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Effect of Temperature on Bacterial Killing by Serum and by Polymorphonuclear Leukocytes

JERRY SEBAG, WILLIAM P. REED,* AND RALPH C. WILLIAMS, JR.

Medical Service, Veterans Administration Hospital, and Department of Medicine, University of New Mexico School of Medicine, Albuquerque, New Mexico 87108*

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Bacterial killing by serum alone and by polymorphonuclear (PMN) leukocytes was studied at 37°C and compared with killing at 39 and 41°C. The test organisms for serum killing were *Staphylococcus aureus* 502A (serum resistant) and *Escherichia coli* O14 (serum sensitive). The organisms used in PMN killing tests were *Streptococcus pneumoniae* type 29 and *E. coli* O86. *S. aureus* was not killed by serum alone at any temperature. Changes in temperature did not affect the rate of serum killing of *E. coli* O14 for the first 60 min, but by 90 and 120 min there was a discrepancy with continued killing at 37°C, but no further killing at 39 and 41°C. PMN phagocytic killing of the pneumococcus was enhanced at 39°C compared with 37°C, and phagocytic killing of *E. coli* O86 was decreased at 41°C when compared with 37°C. Therefore, it appears that under certain circumstances fever may aid the host PMNs in destroying organisms, whereas under other circumstances it may interfere with such destruction.

For centuries fever has been recognized as a cardinal sign of disease, and more recently as a sign of infection. The assumption that fever is somehow beneficial to us in overcoming infections has led in years past to the use of induced fever as effective therapy for gonorrhea and neurosyphilis and as therapy of questionable value for a variety of other conditions (2). The belief that fever can aid the host in killing germs has been supported by the observation that a few bacteria such as gonococci survive poorly at temperatures above 37°C (4) and has been further supported by experimental animal infections with selected organisms. For instance, strains of type 3 pneumococcus that do not survive at 41°C are avirulent in rabbits unless hypothermia is induced, whereas strains that survive 41°C may be lethal for rabbits even when their temperature is not lowered (7, 20). There was also evidence in humans in 1938 that artificial fever could greatly reduce the number of viable bacteria in type 3 pneumococcal meningitis, but patients treated in this manner still died (21).

In addition to the detrimental effect on a few bacteria, it has also seemed possible that fever may enhance the body's defense mechanisms. For example, antibody production may be more effective at higher body temperature in cold-blooded animals such as frogs (3) and in mice (9). Also, as early as 1908 there were data suggesting that phagocytic activity was enhanced by hyperthermia (12), and this was again described in 1942 for the phagocytosis of staphylococci by both guinea pig and human leukocytes (6).

However, contrary evidence suggests that fever may be detrimental. Fever increases the rate at which passively infused antibodies disappear in rabbits (6). Also, sulfonamide-treated mice with pneumococcal infection survive better when kept at room temperature than when kept in an incubator that elevates the body temperature (11). It is also well known that fever may cause fever blisters, probably by promoting the local spread of herpes simplex virus.

Thus it is not clear whether fever is more beneficial or detrimental to an infected host. We have evaluated the effect of temperature on one aspect of host defense – serum and phagocytic killing of selected organisms.

MATERIALS AND METHODS

Bacteria. Two bacterial species were used in the serum killing experiments. *Escherichia coli* O14 is rapidly killed by normal human serum at 37°C and was used especially to determine whether killing was impaired at elevated temperatures. *Staphylococcus aureus* 502A resists killing by serum at 37°C and was used to see if serum killing could improve at elevated temperatures.

Phagocytic killing experiments were done with *E. coli* O86, which resists killing by serum alone but is killed by polymorphonuclear (PMN) leukocytes in the presence of immunoglobulin as the opsonin. *Streptococcus pneumoniae* type 29, which requires complement for opsonization, was also employed.

Phagocytic and serum killing tests. This study used the phagocytic bactericidal test of Maaløe (13) as modified by Hirsch and Strauss (8), in which 5×10^6 organisms were incubated with an equal number of washed human PMN leukocytes in the presence of diluted normal human serum as a source of opsonins.

Organisms were grown overnight in tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) and suspended in 0.1 ml of sterile normal saline. Serum was diluted as indicated below, and 0.1 ml was added to the mixture. PMN leukocytes were obtained by sedimentation of fresh blood in 6% dextran 75 (Travenol, Morton Grove, Ill.) for 50 to 55 min and, after counting, were placed in the mixture. The reaction took place at the temperature specified in each experiment in gel Hanks solution (1% gelatin), which was added to make a final volume of 1.0 ml. Samples were taken at 0, 30, 60, and 120 min by removing 0.001 ml with a calibrated platinum loop and suspended in 1.0 ml of sterile hypotonic saline (0.63 g of NaCl per dl). Then, 0.1 ml of this suspension was mixed with brain heart infusion agar (Difco) and incubated overnight at 37°C in the presence of 5% CO_2. Colonies were electronically counted (Biotron automated colony counter, New Brunswick Scientific). Serum-sensitive *E. coli* O14 and *S. aureus* were reacted with serum in the absence of PMNs, thus constituting a test of serum killing activity. Studies were repeated when they showed an effect of temperature on killing, and the results are reported as the average of the two determinations.

The PMN leukocytes were also evaluated for their ability to ingest latex particles and for their capacity to reduce nitroblue tetrazolium (NBT) after incubation at 37, 39, and 41°C. The latex particle uptake was determined microscopically as the mean number of particles ingested by 100 PMN leukocytes after incubation of 2.5×10^6 PMN and 0.05 ml of 0.81-μm-diameter latex particles (Difco) in 1 ml of Hanks basic salt solution. Quantitative NBT reduction was determined by the method of Baehner and Nathan (1) after 2 h of incubation at the three test temperatures. Results reported are the average of two determinations.

Temperature regulation. The reaction mixtures were prepared in a water bath set at the appropriate temperatures (37, 39, or 41°C) and were maintained in this water bath during the samplings. Between samplings, all mixtures were incubated at the specified temperature with end-over-end rotation at 12 rpm.

Serum dilutions. Full-strength normal human serum was found to decrease the viability of some organisms too rapidly to study the effect of hyperthermia. For instance, in the case of serum-sensitive *E. coli* O14, organisms were killed within minutes of exposure to full-strength serum, but killing could be slowed by diluting the serum. Dilutions were selected to produce nearly complete killing in 2 h at 37°C when possible. This allowed for detection of either an increased or a decreased rate of killing by using the same dilution of serum at different temperatures. The serum dilutions found to yield desirable results were: *S. aureus*, undiluted; *E. coli* O14, 1:48; pneumococcus, 1:4; and *E. coli* O86, 1:16.

Residual complement after incubation. Serum was diluted to the concentration used in the serum killing and phagocytosis tests and was incubated without bacteria. Residual complement via classical and alternative pathways was measured.

The activity of the classical pathway was assessed by measuring total 50% hemolytic complement units as described by Kabat and Mayer (10).

A functional assay for the overall activity of the alternative pathway used serum-sensitive *E. coli* O14, which can be killed through the alternative complement pathway. Washed organisms (4×10^9 per ml) were incubated for 30 min at 37°C with an equal volume of immunoglobulin G (IgG) (75 mg/100 ml) as Cohn fraction II (Sigma Chemical Co.). The organisms were then washed, resuspended to the previous concentration, and further diluted 1:50 in saline. Analogous to the assay for hemolytic complement, 0.1 ml of diluted test sera (1:10, 1:50, 1:100) was incubated with 0.1 ml of sensitized *E. coli*, 0.1 ml of 0.01 M ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N'*-tetraacetic acid (Sigma) and $MgSO_4$, and 0.7 ml of saline for 30 min at 37°C. Thus, the classical pathway was blocked, and killing could occur only through the alternative pathway. A 0.001-ml sample of the reaction mixture was removed with a calibrated loop and diluted in 1.0 ml of saline, and then 0.1 ml was plated in warm Penassay agar (Difco). Bacterial counts were determined at 48 h with a Biotron automatic colony counter and were compared with the count of a sample in which saline replaced diluted serum to indicate the number of viable *E. coli* in the original reaction mixture. Percent killing by each serum dilution replaced percent hemolysis in the standard hemolytic complement assay calculations, so that results were given as the serum dilution sufficient to kill 50% of IgG-coated *E. coli* rather than as the serum dilution sufficient to produce 50% hemolysis of sensitized erythrocytes. The 50% serum dilution required for killing 50% of 8×10^6 IgG-coated *E. coli* O14 was used to emphasize the conceptual similarity between bactericidal and hemolytic complement assays.

RESULTS

Effect of temperature on growth and viability. Growth rates varied when some of the test organisms were incubated at different temperatures in the absence of serum and PMN leukocytes (Fig. 1 and 2). The difference was most apparent with the serum-sensitive *E. coli* O14, which had a three- to fourfold increase in growth rate at 39 and 41°C compared with 37°C. The changes were smaller with the other organisms, and in no case was there a decrease in viability at the higher temperature.

Effect of temperature on serum killing. Serum-sensitive *E. coli* O14 was tested with a 1:48 dilution of serum (e.g., serum diluted 1:480 in the final incubation mixture). The survival curves for the three temperatures are illus-

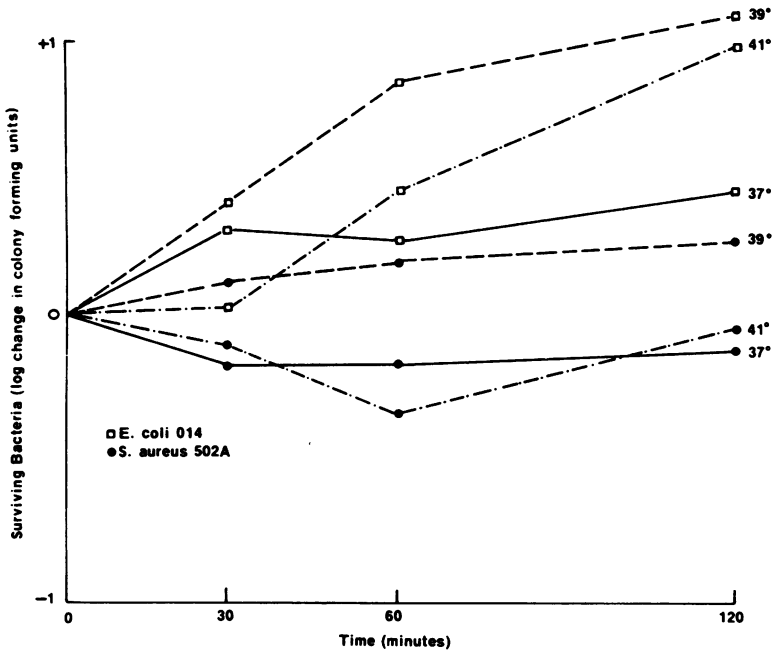

FIG. 1. *Viability of E. coli O14 and S. aureus 502A with serum omitted from the serum killing reaction mixture. Incubations were carried out at 37°C (——), 39°C (-----), and 41°C (-·-·-·). E. coli O14 grew under these conditions, especially at 39 and 41°C, and had nearly one log of growth at 120 min.*

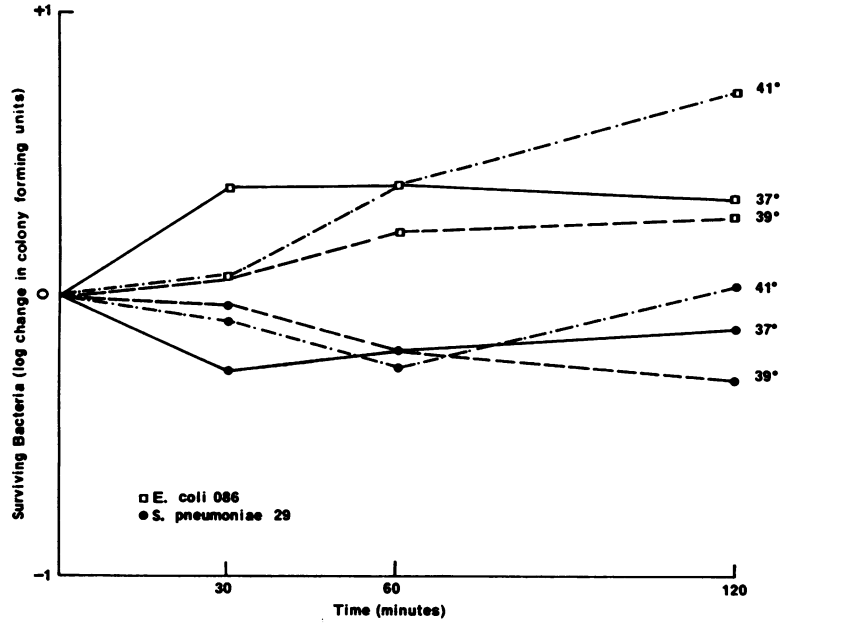

FIG. 2. *Viability of E. coli O86 and S. pneumoniae 29 with serum and PMNs omitted from the phagocytosis reaction mixture. Incubations were carried out at 37°C (——), 39° C (-----), and 41°C (-·-·-·). There was growth of E. coli O86 under these conditions, but the rate of growth was not clearly temperature dependent.*

trated in Fig. 3 and showed nearly similar rates of killing at different temperatures for the first 60 min; however, by 2 h the tubes incubated at 39 and 41°C showed no further killing, and there appeared to be some growth of the organisms. This was the only test organism for which a discrepancy occurred between the results at different time intervals.

The serum-resistant *S. aureus* 502A was tested with undiluted serum (e.g., 1:10 dilution in final incubation mixture) to see if higher temperatures would promote its killing by serum, but no killing occurred even at the higher temperatures.

Effect of temperature on PMN killing. Pneumococci were killed more readily when incubated at 39°C than at 37°C in the presence of serum and PMN leukocytes. To verify this observation, the opsonin-containing serum was further diluted so that no killing occurred at 37°C, but killing still did occur at 39°C (Table 1). Results are expressed as the ratio of organisms surviving at each temperature in Fig. 4 since it is this relationship, rather than the absolute killing curves, that is meaningful. In contrast, there was no enhancement of killing at 41°C compared with 37°C.

The serum-resistant *E. coli* O86 was killed at a similar rate by serum and PMN leukocytes at 37 and 39°C but was killed less well at 41°C (Table 1 and Fig. 4). An overview of bacterial survival under each of the conditions studied is presented in Fig. 5.

Effect of temperature on PMN phagocytosis. The mean number of latex particles ingested per PMN after 60 min of incubation was 15 at 37°C, 14 at 39°C, and 13 at 41°C. After 2 h, most PMNs had ingested >25 particles at all

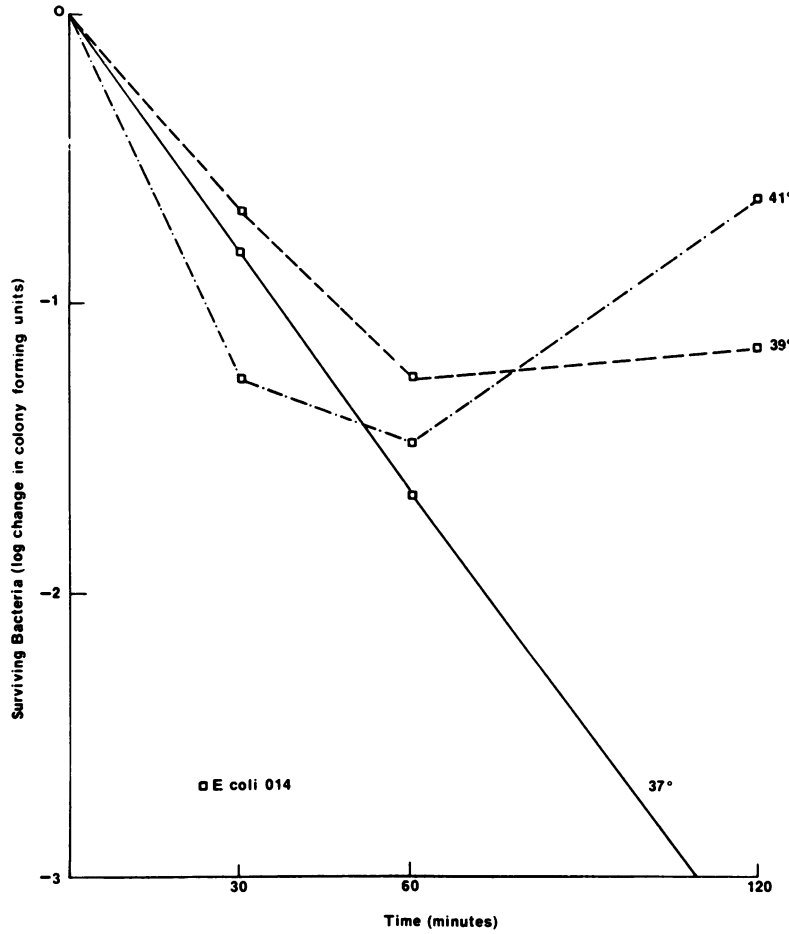

FIG. 3. *Survival of E. coli O14 in the serum killing test at 37, 39, and 41°C. The rate of killing was not affected by temperature at 30 or 60 min. By 120 min there was further killing at 37°C, but no further killing at 39 and 41°C.*

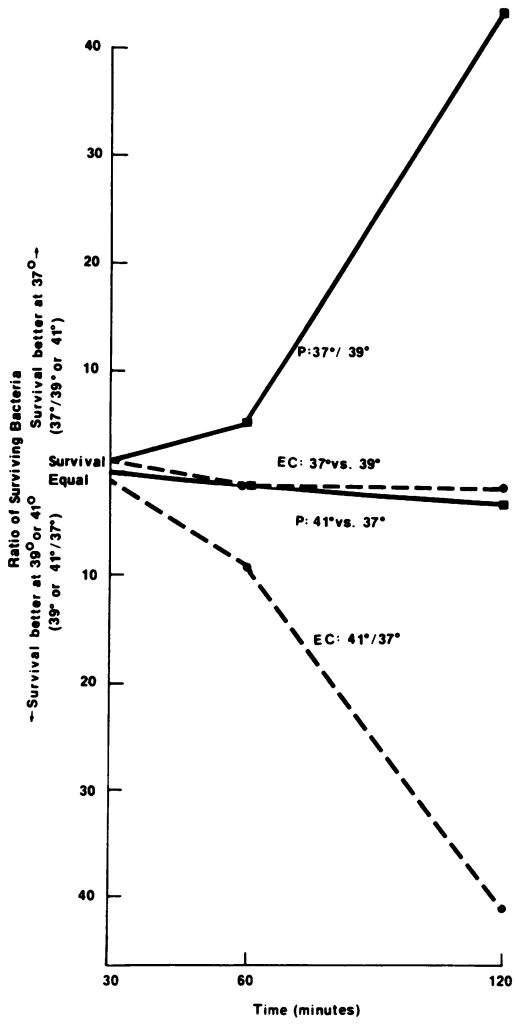

FIG. 4. *Comparison at each time interval between survival at 37°C and elevated temperatures in the phagocytic killing test. The ordinate represents relative bacterial survival expressed as the ratio of surviving bacteria at normothermic and hyperthermic conditions. Enhanced phagocytic killing at higher temperatures is equivalent to poorer survival at the higher temperature and is plotted in the upper part of this chart as the ratio of survival at 37°C to survival at the hyperthermic temperature. Killing of the pneumococcus (labeled P) was more effective at 39°C than at 37°C as shown by this high ratio of survival at 37°C to survival at 39°C, which increased over the course of 2 h. Poorer phagocytic killing at higher temperatures is equivalent to better bacterial survival at the higher temperature and is plotted in the lower part of this chart as the ratio of survival at the hyperthermic temperature to survival at 37°C. Phagocytic killing of E. coli O86 (EC) was less effective at 41°C than at 37°C as shown by this high ratio of survival at 41°C to survival at 37°C, which increased over the course of 2 h.*

temperatures. Both resting and phagocytically stimulated NBT reductions by PMN leukocytes were determined at each temperature by measuring formazan activity as the optical density at 515 nm. The NBT reduction appeared similar at each temperature after 30, 60, and 120 min of incubation (Table 2).

Residual complement after incubation. Since phagocytic killing of *E. coli* O86 was impaired at 41°C, the diluted serum (1:16) that was used as an opsonin source was tested for residual complement activity after incubation at various temperatures. Classical pathway activity determined as total hemolytic complement was reduced to about one-half the initial value after 2 h of incubation at all temperatures (Table 3). Alternative pathway activity determined as bactericidal complement in the presence of ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid and Mg^{2+} was reduced even less (Table 3). Similar results were obtained with serum diluted 1:48, as was used in the serum killing test for *E. coli* O14.

DISCUSSION

The killing of microorganisms by phagocytes is thought to be a major host defense mechanism that aids in the recovery from established infections. The present in vitro study of phagocytic killing at different temperatures indicates that there is no simple answer regarding the role of fever in this form of host defense. It appears that results vary with the temperature and with the bacterial strain involved. The complexities of the situation are further increased when one recalls that phagocytic killing is only one aspect of host defense, and other defense mechanisms may behave differently at elevated temperatures.

The results reported in this study are actually a balance between effects of temperature on growth of the organism and its effects on the killing system. For instance, the serum-sensitive *E. coli* O14 proliferated more rapidly at higher temperatures (Fig. 1), which may have contributed to its somewhat improved survival in the presence of serum at higher temperatures. However, as seen in Fig. 5, the effect of temperature on growth did not entirely account for the impaired bacterial killing of this strain.

The apparent cessation of serum killing of *E. coli* O14 and growth of the organism after 60 min at elevated temperatures may be related to the high dilution of serum in the test system. Complement was present only in low levels and may have been further reduced by consumption in the presence of the organisms. Bactericidal complement did not deteriorate after 2 h at the

TABLE 1. *Survival of bacteria at selected temperatures at which phagocytic killing differed from results at 37°C*

Bacteria and temp at (°C)	No. of surviving bacteria (colony-forming units/ml) at:			
	0 min	30 min	90 min	120 min
Pneumococcus type 29				
37	5×10^6	8.2×10^6 (164)[a]	1.1×10^7 (231)	1.6×10^7 (328)
39	5×10^6	4.8×10^6 (95)	2.6×10^6 (52)	4.5×10^5 (9)
E. coli O86				
37	5×10^6	3.4×10^6 (68)	1.2×10^6 (23)	7×10^5 (14)
41	5×10^6	4.5×10^6 (90)	9.6×10^6 (191)	2.9×10^7 (574)

[a] Numbers in parentheses are percentages.

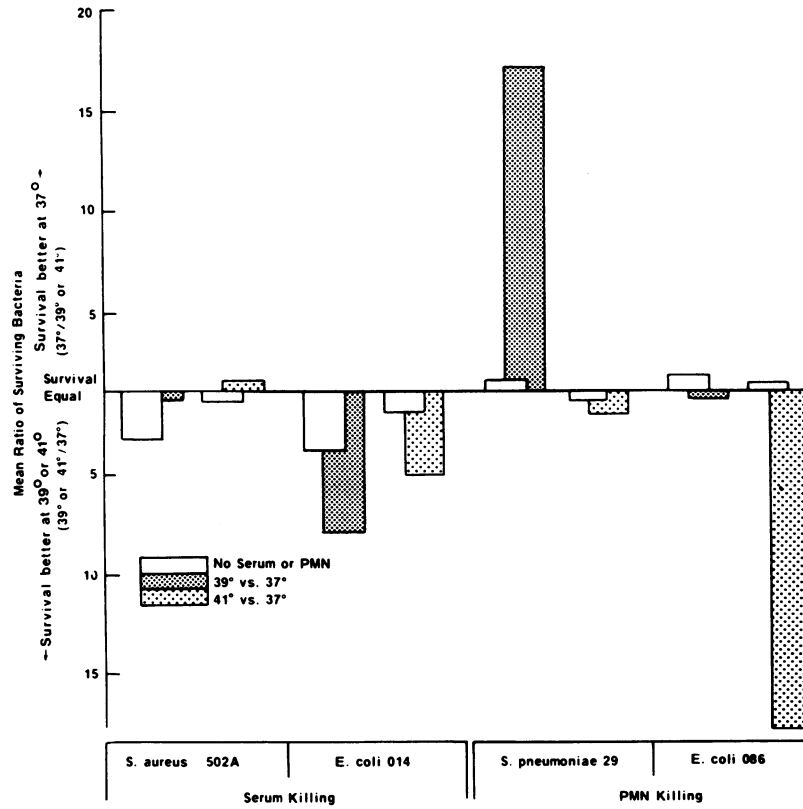

FIG. 5. *Comparison between survival at 37°C and elevated temperatures in phagocytic and serum killing tests. All bars represent the mean values obtained from the three time intervals. The unshaded bars represent organism viability in the absence of serum and PMNs. As in the previous figure, increased bacterial killing at elevated temperature is indicated by a bar directed upwards, whereas decreased killing at an elevated temperature is indicated by a bar directed downwards. It can be seen that elevated temperatures seemed to impair serum killing of E. coli O14 more than they promoted growth of the organism. Temperatures elevated to 39°C aided PMN killing of the pneumococcus, whereas 41°C hindered PMN killing of E. coli O86.*

test temperatures in the absence of organisms.

Some of the dilutions of serum used were high, with resulting unphysiologically small concentrations of antibody and complement. The dilutions used were sought as a means of reaching borderline levels for serum killing and opsonization so that small differences in killing at different temperatures might be detected. However, certain infections may be associated with low levels of complement, and then the results may be directly applicable to patients. For instance, serum complement may be de-

TABLE 2. *Quantitative NBT reduction by PMN leukocytes incubated at various test temperatures*

Incubation temp (°C)	Formazan activity (optical density at 515 nm)					
	Resting cells			Phagocytosing cells		
	30[a]	60	120	30	60	120
37	0.235	0.272	0.328	0.316	0.345	0.374
39	0.235	0.329	0.321	0.284	0.330	0.383
41	0.168	0.191	0.291	0.321	0.358	0.347

[a] Minutes of incubation.

TABLE 3. *Residual complement activity after incubation of serum diluted 1:16 at various test temperatures*

Incubation temp (°C)	Residual complement activity							
	Hemolytic complement[a]				Bactericidal complement[b]			
	0[c]	30	60	120	0	30	60	120
37	2.8	1.6	1.5	1.4	5.2	5.4	5.5	5.0
39	2.8	1.7	1.5	1.4	5.2	5.2	5.0	5.2
41	2.8	1.6	1.5	1.2	5.2	6.0	4.5	4.5

[a] Expressed as 50% hemolytic complement units, classical pathways.

[b] Expressed as 50% bactericidal complement units, alternate pathway.

[c] Minutes of incubation before determination.

pressed during shock associated with gram-negative bacteremia (14) and pneumococcal sepsis (18), thus creating an in vivo situation that resembles the in vitro condition of these studies.

Phagocytic killing of staphylococci has been studied on several occasions with varying results. Ellingson and Clark (6) reported increased killing of staphylococci at elevated temperatures. Mandell (16), as well as Craig and Suter (5), found no effect on staphylococcal killing in the temperature range used in these studies, and Peterson and co-workers (17) found decreased killing at elevated temperatures. The variation in results may be due to different bacterial strains or different experimental methods. The technique used in this study resembles that of Peterson (17), so the results may be comparable.

It appears that phagocytosis of type 29 pneumococci is enhanced at 39°C, whereas higher temperatures afford no advantage over normothermia. Therefore, insofar as phagocytic killing of organisms is concerned, fever of moderate degree may be beneficial to the host in pneumococcal pneumonia, but fevers in the range of 41°C may be harmful to the host in *E. coli* infections and may contribute to rampant bacteremia. These results correspond with previous studies in which animals maintained at high ambient temperatures resisted pneumococcal infection (15) but had a high mortality from coliform infections (19).

Since there was little change in phagocytic ingestion of latex particles or in NBT reduction at the elevated temperatures, the mechanisms for the effects on phagocytic killing remain unknown. An unexplored possibility may be an effect of temperature on opsonization or on attachment of the opsonized particle to PMN leukocytes. Further investigation would be required to clarify the mechanism through which fever alters phagocytic killing of test strains.

Phagocytosis is only one aspect of host defense, so the available data do not warrant a conclusion regarding the effect of fever on human infections with these organisms. Since it is currently a common practice to reduce fever in many diseases, it would be useful to have further information regarding the benefit or harm of such therapy.

ACKNOWLEDGMENTS

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