Br. J. exp. Path. (1978) **59**, 76

EFFECTS OF FEVER ON HOST DEFENCE MECHANISMS AFTER INFECTION IN THE LIZARD *DIPSOSAURUS DORSALIS*

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Received for publication October 3, 1977

Summary.—Fever has never been proven beneficial in mammals, although it enhances survival in the lizard *D. dorsalis* infected with *Aeromonas hydrophila*. We examined the course of the infection and the function of host defence in febrile (41°) and afebrile (35° or 38°) animals using this model. Infected, febrile lizards had sterile blood cultures, and 1–2 logs fewer bacteria in body tissues 6–12 h after infection. Granulocytes appeared early and in large numbers at the site of inoculation in febrile, but not afebrile, animals. We were unable to demonstrate effects of this small range of temperatures on *in vitro* growth rates of bacteria, on lizard granulocyte chemotactic or phagocytic functions, or upon serum antibody levels. Our results suggest that fever enhances some aspect of the early inflammatory response, leading to increased leucocyte emigration at the local site and containment of the infection.

ALTHOUGH fever occurs regularly as a response to infection, its value in host defence has never been satisfactorily established in mammals. Some heat-sensitive microorganisms that produce disease, such as those causing syphilis and gonorrhoea, can be killed *in vivo* by artificially induced fever, but natural infections with these organisms almost never produce high fevers (Bennett and Nicastri, 1960). In rabbits (Atwood and Kass, 1964), rats (Porter and Kass, 1962) and mice (Connor and Kass, 1961) there is a decrease in the LD_{50} of endotoxin when these animals are made hyperthermic by physical or pharmacological means. However, data from these experiments do not show whether naturally acquired fevers benefit the host, particularly during infectious states. In experimental infections, fever can be suppressed by antipyretic drugs or by low ambient temperatures. However, these measures are known to alter other aspects of host resistance besides body temperature.

Recent studies have shown that lower vertebrates such as lizards (Vaughn, Bernheim and Kluger, 1974), amphibians (Kluger *et al.*, 1977) and fish (Reynolds, Casterlin and Covert, 1976) normally develop fever after infection. Unlike mammals, these animals lack the capacity to conserve or generate body heat by intrinsic mechanisms. Instead, they regulate body temperature, and also produce fever after infection, by seeking appropriate environmental temperatures. Prevention of fever in lizards can thus be accomplished simply by altering the external temperature available to the animals. Using this model, Kluger, Ringler and Anver (1975) recently demonstrated the survival value of fever in the desert iguana *Dipsosaurus dorsalis*.

Animals infected with *Aeromonas hydrophila*, a natural Gram-negative pathogen for lizards, were placed at environmental temperatures between 35° and 42°. Almost all animals allowed to develop the normally occuring fever of 40° or 42° survived, whereas most infected animals prevented from developing a fever died. It seems possible that the reason fever benefits infected lizards may be related to as yet unknown beneficial effects of fever in mammals. We therefore studied the

development of infection and the function of various host defences in febrile and afebrile lizards.

MATERIALS AND METHODS

Animals.—Lizards (*Dipsosaurus dorsalis*) weighing 25–50 g (East Bay Vivarium, Oakland, California) were housed at an ambient temperature of 22–24° in circular cages and fed mealworms, lettuce, and water *ad libitum*. The cages were kept on a 12 h light and 12 h dark photoperiod (12L : 12D) and had a small area of sand heated to a temperature of over 50° by a 250 W heat lamp that was also on the 12L : 12D cycle.

Bacteria.—*Aeromonas hydrophila* was grown in nutrient broth for 16–18 h. The bacteria were then centrifuged at 1800 *g* for 30 min and washed twice with saline. Concentrations of bacteria were adjusted by measuring optical density at 600 nm on a spectrophotometer (Spectronic 20-Bausch and Lomb) and compared with a previously constructed standard curve.

For estimation of the growth rate of *A. hydrophila*, 6 identical flasks were prepared containing 3×10^4 bacteria from an 18-h broth culture in 50 ml of nutrient broth. Two flasks each were incubated at 35°, 38°, and 41° for 6 h. One-ml samples were taken from each flask at 0 h and at 1-h intervals thereafter. Agar pour plates were prepared, after dilution in saline as required, and numbers of bacteria/ml of broth were calculated from colony counts.

Studies of infected lizards.—Six to nine desert iguanas were injected into the subcutaneous dorsal lymphatic space with 1×10^8 live *A. hydrophila* organisms. Groups of 2–3 animals were then placed in incubators at 35°, 38°, or 41° for 6–18 h. Just before killing, heart blood was obtained by percutaneous puncture of the thorax, and 20 μl transferred to 1-ml samples of sterile thioglycollate broth. 0·5 ml as well as 1 ml of a 1 : 100 dilution of this solution was plated using agar pour plates and subsequently incubated. The animals were then killed by decapitation, blood was obtained from head and neck, centrifuged in plastic microfuge tubes (Beckman Instruments, Inc. Mountainside, N.J.), serum removed and stored at −20°. Samples of lung, liver, and dorsal skin at the site of injection were then removed, and a portion was fixed in Helly's solution (Askenase, Haynes and Hayden, 1976) for histolocial examination. The rest of the samples were weighed, minced, and liver and lung tissue homogenized in 2 ml of saline using a Tri-R Stir-R homogenizer with glass tubes and pestles. Skin samples were ground in 2 ml of saline using porcelain mortars and pestles. Supernatants from tissue samples were diluted serially 10–100-fold as required in saline, and agar pour plates prepared and incubated for 24 h. Numbers of bacteria per mg of tissue were calculated from colony counts. Duplicate plates were prepared in most experiments for estimation of bacterial content of tissues. Similar techniques were used to sample bacterial contents of liver, lung, and skin of an uninfected saline-injected lizard. All counts were sterile, except for those from skin, which contained $<10^3$ colonies/mg. Therefore, bacterial colonies in pour plates were assumed to be *A. hydrophila* on the basis of colonial morphology and occasional Gram-stain determinations.

In vitro *studies of chemotaxis and phagocytosis of lizard white cells.*—Lizards were injected into the coelomic cavity with 15 ml of a 1 : 2 dilution in saline of filtered broth from 18-h cultures of *A. hydrophila* after addition of 100 u penicillin and 100 μg/streptomycin/ml, and kept at 37° overnight. After killing, coelomic fluid containing cells was aspirated with a sterile Pasteur pipette, and additional washings made using sterile pyrogen-free saline with 10 u heparin/ml. The suspension was centrifuged at 900 *g* at 4° for 15 min, washed twice and resuspended in saline, cultured, numbers of cells determined in a haemocytometer, and a differential count made from cover-slip smears stained with Giemsa stain. The average yield of leucocytes/lizard was $15–20 \times 10^6$; about 80% were granulocytes.

For chemotaxis experiments, $2–3 \times 10^6$ leucocytes were placed on one side of a modified Boyden chamber which contained a 1·2-μm millipore filter. A 1 : 4 dilution in saline of filtered broth of *A. hydrophila* culture, a positive chemotactic stimulus, or Gey's solution, the control buffer, was added to the other side. The chambers were then incubated in a moist atmosphere at 37° or 41°. After 1 or 2 h, the chambers were removed, and the filters fixed and processed by techniques described by others (Zigmond and Hirsch, 1973). The measure of leucocyte migration was the distance the leading edge of cells progressed into the membrane (Zigmond and Hirsch, 1973). Duplicate chambers were used in all experiments. For the phagocytosis experiments, leucocytes were suspended in 3 ml Krebs–Ringer phosphate buffer containing 2% autologous serum, and live *A. hydrophila* added at ratios varying from 4 to 12 bacteria per leucocyte. Control flasks contained serum, buffer, and bacteria but no cells. The flasks were then incubated with shaking (120 oscillations/min) in Dubnoff incubators at 35° or 41° for 2 h. Samples of 0·1–0·2 ml were taken at ½, 1 and 2 h, diluted in iced saline, and estimates of live supernatant and cell-associated bacteria were made by techniques previously reported (Malawista and Bodel, 1967).

Antibody determinations.—Agglutinating antibody titres were measured by the following technique: 0·1-ml samples of serial two-fold dilutions of serum collected from one or several lizards were mixed on a slide with approximately

9×10^6 heat-killed *A. hydrophila* suspended in 0·05 ml of saline. Suspensions were mixed by rocking, then incubated in a moist Petri dish for 3 h at 37°. The degree of agglutination was scored by microscopic examination of bacterial clumping, visible at 450× magnification. A score of 1+ was used as the end-point.

White blood cell counts.—Lizards which had been infected by techniques detailed above and uninfected lizards were placed in incubators at 37° or 41° for 12 h. Blood obtained after decapitation was drawn into a white or red cell pipette and diluted at once with either filtered Giemsa stain diluted 1 : 2 in saline (for white cell counts) or 7% solution of methylene blue diluted in 100% ethanol (for red cell counts). Cells were counted in a haemocytometer.

Studies of cutaneous inflammation to non-infectious agents.—The dorsal lymphatic space and one hind paw of lizards were injected with 0·1 ml phosphate-buffered saline containing 20 mg of phytohaemagglutinin (PHA-P, Difco, Detroit, Michigan), while the other hind paw was painted with a solution of 50% croton oil (Fisher Scientific Co., Pitts., Pa.) made in 4 : 1 acetone–olive oil. After groups of lizards had been kept at 35° or 41° for 24 h animals were then killed, and skin test sites were excised for histological examination. Micrometer measurements in 0·00125-in units were taken of the hind paws of each lizard, and results compared with measurements made at the beginning of the experiment, before injection or painting.

Detection of heat-sensitive exotoxins from A. hydrophila.—Coelomic leucocytes were obtained from lizards by techniques described above except that the filtered broth was autoclaved before coelomic infusion. A separate overnight culture of *A. hydrophila* was centrifuged, bacteria were resuspended in saline, and their numbers estimated using the spectrophotometer (see above). The broth from this culture was then filtered through a 0·20-μm Millipore filter and half was autoclaved for 20 min. Saline dilutions of both normal and autoclaved broth were then made so that individual tubes received 2 ml of either normal or autoclaved broth potentially containing exotoxins from either 1×10^9 or 1×10^7 bacteria. Four $\times 10^6$ white cells (98% viability) were then added to each tube along with penicillin 100 u, streptomycin 100 μg, and heparin 10 u/ml. The tubes were then incubated at 22°, 35°, or 41° for 6 h. All samples were run in duplicate. At the end of incubation leucocyte viability of each tube was assayed using the criterion of exclusion of 1% neutral red dye.

RESULTS

Bacterial counts in infected lizards

Blood cultures were obtained from infected lizards maintained at 35°, 38°, or 41° for 12 h after infection. The results of 3 such experiments are shown in Table I. Only one out of 12 lizards kept at 41° had a positive blood culture, while all the lizards kept at either 38° or 35° had positive blood cultures, usually with 10^5–10^6 organisms/ml. In a single experiment in which animals were killed 6 h after infection, the results obtained were similar; blood samples from 4 febrile animals (41°) were sterile, while those from 3 afebrile animals (35°) were positive.

TABLE I.—*Blood Cultures from Lizards 12 h after Inoculation with* A. hydrophila

Lizard temperature	Positive cultures/ total (12 h)	Colonies/ml blood
41°	1/12	$0 \cdot 3 \pm 0 \cdot 2$*
38°	8/8	$1 \cdot 4 \times 10^6 \pm 1 \cdot 0 \times 10^6$
35°	10/10	$5 \cdot 4 \times 10^5 \pm 0 \cdot 8 \times 10^5$

* s.e. mean

We next examined the numbers of live bacteria present in various body tissues of infected animals maintained at these different temperatures, and killed 12 h after infection. The results of these studies are shown in Fig. 1. Significant differences in bacterial counts of lung, liver, and skin at the site of injection are apparent. Tissues from lizards kept at 35° or 38° had bacterial counts that were 1–2 logs higher than those taken from lizards kept at 41°. It is noteworthy that animals kept at 41° had sterile blood cultures (see Table I), but contained live bacteria in liver and lung tissue.

It was possible that the differences in numbers of live bacteria present in blood and tissues of animals maintained at different temperatures resulted from different growth rates of the organism under the different conditions. We therefore examined growth rates *in vitro* of *A. hydrophila* at temperatures of 35°, 38°, and 41° during a 6-h incubation period. The results of a representative experiment are shown in Fig. 2. Our findings confirm observations reported by others (Kluger *et al.*, 1975; Reynolds *et al.*, 1976) that no

significant differences could be detected in *in vitro* growth rates of the organism at these temperatures.

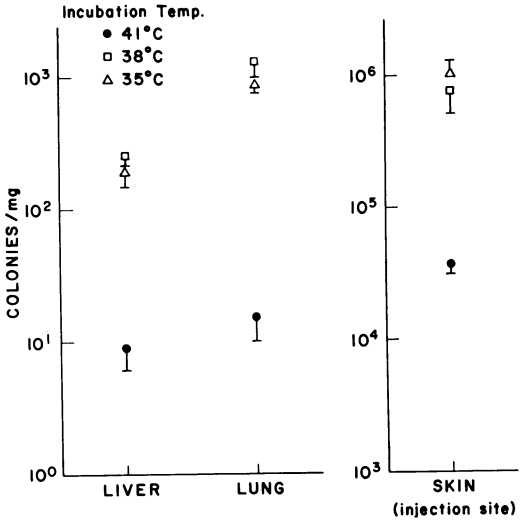

FIG. 1.—Bacterial colonies per mg tissue of liver, lung, and skin at the site of inoculation of lizards with *A. hydrophila*. Different symbols denote incubation temperature of lizards during 12 h after infection and before killing. Average results ± s.e. mean of 6–9 lizards in each group are shown.

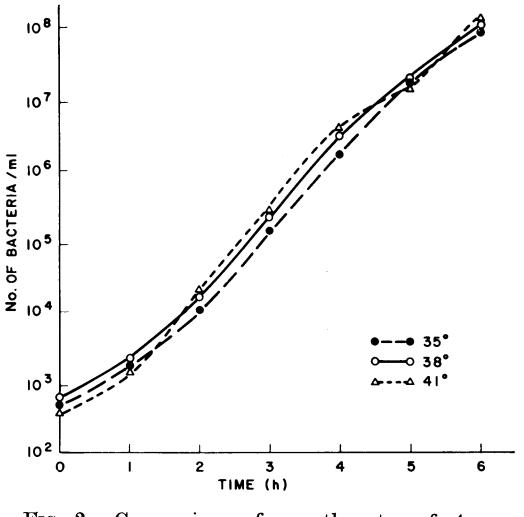

FIG. 2.—Comparison of growth rates of *A. hydrophila* in broth cultures kept at 35°, 38° or 41° for 6 h. Numbers of bacteria/ml were estimated using pour plate technique. Points represent average results of duplicate samples.

Histology of the injected site

Since it seemed likely that host factors were responsible for the lower numbers of bacteria in tissues of febrile animals, we next examined the histology of the injection sites 6 h after infection. Fig. 3 shows representative low-power views of two skin injection sites, one obtained from a febrile (41°) animal (3a), and one from an afebrile (35°) animal (3b). Striking differences in the cellular responses are apparent. There are almost no leucocytes in the subcutaneous tissue in Fig. 3b, whereas many white cells are present in Fig. 3a (see arrows). Not visible in Fig. 3b, but apparent under microscopic examination of the original section, are numerous clumps of bacteria in the tissue, whereas rare free bacteria but numerous leucocyte-associated bacteria are present in the tissue depicted in Fig. 3a. These differences in local infiltration of leucocytes and numbers of free bacteria were consistently observed in all experiments, and were seen in sections taken both 6 and 12 h after infection. In addition, in tissues from febrile animals, leucocytes were frequently clustered at the margin of blood vessels, whereas tissues from the lizards kept at lower temperatures showed dilated vessels containing many erythrocytes, but apparently few marginating granulocytes.

These differences in numbers of leucocytes observed at the site of inoculation suggested differences in rates of mobilization of leucocytes from blood to tissue site. This, in turn, might be a consequence of changes in numbers of intravascular leucocytes. We therefore examined both red and white cell blood counts in representative animals. Infected lizards kept at 41° (n=10) had blood counts (RBC= 730,000 WBC=5700) which were nearly the same as those of lizards kept at 35° (n=8) (RBC=760,000, WBC=5500). Also, control uninfected animals which were kept at either 37° or 41° had counts essentially identical to those of infected animals. Thus, we could observe no differences in numbers of leucocytes available for mobilization from blood as a result

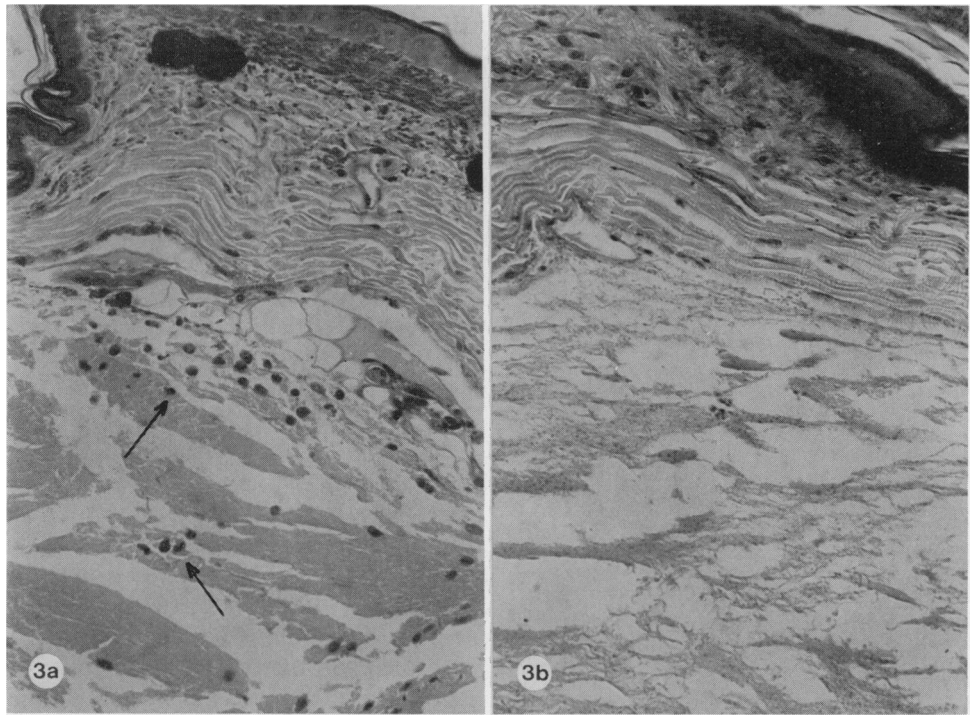

FIG. 3.—Sections of skin infection sites 6 h after inoculation with *A. hydrophila* from a lizard maintained at 41° (3a) and 35° (3b). Tissues were fixed in Helly's solution and stained with haematoxylin and eosin. Arrows point to lizard granulocytes. Final magnification ×480.

of either on-going infection or environmental temperature.

Since numbers of circulating white cells were the same in infected lizards kept at 41° and 35°, we next tested the possibility that febrile temperatures may increase *in vivo* chemotaxis and diapedesis of leucocytes to various stimuli. We incited nonspecific cutaneous inflammatory responses, in one case anticipating infiltration by mononuclear cells as a result of painting lizard foot pads or injecting with PHA, or in the other case a response rich in granulocytes by painting the foot pads with croton oil. The paws which were painted with PHA showed an increase in thickness of 15% or 10% at 4 and 24 h respectively, while the paws painted with croton oil showed an increase in thickness of only 6% and 1%. All of these responses, however, were the same in animals kept at 41° and 35°. Histological examination of the area of skin injected with PHA from lizards kept at the different temperatures showed no difference in the degree of leucocyte infiltration after 24 h. Croton oil produced only a very mild inflammatory response in these animals, with minimal leucocyte infiltration in all cases.

In Vitro *studies*

In order to determine the effects of temperature on leucocyte function *in vitro*, we examined chemotaxis and phagocytosis by lizard exudate granulocytes, at 35°, 37°, or 41°. In the chemotactic experiments, cells exposed only to a buffer solution in the Boyden chambers moved about 40 μm over a 2-h period, and those exposed to filtered broth which contained chemotactic factors moved about 100 μm (see Fig. 4). There was no difference between cells maintained at 37° compared to 41°. Therefore, we observed no effect of this small range in temperature on either

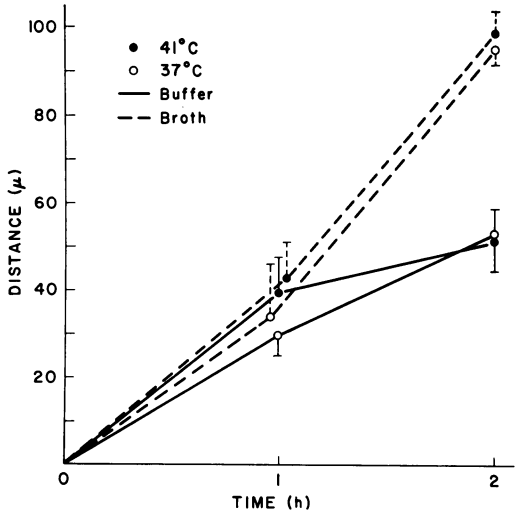

FIG. 4.—Distance moved by lizard exudate leucocytes into a 1·2 μm pore-size filter in response to Gey's buffer (——) or a dilution of filtered broth from a culture of *A. hydrophila* (– –). Symbols indicate different temperatures at which cells were incubated. Average results of two experiments are shown in which filters from duplicate chambers were analysed using 10 different microscopic (490×) fields in each case.

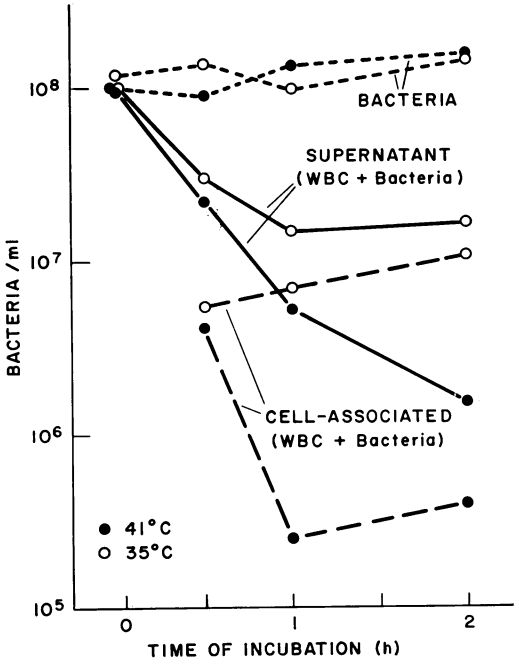

FIG. 5.—Phagocytosis of *A. hydrophila in vitro* by lizard exudate leucocytes, incubated at 35° or 41°. Solid lines show numbers of bacteria/ml in supernatant of flasks containing bacteria and leucocytes, and dotted lines show numbers of cell-associated bacteria in the same flask. Number of bacteria incubated alone are shown at the top. Average results of duplicate determinations from one representative experiment are given.

random migration or chemotaxis of lizard granulocytes *in vitro*.

In vitro phagocytosis by coelomic leucocytes was found to be slightly influenced by temperature (Fig. 5). After a 2-h incubation, supernatants of flasks containing leucocytes and bacteria incubated at 41° had 1 log less of live bacteria than did those of flasks incubated at 35°, indicating that phagocytosis and killing had occurred somewhat more rapidly at the higher temperature. However, the difference in terms of absolute numbers of bacteria removed is small, since over 90% of the organisms were removed under both conditions. Furthermore, when we reduced the multiplicity of bacteria: leucocytes (not shown) we could not detect any significant differences. The higher number of cell-associated live bacteria in the flasks incubated at 35° (Fig. 5) may be due to intra- or extra-cellular organisms, since our initial attempts failed to establish the actual location of the bacteria. Numbers of bacteria, incubated alone in control flasks, did not change significantly over the 2-h period.

Antibody levels

We also considered the possibility that in afebrile lizards there was a failure of a temperature dependent, early (<6–12-h) immune response. We therefore measured the levels of agglutinating antibody to *A. hydrophila* in sera taken from lizards 12 h after infection. As shown in Table II, all sera of infected lizards, regardless of the temperature at which they had been maintained, had titres of either 1 : 64 or 1 : 128. These titres were entirely similar to the titres present in normal uninfected lizards, and presumably represent previ-

TABLE II.—*Agglutinating antibody titres against* A. hydrophila *of sera from normal lizards or infected lizards maintained at 35°, 38° or 41°*

	Agglutinating antibody titres	
Sera	64	128
Normal lizards	+	+
Infected lizards		
35°	+	+
	+	
38°	+	+
	+	
41°		+
		+
		+
		+

ously acquired specific or cross-reacting antibody to this organism.

Exotoxin effects on leucocytes

Since differences in numbers of tissue leucocytes seen in afebrile and febrile infected lizards might be due to the presence of heat-sensitive exotoxins produced by *A. hydrophila* (Wadström, Ljungh and Wretland, 1976; Scholz, Schormann and Blobel, 1974), we set up an *in vitro* experiment to detect such toxins which might damage lizard leucocytes (see Methods). The results indicated that all leucocytes had some decrease in viability when incubated for 6 h with unheated broth derived from 1×10^9 organisms (23% mortality for leucocytes incubated in normal broth compared to 5% mortality for cells incubated in autoclaved broth). However, the effects of the normal broth were no different when the tubes were incubated at 22°, 35°, or 41°. When we used 100-fold lower dilutions of the broths, no significant leucocidal activity was apparent at any temperature.

DISCUSSION

Fever occurs naturally in infected desert iguanas (*Dipsosaurus dorsalis*). When the lizards are prevented from achieving febrile temperatures, mortality from infection with *A. hydrophila* is markedly increased (Kluger *et al.*, 1975; Bernheim and Kluger, 1976). No studies have previously examined the reasons why fever is beneficial in this model. The present studies were therefore undertaken to examine in some detail the course of the infection and the function of host defences in infected febrile and afebrile lizards.

We observed marked differences in the manner in which the infection spread, depending on the temperature at which the lizards were kept. Febrile lizards had sterile blood cultures as early as 6 h after inoculation of bacteria into the dorsal lymphatic space whereas afebrile lizards had bacteraemia both 6 and 12 h afterwards. This continuing bacteraemia presumably explains the significantly higher bacterial counts which were present in the tissues of the animals kept at lower temperatures. Since some bacteria were present 12 h after infection in tissues of the febrile but abacteraemic animals (unlike control, uninfected lizards), it appears that an early spread of bacteria from the dorsal lymphatic space occurred in all animals. In the febrile lizards, however, the infection was apparently rapidly contained, so that subsequent bacteraemia was prevented, and numbers of bacteria at the site of inoculation slowly decreased.

The containment of the infection in febrile but not afebrile animals appears to be related to the fact that granulocytes appeared early and in large numbers at the site of inoculation in animals kept at 41°, but were almost entirely absent at 6 h in lizards kept at lower temperatures. Thus there seems to be some alteration in the ability of afebrile lizards to mobilize leucocytes to an infected site. When we examined the effect of temperature on leucocyte mobilization in response to non-infectious inflammatory stimuli (PHA and croton oil) we did not note any differences. This could be due to the time at which the tissues were examined, since in order to allow time for full development of the reaction, samples were not taken until 24 h after challenge with these materials. Also, an infectious agent such as *A. hydrophila* may produce stronger chemotactic factors than PHA or croton

oil at the injection site and could magnify early differences in leucocyte infiltration at different temperatures.

The striking difference in the early cellular response between febrile and afebrile animals infected with *A. hydrophila* was not satisfactorily explained by a difference in circulating white cell counts at 37° or 41°, nor by differences in lizard granulocyte function in two *in vitro* tests. We found no effect of different temperatures (35°, 37°, and 41°) on *in vitro* chemotaxis to filtrates of the infecting organism, and only minimal effects on phagocytosis. We also found no differences in serum antibody levels in febrile or afebrile lizards. Since various heat-labile toxins are elaborated by *Aeromonas* species (Scholz *et al.*, 1976; Wadström *et al.*, 1976), we tested leucocidal activity of broth culture medium, and at the range of temperature we studied we could detect no temperature-dependent toxicity. We did not measure blood flow in our experimental animals. However, since these lizards are poikilothermic and regulate their body temperature chiefly by behavioural and not physiological means (De Witt, 1967; Norris, 1953), there is no reason to suppose that skin blood flow is altered selectively as a result of lowered temperature. It is possible that changes in cardiac output may occur between 35° and 41° (Bartholomew, Tucker and Lee, 1965) and that they alter the ability of leucocytes to enter inflammatory sites. Further studies are needed to examine this possibility.

It has recently been demonstrated in mice (Gershon, Askenase and Gershon, 1975) that migration of inflammatory cells to tissue sites of delayed hypersensitivity *in vivo* probably depends on both chemotactic agents and on alterations in the local vasculature caused by vasoactive substances. In this form of inflammatory response, therefore, chemotactic factors are considered necessary but not sufficient for the diapedesis of leucocytes. This may also pertain to the cellular response to infection in lizards. Thus, the ability of leucocytes to cross endothelial cells may be impaired in afebrile, infected lizards either because of lack of production of chemotactic factors, or by a failure of the host to produce vasoactive substances. To our knowledge, the relation of small changes in body temperature to the generation of chemotactic factors or the release of vasoactive substances *in vivo* has not yet been investigated in any model.

There are certain significant similarities in the immune systems of mammals and reptiles. Lizards demonstrate transplantation immunity (Borysenko, 1970), produce immunoglobulins, and exhibit anamnestic humoral immune responses to various antigens (Wetherall and Turner, 1972; Kanakambika and Muthukkaruppan, 1972). The classification of lizard blood cells has been attempted (Efrati, Nir and Yoari, 1970) and, although there is disagreement on how many types of granulocytes exist, it is clear that lizards have both circulating granulocytes and mononuclear cells which respond to chemotactic stimuli, and phagocytize and kill bacteria *in situ*. Because of these similarities in the immune and leucocyte host defence systems of reptiles and mammals, it seems likely that both groups of animals share some basic defence responses to infections. If so, it seems possible from our observations that fever benefits mammals in certain situations, as it does in lizards, by facilitating the early inflammatory response in an infected host.

We thank Karen Wenc and Betty Hayden for technical assistance.

This work was supported by grants from the National Institutes of Health AI-01564, AI-12211, AI-70829, CA-14655 and CA-07019.

P.W.A. is an Allergic Disease Academic Awardee.

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