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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM		
1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A125590	3. RECIPIENT'S CATALOG NUMBER		
4. TITLE (and Subtitle) Role of Gram-Negative Bacteria and their Endotoxins in Rat Death from Heat Stress of Intestinal Origin in Rat Heat Stress Mortality		5. TYPE OF REPORT & PERIOD COVERED		
7. AUTHOR(s) D. A. DuBose, K. Basamania, L. Maglione, and J. Rowlands		6. PERFORMING ORG. REPORT NUMBER M-4/81		
9. PERFORMING ORGANIZATION NAME AND ADDRESS US Army Research Institute of Environmental Medicine, Natick, MA 01760		8. CONTRACT OR GRANT NUMBER(s)		
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command. Ft. Detrick, MD 21701		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS		
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Same		12. REPORT DATE 26 Feb 81		
		13. NUMBER OF PAGES		
		15. SECURITY CLASS. (of this report) Unclassified		
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE		
16. DISTRIBUTION STATEMENT (of this Report) Distribution of this document is unlimited				
<table border="1"> <tr> <td>DISTRIBUTION STATEMENT A Approved for public release; Distribution Unlimited</td> </tr> </table>				DISTRIBUTION STATEMENT A Approved for public release; Distribution Unlimited
DISTRIBUTION STATEMENT A Approved for public release; Distribution Unlimited				
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) N/A				
18. SUPPLEMENTARY NOTES N/A				
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Heat stress, endotoxin, Limulus test, endotoxemia				
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Stress which induces an increase in the gram-negative bacterial count of the upper intestinal tract can be associated with extra-intestinal invasion of gram-negative bacteria and their endotoxins. The <u>Limulus</u> amoebocyte lysate (LAL) test and standard microbiological procedures were used to determine if duodenal and extra-intestinal invasion occurred in rat heat stress. Because gram-negative bacteria were found in extra-intestinal tissues (lung, liver and spleen) of some non-heated controls of the first rat group studied, (cont'd)				

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the experiments were repeated in rats found to be free of extra-intestinal gram-negative bacteria (group II). After heat stress, short (≤ 10 h) and intermediate (> 10 h but < 72 h) survivors of group I had a significantly increased incidence of duodenal invasion (gram-negative bacterial count $\geq 1.5 \times 10^4$ /g), as compared to controls. Intermediate survivors also had extra-intestinal invasion (a significantly increased incidence of gram-negative bacteria or endotoxins in liver, spleen or blood). These signs of invasion were generally associated with low bacterial count and LAL activity. Short and intermediate survivors of group II had duodenal, but not extra-intestinal invasion. No significant differences in the mean survival times of groups I and II were found. Duodenal invasion did occur after heat stress. This did not result in extra-intestinal invasion in group II and even the extra-intestinal invasion noted in group I had no significant impact on length of survival. Signs of extra-intestinal invasion in group I were likely the result of factors present prior to heating. Therefore, extra-intestinal invasion of gram-negative bacteria and their endotoxins did not appear to play a mediating role in rat death after heat stress.

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Role of bacterial endotoxins of intestinal origin in rat heat stress mortality

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DuBOSE, D. A., K. BASAMANIA, L. MAGLIONE, AND J. ROWLANDS. Role of bacterial endotoxins of intestinal origin in rat heat stress mortality. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* 54(1): 31-36, 1983.—Using unanesthetized rats, the effect on heat stress mortality of endotoxin tolerance or zymosan treatment was determined. In addition, the incidence of invasion by gram-negative bacteria and their endotoxins was studied to evaluate the role of gut-derived bacterial endotoxins after heat stress. Endotoxin tolerance resulted in heat stress resistance. The estimated mean total thermal area, which induced an LD₅₀ in endotoxin-tolerant rats (61.85°C·min) was significantly greater ($P < 0.001$) than that for non-tolerant rats (44.03°C·min). Rats were significantly ($P < 0.005$) more sensitive to endotoxin after zymosan treatment, but this treatment did not alter the heat stress mortality rate. The *Limulus* amoebocyte lysate test indicated that endotoxemia did not occur as a result of heat stress. Though a significantly increased incidence of high gram-negative bacterial count in the duodenum was noted, extraintestinal invasion was not found. It was concluded that resistance to heat stress may not be due to protection from gut-derived bacterial endotoxins, but resistance may possibly be associated with the ability of endotoxin tolerance to protect from shock syndromes. Thus bacterial endotoxins of intestinal origin did not appear to have a significant role in rat heat stress mortality.

endotoxin tolerance; zymosan treatment; *Limulus* amoebocyte lysate test; gram-negative bacterial invasion; endotoxemia

ENDOTOXINS or lipopolysaccharides are complex substances associated with the cell walls of gram-negative microorganisms. The release of endotoxins into the circulating blood may ultimately lead to a state of shock (6). A natural source or pool of gram-negative bacteria and their endotoxins is the intestinal tract. Increased numbers of gram-negative bacteria in the upper intestinal tract are associated with extraintestinal invasion in cases of enteritis (16) and irradiation stress (17). The intestinal tract is also thought to be the source of the extraintestinal invasion noted in hyperbaric stress (10), burn injury (18), and graft-vs.-host disease (22). A few isolated reports associate plasma endotoxins with the circulatory collapse and coagulative disorders noted in human heatstroke (5, 11). Furthermore, treatment to reduce gut flora increases the incidence of 18-h survival in experimental dog heatstroke (4). To determine the role of gut-derived bacterial endotoxins in rat heat stress mortality, the present study examined the effect of endotoxin tolerance and deter-

mined the incidence of duodenal and extraintestinal invasion by gram-negative bacteria and their endotoxins. In addition, the heat stress sensitivity of endotoxin-sensitive rats was evaluated.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Charles River, Wilmington, MA) were used throughout the study. Rats were individually caged and given food (Charles River chow) and water ad libitum. Five to six days prior to subjection to experimental heat stress, rats were placed in an environmental chamber maintained at 26°C and approximately 50% relative humidity.

Experimental Heat Stress

The experimental heat stress followed the procedures of Hubbard et al. (13, 14). After fasting for 18-24 h, unanesthetized rats were placed in small individual restrainer cages. A copper-constantan thermocouple was inserted 6.5 cm into the rectum to measure core temperature using a multipoint temperature scanning system (Leeds and Northrup, North Wales, PA). Rats were then placed in a heat stress chamber (Napco incubator, Portland, OR) that was maintained at $41.5 \pm 1^\circ\text{C}$. Core temperatures were determined at 1-min intervals, and thermal area (13) was calculated when core temperature exceeded 40.4°C. Restrained rats were subjected to heat stress until a desired core temperature or thermal area was obtained. At this time, rats were removed from the heat stress chamber and restrainer cages and allowed to cool passively at an ambient temperature of 26°C. In all cases, rats were observed for mortality over a 72-h period after heat stress.

Endotoxin-Tolerant Rats

The treatment to induce endotoxin tolerance was initiated in rats weighing between 500 and 530 g. Nine increasing doses of endotoxin (*Escherichia coli*, 0127:B8, Difco Lab, Detroit, MI) were administered intravenously into the lateral tail vein over a 3-wk period. Each endotoxin dose was contained in 0.5 ml of saline and was administered in the following sequence: 0.5, 0.8, 1.2, 1.6, 4.0, 6.0, 8.0, 8.0, and 12.0 mg/kg body wt. A 48-h rest period followed each endotoxin injection, except for the third and sixth injections in which rats rested for 72 h.

Rats were also rested 72 h between the last injection and the beginning of the heat stress procedure. Control rats weighing between 380 and 420 g received saline injections (0.5 ml) following the same sequence as the rats receiving endotoxins.

Endotoxin tolerance was determined by challenging the endotoxin- or saline-injected rats with 40 mg/kg, twice the lethal dose at which 100% of the rats died ($2 \times LD_{100}$), and 20 mg/kg ($1 \times LD_{100}$) of endotoxin. This challenge occurred 72 h after the last endotoxin or saline injection.

Endotoxin-Sensitive Rats

Injection of zymosan was used to increase the sensitivity of rats to endotoxin. Zymosan was suspended in saline by sonication at 40 W of power for 4 min, using a Lab Line Ultratip Labsonic system (Lab Line Instruments, Melrose Park, IL). Rats received one daily injection of the suspension (10 mg/kg body wt) for a 3-day period. Control rats received saline injections. Twenty-four hours after the last injection, groups of zymosan- and saline-treated rats were challenged with endotoxin (6 mg/kg body wt) to determine their level of sensitivity to endotoxin shock. Other groups of zymosan- and saline-treated rats were subjected to experimental heat stress.

Invasion of Gram-Negative Bacteria and Their Endotoxins

Groups of heated and nonheated rats, which had not received any prior endotoxin or zymosan treatment, were examined for the presence of endotoxins and gram-negative bacteria in blood, liver, spleen, and lung samples. Gram-negative bacterial counts and endotoxin activity in the duodenum were also determined.

Sample collections. All blood samples were heparinized using heparin derived from beef lung (Upjohn, Kalamazoo, MI). Lateral tail vein blood samples were drawn before heating and after removal from the heat stress chamber. Agonal blood samples were collected from the heart. Tissue samples were obtained immediately after death using sterile surgical techniques and endotoxin-free surgical instruments. Nonheated controls were anesthetized by injection (0.3 ml/500 g body wt) of pentobarbital sodium (Abbott Laboratories, North Chicago, IL) into the lateral tail vein before sample collection.

Sample preparation. Blood samples for endotoxin assay were centrifuged (20 g) for 10 min to obtain platelet-rich plasma. This was then diluted 1:3 in sterile endotoxin-free water (Travenol Laboratories, Mansfield, MA). A laminar-flow biohazard safety hood (Baker, Sanford, ME) was used to provide a suitable sterile environment for the mincing of tissue samples. Liver (1:2), spleen (1:6), lung (1:6), and duodenum (1:5) samples were then diluted with sterile pyrogen-free saline (Travenol Laboratories). In the biohazard hood, liver, spleen, and lung samples were homogenized using endotoxin-free Teflon-tip grinders and glass grinding vessels. Dilutions of 1:6, 1:12, and 1:24 of these tissue homogenates were placed in endotoxin-free screw-cap glass tubes for extraction before endotoxin testing. Minced and diluted duodenal samples were blended in a vortex mixer for 1 min and then

centrifuged for 1 min at 20 g to remove debris. Serial dilutions of the supernatant were then made.

Endotoxin assay. The *Limulus* amoebocyte lysate (LAL) test (20) was used to detect the presence of endotoxins in the blood, tissue, and duodenal samples. The dilution + heating extraction procedure (7) was employed to remove protein LAL inhibitors from the samples before testing. All samples for LAL testing were heat extracted except duodenal samples, which required no extraction due to their high level of dilution. Pyrotell (Associates of Cape Cod, Woods Hole, MA) LAL was used in a 0.1-ml volume with 0.1 ml of test sample. In addition, all blood and tissue samples were retested with Pyrotell lysate (Difco, Detroit, MI). All readings were made after 1 h of incubation in a 37°C water bath. A test was considered positive by formation of a firm gel by the reactants. Lysate sensitivity was determined by testing serial dilutions of *E. coli* endotoxin (Associates of Cape Cod). Appropriate positive and negative controls were employed.

Gram-negative bacterial analysis. Both aerobic and anaerobic blood cultures were prepared from agonal blood samples (1–5 ml) using trypticase soy and thioglycolate broth (Becton-Dickinson, Rutherford, NJ). These were incubated at 37°C and tested over a 21-day period for the presence of gram-negative bacteria. Aerobic gram-negative bacteria were isolated from lung, liver, and spleen homogenates by placing 1–2 ml of the homogenate in brain-heart infusion broth (Difco) and then after 24 h of incubation inoculating both selective (MacConkey, Difco) and nonselective (5% sheep blood agar) plating media. A pre-reduced anaerobically sterilized chopped meat broth (Carr-Scarborough, Stone Mountain, GA) was used to isolate anaerobic gram-negative bacteria from lung, liver, and spleen homogenates. Gram-negative bacterial count per gram of duodenal sample was determined from colony counts made from five replicate MacConkey plates prepared from each dilution of the duodenal sample.

Statistical analysis employed chi-square and Student's *t* tests. Differences in the heat stress mortality rate between endotoxin-tolerant and control rats were analyzed by estimation of the lethal dose at which 50% died (LD_{50}) using the method of Reed and Muench (23). The standard error was determined by the procedure of Pizzi (23).

RESULTS

Table 1 indicates that rats receiving a sequence of endotoxin injections were significantly more resistant to

TABLE 1. Comparison of endotoxin resistance between endotoxin- and saline-injected rats

Injection type	Survival Rate After Endotoxin Challenge	
	$2 \times LD_{100}$ 40 mg/kg	$1 \times LD_{100}$ 20 mg/kg
Endotoxin	5/5* (100%)	5/5* (100%)
Saline	0/5 (0%)	0/5 (0%)

Endotoxin, *E. coli* (0127:B8). LD_{100} , lethal dose at which 100% of rats died. * Differs significantly from saline-treated rats ($P < 0.05$).

TABLE 2. Comparison of heat stress mortality rate between endotoxin-tolerant and nontolerant rats

Heat Stress Group	n	Maximum Rectal Temp, °C	Total Thermal Area, °C·min	Fasted Weight, g	Percent Mortality
1 T	21	42.23 ±0.12	26.52 ±2.50	410.57† ±38.05	0.0
1 NT	8	42.23 ±0.15	28.48 ±1.75	462.25 ±24.79	12.5
2 T	26	42.34 ±0.20	34.88 ±3.11	421.54† ±31.45	0.0§
2 NT	32	42.49 ±0.17	34.23 ±2.97	456.31 ±25.60	28.1
3 T	13	42.54 ±0.17	44.75 ±2.86	427.46 ±28.44	15.4‡
3 NT	15	42.64 ±0.28	44.87 ±3.19	433.40 ±24.85	66.7
4 T	23	42.69 ±0.28	54.19 ±2.47	426.39 ±34.47	26.1‡
4 NT	23	42.53 ±0.47	54.89 ±2.50	433.43 ±21.87	73.9
5 T	16	42.66 ±0.43	63.77 ±2.61	440.00 ±36.14	50.0
5 NT	19	42.44 ±0.45	64.50 ±2.63	439.84 ±22.71	68.0
6 T	15	42.53 ±0.36	75.73 ±3.02	427.47§ ±21.93	73.3
6 NT	12	42.41 ±0.37	75.05 ±3.39	454.67 ±33.79	91.7

n, No. of rats. T and NT, endotoxin-tolerant and nontolerant rats, respectively. * Values are means ± SD. † P < 0.01. ‡ P < 0.005. § P < 0.02.

endotoxin than those treated with saline alone. All endotoxin-treated rats survived the 40 and 20 mg/kg endotoxin challenge, whereas the saline-treated group experienced a 100% mortality.

Table 2 shows a comparison of mortality rates between endotoxin-tolerant and nontolerant rats subjected to six different heat exposures. In each heat stress group, there were no significant differences in the mean maximum rectal temperature and total thermal area between the tolerant and nontolerant rats. Endotoxin-tolerant rats were found to have a reduced mortality rate in all heat stress groups. Groups 2-4, exposed to a moderate form of heat stress (thermal area <60°C·min), were noted to have a significantly reduced mortality rate, whereas groups 5 and 6 exposed to extreme heat stress (thermal area >60°C·min) did not.

Table 2 also illustrates the weight loss incurred by the rats receiving endotoxin injections. All rats were above 500 g at the start of the injection sequence, but mean fasted weights were <450 g just prior to the heat treatment. Rats receiving saline continued to gain weight during the 3-wk period of injections. This resulted in heat stress groups 1, 2, and 6 with endotoxin-tolerant rats with mean fasted weights significantly less than that for the nontolerant rats. Because both significant and insignificant reductions in heat stress mortality could be found in these heat stress groups, the differences in weight did not appear to affect the outcome of the heat treatment.

A comparison of mortality rate between endotoxin-tolerant and nontolerant rats after analysis by Reed and Muench (23) is illustrated in Fig. 1. The estimate of the heat exposure needed to induce an LD₅₀ in endotoxin-tolerant rats (61.85°C·min) was significantly greater (P < 0.001) than the estimate for nontolerant rats (44.03°C·min).

As shown in Table 3, prior treatment with zymosan significantly (P < 0.005) increased the sensitivity of rats to the toxic effects of endotoxin. But zymosan-treated rats were not found to be any more sensitive to either extreme or moderate heat stress (Table 4). This finding was not due to differences in the heat treatments between

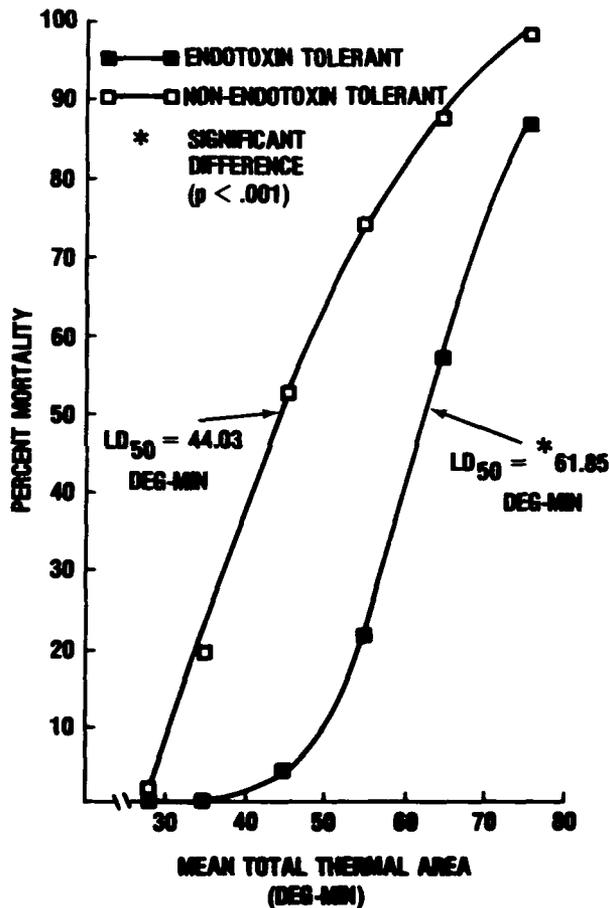


FIG. 1. Comparison of dose-response curves (% mortality vs. mean total thermal area) between endotoxin-tolerant and nontolerant rats. Data calculated by method of Reed and Muench (23).

TABLE 3. Survival rate after endotoxin challenge

Treatment	Survival Rate After Endotoxin Challenge
Zymosan	0%* (0/10)
Saline	80% (8/10)

Endotoxin challenge, 6 mg/kg iv. * Differs significantly from saline-treated rats (P < 0.005)

the zymosan- and saline-treated rats, for there were no significant differences in maximum rectal temperature or thermal areas in any of the heat stress groups studied. There were also no significant differences in body weight between zymosan- or saline-treated rats.

The incidence of invasion of gram-negative bacteria and their endotoxins in nonsurviving rats after experimental heat stress can be found in Table 5. Both moderate and extreme forms of heat stress were examined to elucidate any possible difference in the incidence between those rats dying after short or intermediate time periods. Extreme heat stress resulted in short (2.25 ± 1.48 h), whereas moderate heat stress resulted in intermediate (20.66 ± 8.21 h) survival times. These groups approximated heat treatments in which endotoxin tolerance was associated with either a significant (thermal area $<60^\circ\text{C}\cdot\text{min}$) or an insignificant (thermal area $>60^\circ\text{C}\cdot\text{min}$) reduction in mortality rate after heat stress (see Table 2). Both groups had a significantly increased incidence of gram-negative bacterial invasion in the duodenum, but

this did not result in a significantly increased incidence of LAL activity in the duodenal samples. With the exception of lung tissue from the extreme heat stressed rats, no significantly increased incidence of invasion by gram-negative bacteria or their endotoxins was noted in blood or extraintestinal tissues from either heat-stressed group. Retesting samples for the presence of endotoxins using Pyrotest lysate did not result in significantly different findings.

DISCUSSION

As indicated by the estimated LD_{50} (Fig. 1), endotoxin tolerance was found to induce significant resistance to heat stress mortality. It was noted that in cases of extreme heat stress (groups 5 and 6, Table 2), endotoxin-tolerant rats were not significantly resistant. Though this may just reflect a typical response to increased heat exposure, it might also be indicative of possible differences in the cause of death between rats exposed to moderate and extreme heat stress. An association of endotoxemia with moderate but not extreme heat stress mortality was considered a possible explanation. This seemed likely, because survival time in moderate heat stress (Table 5) appeared to be of sufficient length for the development of endotoxemia. However, an examination for signs of extraintestinal invasion by gram-negative bacteria and their endotoxins did not indicate that endotoxemia resulted after either extreme or moderate heat stress, although duodenal invasion did occur (Table 5).

These findings were confirmed by the retesting of samples with the Pyrotest lysate. This lysate is more sensitive to gut-derived endotoxins, since it will respond to endotoxins of an impure state (21). In the present study, testing for endotoxemia was limited by the low number of blood samples that could be collected. However, on those occasions (long-term survivors) when additional lateral tail vein blood samples could be obtained, positive LAL tests were generally not found. In addition, it has been reported that even when the blood endotoxin concentration is below detectable levels, an antemortem endotoxemia can be determined by the finding of positive LAL tests in liver samples (8). Using both lysates, there were not significant numbers of rats with positive liver

TABLE 4. Comparison of heat stress mortality rate between zymosan- and saline-treated rats

Heat Stress Group	n	Maximum Rectal Temp,* °C	Total Thermal Area,* °C·min	Fasted Weight,* g	Percent Mortality
1 Zym	12	42.53 ±0.19	35.57 ±2.17	480.25 ±12.24	25.0
	Sal	16	42.45 ±0.23	35.85 ±2.32	474.38 ±15.14
2 Zym	14	42.55 ±0.28	43.24 ±3.02	477.57 ±19.33	57.1
	Sal	17	42.64 ±0.12	44.13 ±2.83	470.82 ±8.61
3 Zym	12	42.59 ±0.27	53.14 ±2.73	469.03 ±15.90	91.6
	Sal	11	42.59 ±0.33	55.03 ±1.38	473.70 ±15.89
4 Zym	10	42.70 ±0.23	64.96 ±2.94	478.70 ±22.84	80.0
	Sal	8	42.53 ±0.37	66.40 ±2.14	470.13 ±9.43

* Values are means \pm SD. Zym and Sal, zymosan- and saline-treated rats, respectively.

TABLE 5. Incidence of invasion of gram-negative bacteria and their endotoxins in nonsurviving rats after heat stress

Heat Stress	n	Heat Treatment		Survival Time,* h	Duodenal Invasion ^b	Invasion ^c			
		Maximum rectal temp,* °C	Total thermal area,* °C·min			Lung	Liver	Spleen	Blood
Extreme	12	42.41 \pm 0.14	78.81 \pm 18.85	2.25 \pm 1.48	91.7 ^d 6.6	33.3 ^d 33.3	0.0 8.3	0.0 0.0	14.3 ^e 16.7
Moderate	15	42.26 \pm 0.13	37.92 \pm 5.52	20.66 \pm 8.21	40.0 ^d 33.3	13.3 13.3	0.0 6.7	0.0 0.0	6.7 20.0
Nonheated controls	18				0.0 5.5	0.0 11.1	0.0 5.5	0.0 0.0	0.0 5.5

* Values are means \pm SD. ^b First value represents % with aerobic gram-negative bacterial count $\geq 1.5 \times 10^4$ /g. Second value represents % with *Limulus* amoebocyte lysate (LAL) activity $\geq 1.0 \times 10^3$ ng of *E. coli* endotoxin/g. ^c First value represents % having gram-negative bacterial isolates. This includes both aerobic and anaerobic test results. Second value represents % having positive LAL tests, using Pyrotest lysate. Retesting with Pyrotest lysate did not indicate any significant differences in test results. ^d Differs significantly from control value ($P < 0.05$). ^e Only 7 tested.

assays (Table 3), indicating that the incidence of endotoxemia after heat stress was insignificant.

Though extraintestinal invasion was not found, duodenal invasion was noted after heat stress. This may have been due to multiplication of the low numbers of gram-negative bacteria normally present in this area or to the depositing of lower bowel gram-negative bacteria into the upper bowel. Isolates in the lungs, from rats subjected to extreme heat stress, may reflect the rat's coprophagic nature and represent spread of gram-negative bacteria from the oral cavity into the lung. It would appear that any endotoxemia resulting from this lung invasion was of little importance, because endotoxin tolerance did not significantly improve survival after extreme heat stress.

Why heat stress-induced duodenal invasion did not result in extraintestinal invasion as previously noted in other forms of stress (16, 17) is not known. Perhaps the level of duodenal invasion was not sufficiently great enough to result in extraintestinal invasion or the rats died before extraintestinal invasion could occur. Thus these findings may just reflect differences in the pathophysiology of different forms of stress.

These findings also do not explain why heat-stressed dogs pretreated to reduce gut flora experience a significantly increased incidence of 18-h survival (4). This may be due to species differences and reflect the rat's natural resistance to endotoxin. However, Grun et al.'s (12) study of the role of endotoxins in galactosamine-hepatitis has established that the rat and the LAL test are suitable for studies of endotoxin invasion originating from the gut (12). Because thermal area was not determined in the dog study (4), it is possible that differences in the incidence of 18-h survival are due to differences in the heat treatment experienced by the pretreated and nontreated dog groups. Also, the pretreatment regime itself may be the important factor in this phenomenon and not actually the reduction in gut flora.

The fact that zymosan treatment did not increase the sensitivity of rats to experimental heat stress, although these rats were significantly more sensitive to endotoxin (Table 3), appeared to substantiate the *Limulus* findings (Table 5). Thus the effect of increased survival associated

with endotoxin tolerance may be due to factors other than protection against endotoxemia.

Endotoxin tolerance is known to induce resistance to a variety of stresses, such as epinephrine shock (15), hemorrhagic shock (15), traumatic shock (1), acceleration stress (19), and oxygen toxicity (9). In the study of shock, there is evidence to indicate that the resistance mediated by endotoxin tolerance is not due to protection from circulating endotoxins originating from the intestinal tract (2, 3, 15, 24). Thus some broad effect, not necessarily associated with endotoxemia protection, may explain the resistance to shock induced by a state of endotoxin tolerance. A similar effect may explain the noted resistance of endotoxin-tolerant rats to experimental heat stress.

To summarize, endotoxin tolerance was found to induce significant resistance to heat stress. The data did not indicate that this resistance was due to protection from gut-derived bacterial endotoxins released as a result of the heat stress. Perhaps endotoxin tolerance resulted in increased resistance to heat stress mortality due to its ability to protect from shocklike syndromes. Thus differences in the protective effects of endotoxin tolerance between moderate and extreme forms of heat stress may indicate that moderate heat stress mortality was associated with heat shock, whereas extreme heat stress mortality resulted from heatstroke involving the central nervous system. Therefore, endotoxin tolerance may adequately protect from heat shock death but not heatstroke.

These findings are of importance for several reasons. First, they provide additional evidence that gut-derived bacterial endotoxins may not be responsible for the shock state noted in stressed animals. Second, they indicate that, as in other forms of shock, a state of endotoxin tolerance can offer significant protection from heat shock mortality.

We acknowledge and appreciate the technical support of Roger Benson and Cecilia Helinski. We also thank Joann Deluca, Pat Basinger, and Julie Cyphers for their excellent typing of the manuscript.

Received 19 January 1982; accepted in final form 15 July 1982.

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