Relationship of Serum $\beta$-Glucuronidase and Lysozyme to Pathogenesis of Tularemia in Immune and Nonimmune Rats

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Received for publication 26 February 1975

A temporal study is reported of the febrile responses, tissue bacterial contents, and serum concentration of the lysosomal enzymes, $\beta$-glucuronidase and lysozyme, in nonimmune rats inoculated with virulent or attenuated strains of Francisella tularensis, and in immune rats challenged with either a high or low dose of virulent organisms. The level of serum $\beta$-glucuronidase appears to be an indicator of hepatocyte damage, whereas serum lysozyme correlates with the appearance, frequency, and severity of pyogranulomatous lesions. Survival of nonimmune rats after a challenge with either virulent or attenuated organisms appears to depend on a balance between dose of bacterial inoculum, celerity of irreversible pathologic events, and the ability of the reticuloendothelial and immune systems to collaboratively mount a response to limit or prevent dissemination of the infection. In immune rats, infection of parenchymal hepatic cells does not occur after a low dose ($10^4$) virulent challenge. Infection of parenchymal hepatic cells, however, does occur in immunized rats when the challenge dose is sufficiently large ($10^6$) so as to overcome the capacity of the reticuloendothelial to clear opsonized organisms.

Previous studies on the pathogenesis of tularemia in the rat have investigated the effects of prior inoculation of various strains of Francisella tularensis on the immune response and survival rate after subsequent virulent challenge (3, 5, 6, 16). Only recently have the pathogenesis and comparative lesions of tularemia in rats after infections with virulent or vaccine strains of F. tularensis been described (14). To further characterize the pathogenesis of tularemia in rats we now report the temporal examination of febrile responses, tissue bacterial contents, and serum concentrations of the lysosomal enzymes, $\beta$-glucuronidase and lysozyme, in nonimmune rats inoculated with virulent or attenuated strains of F. tularensis and in immune rats challenged with either a high or low dose of virulent organisms. A correlation between serum lysozyme levels with frequency and severity of pyogranulomatous lesions is reported. Marked alterations in serum $\beta$-glucuronidase occurred only in rats which did not survive. These data are discussed relative to proposed sequences of pathogenic events.

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MATERIALS AND METHODS

Cultures of virulent (SCHU S4) and attenuated (LVS) strains of F. tularensis were prepared as previously described (14).

Male, Fisher-Dunning white rats weighing approximately 200 g were supplied by Microbiological Associates, Inc., Bethesda, Md. Rats were fed Purina pellets and maintained in light- and temperature-controlled quarters.

Experimental rats were inoculated intraperitoneally with 1-ml aliquots of either $10^4$ LVS (NA4) or SCHU S4 (NV4). (A three-symbol designation is assigned to each experimental group. The immune status of animals, represented by the first letter, is indicated by either N [nonimmune] or I [immune]. Strain of F. tularensis employed, represented by the second letter, is indicated by either A [attenuated] or V [virulent]. The log dose of administered challenge organisms is indicated by the third symbol, a number.) Two additional groups of rats were vaccinated with $10^4$ LVS 8 weeks before intraperitoneal challenge of $10^4$ (IV4) or $10^6$ (IV6) virulent SCHU S4. Pair-weighted control rats were injected with 1 ml of tryptose-saline intraperitoneally. Since NV4 rats became anorectic 24 h postinoculation, their controls were pair fed. All other experimental groups maintained preinoculation dietary intake; thus their controls were fed ad libitum. Rectal temperatures were obtained with a Yellow Springs telethermometer.

At appropriate intervals, six rats per group were
RESULTS

Interactions of nonimmune rats with virulent and attenuated F. tularensis. All immune rats inoculated with virulent F. tularensis (SCHU S4) succumbed to the infection; death occurred after as few as 100 organisms (Table 1). A linear relationship was found between median time to death and the log dose of challenge SCHU S4 organisms. Median time to death for NV4 rats was 72 h, thus limiting experimental observations to 3 days.

A febrile response was observed as early as 12 h postinoculation (Fig. 1, left column) with SCHU S4. Fever continued to increase until 60 h, at which time a precipitous drop in body temperature signaled imminent death. Mortibund rats were bacteremic and failed to develop agglutinating antibody titers to F. tularensis.

Continuous growth of organisms was observed in liver and spleen of these rats, respectively, approaching a concentration of 10⁶ and 10⁸ organisms per g of tissue by day 3. Precipitous increases in serum β-glucuronidase and lysozyme were observed. However, the increase in lysozyme activity lagged behind that of β-glucuronidase by approximately 12 h.

All rats challenged with 10⁴ attenuated organisms survived; some, however, died when larger doses were employed (Table 1). NA4 rats (Fig. 1, right column) did not become significantly bacteremic but did develop an agglutinating titer of 1:320 by day 7. Growth of LVS and spleen was observed during the first 2 days, followed by a gradual reduction in total bacterial content. No evidence of organisms in either organ was found 8 weeks after inoculation.

Fever was observed in this group during days 2 and 3 of infection. Serum lysosomal enzymes were elevated by day 3. Lysozyme, in contrast to β-glucuronidase, remained elevated through day 7.

Dose-related responses with attenuated F. tularensis. When nonimmune rats were infected with 10⁴ LVS organisms, serum β-glucuronidase activity was maximal on day 2; lysozyme, however, increased steadily throughout the experimental period (Table 2). Infecting doses of 10⁸ attenuated organisms resulted in

### Table 1. Effect of F. tularensis dosage on survival of immune and nonimmune rats

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*Heat inactivated.
75% mortality and was marked by steady and substantial increases in the serum activity of both lysosomal enzymes.

**Interactions of immune rats with virulent F. tularensis.** Immunized rats were protected against challenge with as many as 10⁸ SCHU S4 organisms. With larger doses, however, immune rats succumbed to challenge (Table 1). IV4 rats (Fig. 2, left column) showed a trend toward an increased agglutinating antibody titer, a low-grade fever at 48 h postinfection, and tissue bacterial contents which were less than 1% of those found in nonimmune rats. Whereas no increases in serum β-glucuronidase were found, lysozyme was slightly elevated during days 4 to 7.

IV8 rats (Fig. 2, right column) were febrile during the 24 to 72 h period after challenge. While a marked elevation in serum lysozyme activity was observed throughout the experi-
TABLE 2. Effect of LVS-challenge dose on serum β-glucuronidase (β-G) and lysozyme (LYS) activity of nonimmune rats

<table>
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<th>Hours post-infection</th>
<th>Activity (%) compared to noninfected controls at challenge doses of:</th>
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<td>48</td>
<td>116 ± 7</td>
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<td>72</td>
<td>146 ± 13*</td>
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*Six rats per group.
*P < 0.01.
*Mean of two surviving animals.

DISCUSSION

Results of various studies indicate that neutrophils and monocytes are the most probable source of serum lysozyme (22), whereas hepatic parenchymal cells most likely account for the major portion of β-glucuronidase released into tissue during the initial 2 days of infection; thereafter, the concentration of organisms in these tissues slowly declined. A typical anamnestic response was also observed in this group.

mental period, β-glucuronidase was elevated only on day 2. In excess of 10^6 organisms per g of tissue were found in liver and spleen during the first 2 days of infection; thereafter, the concentration of organisms in these tissues slowly declined. A typical anamnestic response was also observed in this group.

DISCUSSION

Results of various studies indicate that neutrophils and monocytes are the most probable source of serum lysozyme (22), whereas hepatic parenchymal cells most likely account for the major portion of β-glucuronidase released into tissue during the initial 2 days of infection; thereafter, the concentration of organisms in these tissues slowly declined. A typical anamnestic response was also observed in this group.
the blood (1, 2, 7). The latter cells contain 20 times as much \( \beta \)-glucuronidase activity as Kupffer cells (9) and contribute over 95% of the total \( \beta \)-glucuronidase content of liver. In patients with different liver disorders, elevated serum \( \beta \)-glucuronidase activity has been shown to be a sensitive indicator of liver parenchymal cell damage (15). In contrast to hepatic parenchymal cells, phagocytes contain high concentrations of lysozyme in their granules and are the most likely source of the enzyme for most tissues of the body (8, 12). Release of lysozyme by neutrophils during phagocytosis and degradation of senescent phagocytes with subsequent release of lysozyme is thought to account for its presence in serum (18); hence, elevations of serum lysozyme levels are found in myeloid and monocytic leukemias and in granulomatous diseases such as tuberculosis (19) and sarcoidosis (18).

In the present study, an increase in activity of the lysosomal enzymes, \( \beta \)-glucuronidase and lysozyme, was observed in serum after inoculation of nonimmune rats with \( F. \) tularen\emph{s}is. Dissimilarity in time of appearance and/or magnitude of enzyme changes indicates that different cell types independently contribute to the elevated serum activity of each hydrolase. Invasion of liver cells by \( F. \) tularen\emph{s}is, intra-cellular proliferation of bacteria, and necrosis of hepatic parenchyma are consistent components in the pathogenesis of tularemia (10, 11, 21). Hence, \( \beta \)-glucuronidase from damaged or necrotic hepatocytes can account for the elevated levels of serum \( \beta \)-glucuronidase of tularemic rats. We further propose that recruitment of phagocytes to necrotizing foci with subsequent phagocytosis of cell debris, and perhaps the organism itself, results in release of lysozyme and other nonspecific mediators of inflammation (e.g., pyrogen). The appearance of pyogranulomatous lesions, characterized by neutrophil and macrophage invasion (14; J. B. Moe et al., manuscript in preparation) within the parenchyma of liver and in spleen; the correlation between serum lysozyme level and number and severity of pyogranulomatous necrotic lesions; and the presence of massive concentrations of bacteria in liver and spleen significantly preceding febrile responses are consistent with the proposed sequence of pathogenic events. Characterization of pyogranulomatous lesions in liver and spleen are reported elsewhere (14; J. B. Moe et al., manuscript in preparation]. The possible contribution by the spleen to the observed increases in serum \( \beta \)-glucuronidase, although undetermined, must be considered minimal since the total content of \( \beta \)-glucuronidase in spleen is low compared to liver. Furthermore, liver, but not spleen, of NV4 rats showed a reduction in total \( \beta \)-glucuronidase at 72 h after challenge [unpublished observation]. Splenic contribution to serum lysozyme, however, may be more significant due to accumulation of inflammatory cells.) Differences in the response pattern of the lysosomal enzymes among the various experimental groups, however, suggests that the sequences of pathologic events in tularemia can be modified depending on the immune status of the host and the virulence of the strain of bacteria employed.

In rats given attenuated organisms (group NA4), only a transient rise in serum \( \beta \)-glucuronidase occurred, suggesting that destruction of hepatocytes was limited. Amelioration of infection in NA4 rats occurred concomitantly with the appearance of specific serum antibody. It is likely, therefore, that appearance of humoral antibody and/or development of cellular immunity prevented the dissemination of organisms by enhancing the clearance of opsonized bacteria by the reticuloendothelial system (RES) before induction of irreversible pathologic changes. The observation that large inocula of attenuated organisms can kill rats indicates that a delicate balance exists between infectious dose, celerity of irreversible pathologic events, and the ability of the RES and immune system to limit or prevent dissemination of the disease.

In nonimmune rats (NV4) infected with the virulent strain, the rise in serum \( \beta \)-glucuronidase that occurred before the increase in lysozyme supports the tenet that hepatocellular damage precedes a phagocytic cell response, and furthermore, in contrast to infection with the attenuated LVS strain, the nearly linear increases in \( \beta \)-glucuronidase activity attests to the fact that damage of hepatocytes is not limited but, rather, is rapid and extensive. This may reflect a greater affinity of SCHU S4 for hepatocytes resulting in an increased number of infectious foci, or an inability of phagocytes to kill this organism rapidly enough to prevent extensive dissemination. The latter interpretation is consistent with published observations that phagocytes in culture inactivate fully virulent strains of \( F. \) tularen\emph{s}is at a much slower rate than less virulent strains (20).

The absence of histopathological lesions and serum \( \beta \)-glucuronidase release IV4 rats suggests that infection of hepatocytes does not occur. Opsonization of organisms by immune serum most likely increases the capacity of the RES to phagocytize the organism and prevents invasion of hepatocytes. The presence of some bacteria in
liver and spleen, and the minor elevations in fever and serum lysozyme activity, however, suggests that the RES cells in these tissues are capable of ingesting and limiting, but not totally preventing, growth of virulent *F. tularensis*.

The early febrile response evoked in immune rats challenged with 10⁴ virulent organisms indicates that the number of opsonized organisms presented to the RES was sufficient to promote rapid release of pyrogen. The increase in serum lysozyme, occurring prior to the rise in β-glucuronidase, represents a sequence of responses unlike that observed in any other experimental groups and suggests that a large number of opsonized organs were rapidly phagocytized. The high tissue bacterial counts and the transient appearance of β-glucuronidase further suggest that the capacity of the RES to clear SCHU S4 was overwhelmed by the large number of bacteria administered; infection of parenchymal cells ensued as demonstrated by the extensive number of necrotizing lesions in both liver and spleen. The resulting phagocytic involvement resulted in substantial release of lysozyme. However, unlike the response observed in nonimmune rats, hepatocellular damage in immune animals did not go unchecked; the spread of infection was probably limited by immune phagocytes culminating in clearance of the infecting organism and survival of the rat.

ACKNOWLEDGMENTS

We thank W. R. Beisel and H. G. Dangerfield for their advice in the preparation of the manuscript. The secretarial support of Patricia Kemp and Rhonda Wetzel, and technical assistance of Eric Henrikson, Eleanor Ayala, and Dan Bowman, are gratefully acknowledged.

LITERATURE CITED