VIRULENCE AS A FACTOR IN SALMONELLA INFECTION IN MICE MAINTAINED IN THE COLD

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(Prepared under Contract AF 41(657)-340 by
G.J. Miraglia and L. J. Berry
Dept. of Biology, Bryn Mawr College
Bryn Mawr, Pennsylvania)

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1. Salmonella
2. Staphylococcus
3. *Infections*
4. Exposure
5. Mice
6. Dosage
7. Iron Compounds - Proferrin

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ABSTRACT

The object of this study was to determine possible differences in the course of salmonellosis in mice maintained at 25° C and others kept at 5° C, and to uncover, if possible, mechanisms responsible for such differences. The LD_{50} dose for mice of Salmonella typhimurium, strain RIA, is 4.6×10^5 for animals individually housed without bedding and maintained at 25° C. It is 3.8×10^3 for animals similarly housed but kept at 5° C. An intravenous injection of 0.1 ml of saccharated iron oxide (Proferrin) two hours prior to infection lowers the LD_{50} to 4.9×10^3 and 4.0×10^1 for mice kept respectively at 25° C and at 5° C. Low environmental temperature and "blockage" of the reticuloendothelial system (RES) lower the resistance of mice to about the same degree, but low temperature and RES impairment together lower resistance as if each is acting independently. Doubling the volume of Proferrin more than doubles the change in susceptibility to infection manifested by the mice but this amount seems to be toxic for mice. Even more important is the incidence of staphylococci found in liver or kidney of mice infected with S. typhimurium and kept at 5° C. Cultures were made on animals that survived infection for a period of 14 days and, except for the largest challenge doses where only a few animals remained, the incidence of staphylococci was proportional to the number of salmonellae injected. At 25° C only a small percentage of mice have staphylococci in tissues and these occur independent of the infectious dose of salmonellae.

PUBLICATION REVIEW

Horace F. Drury

HORACE F. DRURY
Director of Research

VIRULENCE AS A FACTOR IN SALMONELLA INFECTION IN MICE MAINTAINED IN THE COLD

SECTION I. INTRODUCTION

Although the literature concerning the physiological effects of low environmental temperatures on various hosts is voluminous, including several recent reviews (Hemingway, 1945; Hardy, 1950, 1961), reports dealing with the effect of cold on the course of bacterial infections are relatively meager.

Nearly a century ago Pasteur reported that the resistance of chickens to anthrax was due to their high body temperature. He found that by lowering their body temperature they did indeed become lethally susceptible. More recently, Junge and Rosenthal (1948) studied the effect of environmental temperature on survival of mice infected with pneumococci and reported an increase in host susceptibility when the temperature was decreased to 18° C. However, it was necessary to treat the mice with sulfadiazine immediately following infection to insure survival of a sufficient number for study.

Muschenheim and collaborators (1943) studied the effect of hypothermia on the resistance to experimental pneumococcal infection in rabbits. It was found that when a highly virulent strain was employed all animals died irrespective of environmental temperature and the only demonstrable effect of cold was a decrease in the local inflammatory reaction. However, when an avirulent strain was used, the induced hypothermia resulted in bacteremia and death in addition to the inhibition of the dermal inflammatory reaction.

The interaction of certain viruses and a variety of hosts as influenced by environmental temperature has received considerable attention in recent years. The incisive investigations of Boring et al (1956) are particularly germane to this report since they allowed within certain limits comparison between viral and bacterial infections in hosts maintained in the cold.

Using a coxsackie virus and the mouse as an experimental model, these investigators found that cold had an adverse effect on the host. Mice were housed 8 to 12 per cage at 4° C without restriction on huddling. Mice in the cold had a viremia through the fourth postinfection day, while the blood was clear of virus by that time in mice at 25° C. The titer of virus in the liver was higher on the fourth day in mice at 4° C than in mice at 25° C.

These experiments indicate that although adult mice possess a natural resistance to the coxsackie virus such that the disease is limited to a

nonfatal infection, this resistance is lost when animals are maintained in the cold. Under these conditions a lethal infection ensues which is characterized by a persisting viremia, high levels of virus in the liver, and lesions demonstrable in other organs.

The mechanism by which cold reduces resistance to this virus is unknown, although the fact that cortisone causes a similar loss of resistance suggests that cold may act through its capacity as a stress agent to cause excessive secretion of adrenocortical steroids.

In a subsequent report Walker and Boring (1958) observed that neutralizing antibody appeared on the fourth day in mice at room temperature but failed to appear in animals in the cold.

Schmidt and Rasmussen (1960) reported that mice maintained at 37° C were more resistant to infection with herpes simplex virus than those held at 25° C. It was concluded that this sparing action was due to the lower viral population in brain tissue at the higher temperature. The mechanism responsible for this decrease in the number of viruses is unknown, but it is thought that a possible explanation for the differences in mortality rates is an alteration of viral multiplication due to an indirect action on the metabolism of host tissue. It has been well established that viral populations can be controlled to some extent by altering host-cell metabolism by means of temperature changes. This subject has been reviewed recently by Lwoff (1959).

The object of this study was to determine possible differences in the course of salmonellosis in mice maintained at 25° C and others kept at 5° C, and to uncover, if possible, mechanisms responsible for such differences. It was not the intention, however, to employ hypothermic mice since the lower environmental temperature to which animals were subjected failed to depress the core temperature below normal limits. Nevertheless, moribund animals in the cold become markedly hypothermic, but similar findings are noted during the last moments of life in mice infected with salmonellae and held continuously at room temperature.

Data to be reported in this study demonstrate an alteration in host susceptibility to salmonella infection as a response to exposure to a low ambient temperature. In addition, and perhaps of even greater importance, is the observation that a second invading organism, presumably from the environment or from the mouse itself, may prey on a host already stressed by cold and a single experimental infection. It has long been believed that primary infections predispose the host to a secondary invasion but, heretofore, experimental evidence for this has not been clear. As far as is known, this is the first description of an experimental situation which permits consistent prediction of the incidence of secondary infection.

SECTION 2. SUMMARY

Proferrin decreased the LD_{50} for mice infected with S. typhimurium, strain RIA, 100-fold at both 25° C and 5° C.

RIA could be isolated from the livers of infected animals with greater frequency and persisted longer in mice maintained at 5° C than those kept at 25° C.

Staphylococci were isolated from livers in 23.3 per cent of "normal" (nonmanipulated) mice kept 14 days in the cold. Staphylococci could not be isolated from a similar group of mice at 25° C.

The percentage of livers positive for staphylococci increased in infected animals kept in the cold to a greater extent than those kept at room temperature. (This was noted in mice infected with both virulent and avirulent salmonellae respectively.) This increase in the number of staphylococci-positive livers was considerably greater than that found in "normal" mice kept in the cold.

SECTION 3. METHODS

Animals. CF_1 female mice (Carworth Farms) weighing 20 to 22 gm were used in all experiments. They were housed in plexiglass cages which were divided into 10 equal compartments per cage. Each compartment measured 3.5 X 7.5 X 9.0 cm. No bedding was used, and mice were housed individually to prevent huddling. The open tops and bottoms of the plexiglass enclosures were covered with 3/8" mesh hardware cloth. All completely assembled cages were placed on wire mesh to keep the animals free from excessive moisture and excreta. Water and Dietrich and Gambrill pathogen-free mouse food were available at all times.

Animal Rooms. Two animal rooms were used. One was maintained at 25° ± 2° C, the other at 5° ± 1° C. An automatic lighting system provided 12 hours of light for each 24-hour period. The mice were kept in the appropriate room continuously for the entire experimental period. Humidity was not controlled but results were reproducible at different seasons of the year when humidity is known to vary.

Inoculum. Two strains of Salmonella typhimurium were used, the highly virulent SR-11 and the relatively avirulent RIA. (The latter was generously provided by Dr. Howard Schneider, Rockefeller Institute,

New York City.) Both strains were cultivated in brain-heart infusion broth (Difco) for 16 hours at 37° C. Decimal dilutions of this culture were plated in triplicate on both nutrient and SS agar plates (Difco) to enumerate the colonies and to insure the uniformity of the culture. Inoculations consisting of the appropriate number of organisms contained in 0.5 ml of saline were administered by the intraperitoneal route.

Organ Culture Technique. Organs selected for bacterial culture were excised from mice immediately after death, observing aseptic technique. Samples were cultured on appropriate media for identification of microflora, using the organ print method.

Miscellaneous. The reticuloendothelial system (RES) of mice was "blocked" by the intravenous injection, via the tail vein, of 0.1 ml saccharated iron oxide (Proferrin of Merck, Sharp and Dohme, Rahway, New Jersey) two hours prior to infection.

SECTION 4. RESULTS

Determination of LD_{50}

The LD_{50} for animals infected with strain RIA and maintained at 5° C and 25° C was determined by the method of Reed and Muench (1938). This was found to be 4.6×10^5 cells per mouse at 25° C and 3.8×10^3 cells per mouse at 5° C, as shown in Table I. The LD_{50} for the highly virulent SR-11 strain was less than seven cells per mouse at room temperature. All observations were terminated after a period of 14 days. Schneider and Zinder (1956) have reported that approximately one cell of SR-11 causes a fatal infection.

In view of the finding that mice were more susceptible in the cold than at room temperature to infection with the avirulent strain of salmonella, two basic questions required answers: (1) Was this observation due to an enhanced virulence of the microbe in the animal maintained in the cold, or (2) Was it due to a decrease in the resistance of the host?

In order to test the possibility that a cold environment enhanced the virulence of the organism (strain RIA), isolates were recovered from the liver and spleen of infected mice held for various periods up to 14 days at 5° C. These isolates were injected into normal mice and the LD_{50} and mean survival times were determined at both temperatures. No differences from the parent strains were noted, nor were there any detectable changes in colonial

morphology or growth rate "in vitro" when compared to the original strain. Using these criteria, it would appear that cold environment does not alter the virulence of the invading organism.

TABLE I

The LD_{50} of S. typhimurium for mice as influenced by bacterial strain, environmental temperatures, and reticuloendothelial "block" with saccharated iron oxide (Proferrin)

Treatment Prior to Inoculation	Inoculum	Temperature	LD_{50}
None	Strain RIA	25° C	4.6×10^5
None	Strain RIA	5° C	3.8×10^3
Proferrin	Strain RIA	25° C	4.9×10^3
Proferrin	Strain RIA	5° C	4.0×10^1
None	Strain SR-11	25° C	7 cells
None	Strain SR-11	5° C	7 cells

Attention was then focused on a study of host defenses. It was reasoned that impairment of the functional capacity of the RES, a major host defense, by colloidal "blockade" might mimic the effect of cold on the animals' response to infection.

Effect of RES Block on LD_{50}

To evaluate the role played by the RES at the two experimental temperatures, a known RES blocking agent, Proferrin, was employed in this phase of the study. Blocking the RES two hours prior to infection lowered the LD_{50} 100-fold in animals injected with RIA and held at 25° C, as compared to normal controls at the same temperature. A similar decrease in the LD_{50} of infected animals treated with Proferrin and maintained at 5° C was also observed. The LD_{50} for mice given Proferrin and infected with RIA was 4.9×10^3 cells per mouse at 25° C (Table I). This is also the LD_{50} of animals similarly infected and kept at 5° C but not given Proferrin. In view of the near identity in the two results, it is tempting to suggest that a depression in the activity of the RES may be a major mechanism responsible

for altering host susceptibility in the cold. It is difficult to reconcile this premise, however, with the observation that Proferrin decreased the LD_{50} 100-fold at both 25° C and 5° C. It would seem more probable that, although the RES is a major protective system in infection, a sufficient amount of RES activity remained in animals at 5° C to permit still further depression when Proferrin was administered. It is also possible that Proferrin and cold act on different target areas of the RES independently of one another.

Table II shows the mortality rates of mice at room temperature and in mice when given graded doses of strain RIA. It is apparent that animals maintained in the cold are not only more susceptible to infection, as judged by the increased mortality rate, but also the initial deaths occur at least several days sooner than those at 25° C. For example, when an LD_{50} dose of strain RIA (4.6×10^5 cells per mouse at 25° C) is injected, a highly significant difference is noted in the mortality ratios between the two temperatures. This difference is significant at the 0.8 per cent level by the rank test (White, 1952).

Indicated in Table III is the effect of temperature on the time of death in mice treated with Proferrin prior to infection. A comparison of this Table with Table I will show that in both groups, i. e. with and without Proferrin, an increase in the number of infecting cells was attended by a corresponding increase in death rate. As stated previously, Proferrin treated animals became 100 times more susceptible to infection as compared to untreated controls at corresponding temperatures. In addition, infected mice pre-treated with Proferrin succumbed to the infection sooner than infected mice not given Proferrin.

Influence of Temperature on the Per Cent of Livers Positive for Bacteria

At the termination of each experiment all animals that survived the 14-day period of observation were killed by cervical dislocation. The livers were immediately excised and cultured on nutrient agar, MacConkey's agar, SS agar, and Staph 110 agar by the print method. The results are shown in Figure 1 for the experiments conducted with strain RIA. It should be noted that the per cent of animals in which salmonella could be isolated from the liver was usually greater in mice kept at 5° C than in those maintained at 25° C. Moreover, as might be expected, the per cent of positive livers increased in proportion to the size of the infectious dose.

However, with inocula exceeding the LD_{50} dose for mice at 5° C, fewer animals were available for culturing and hence a new population was selected, that is, the highly resistant animals destined to survive. Thus, the trend previously noted became less apparent and even unpredictable, as indicated by the graph plotted from the 10^4 and 10^5 inocula.

TABLE II

The effect of temperature on the distribution of deaths with time in mice infected with graded doses of S. typhimurium, strain RIA

Post Infection Day	Number of S. typhimurium injected intraperitoneally into mice kept at the temperatures indicated							
	4.8×10^2		4.8×10^3		4.8×10^4		4.8×10^5	
	25°	5°	25°	5°	25°	5°	25°	5°
1								
2					1			
3					1			2
4								4
5		1			1		3	5
6				2			5	2
7		3		3		1	1	3
8	1			1		2		3
9				1		1		
10				2		2		
11		1		1		1		
12						1		
13						1		
14				1				
Dead / Tested	1/10	5/10	0/30	11/30	0/30	12/20	9/20	19/20

TABLE III

The effect of temperature on the distribution of deaths with time in mice given Proferrin and infected with graded doses of S. typhimurium, Strain RIA

Post Infection Day	Number of S. typhimurium injected intraperitoneally into mice kept at the temperature indicated									
	4.8×10^1		4.8×10^2		4.8×10^3		4.8×10^4		4.8×10^5	
	25°	5°	25°	5°	25°	5°	25°	5°	25°	5°
1						1		2	4	10
2									2	
3		1						1	3	
4				3	2	1	5	4	1	
5	1	5	1	2	1	2	3	3		
6	1	3	2	3		4	2			
7				1	1	1				
8		1								
9		1								
10		2								
11		1				1				
12					1					
13										
14										
Dead / Tested	2/20	14/20	3/10	9/10	5/10	10/10	10/10	10/10	10/10	10/10

Despite the tendency toward selection of atypical survivors, the data obtained with livers cultured for staphylococci continued to show a substantially higher percentage of recovery from mice in the cold than from animals at room temperature.

The Effect of Low Temperature on Mice Carrying Salmonella

Results similar to those obtained with strain RIA were noted with the highly virulent strain SR-11 in experiments with mice which proved to be typhoid carriers. Figure 2 shows the results of this study, using mice which arrived from the supplier with feces which yielded positive cultures for salmonellae. These mice were termed "natural salmonella carriers." Once again it will be noted that liver cultures positive for both salmonellae and staphylococci were more prevalent in animals at the lower temperature, except that when high dosages of strain SR-11 were employed, the per cent of positive livers at both temperatures were nearly alike. Attention is called to the fact that these mice were able to withstand 7000 LD_{100} of strain SR-11 at room temperature and that 4 out of 10 animals died from this dose at 5° C.

It is apparent that animals in the carrier state enjoy a substantial protection against an organism that is usually fatal to the host when injected in a dose of only a single cell per mouse. Even with this high level of protection, however, host defenses at 5° C could not prevent some deaths following a sufficiently large infectious dose even though no animals succumbed at 25° C. This points out once again an apparent enhancement in the susceptibility of the host at low temperatures.

In line with the high immunity observed in carrier mice were the observations of Previte (unpublished) who noted a profound resistance of these mice to endotoxin derived from salmonellae, and the observations of Krause (unpublished) who found that a dose of endotoxin, which lowers completely the urinary nitrogen excreted in response to ACTH in normal mice, failed to do so when administered to natural salmonella carrier mice. In fact the studies of Krause are in accord with the report of Berry and Smythe (1959) for homologous immune mice. Thus, carrier mice behave like immune mice if judged by the urinary nitrogen block technique of Berry and Smythe (1961).

As was noted previously with the avirulent salmonella, the rise in staphylococci-positive livers from carrier mice in the cold varied directly with the infecting dose of SR-11. It might be suggested that as increasing numbers of salmonellae are injected, progressively mounting stress is applied to a host already stressed by cold, so that a strain of staphylococcus

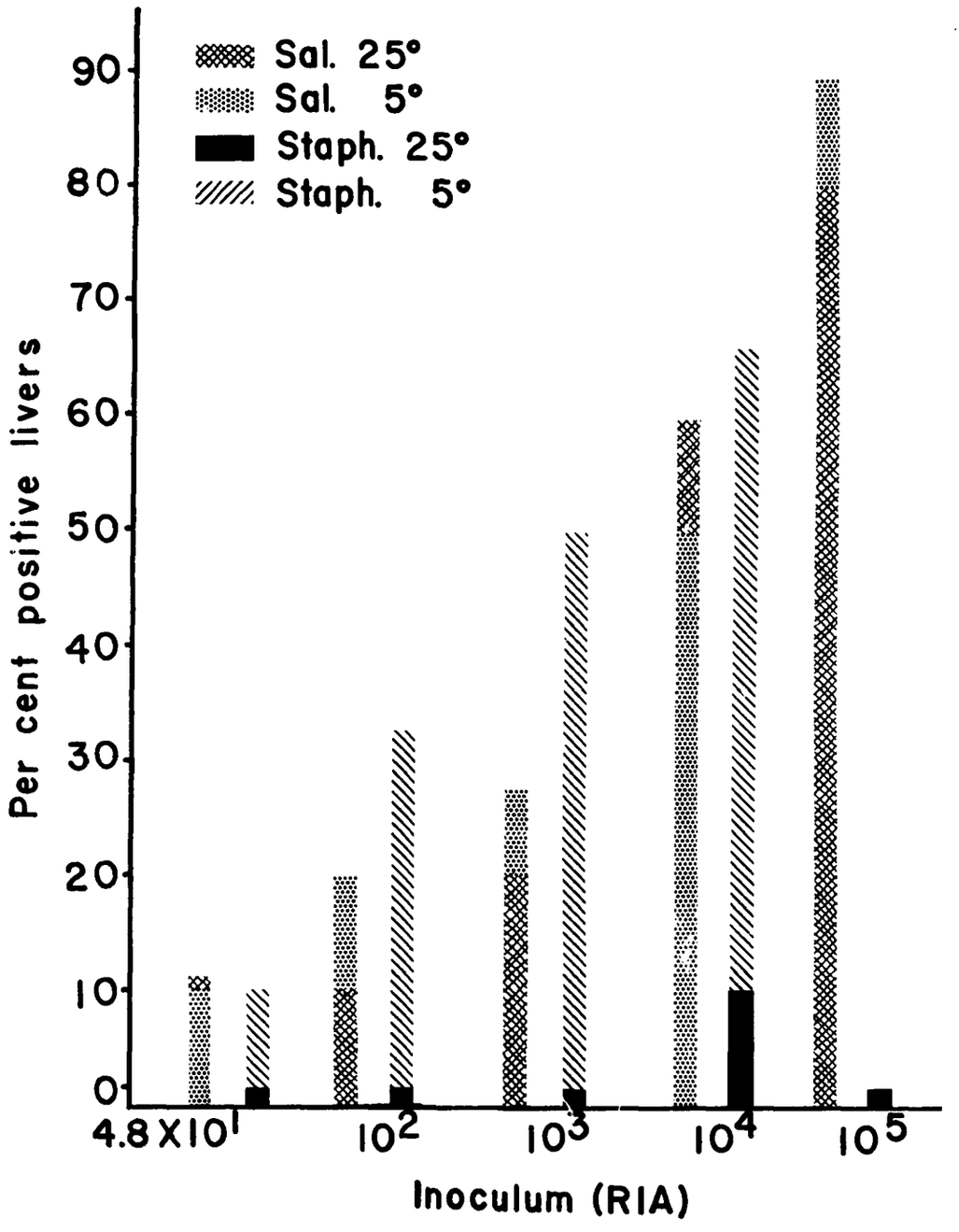

FIGURE 1

The per cent of liver cultures positive for salmonella and/or staphylococcus in mice at 5° C and 25° C as related to the infecting dose of S. typhimurium, strain RIA. All liver cultures were made 14 days postinfection.

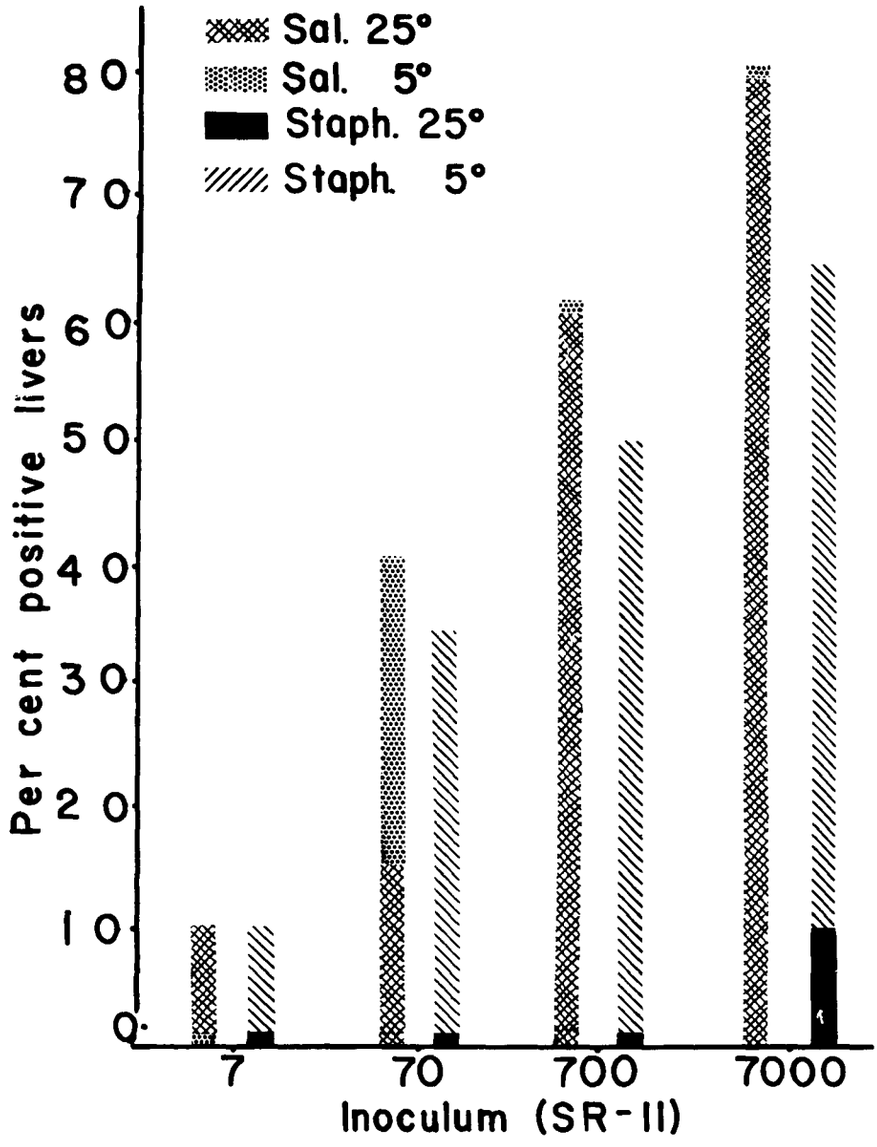

FIGURE 2

The per cent of liver cultures positive for salmonella and/or staphylococcus in natural salmonella carrier mice at 5° and at 25° C as related to the infecting dose of S. typhimurium, strain SR-11. All liver cultures were made 14 days postinfection.

already in the host or its environment now becomes established in its tissues. Staphylococci were not present in livers cultured from mice maintained at 25° C except at the highest dosage of strain SR-11, and even here only one out of 10 mice had a positive culture.

Noteworthy also was the observation that no salmonella and 23.3 per cent staphylococci-positive livers were cultured from a group of 30 non-infected controls held at 5° C for 14 days. A similar number of control mice at 25° C had negative liver cultures for both salmonellae and staphylococci. This lends further support to the contention that animals in the cold have a decreased capacity to resist infection.

SECTION 5. DISCUSSION

That host defenses are breached in animals maintained in the cold cannot be denied. Immunologists recognize at least two levels of cellular defense, one comprised of the more peripheral wandering phagocytes and the other the deeper fixed tissues of the RES. The effect of cold or of hypothermia on each of these has been reported in the literature. Halpern et al (1951) studied the activity of the RES, as judged by its ability to clear colloidal carbon, in hypothermic rats. A decided slowing down of the activity of the RES was noted. In rats at normal temperatures 90 per cent of the carbon was "fixed" in the RES in 35 minutes, while in the hypothermic animal only 29 per cent was sequestered.

Frohlich (1938) in his studies of wandering phagocytes found that in hypothermic rabbits, polymorphonuclear leucocytes did indeed increase in number as noted by others, but up to 65 per cent of these cells were either injured or were atypical. Similarly, Taylor and Dyrenforth (1938) reported an impairment of phagocytic activity of fixed tissue cells in human subjects immersed in water at 68.5° F. Moreover, it was claimed that low environmental temperatures predispose the host to infections, especially in the upper respiratory region, but the evidence for this was not convincing, primarily on the basis of sample size.

A decrease in the blood content of complement and opsonin was found by Wildfuhr (1950) in persons exposed to cold. Thus, both humoral and cellular defense systems are altered in the cold.

It is clear that in most host-parasite systems low ambient temperatures are generally deleterious and seem to enhance the infectious process by presumably altering homeostatic mechanisms in the host.

Attempts to compare data obtained from various laboratories suffer, unfortunately, from the lack of adequate standardization in experimental design. That different host-parasite models are used assumes little importance in face of the realization that not all investigators report the duration of the photoperiod per day and housing conditions employed. Furthermore, the term cold, depending on the investigator, frequently spans great temperature ranges, as noted in the literature cited. Admittedly, the conditions used in these studies were quite artificial and may not have a counterpart in nature. Animals were subjected to a constant and unfluctuating cold forcing them to live at a level of high energy expenditure for long periods, a condition seldom known to occur with any certainty in the field. Moreover, the photoperiod was an unchanging 12 hours of light per day and the light intensity was constant. This too, of course, is contrary to the natural state. In spite of these apparent shortcomings, the results were constant and reproducible, and the evidence for a decreased host resistance in the cold to both a virulent and an avirulent strain of S. typhimurium seems convincing and becomes even more reasonable to accept in view of the increased incidence of secondary infection with staphylococci in mice maintained in the cold.

The differences in host behavior at room temperature and in the cold in response to salmonella infection are more apparent at infectious dosages below or at the approximate LD_{50} level. With heavier inocula, homeostatic balance in animals at both temperatures is overcome and the previously noted differences become erratic. This is especially true in experiments in which attempts were made to culture organs for bacteria at the fourteenth post-infection day. In this regard, the data indicate that while the per cent of salmonella positive livers is greater in animals held at 5° C than in those at room temperature, this difference becomes less pronounced as heavier inocula are employed. The per cent of livers positive for staphylococci, however, continues to increase with increasing dosages of both strains of salmonella.

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Rpt. AAL-TDR-62-7. VIRULENCE AS A FACTOR IN SALMONELLA INFECTION IN MICE MAINTAINED IN THE COLD. June 1962. 15 p. incl tables, illus., 20 refs.

Unclassified Report

The object of this study was to determine possible differences in the course of salmonellosis in mice maintained at 25° C and others kept at 5° C, and to uncover, if possible, mechanisms responsible for such differences. The LD_{50} dose for mice of Salmonella typhimurium, strain RIA, is 4.6×10^5 for animals individually housed without bedding and maintained at 25° C. It is 3.8×10^3 for animals similarly housed but kept at 5° C. An intravenous injection of 0.1 ml of saccharated iron oxide (Proferrin) two hours prior to infection lowers the LD_{50} to 4.9×10^3 and 4.0×10^1 for mice kept respectively at 25° C and 5° C. Low environmental temperature and "blockage" of the reticuloendothelial system (RES) lower the resistance of mice to about the same degree, but low temperature and RES impairment together lower resistance as if each is acting independently. Doubling the volume of Proferrin more than doubles the change in susceptibility to infection manifested by the mice but this amount seems to be toxic for mice. Even more important is the incidence of staphylococci found in liver or kidney of mice infected with S. typhimurium and kept at 5° C. Cultures were made on animals that survived infection for a period of 14 days and, except for the largest challenge doses where only a few animals remained, the incidence of staphylococci was proportional to the number of salmonellae injected. At 25° C only a small percentage of mice have staphylococci in tissues and these occur independent of the infectious dose of salmonellae.

1. Salmonella
2. Staphylococcus
3. Infections
4. Exposure
5. Mice
6. Dosage
7. Iron Compounds - Proferrin

I. Project 8241-32
II. Contract AF 41(657)-340
III. Bryn Mawr College, Bryn Mawr, Pa.
IV. Miraglia, G.J. and L.J. Berry
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