U/L (LDH); 94 ± 35 U/L

THE TABLE. *Serum levels of lactate dehydrogenase (LDH), glutamate-pyruvate transaminase (GPT), glutamate-oxalacetate transaminase (GOT), and alkaline phosphatase (AlkP) in NIH normal (N) and* M. lepraemurium*-infected (L) mice.*[a]

Months of infection	LDH			GPT			GOT			AlkP		
	N	L	R	N	L	R	N	L	R	N	L	R
0	909 ± 112			94 ± 35			222 ± 89			36 ± 4		
5.5	1,011	7,564	7.5	122	244	2.0	266	1,229	4.6	40	43	1.0
6.0	817	7,096	8.7	73	257	3.5	296	1,353	4.6	38	30	0.8
7.0	914	10,462	11.5	97	219	2.3	281	1,436	5.1	32	33	1.0

[a] Each animal received, i.p., 1.5×10^8 bacilli. Values are the mean, in units per liter, of three individual mice. R = ratio of activity in the infected (L)/activity in the control (N) mice.

(GPT); 222 ± 89 U/L (GOT), and 36 ± 4 U/L (AlkP).

DISCUSSION

Mice injected i.p. with an infective dose of *M. lepraemurium* similar to the one used in the present study (1.5×10^8 bacilli) develop, as time progresses, a state of cell-mediated immunity (CMI) that leads to biochemical activation of the host's macrophages and that peaks around 50 days post-inoculation, vanishing thereafter (unpublished observations and Poulter and Lefford [6]).

While trying to demonstrate the existence of infection at a time previous to the one in which symptoms are evident (in our system, this happens around 7–8 months post-inoculation), and being aware of the liver involvement, we used serum analysis to look for nonspecific biochemical alterations caused by the disease. We found that the disease affected the LDH, GOT, and GPT levels in that order, while the levels of AlkP were not altered. Based on human disease, one might have expected an increase of both transaminases and AlkP if the enzyme levels signified liver disease. The fact that the highest level reached was that of LDH, an enzyme which is more associated with cardiac muscle, also attracted our attention. Rodents, however, have a different enzyme distribution; alkaline phosphatase (not modified by the infection), for example, is found in the liver (0.3), kidney (100), spleen (1.5), skeletal muscles (0.2), etc., while LDH (the most altered enzyme activity) exists in the liver (96), kidney (31), spleen (40), heart (82), skeletal muscles (100), etc. (Figures in parentheses indicate the amount of each enzyme in a given wet weight of tissue which is shown as 100 [4].)

Therefore, our results are in harmony with what is known of the murine tissue distribution of the enzyme activities studied in this paper. They support the known fact that the liver is importantly affected by the disease caused by *M. lepraemurium* and, hence, they allow us to propose the enzyme analysis of serum for detection of the ongoing infection well before the appearance of clinical signs. We feel that having other conditions controlled (appropriate hygiene and nourishment, adequate environmental conditions, absence of obvious unwanted infections, etc.) and measurement of the LDH (at least) and GOT levels may be a useful way to monitor the progress of this murine mycobacterial disease.

When death of the host has to be avoided, from 0.3–0.4 ml of blood is taken aseptically with a prepared Pasteur pipette via the retro-orbital venous sinus, leaving the animal alive and practically unharmed for future manipulation. From this volume of blood, it is possible to obtain from 0.1–0.2 ml of serum, more than enough for the proposed enzyme determinations.

SUMMARY

Mice injected interperitoneally with 1.5×10^8 *Mycobacterium lepraemurium* develop progressive visceral alterations that are reflected in the lactic dehydrogenase (LDH), glutamate-pyruvate transaminase (GPT), and glutamate-oxalacetate transaminase (GOT) levels. The rise in GPT and GOT levels starts earlier (about 30 days post-infection) than the rise in LDH activity (about 70 days), but the latter shows the

most impressive increases. Differences between infected and control groups, however, reach statistical significance only at 75 days (LDH), 90 days (GOT), and 150 days (GPT) post-inoculation, still well before the appearance of obvious external signs of infection (about 240 days in our model). It is suggested that the ratio of enzyme levels in infected to enzyme levels in uninfected animals could be taken as a reliable index to follow the progress of the infection with *M. lepraemurium.*

RESUMEN

Los ratones infectados i.p., con 1.5×10^8 *Mycobacterium lepraemurium,* desarrollan alteraciones viscerales progresivas que se reflejan en los niveles séricos de las enzimas LDH, TGP, y TGO. El incremento en los niveles de TGP y TGO, comienza más temprano (ca. 30 días post-infección) que el incremento en LDH (ca. 70 días) pero ésta última muestra los incrementos más marcados. Sin embargo, las diferencias entre los grupos control e infectado sólo alcanzan significancia estadística hasta los días 75 (LDH), 90 (TGO), y 150 (TGP) post-inoculación; todavía mucho antes de la aparición de los signos externos de la infección (ca. 240 días en nuestro sistema experimental). Se sugiere que la relación entre los niveles enzimáticos de los animales infectados y los controles, podría tomarse como un índice confiable de infección, útil en el seguimiento de la enfermedad.

RÉSUMÉ

Des souris injectées par voie interpéritonéale par $1{,}5 \times 10^8$ *Mycobacterium lepraemurium* ont développé des altérations viscérales progressives entraînant une modification des taux de la déhydrogénase lactique (LDH), de la glutamate-pyruvate transaminase (GPT), et de la glutamate-oxalacetate transaminase (GOT). L'élévation des taux a débuté plus précocement pour la GPT et pour GOT (environ 30 jours après l'infection) que pour l'activité en LDH (celle-ci débutant environ 70 jours après l'infection). Cependant l'activité en LDH a présenté l'augmentation la plus impresionnante. Des différences statistiquement significatives entre les animaux injectés et les groupes témoins n'ont cependant pu être mis en évidence que 75 jours après l'inoculation pour la LDH, 90 jours pour la GOT, et 150 jours pour la GPT. Ces délais sont cependant bien inférieurs à ceux notés pour l'apparition de signes externes indubitables d'infection; ces manifestations externes dans ce modèle expérimental ne surviennent qu'après environ 240 jours. On suggère dès lors que le rapport des taux d'enzymes chez des animaux infectés et chez des animaux non infectés pourrait constituer un indice fiable pour suivre la progression de l'infection par *M. lepraemurium.*

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