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STUDIES IN STAPHYLOCOCCAL FEVER.
VI. RESPONSES INDUCED BY CELL WALLS AND VARIOUS FRACTIONS OF STAPHYLOCOCCI AND THEIR PRODUCTS†

In earlier papers of this series[1-5] we have shown that cultures of *Staphylococcus aureus* when injected intravenously into rabbits produce fever by two different mechanisms. The cell bodies of this organism, in common with those of other Gram-positive bacteria, caused fevers that appeared after a characteristic latency of 45 to 60 minutes. The febrile responses were roughly proportional to the number of organisms injected, above a minimal pyrogenic dose of 1 x 10^7. Since autoclaved bacteria were equally pyrogenic, fever was clearly independent of infection and appeared to be caused by phagocytosis of the injected bacteria by circulating leukocytes.

A second pyrogenic factor was found to be present in culture filtrates of staphylococci belonging to certain phage types. Although the responses to this agent were indistinguishable from those induced by the cell bodies, several lines of evidence indicated that the soluble agent was an antigenic protein that produced fever by means of a naturally acquired or experimentally induced hypersensitivity of the delayed type.[2-5]

The present studies were carried out with several partially purified fractions of staphylococci of two strains (Giorgio and 80-81) in an attempt to determine further the relation between these pyrogenic factors and the mechanisms by which they produce fever.

MATERIALS AND METHODS

Cell walls and their constituents were prepared from the 80-81 strain of *Staphylococcus aureus* (NYH-6) by methods previously described in detail.[6] In brief, suspensions of acetone-dried organisms were disrupted in the Mickle disintegrator and the cell walls were deposited by centrifugation at 10,300 x G for 10 minutes. The crude cell walls (about 20 per cent of the dry weight of the original cells) were then treated with trypsin, pepsin, and ribonuclease to remove adherent bacterial cyto-

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plasm and then resuspended in pyrogen-free saline or distilled water to a concentration of 100 or 500 μg/ml. Cell membranes were harvested from the 10,300 x G supernatant by further centrifugation at 13,000 x G for 30 minutes.

Mucopeptide was prepared by heating cell walls at 90° C. for 15 minutes in 5 per cent trichloracetic acid (TCA). This procedure liberates into solution over 95 per cent of the cell wall teichoic acid without extensive solubilization of the mucopeptide "skeleton." The mucopeptide residue, which represented 75 per cent of the weight of the initial cell wall preparation, was isolated by centrifugation, washed with distilled water and resuspended in a volume equal to that of the original cell wall suspension. Thus, equivalent doses of cell walls and mucopeptide could be tested by injection of equal volumes of each material.

Although *Staphylococcus aureus* cell walls are resistant to the action of lysozyme (muramidase), the mucopeptide is sensitive. Incubation of mucopeptide at a concentration equivalent to 500 μg of cell walls/ml. with 50 μg of lysosyme/ml. resulted in an 80 per cent reduction of the optical density of the suspension after 18 hours at 37° C.

Cell walls, mucopeptide, and lysozyme digests of the mucopeptide were also prepared from strain Giorgio (phage type 83, previously called VA_4) in a similar fashion. It was of note that only 60 per cent of the mucopeptide prepared from this strain was solubilized by lysozyme under conditions in which 80 per cent of the 80-81 mucopeptide was brought into solution.

Supernates of both cultures and disrupted cells of the 80-81 strain were initially lyophilized. Before injection, saline was added to give solutions of 100 μg per ml.

Teichoic acid was isolated from cold TCA extracts of the 80-81 strain by methods described previously.[6]

All materials and procedures relative to overnight culture of the Giorgio strain in broth, as well as the technique for disintegration of the microorganisms in a Mickle disintegrator, have been described.[1-5] Determinations of bacterial protein (both before and after disruption of cells) were done as previously[5] by the technique of Lowry.*

Tolerance to bacterial pyrogen was induced as before[1] by at least seven daily injections of 1.5 ml. of a 1:10 dilution of stock typhoid vaccine.** Techniques to avoid incidental contamination of solutions and equipment with bacterial pyrogens, selection of rabbits, measurement of temperatures, and calculation of fever indices were similar to those presented previously.[1]

RESULTS

Fever induced by staphylococcal cell walls

Figure 1 shows the mean fevers induced by three daily intravenous injections of 500 μg of cell walls (prepared from the 80-81 strain of

* Kindly performed by Dr. David Seligson, Director of Clinical Laboratories, Yale-New Haven Hospital.

** Typhoid vaccine (monovalent reference standard NRV-LS No. 1) made from *Salmonella typhosa* V-58. Generously supplied by the Army Medical Service Graduate School, Walter Reed Medical Center, Washington, D.C.

Staphylococcus aureus)* in two groups of rabbits, one normal and the other previously rendered tolerant to the fever-inducing effects of Gram-negative bacterial endotoxin by typhoid vaccine (see METHODS). The following features are noteworthy: (1) The relatively prolonged latent period of nearly one hour before onset of fever; (2) the vigorous biphasic responses which are similar in the normal and endotoxin-tolerant animals; (3) the development in both groups of a partial tolerance (evident in

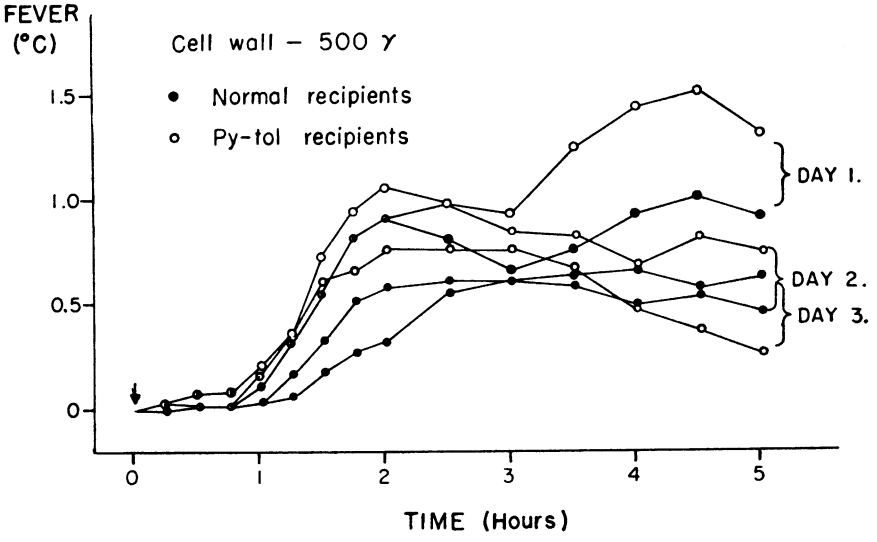

FIG. 1. Fevers induced by 3 daily injections of 500 μg 80-81 cell walls (contained in 5 ml.) in normal and pyrogen-tolerant recipients. Each curve represents the average response of 3 rabbits.

reduction of the second fever peak) on the third day. The prolonged latency of action, the production of equal responses in normal and pyrogen-tolerant recipients, as well as the minimal tolerance appearing with daily inoculations, are similar to the effects produced by whole staphylococcal cells[1] and clearly separate this staphylococcal cell wall preparation from the endotoxins that are present in the cell walls of Gram-negative bacteria.

Fever induced by cell walls after acid and lysozyme

Because the fevers induced by cell walls and intact staphylococcal cells were similar, it seemed appropriate to determine next which constituent of the cell wall was the pyrogenic agent. Accordingly, the cell walls were

* This dosage of cell walls is derived from 4 x 10^9 cells.

extracted with hot trichloracetic acid to remove the teichoic acid component (see MATERIALS AND METHODS) and the mucopeptide residue was then injected in dosages equivalent to the untreated preparations. As is evident in Figure 2, this treatment of the cell walls did not affect their pyrogenicity, which appears, therefore, to be independent of the species-specific antigens originally present in the cell wall.

In the next experiment, these acid-extracted preparations (in dosages equivalent to 500 μg cell wall) were incubated with lysozyme to disrupt the mucopeptide residue, an effect that was grossly evident by clearing of the previously turbid suspensions of cell walls (see MATERIALS AND METHODS). This procedure virtually abolished the capacity of the cells

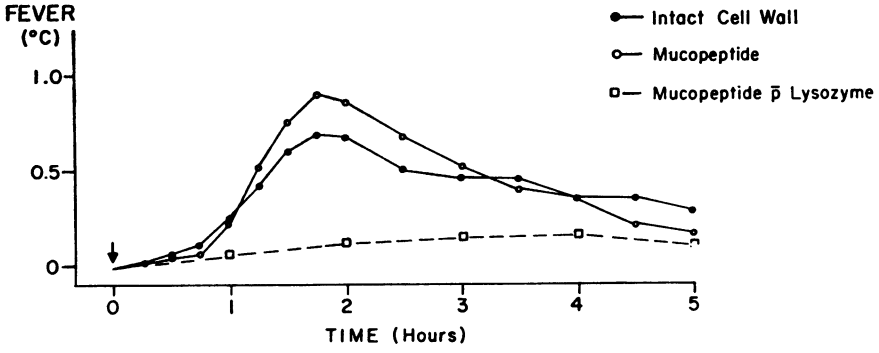

FIG. 2. Mean fevers induced by 1 ml. intact cell walls, mucopeptide and mucopeptide (80-81 strain) after treatment with lysozyme. All injections were given in the same equivalent dosage (500 μg) to the same 5 recipients. To avoid the possible effects of tolerance, the lysozyme digest of cell walls was inoculated the day before the mucopeptide and the intact cells were given 5 days later.

to induce fever, as shown in Figure 2. Of 10 rabbits given the lysozyme-treated preparations, only two had febrile responses (transient elevations of 0.8 and 0.9° C. at 2 hours). These data accordingly suggest that cell walls, like intact cells, produce fever by virtue of their particularity rather than because of any biochemical component on the cell surface.

Cell walls and mucopeptide prepared from strain Giorgio induced fevers comparable to those seen with the products prepared from the 80-81 strain. However, the mucopeptide residue from this strain was less susceptible *in vitro* to lysozyme (see MATERIALS AND METHODS), as was evident *in vivo* by its unimpaired ability to produce fever (in a dosage equivalent to 500 μg of Giorgio cell walls) after incubation with lysozyme. Lower doses of mucopeptide (equivalent to 100 μg cell wall), which produced monophasic fevers of 0.7 to 1.1° C. in three rabbits, lost their pyrogenicity after similar treatment with lysozyme.

Pyrogenicity of staphylococcal cell walls and culture supernates

To determine the possible relation of pyrogenic agents in staphylococcal cell walls with those present in supernates of growing cultures, a group of rabbits were given three successive daily inoculations of the culture supernate (strain 80-81). By the third day the recipients had developed almost complete tolerance to the pyrogenic agent in the supernate, as shown in Figure 3. However, when the rabbits were given an intravenous

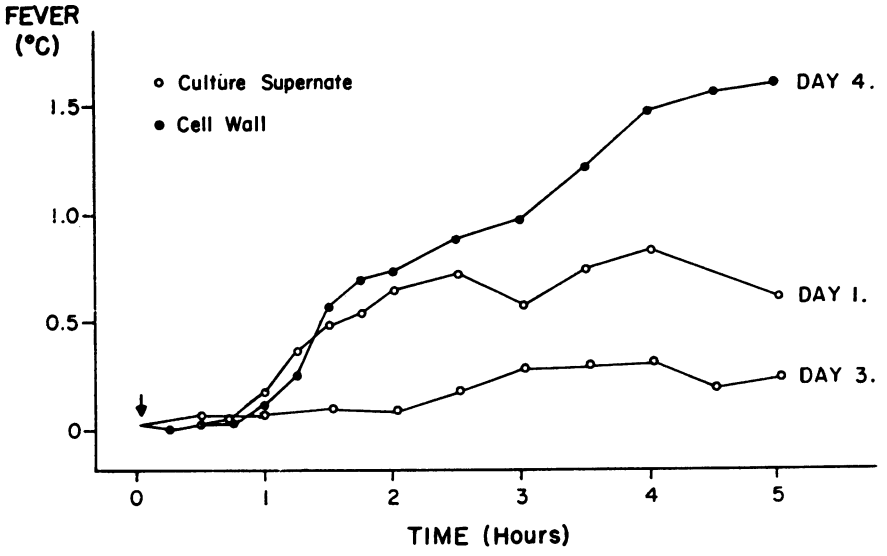

FIG. 3. Responses produced by 5 ml. 80-81 culture filtrate (containing 500 μg) on the 1st and 3rd days of 3 successive, daily injections. On the 4th day, the same recipients received 5 ml. 80-81 cell walls (500 μg). Each curve represents the average of 3 rabbits.

injection of cell walls from the same strain on the fourth day, they had high biphasic fevers, indicating a lack of cross-tolerance between these two factors. A similar lack of cross-tolerance has been previously demonstrated between the soluble pyrogen present in culture filtrates and intact staphylococcal cells of the Giorgio strain,[8] thus further suggesting that the intact cell and cell wall produce fever by a similar mechanism.

Tolerance induced by filtrates from disrupted intact cells

In order to determine whether intact staphylococci contained an agent similar to the material that appeared in filtrates of growing cultures of this microorganism, a suspension of washed Giorgio cells was placed in a

Mickle disintegrator and the cells mechanically disrupted (see METHODS). Filtrates of these suspensions produced fevers of delayed onset similar in appearance to those caused by culture filtrates (or supernates). Similarly, a single inoculation of this agent induced an almost complete pyrogenic tolerance on the second day, as shown in Figure 4.

Cross-tolerance between the pyrogenic agent in filtrates of cultures and in disrupted cells

To determine the relation in fever-inducing activity between the pyrogens present in culture filtrates and in filtrates of disrupted cells, an attempt was

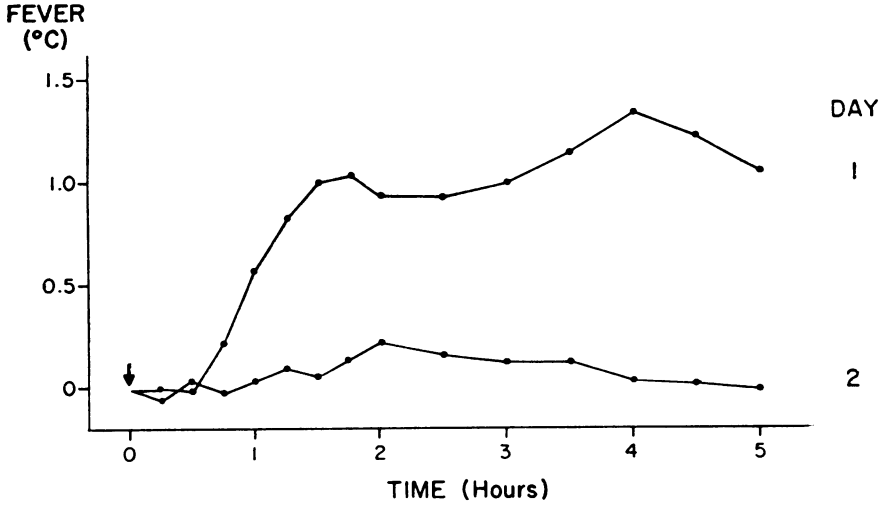

FIG. 4. Mean responses of 3 rabbits given 8 ml. filtrate of disrupted cells (Giorgio) on two successive days. Each dose contained 640 μg protein.

made to induce cross-tolerance between these agents. A group of rabbits were given an initial injection of the filtrate of "Mickled" cells (strain Giorgio). Two days later, they received the first of four daily intravenous inoculations of culture filtrate from the same strain. On the day following the last of these injections, when the animals had developed almost complete tolerance to the culture filtrate, they were given a second inoculation of the filtrate from disrupted cells. As shown in Figure 5, a significant degree of cross-tolerance was evident between these two agents. Rabbits that had initially responded to the filtrate of "Mickled" cells with high biphasic fevers developed only brief, monophasic responses to the same material after they had been rendered tolerant to the culture filtrate from the same

microorganism. As previously shown in tests of tolerance to Giorgio culture filtrate,[8] cross-tolerance to the disrupted cell material was transient. When tested six days later, all animals had recovered their initial responsiveness to the Mickled cell filtrate.

Similar attempts to demonstrate cross-tolerance between the different fractions of the 80-81 strain were also carried out. A group of rabbits were given three daily injections of the intact cell wall and then challenged with supernates of disrupted cells on the fourth day. As shown by the

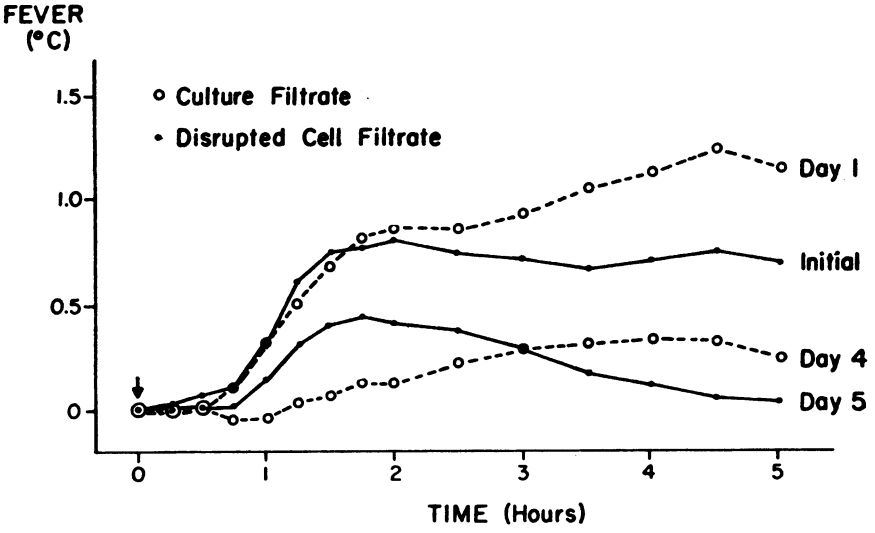

FIG. 5. Mean febrile responses of 6 rabbits to 1 ml. Giorgio culture filtrate on the 1st and 4th days of 4 daily injections. Responses of the same rabbits to 4 ml. "Mickled" filtrate of disrupted cells (Giorgio) on the 5th day are shown and compared with initial responses to the same material, given several days before the onset of the experiment. Each dose of the Mickled material contained 600 μg protein.

fever indices in Table I, there was no evident reduction in the responses of this group to the disrupted cell material (as compared with a group of control rabbits) despite development of a significant tolerance to the cell wall. However, another group of rabbits that were given the "Mickled" material for three successive days demonstrated a marked tolerance to the culture supernate on the fourth day as compared with a similar group of controls (see Table I).

These experiments suggest, therefore, that supernates of growing cultures and of disrupted cells contain substances that are similar in their pyrogenic activity and in their ability to induce cross-tolerance, but differ from the agent(s) present in the intact cell wall.

Pyrogenic responses of "new" and "old" rabbits

Since cross-tolerance was not complete between filtrates of staphylococcal cultures and disrupted cells, the pyrogenic agents in these two materials, though similar, did not appear to be identical. Further confirmation of this inference was obtained in the responses of two different groups of rabbits to these agents. One group, designated "old," were animals that had received an intensive series of injections of staphylococcal products, both culture filtrates and filtrates of disrupted cells, over the previous 2½ months (in one rabbit, these injections had extended over a period of nine months). The other group ("new") had recently come into the colony

TABLE 1. AVERAGE 5-HOUR FEVER INDICES OF RABBITS GIVEN VARIOUS FRACTIONS OF 80-81 PHAGE TYPE STAPHYLOCOCCI AND THEIR PRODUCTS

	Febrile response			*Febrile response*	
First material	*Initial, day 1*	*Tolerant, day 3*	*Second material*	*Cross-tolerant, day 4*	*Control*
Cell wall	37.5 (3)	27.0 (3)	Supernate of disrupted cells (Mickle)	26.5 (3)	22.5 (5)
Supernate of disrupted cells (Mickle)	22.5 (5)	15.0 (5)	Supernate of culture	12.5 (5)	29.0 (3)

NOTE: Numbers in parentheses indicate number of rabbits from which each value was derived. All dosages = 500 μg.

and had received no previous injections. These two groups responded similarly to a small dose of staphylococcal culture filtrate (strain Giorgio). On the other hand, the filtrate of disrupted cells from the same strain produced significant fevers in the "old" group but no response in the new rabbits (see Fig. 6). These results suggest that the disrupted cell contains one or more antigens that will induce fever only in rabbits that have been highly sensitized to staphylococci and their products, whereas the antigens present in culture filtrates may evoke equally vigorous responses in rabbits presumably naturally sensitized by inapparent infection.[8]

Responses to filtrates of phage-lysed staphylococci

In an effort to test the pyrogenicity of material from cells disrupted by an enzymatic rather than mechanical process, staphylococci (strain Giorgio) were plated in soft agar and incubated with specific bacteriophage (83) according to standard techniques.[7] The soft agar layers from two

plates containing the phage-lysed cultures were then scraped up, washed into a tube with pyrogen-free broth and shaken to liberate the phage and soluble contents of the cells. After centrifugation to sediment the fragments of agar, the supernatant was passed through a sintered glass filter.*

When injected into rabbits, these phage-lysed culture filtrates (containing titers of bacteriophage of 1 x 10^4) produced delayed fevers identical in appearance to those induced by filtrate of cells disrupted by "Mickling."

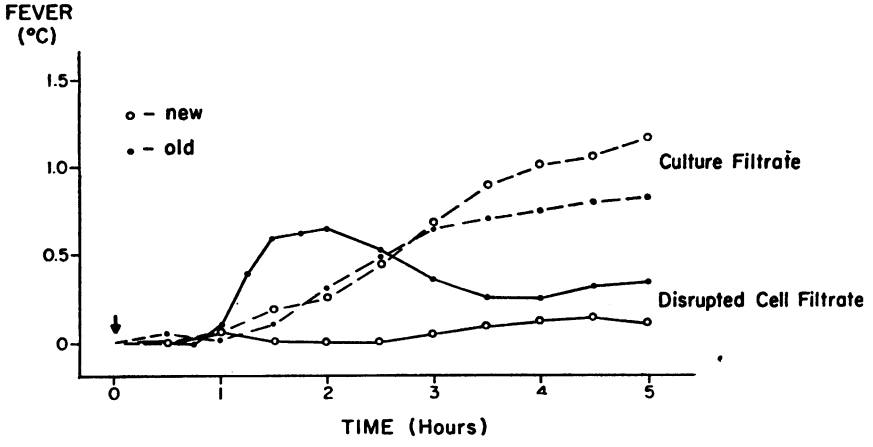

FIG. 6. Mean responses on 4 "old" and 4 "new" rabbits given 4 ml. filtrate of disrupted cells (Giorgio), containing 448 μg protein. Mean fevers of the same 2 groups of rabbits are shown in 2 ml. Giorgio culture filtrate (1:10 dilution) given several days later (see Text for details).

Similarly, the rabbits developed complete tolerance to the phage-lysed material after two or three injections on successive days.

Filtrates of staphylococcal cultures grown and collected under identical conditions but without added phage produced similar but somewhat lower fevers.[5] The similar nature of at least some of the agents released by growing and phage-lysed cultures of staphylococci was evidenced by the following experiment. Two groups of rabbits were injected with a small dose of Giorgio culture filtrate. The next day the experimental group was given the first of three daily injections of phage-lysed culture filtrate to which a pyrogenic tolerance was rapidly induced. On the day following the last injection, both the experimental and control groups received a second injection of the culture filtrate given initially. Whereas the control

* Morton bacteriological filter apparatus with ultrafine fritted disc (Corning).

group had nearly identical responses to the two spaced inoculations of staphylococcal culture filtrate, the experimental group had been rendered completely tolerant to the culture filtrate by the preceding injections of phage-lysed material (see Fig. 7). As demonstrated previously in other experiments with culture filtrate,[8] the tolerance was transient and most animals had recovered a significant responsiveness to the filtrate when reinjected one week later.

Fevers induced by a membrane fraction

Three rabbits injected with a partially purified membrane fraction (500 μg) developed prolonged monophasic fevers that reached a maximum

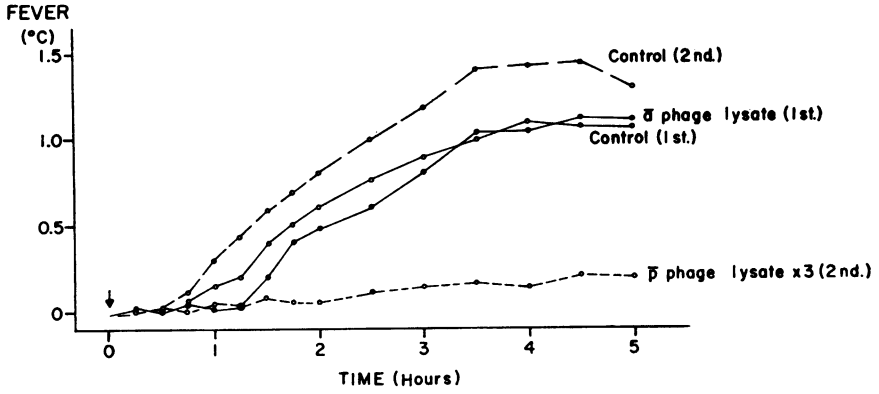

FIG. 7. Mean responses of 2 groups (5 rabbits each) to 0.5 ml. culture filtrate (Giorgio) given on days 1 and 5. Recipients in the experimental group were injected with 1 ml. phage-lysed filtrate (bacteriophage titer 1:10,000) on days 2-4. The control group received the 2 spaced injections of culture filtrate only. By day 11, the experimental group had partially recovered responsiveness to the culture filtrate. (Mean fever of 0.7° C. at 5 hours). See Text for details.
1st = day 1; 2nd = day 5.

of 0.7 to 1.1° C. in 2½ to 3 hours. These responses appeared after a one hour latency similar to those seen with the other staphylococcal products. Because of the small amounts available, no cross-tolerance experiments were performed to determine the possible immunological relationship of this fraction to those already described.

Fevers induced by purified teichoic acid

Five-hundred micrograms of teichoic acid derived from staphylococcal cell walls was tested for its pyrogenicity. Four rabbits were used as recipients: two were older animals that had been in the colony for nine

months and had received inoculations of live organisms, as well as a series of injections of autoclaved cocci; the other two animals had been purchased less than a month before and had received only five or six injections of various cell fractions. The responses of these two groups were markedly different. The "new" animals were unresponsive to the teichoic acid, but the two "old" rabbits developed prolonged monophasic fevers of 1.25° C. after a delay of one hour (Fig. 8). These preliminary observations suggest that the febrile response to this complex carbohydrate depends

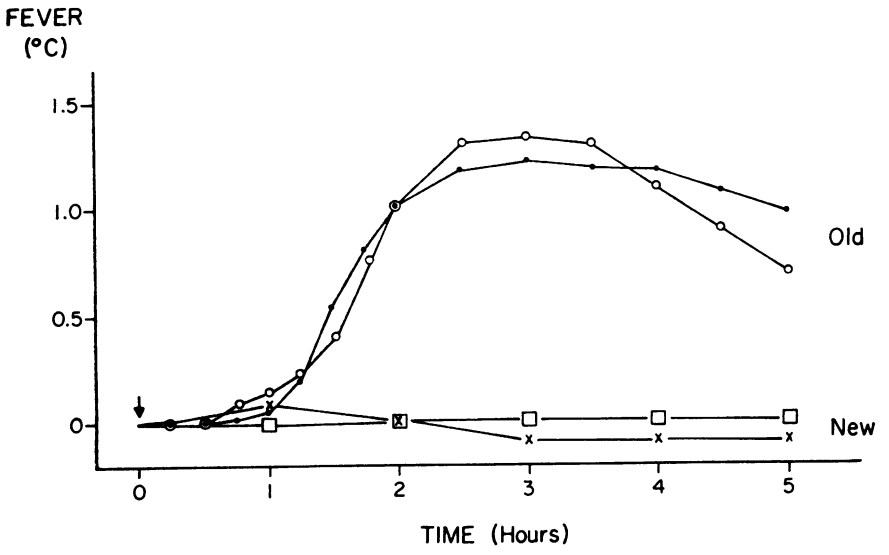

FIG. 8. Individual responses of 4 rabbits (2 "old" and 2 "new") to 500 μg teichoic acid (see Text for details).

upon previous sensitization, as with materials derived from filtrates of growing cultures or disrupted cells.[2-5]

DISCUSSION

These studies were designed to supplement earlier work[1-5] which indicated that particles and culture filtrates of staphylococci are both capable of inducing fever in rabbits. Since the intravenous inoculation of washed, bacterial cells was immediately followed by a transient but brisk leukopenia, and circulating staphylococci are known to be rapidly phagocyted by granulocytes,[8] it was inferred that phagocytosis had evoked the release of an endogenous pyrogen (EP) from the leukocytes of the host. The second pyrogenic agent was present in filtrates of staphylococcal cultures. Unlike

the cells, it caused higher febrile responses in specifically sensitized rabbits. This agent was determined to be a protein antigen which produced specific immunologic reactions of the delayed type that also resulted in the release of a circulating EP.[2,4,5]

The data presented here further confirm the basic difference in the mechanism by which bacterial cells and filterable antigens produce fever. Staphylococcal cell walls, free of surface and cellular protein caused fever in unsensitized rabbits. Treatment of the cell walls with hot TCA removed teichoic acid, the species-specific carbohydrate antigen present in the cell wall. The residual structured mucopeptide possessed the full pyrogenicity of the original cell walls. However, fever-inducing properties of the mucopeptide were destroyed by lysozyme digestion.

These findings suggest that staphylococcal cell walls are pyrogenic by virtue of their particularity, and also that phagocytosis is the mechanism for EP production. It is of interest that the mucopeptide of bacterial cell walls has been recently reported to have immunologic activity.[9] If the lysozyme-susceptible muramic acid-glucosamine bond is involved in the antigenicity of the mucopeptide, an immunologic mechanism might play a role in "activating" leukocytes to release EP by promoting phagocytosis of these particles. Since the mucopeptides of both Gram-positive and Gram-negative organisms are similar in chemical composition, cross-reacting antibodies to mucopeptide may be present in normal animals. Under these circumstances, therefore, specific sensitization would not be required to set in operation the chain of events leading to EP production and fever.

Although phagocytosis of heat-killed pneumococci[10] as well as staphylococci[11] by blood leukocytes *in vitro* results in release of EP, it has been reported that certain agents may be phagocyted without activating leukocytes to produce this substance. Preparations of polystyrene latex particles of the same approximate size as staphylococci, treated to remove contaminating bacterial pyrogen, are not pyrogenic when injected in equal amounts intravenously[10,12] and, similarly, do not produce detectable amounts of EP when they are incubated with and presumably phagocyted by leukocytes *in vitro*.[10] Why phagocyting cells are activated to release EP in certain conditions but not others is unknown. Further studies employing other inert particles will be necessary to determine whether previously mentioned immunological agents, present in microbial particles, play a critical role. In instances where soluble antigens are activators of cells, it seems likely that EP production, on the other hand, may occur independently of some of the metabolic activities associated with phagocytosis and granule lysis.

The preliminary data presented here with teichoic acid, a main constituent of the staphylococcal cell wall and an antigen, suggest that immunological factors associated with the particle could perhaps contribute to fever in specifically sensitized recipients. Highly immunized rabbits reacted to this agent with delayed fevers similar to those induced by intact cells, whereas relatively unexposed rabbits were unresponsive. It is difficult to evaluate the contribution of this agent to producing the febrile responses to intact cells, however, because the dosage of carbohydrate injected was derived from a large number of cells (2×10^{10}) as compared with the minimal pyrogenic dose of intact cocci, which is only $1\text{-}5 \times 10^{7}$.[1]

There are certain features that clearly distinguish the febrile response to culture filtrates and particles. When given daily, culture filtrates rapidly induce a nearly complete pyrogenic tolerance (see Fig. 3) as compared with the slight reduction in pyrogenic response brought about by daily administration of cell walls (Fig. 1), a response similar to that shown previously with intact cocci or Gram-positive bacilli.[1] More conclusively, rabbits made tolerant to culture filtrates react normally to the cell wall preparations (see Fig. 3). Since tolerance to culture filtrates has been previously shown to be due to desensitization,[3,4] these data provide additional evidence that antigens present in these media, at least, do not play a significant role in particle fever.

The pyrogen derived from filtrates of cells disrupted either mechanically or enzymatically appeared to be similar to the agent in culture filtrates, both in its pyrogenic action and in its ability to induce an almost complete tolerance when given daily (see Fig. 4). By utilizing the device of pyrogenic cross-tolerance for detecting immunological relationships,[4,13] these studies confirm earlier work[5] that the agent or agents within disrupted staphylococci are antigens similar, but not identical, to those released by growing cultures (see Table I and Fig. 5). Previous biochemical evidence and cross-tolerance data suggested that the pyrogen appearing in staphylococcal broth culture filtrates was a protein antigen with broad specificity among human pathogenic strains, including both Giorgio and 80-81 strains used here.[5] The pyrogen released by disrupted cells, however, appeared to be predominantly nucleoprotein and in the studies reported here produced fever only in intensively immunized rabbits (see Fig. 6).

In summary, these studies indicate that staphylococci evoke two basic defense mechanisms of the host—phagocytosis and the immune response —that are capable of inducing fever. Natural infection in rabbits presumably sensitizes these hosts to soluble antigens liberated by growing or disrupted microorganisms. On the other hand, phagocytosis of bacterial

cells, which also occurs in the course of infection, causes fever by a mechanism that appears to be largely or wholly independent of immunity. Whether the pyrogenic lipopolysaccharide "endotoxins" which constitute the cell wall of virtually all Gram-negative microorganisms also produce their characteristic signs of toxicity, including fever, by acting as antigens in hosts naturally sensitized by the presence of these bacteria in the gastrointestinal tract, remains an attractive but as yet unproven hypothesis.[14] The more rapid onset of fever following intravenous inoculation of endotoxins as compared with known antigens, both bacterial and nonbacterial, suggests, however, that endotoxins may produce some of their effects, at least, by a direct toxic effect on host tissues.[15] Since animals with induced tolerance to endotoxins respond fully to small doses of either cell walls (see Fig. 1) or culture filtrates of staphylococci,[8] it seems unlikely that the fever-inducing activity of these Gram-positive microorganisms can be in any way attributed to endotoxin-like agents, either associated with the cell surface or within the cytoplasm of the cell.

SUMMARY

Further studies have been carried out with two strains of staphylococci (Giorgio and 80-81) to elucidate the mechanisms by which these microorganisms cause fever in rabbits.

On intravenous injection, staphylococcal cell wall preparations, as well as several soluble fractions derived from staphylococci, were found to produce similar fevers after a latency of 45 to 60 minutes.

Since disruption of the mucopeptide structure of the cell walls abolished their ability to cause fever, it was inferred that phagocytosis of the particles was essential for their pyrogenicity.

Pyrogenic cross-tolerance studies indicated that soluble agents liberated by disrupted staphylococci were similar to those released by growing cultures. Lack of cross-tolerance between the cell walls and these soluble agents, on the other hand, supplies additional evidence that the staphylococcus produces fever by means of at least two different mechanisms—one involving phagocytosis of the intact cell, and the other, an immunological reaction to a protein antigen present in the cell cytoplasm and released by multiplying bacteria into the culture medium.

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