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Sequential Metabolic Alterations in the Myocardium During Influenza and Tularemia in Mice

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Mice with generalized influenza or tularemia of similar lethality were studied in an effort to compare biochemical responses of the myocardium during infections of viral and bacterial etiology. A progressive loss of body weight characterized the course of both infections. Accompanying this, the myocardial content of protein and the activities of lactate dehydrogenase, citrate synthase, and cytochrome *c* oxidase all decreased. However, myocardial protein degradation appeared earlier and was more pronounced in influenza, and the protein changes were accompanied by a rapid decline of myocardial RNA. Activation of acid hydrolases, such as cathepsin D and β-glucuronidase, occurred in tularemia but not in influenza, whereas leakage of β-glucuronidase into the plasma occurred in both infections. Conversely, there was a considerably greater activation of myocardial catalase in influenza. These findings suggested that different control mechanisms or metabolic pathways were operative in the degradation of myocardial constituents in influenza as compared with tularemia. The absence of histological signs of myocarditis in either infection appeared to exclude any direct local effects of an inflammatory process on myocardial cells. Since the infections were of comparable lethality (based upon the inoculated dose of organisms), the observed differences in pattern and extent of metabolic responses of the myocardium to these infections may be attributed to different pathophysiological mechanisms evoked by the different microorganisms.

Myocarditis is a common complication in enterovirus infections (21, 39) and has been reported at variable frequencies in other viral infections as well, including influenza (40). Bacterial infections are less frequently associated with myocarditis (6). Even in the absence of myocarditis, the physical performance capacity of the host is often impaired after various acute infectious diseases (9, 13). Little is known, however, about the effects of generalized infections or myocardial metabolism. In the absence of a localized myocarditis, Newcastle disease virus infection in chicks causes a decrease in heart size and in the myocardial contents of protein, RNA, and DNA (28). Only recently was it demonstrated that a bacterial infection without myocarditis, i.e., one due to *Francisella tularensis*, could also cause myocardial protein degradation in rats concomitant with a decreased physical performance capacity of skeletal muscles (15, 16). In tularemia, there is also evidence to suggest involvement of the lysosomal enzyme system of the rat myocardium (16).

The purpose of the present study was to compare degradative processes, and the metabolic consequences for the myocardium, of a generalized viral infection with those of a bacterial infection of similar lethality. Histological examination was performed to exclude the development of myocarditis and the possible confounding metabolic effects of a localized inflammatory reaction in the myocardium.

MATERIALS AND METHODS

Animals. Male Swiss-Webster mice were used (Harlan, Sprague-Dawley Inc.). Mice were maintained on a commercial diet (Wayne Lab Blox, Allied Mills, Chicago, Ill.) until the beginning of an experiment and were housed in rooms maintained at 23 ± 1°C. The initial mean body weights (± standard deviation) of the mice were 29.2 ± 1.5 g for the influenza infection and 27.9 ± 1.0 g for the tularemia infection. Food and water were supplied ad libitum. Since inoculating doses were identified in preliminary studies to achieve median lethality after 7 days of influenza or after 6 days of tularemia, mice were preassigned at random to take part in the experiments for a total of 2, 4, or 7 days in the influenza infection and for 2, 4, or 6 days in the tularemia infection (study 1).

Additional mice were randomized in similar groups in each infection, and after 6 days of influenza or 4 days of tularemia, tissue samples were excised for histopathological examination (study 2). Infected and noninfected control mice were studied simultaneously in each infection. Groups were sized to allow for losses due to lethality.

Infection. On day 0, mice were inoculated intranasally with 0.1 ml of a 10-fold dilution of a mouse-virulent suspension of influenza virus, $10^{8.7}$ egg median infectious doses of strain A/Aichi/2/68 (H3N2) per ml (20), or intraperitoneally with a 0.2-ml suspension of 3.15×10^4 CFU of nonwashed *F. tularensis* live vaccine strain per ml that had been grown on solid fortified glucose-cysteine-blood agar (25). The influenza virus suspension was delivered to the nose pad, encompassing both nares, to permit its inhalation during light anesthesia with halothane. Control mice were administered similar volumes of heart infusion broth in the influenza trial (intranasally) and sterile tryptose saline (Difco Laboratories, Detroit, Mich.) in the tularemia trial (intraperitoneally). Body temperatures were recorded by a rectally inserted thermocouple before blood and tissues were sampled on each of days 2, 4, and 6 or 7.

Sampling. (i) Study 1. On days 2, 4, and 7 in influenza and 2, 4, and 6 in tularemia, eight randomly preassigned mice from each group were anesthetized with halothane. The thoracic cavity was opened, the caval vein was severed, and blood was collected from the right pleural cavity by using heparinized pipettes. The heart and spleen were removed and put into ice-cold homogenization buffer. The myocard-

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ium was rapidly opened; atria, vessels, and blood were removed, and the entire remaining ventricular muscle was weighed and cut into small pieces with a pair of scissors. These pieces were homogenized in ice-cold homogenization medium (150 mM KCl, 50 mM $KHCO_3$, and 6 mM EDTA [pH 7.4]) with all-glass Potter-Elvehjelm homogenizers operated manually. The entire procedure was performed at 0 to 4°C. The homogenates for acid hydrolase assays were made 0.1% with respect to Triton X-100 concentration. The spleen was cleansed of connective tissue, rinsed, blotted on filter paper, and weighed.

(ii) Study 2. On day 6 in influenza and on day 4 in tularemia, mice were killed by the same procedure as in study 1, and the myocardia were similarly excised and placed in 10% Formalin for routine histological processing and preparation of hematoxylin and eosin slides.

Assays. (i) Plasma. In study 1, blood plasma was held in wet ice until used in individual analyses of β-glucuronidase (GUase: EC 3.2.1.31) activity (Sigma Analytical Kit, Sigma Chemical Co., St. Louis, Mo.).

(ii) Myocardium. In study 1, in all-heart homogenates, total protein (after incubating 0.1 ml of homogenate with 0.1 ml of 20% KOH at 80°C for 60 min) (22), RNA, and DNA were measured (36). Further, the activities of lactate dehydrogenase (LDH: EC 1.1.1.27) (4), citrate synthase (CS: EC 4.1.3.7) (29), cytochrome *c* oxidase (CYTOX: EC 1.9.3.1.) (32), cathepsin D (Cat D: EC 3.4.23.5) (11), GUase (3), and catalase (EC 1.11.1.6) were determined. The activity of catalase, as expressed by oxygen production, was measured with an oxygen electrode, but otherwise essentially as previously described (5).

All assays were performed immediately, except those for plasma GUase and tissue protein, RNA, and DNA. The latter tissue studies were performed on homogenates that had been frozen and thawed. All measured tissue variables were calculated and expressed as activity (or content) in the entire ventricular muscle and per gram of "wet" tissue.

In study 2, histopathological examination of serial sections of the ventricular myocardium was performed to detect any myocardial necrosis, inflammation, or other lesions.

Statistics. The effect of each infection was calculated for each variable by means of a two-way analysis of variance by comparing results in infected groups with those of noninfected controls on days 2, 4, 6, and 7. Correlations were estimated and tested for significance by the least-squares method.

RESULTS

The disease intensity was similar in both infections in terms of lethality rates, the median lethality occurring at 7 days in influenza and at 6 days in tularemia.

There was progressively increased involvement of tissues throughout the course of the infections, as reflected by alterations in body, heart, and spleen weights (Fig. 1). The tissue weights and biochemical data of the ventricular myocardia in sham-inoculated control mice are given in Table 1.

Study 1. As a result of influenza or tularemia, heart and total body weights dropped. The general wasting of body tissues was more pronounced in influenza than in tularemia, but the relative effect on the myocardial weight was similar in the two infections. Spleen enlargement occurred in tularemia but not in influenza. Plasma GUase activity increased in both infections, and subnormal rectal temperatures were recorded throughout the influenza infection and even late in tularemia (Fig. 1).

The myocardial content of protein and RNA and the total myocardial activities of LDH, CS, and CYTOX were reduced in the course of each infection (Fig. 2 and 3). However, a clear difference was evident in that the degradation appeared earlier, developed faster, and became more severe in influenza (Fig. 2 and 3). RNA increased early in tularemia, a trend that was later reversed, whereas in influenza, RNA dropped progressively (Fig. 2). The DNA content was unaffected in either infection (Fig. 2). In tularemia, the activities of the mitochondria-associated oxida-

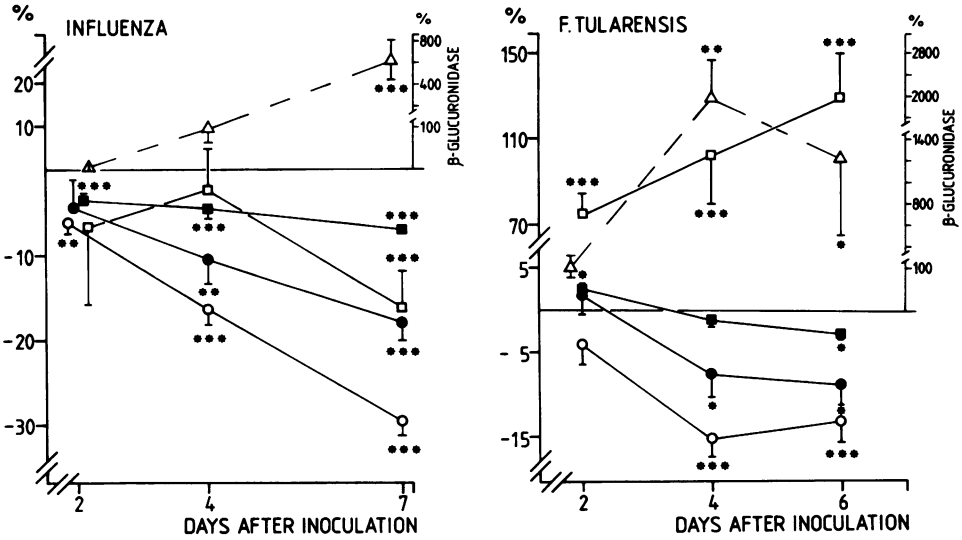


FIG. 1. Alterations in rectal temperature (■) and GUase activity in plasma (△), and weight of body (○), heart (●), and spleen (□) in influenza- and *F. tularensis*-infected mice at different times after inoculation, expressed as percent deviation from corresponding values in sham-inoculated control mice (units of variables are given in Table 1). Means ± standard errors are given. Asterisks denote statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between infected and noninfected mice.

TABLE 1. Tissue weights and normal enzyme activities and concentrations of protein, RNA, and DNA in myocardia of all (n = 48) sham-inoculated control mice (study 1)

Component	Unit	Mean ± SD
Body wt	g	28.1 ± 1.7
Spleen wt	mg	108 ± 21
Heart wt	mg	110 ± 6
Plasma GUase	U/ml	4.92 ± 2.20
Heart tissue variables (units per g of "wet" muscle)		
Protein	mg	166 ± 13
RNA	mg	3.25 ± 0.49
DNA	mg	1.82 ± 0.21
Glycolytic enzyme LDH	μmol/min	210 ± 20
Oxidative enzymes		
CS	μmol/min	178 ± 32
CYTOX	μmol of O_2 per min	5.69 ± 0.81
Lysosomal acid hydrolase enzymes		
GUase	nmol/min	11.9 ± 3.8
Cat D	μg of albumin per min	1,124 ± 157
Peroxisomal enzyme (catalase)	μmol of O_2 per min	35.7 ± 7.4

tive enzymes CS and CYTOX decreased more than the activity of cytoplasmic LDH, but in influenza, no such difference was evident; these enzymes decreased their activities in parallel (Fig. 3).

The lysosomal enzymes GUase and Cat D were activated only in tularemia; a consistent decrease of GUase activity was found in influenza (Fig. 4). Conversely, the myocardial activity of catalase showed a major increase only in influenza (Fig. 4). The catalase increase correlated negatively with the activity of CYTOX ($R = -0.720$; $P < 0.01$, infected mice on days 4 and 7 combined). No similar correlation was observed in noninfected or tularemia-infected mice. When expressed as activity per milligram of myocardium, Cat D showed an increase even in influenza ($P < 0.001$ on day 7), whereas the decrease of GUase remained significant ($P <$ 0.01 on day 4 and <0.001 on day 7).

Study 2. No histological signs of necrosis of myocytes or infiltration of inflammatory cells could be found in the myocardium in any of the influenza- or *F. tularensis*-infected or sham-inoculated mice.

DISCUSSION

The present study, in which one viral infection (influenza) and one bacterial infection (tularemia) without myocarditis were compared, showed that influenza caused a more pronounced tissue wasting, including the ventricular myocardium, than did tularemia of similar lethality. In influenza, alterations occurred earlier and progressed farther than in tularemia. The myocardial changes included a decrease in total protein and glycolytic and oxidative enzyme activities in both infections. In influenza, the protein synthetic capacity as assessed by RNA content was depressed, but in tularemia, RNA was initially stimulated. Different mechanisms or pathways for the degradation of myocardial con-

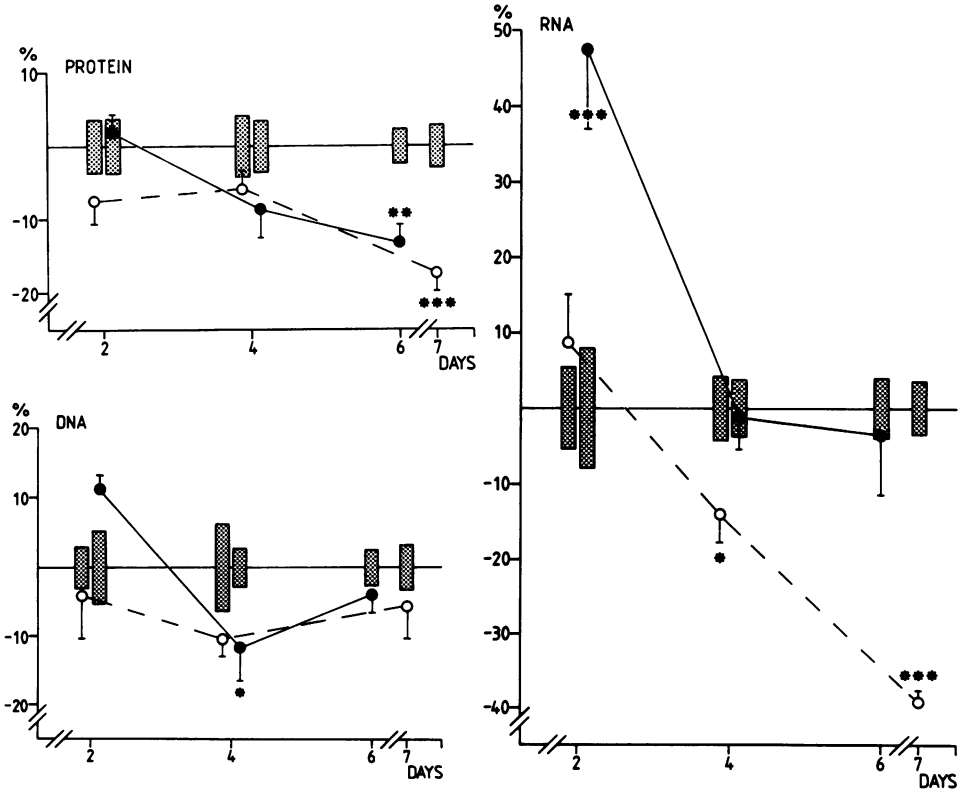
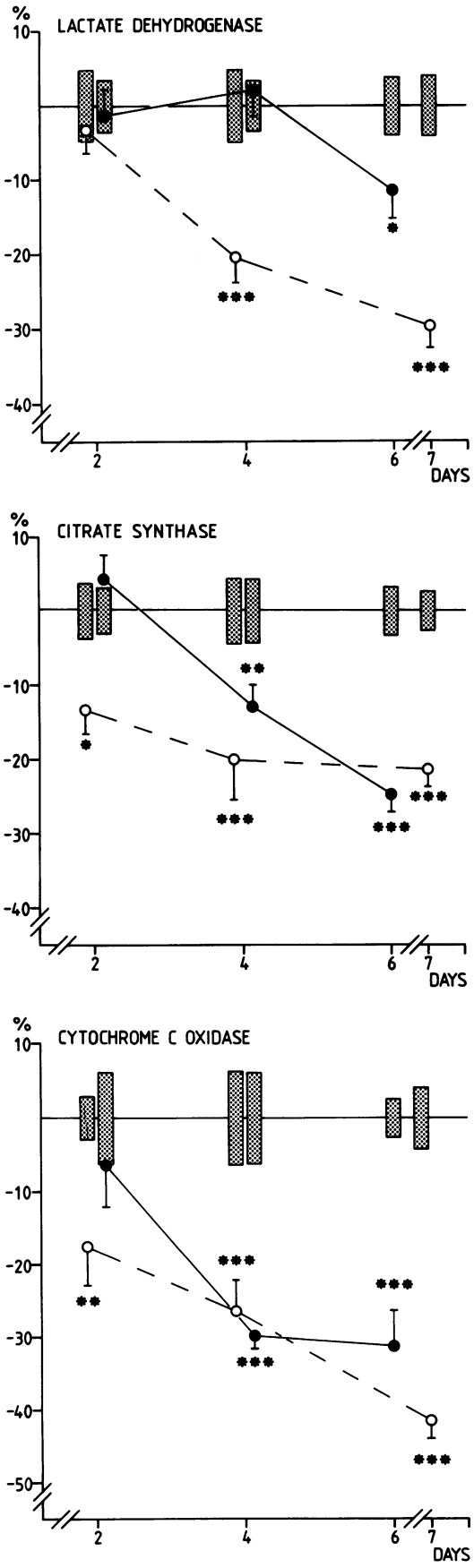


FIG. 2. Total myocardial contents of protein, RNA, and DNA in influenza- (○) and *F. tularensis* (●)-infected mice at different times after inoculation, expressed as percent deviation from corresponding values in sham-inoculated control mice. Units of variable contents are given in Table 1. Means ± standard errors are given. Asterisks denote statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between infected and noninfected mice.



stituents in viral as compared with bacterial infections are suggested by the different patterns of activations of myocardial tissue-degrading enzymes observed in these two infections.

The intensities of the two infections were comparable since similar lethality rates were recorded. In tularemia in rats, spleen weight is useful for estimating the progression of the infection (24), and, not unexpectedly, a progressive increase of spleen weight was recorded in the present mouse tularemia model, whereas in influenza there was a significant decrease in spleen weight. Similarly, plasma GUase activity is known to increase in tularemia, the enzyme probably originating in the liver (12). We observed an increased GUase activity even in influenza. The plasma activity of this enzyme does not seem to have been studied in viral infections before.

Cardiac changes known to be associated with some viral infections, such as coxsackievirus infections, include histological signs of myocarditis, i.e., myofiber necrosis, infiltration of inflammatory cells (39), acute increase of heart weight (21, 31) and subsequent fibrosis (18). Multiplication of virus particles within the myocardium is believed to initiate this process (18, 39). Such an ingress of inflammatory cells would alter the myocardial content of DNA, RNA, and protein and make it difficult to identify any metabolic changes in myocardial cells per se. Since no histological signs of myocarditis were observed in the present infections, the recorded biochemical alterations should be considered part of the systemic catabolic response of the host to each of these infections rather than effects of invasion by the specific microorganisms.

In both viral and bacterial infections, there is degradation of skeletal muscle protein, the largest somatic protein pool of the body (8), and the heart muscle seems not to be spared in this process, as shown in this (Fig. 1 and 2) and previous studies (16, 28). In previous metabolic balance studies in humans suffering from viral or bacterial disease, there was a remarkably similar, almost stereotyped pattern of metabolic responses, and the magnitude of change was related to the severity of illness (8). However, no studies seem to have been published in which catabolic responses of visceral or somatic organs have been compared in two or more infections. The metabolic consequences of generalized infections for the myocardium are important since this would partly explain the deterioration in physical performance capacity after acute infectious disease (13).

The altered myocardial RNA content may have reflected an initially increased protein synthetic capacity in tularemia that was later normalized. Conversely, RNA showed a progressive decrease below base-line values in influenza, probably indicating a deceleration of the rate of protein synthesis (23). Indirect evidence has previously been presented of a decreased protein synthetic capacity of somatic proteins in *Salmonella typhimurium* infection (19, 41).

The control mechanisms that initiate altered myocardial cell metabolism during generalized infections have yet to be identified. Infections are accompanied by multiple hormonal

FIG. 3. Total myocardial enzyme activities of LDH, CS, and CYTOX in influenza- (○) and *F. tularensis* (●)-infected mice at different times after inoculation, expressed as percent deviation from corresponding values in sham-inoculated control mice. Units of enzyme activities are given in Table 1. Means ± standard errors are given. Asterisks denote statistically significant differences (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$) between infected and noninfected mice.

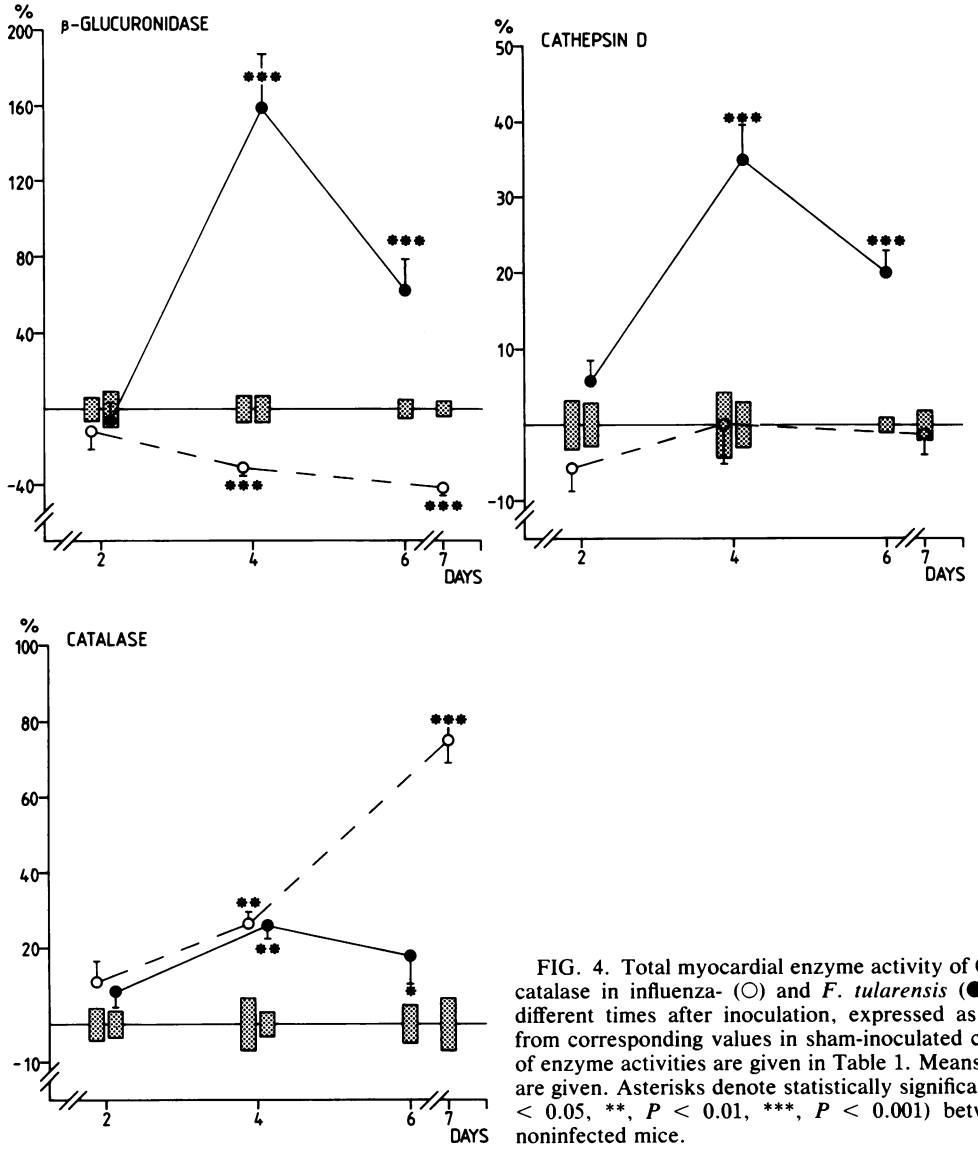


FIG. 4. Total myocardial enzyme activity of GUase, Cat D, and catalase in influenza- (○) and *F. tularensis* (●)-infected mice at different times after inoculation, expressed as percent deviation from corresponding values in sham-inoculated control mice. Units of enzyme activities are given in Table 1. Means ± standard errors are given. Asterisks denote statistically significant differences (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$) between infected and noninfected mice.

responses, and by the secretion by phagocytic cells of endogenous mediators of fever and other physiological responses. One of these, leukocytic endogenous mediator, is released by activated phagocytic leukocytes early in bacterial infections and to a lesser extent in viral infections (37). Leukocytic endogenous mediator stimulates RNA replication and subsequent protein formation in the liver (38), but its effect on skeletal muscle protein may be catabolic (7). Additional studies will be required to determine whether an interplay of hormonal and mediator effects can explain the proteolytic and enzymatic changes in myocardial tissue during generalized bacterial and viral infections. Similarly, the myocardial changes could be influenced to some degree by the sizable acute nutritional losses of body mass observed during each of these mouse infections.

In tularemia, the loss of mitochondrial enzyme activity, i.e., of CS and CYTOX, preceded the decrease in LDH located in the cytoplasm, whereas in influenza all of these enzyme activities decreased early (Fig. 3). Thus, mitochondrial involvement occurred early in both infections. In previous studies of mitochondrial and cytoplasmic enzymes in skeletal muscle in viral, mycoplasma, and bacterial infections in humans (1, 2) and rats (15), the membrane-attached enzyme CYTOX was considerably more resistant to the catabolic stimulus than were cytoplasmic enzymes such as LDH. It is noteworthy that in skeletal muscle, isoenzyme 1 of LDH, which predominates in myocardium (27), is less affected than the other LDH isoenzymes by similar infections (17). Thus, even minor biochemical differences in enzyme structure and intracellular environment seem to influence the sensitivity of an enzyme to stresses such as those of an infection.

In tularemia, the lysosomal enzyme system was activated in the myocardium, as indicated by the increase in total activity of acid hydrolases, such as Cat D and GUase. Similarly, these and other lysosomal enzymes showed increased activity in rat myocardium and skeletal muscle in tularemia (15, 16) and in *S. typhimurim* myocarditis (N.-G. Ilbäck, G. Friman, R. L. Squibb, A. J. Johnson, D. A. Balentine, and W. R. Beisel, Acta Pathol. Microbiol. Scand.

Sect. A, in press). Further, increased activity of these enzymes may also be recorded in skeletal muscle undergoing necrosis as a result of overloading (33). However, histological signs of myocarditis were absent in tularemia- and influenza-infected mice. Thus, the lysosomal enzyme system was involved in the degradative turnover of various tissue components in normal and pathological states (10, 33). It may have mediated myocardial proteolysis in tularemia.

In influenza, on the other hand, the total activity of Cat D was unchanged, and that of GUase even decreased. In contrast, when expressed as tissue concentration, the activity of Cat D, but not that of GUase, was significantly elevated. In degenerative processes such as those following vitamin E deficiency or irradiation, decreased GUase activity has been recorded (35). The mechanisms of protein degradation in influenza are not clear, but reduced protein-synthesizing capacity, as reflected by the abrupt decrease of RNA at a normal or even slightly increased protein degradation rate, seems to fit our data best. However, alternative or additional factors cannot be excluded, such as damage of mitochondrial membranes or leakage of macromolecules through altered myocyte membranes. Macromolecules of molecular weight 80,000 have previously been demonstrated to leak from muscle into serum in patients suffering from influenza without cardiac complications (14). Leakage of GUase into the plasma was evident in both of the present infections, although the myocardial content of this enzyme decreased only in influenza.

Lipid peroxidation and hydrogen peroxide formation is the basic mechanism of destruction of unsaturated fatty acid chains in biological membranes. If the mechanism of regulation is unbalanced, detrimental effects, such as increased membrane permeability, enzyme solubilization, or inactivation may occur (34). This process is associated with hydrogen peroxide formation, which may exert cytotoxic effects, but hydrogen peroxide is degraded by catalase (5). Increased catalase activity has been proposed to be a valid indicator of ongoing skeletal muscle wasting (30). One intracellular location of catalase in myocardium has been shown to be the mitochondrial matrix (26). Thus, the present finding of a negative correlation between catalase and CYTOX activity in influenza may indicate that increased hydrogen peroxide formation is involved in mitochondrial damage and the more pronounced wasting of myocardial tissues and cellular constituents in this infection than in tularemia.

Thus, the present results suggest different pathways for myocardial degradation in influenza and tularemia. As both infections were of the same lethality, the different metabolic responses to these infections may be explained by a different pathogenic mechanism associated with these specific microorganisms. It remains to be established whether these differences are characteristic of viral versus bacterial infections in general.

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