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STUDIES IN STAPHYLOCOCCAL FEVER

V. Staphlococcal Filtrate Pyrogen†

Broth culture filtrates from several strains of *Staphylococcus aureus* produce fever when injected intravenously into normal or previously sensitized rabbits.[1] This response appears to depend upon a naturally acquired state of delayed hypersensitivity, which may be increased with specific infection.[2] Rabbits made unreactive (tolerant) to filtrate from one staphylococcal strain were tolerant to filtrate from two other strains,[2] so that a common antigen may be responsible for the pyrogenic activity of different staphylococcal filtrates. Since only actively multiplying staphylococci release pyrogen,[1] this agent may be an "exotoxin."

Many staphylococcal toxins have been identified, yet few influence the manifestations or pathogenesis of staphylococcal infection.[3] Furthermore, although staphylococcal infection in man produces both circulating antibody and a state of delayed hypersensitivity to various staphylococcal antigens, the influence of these host responses on either the development or the symptomatology of staphylococcal disease is not clear.[4] Since fever is such a prominent manifestation of both infection and hypersensitivity, a staphylococcal product that produces fever by a hypersensitivity reaction might also have other effects on the course of staphylococcal infections. It seemed of interest, therefore, to study further the pyrogen produced in staphylococcal filtrates in an attempt to characterize its chemical nature, its relation to known staphylococcal antigens, and to known filtrable products or toxins of the staphylococcus.

MATERIALS AND METHODS

All materials and techniques were similar to those previously employed[5] unless otherwise stated. All filtrates from Giorgio strain cultures were tested in normal rabbits; those from 80-81 strain cultures were tested in rabbits previously sensitized by infection with 80-81 strain staphylococci.[2]

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Cultures. Two strains of *Staphylococcus aureus* were used, belonging to the following phage types: (a) 83 (VA-4) strain Giorgio, and (b) 80-81. The culture characteristics of these strains have been previously described.[5] Broth filtrates were prepared from 18-hour cultures of bacteria in pyrogen-free beef heart infusion broth. Cultures grown in the presence of CO_2 included both stationary tubes in an atmosphere of 5 per cent CO_2, and cultures through which 5 per cent CO_2—95 per cent O_2 was bubbled for 18 hours.

Disintegrated cell filtrate. Eighteen-hour broth cultures of bacteria were centrifuged and the cells washed once in pyrogen-free saline. The cells were then resuspended in a small volume of saline (one fourth that of the original culture). Seven and one half milliliters of this suspension were transferred to each Mickle glass vessel, and 5 gm. of No. 080 glass beads added. The vessels were closed with rubber stoppers (previously autoclaved for two hours), and vibrated on the Mickle disintegrator* for one half to one hour. When disintegration was complete (as indicated by gram stain), the filtrate was recovered by passage through an ultrafine sintered glass filter.** The filtrate usually contained one third to one half the total bacterial protein,*** as determined by the method of Lowry.[6]

Fractionation of broth filtrate. The following modification of Verwey's method was used.[7] An 18-hour broth filtrate was adjusted to pH 3.5 with 0.1N HCl. After standing at 4° C. for one to 18 hours, 50 per cent trichloracetic acid was added to a final concentration of 15 per cent. The precipitate was removed by centrifugation, dissolved in a small volume of saline, adjusted to pH 6 to 7 with 0.1N NaOH, and 4 to 6 volumes of absolute alcohol added. After standing overnight at 4° C., the precipitate was recovered by centrifugation, dissolved in saline, and the pH adjusted to 7. A nonprotein fraction from the trichloracetic acid supernate was recovered when the pH was adjusted to 6 to 7 with 0.1N NaOH, 6 to 8 volumes of alcohol added, and the precipitate allowed to form for two to three days at 4° C. This precipitate was dissolved in saline, and sometimes reprecipitated with alcohol.

Extraction of broth filtrate with phenol. Thirty milliliters of filtrate was lyophilized, and reconstituted with 5 ml. of distilled water. To this was added 5 ml. of 50 per cent phenol in H_2O (w/v), and the mixture incubated at 65° C. for half an hour. The two phases were separated while the tube remained in the water bath (a small ring of precipitate at the interface was discarded). To each solution, six volumes of absolute alcohol were added, and the precipitates allowed to form at 4° C. overnight. These were recovered by centrifugation, redissolved in 5 ml. H_2O, and reprecipitated with alcohol. Following the second precipitation, complete recovery of material from the aqueous phase required centrifugation at 20,000 g. for one hour. The final precipitates were dissolved in saline, adjusted to pH 7, and passed through an ultrafine sintered glass filter.

Proteolysis and periodate oxidation. The methods used were those of Rafter, Collins, and Wood[8] with the following changes. One milliliter of broth filtrate undiluted or diluted 1:4 was used as starting material. Dialysis of the periodate mixture after

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* H. Mickle, Hampton, Middlesex. Distributor Brinkman Instruments, Westbury, N. Y.

** Morton bacteriological filter apparatus with ultrafine fritted disc (Corning).[5]

*** Kindly determined by Dr. David Seligson, Director of Clinical Laboratories, Yale-New Haven Medical Center.

TABLE. 1. GIORGIO FILTRATE PYROGEN

Filtrate Volume injected (ml.)	*Procedure**	*Average* fever index*	*No. of rabbits*
1.0	None	31.3	10
2.0**	TCA precipitin	39.5	2
6.0**	TCA supernatant (alcohol precipitate)	1.7	1
3.0**	Phenol soluble	25.8	2
6.0**	Phenol insoluble	0.8	2†
1.0	Control for trypsin (pH 8.0)	27.0	2
1.0	Trypsin (pH 8.0)	4.1	2†
0.5	KIO_4 (pH 5.2)	34.4	2
0.25	Pepsin (pH 4.0)	21.7	2
0.5	None	25.5	4
0.5	56° for 30 min.	15.4	4†
0.5	56° for 1 hr.	22.2	4†
0.5	pH 8.5 at 37° for 18 hrs.	15.0	4†
1.0	None	29.0	2
1.0	pH 2.0 at 37° for 18 hrs.	26.8	2†
0.25	None	22.5	2
1.0	Anaerobic growth	2.1	2†
5.0	Anaerobic growth	27.8	2†
0.5	None	27.6	2
0.5	Growth with CO_2	14.3	2†

* See Materials and Methods.
** Represents the initial volume of filtrate from which the injected material was derived.
† The same rabbits as in the preceding experiment.

incubation was omitted. Control tubes contained no enzymes (or periodate). The pepsin,* trypsin,** and periodate preparations had previously been shown to be non-pyrogenic by injection of equal amounts into rabbits.

Coagulase titer. Coagulase titer of broth filtrates was determined by addition of 0.3 ml. of reconstituted rabbit citrated plasma (Baltimore Biol. Labs.) to 0.5 ml. of serial twofold dilutions of filtrate in saline. Tubes were incubated overnight at 37° C., and clotting measured as 1 to 4+. Titer was expressed as the reciprocal of the dilution giving a 1+ clotting reaction.

Fever index. All calculations were done using the method of Beeson.[9] Fever curves were plotted on one-sixth inch graph paper, with ordinate 1 inch/0.6° C. and abscissa 1 inch/hour. The area measured was from injection to four hours after injection.

* Pepsin, 3x crystallized, Nutritional Biochemicals, Cleveland, Ohio.
** Trypsin, 2x crystallized, Worthington Chemical Co., Freehold, N. J.

RESULTS

Several different procedures were carried out to examine the chemical behavior of the staphylococcal pyrogen and the conditions of microbial growth required for its production. The results are summarized in Table 1. These studies were done with filtrates from the Giorgio strain, since large amounts of pyrogen were present in this filtrate and could be detected by injection into normal rabbits.[1] Most of the same procedures were also carried out with culture filtrates from 80-81 staphylococci ordinarily pyrogenic only in specifically sensitized rabbits.[2] Without exception, the pyrogens from the two different strains behaved similarly.

Chemical and physical properties

Pyrogen was nondialyzable and could be recovered in successive precipitations with 4 to 6 volumes of alcohol. Verwey's fractionation (see METHODS) was used in an attempt to obtain protein, nucleoprotein and carbohydrate fractions. Acidification of filtrate to pH 3.5 produced no precipitate, but when trichloracetic acid was added, a dense precipitate formed. This material, when dissolved in saline and injected into normal rabbits, appeared to contain roughly the same amount of pyrogen as was present in the whole original filtrate (Table 1). The latent period and the shape of the fever curve of this material were identical with that of the whole filtrate. Alcohol precipitation of the trichloracetic acid supernate produced no additional pyrogen (Table 1).

During some early studies it was noted that filtration of some saline solutions of trichloracetic acid-precipitated material through ultrafine sintered glass filters removed the pyrogen. Since a variety of nonfiltrable particles can produce fever,[5] it seemed possible that the trichloracetic acid-precipitated material might be causing fever on this basis. However, when the saline-dissolved material was adjusted from pH 4.5 to pH 7.5 and warmed, pyrogen passed through the filter. This observation suggested that the pyrogen molecules aggregate at low pH.

Attempts to recover pyrogen from staphylococcal filtrates by extraction with phenol were initially unsuccessful, since both phenol-soluble and phenol-insoluble fractions had no pyrogenicity. However, when filtrate was concentrated before extraction (see METHODS) pyrogen was recovered in the phenol phase but not in the aqueous phase (Table 1).

Further proof of the protein nature of the pyrogen was obtained by trypsin inactivation. Several attempts to inactivate it with pepsin were unsuccessful. Treatment with potassium periodate did not remove the pyrogenicity (Table 1).

The stability of the pyrogen to heat and to pH changes was next investigated. The same group of rabbits was injected in turn with one milliliter of a 1 : 2 dilution of Giorgio filtrate unheated, heated at 56° C. for 30 minutes, and at 56° C. for 1 hour. The injectons were given at 0, 3, and 7 days to prevent development of tolerance in the recipients. As seen in Table 1, only slight loss of pyrogenicity resulted from heating. There was apparently greater loss of activity by heating at 30 minutes than by heating for one hour. This paradoxical heat sensitivity has been described for other staphylococcal proteins, including alpha and beta hemolysins.[4] Autoclaving for ten minutes has previously been reported to reduce pyrogenic activity of Giorgio filtrate to 1/5—1/10 of its original value.[1] Trichloracetic acid-precipitated pyrogen from filtrate showed little loss of activity when heated at 56° C. for one hour.

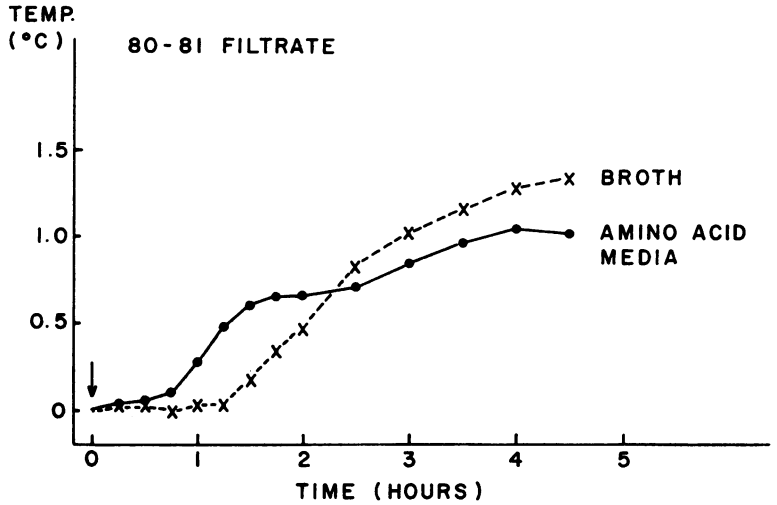

FIG. 1. Average fevers of three rabbits to injection of 1 ml. of 80-81 broth culture filtrate, and 3 or 5 ml. of 80-81 amino-acid media culture filtrate.

Broth filtrate incubated at 37° C. for 18 hours at pH 8.5 showed significant loss of pyrogenic activity. No inactivation of pyrogen occurred with similar incubation at pH 2.0 (Table 1).

Pyrogen production

Cultures grown in an atmosphere of 5 per cent CO_2, or with 5 per cent CO_2 bubbled through them did not show an increase in pyrogen over the amount in usual broth filtrates in spite of comparable or higher colony

counts. Under anaerobic conditions of growth, pyrogen production by staphylococci was much reduced. Pyrogen could be detected, however, when five times the usual dosage of filtrate was injected (Table 1). Although the colony count of the anaerobic culture was only 2 x 10^8/ml., this number of organisms growing normally in broth or serum has, in previous studies, produced nearly maximum amounts of pyrogen.[1] Thus, anaerobic conditions partially inhibited pyrogen production.

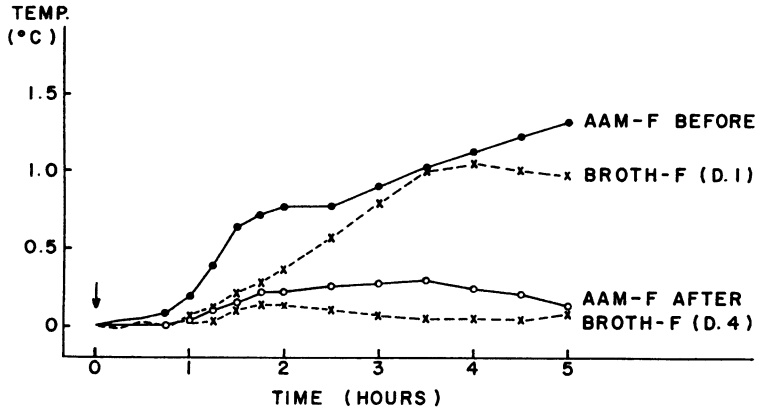

FIG. 2. Average fevers of a group of three rabbits. The curves with dotted lines indicate the responses to 80-81 broth culture filtrate on days 1 and 4 of a series of four daily injections. Two rabbits received 2 ml. of filtrate repeatedly, and the other 4 ml. The curves with solid lines indicate the responses to 3 or 5 ml. of 80-81 amino acid media filtrate (AAM-F) before and one day after the last injection of broth filtrate.

An attempt was made to obtain the filtrate pyrogen in a nonprotein medium to characterize it further. For this purpose, an amino-acid medium was prepared, as described by Fildes, *et al.*[10] and Gladstone,[11] containing only amino acids, salts, nicotinic acid, thiamine, and glucose. In five milliliter dosages, this medium was nonpyrogenic. Both 80-81 and Giorgio staphylococci grew in this medium, although the bacterial counts after one to three days' growth usually did not exceed 5 x 10^7 per ml. Pyrogen was consistently present in the 80-81 culture filtrate after two to three days incubation, and was occasionally present in Giorgio filtrate. Three to five milliliters of amino-acid media culture filtrate produced about the same fever as 1 milliliter of 80-81 broth-culture filtrate. As seen in Figure 1, the fever curves produced by the amino-acid media culture filtrate had a somewhat different shape from those produced by broth-culture filtrate. Fever frequently began earlier, and an initial peak of fever was noted at

about 1 to 1½ hours. Nevertheless, the similarity of the pyrogens from these two culture filtrates was shown by a cross-tolerance experiment (Fig. 2). In this small group of rabbits, cross-tolerance was nearly complete.

The pyrogen produced in amino-acid media appeared to have certain characteristics similar to those of a pyrogen obtained from disrupted 80-81 staphylococcal cells. This pyrogen almost always produced fever that

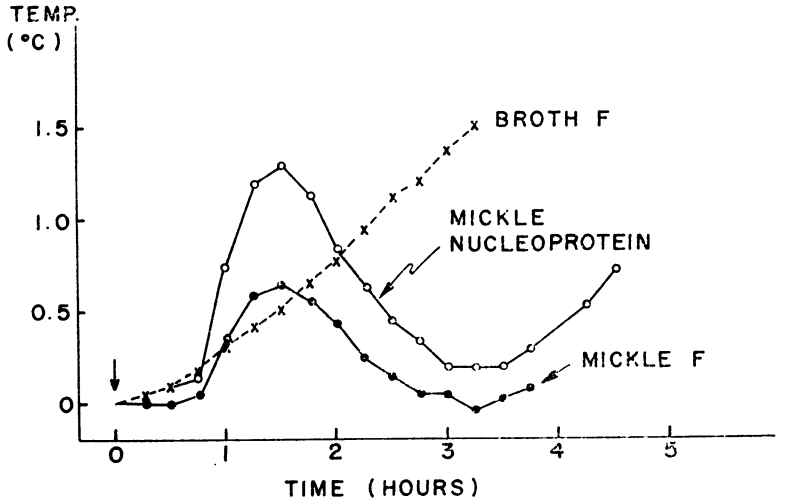

FIG. 3. Febrile responses of one rabbit, infected several months before with 3 x 10^7 80-81 staphylococci intravenously, to injection of three filtrates derived from 80-81 staphylococci. These are 1 ml. of broth culture filtrate, 2 ml. of Mickle filtrate, and the precipitate at pH 3.5 from 3 ml. of Mickle filtrate, redissolved in saline (Mickle nucleoprotein).

began before one hour and was maximal at one to two hours (Fig. 3). Certain characteristics of the staphylococcal pyrogens obtained from these three sources—broth, amino-acid media, and Mickle filtrate—are listed in Table 2 for comparison. The pyrogens from the second and third sources resemble each other in duration of latent period before fever and in the shape of the fever curve. In addition, Mickle filtrate or amino-acid media filtrate produced variable responses in rabbits that were tolerant (by daily inoculation) to broth filtrate. Although most rabbits were unreactive (tolerant), occasional rabbits had small or even normal fevers, indicating lack of complete cross-tolerance at these dosages. Pyrogen from Mickle filtrate precipitated almost entirely at pH 3.5, a behavior characteristic of nucleoprotein, in contrast to the broth-filtrate pyrogen, which precipitated

only with trichloracetic acid. No precipitate was ever obtained from amino-acid media filtrate.

Amino-acid media pyrogen was not affected by heating at 56° for one hour. A protein determination by the method of Lowry[6] was carried out on amino-acid media culture filtrate previously dialyzed against water to remove all free tyrosine. Less than 250 μg/ml. (the lowest standard used) was present.

TABLE 2. STAPHYLOCOCCAL FILTRATE PYROGENS

Source	*Strain*	*Latent period preceding fever**	*Time of maximum height of fever**	*Cross-tolerance with broth filtrate, same strain*	*Precipitated by*
Broth	Giorgio, 80-81	1 hr.	4-5 hrs.	complete	Trichloracetic acid
Amino-acid media	80-81	30-45 min.	1½ hrs + 4-5 hrs.	(?) partial	—
"Mickle" disrupted staphylococci	Giorgio, 80-81	30-45 min.	1½ hrs. (large amounts also 4-5 hrs.)	partial	HCl pH 3.5

* Estimated from fever responses of approximately equal F.I.

Fifty milliliters of 80-81 amino-acid media filtrate was dialyzed against pyrogen-free water, lyophilized, redissolved in five milliliters of saline, and an ultra-violet absorption spectrum obtained* (Fig. 4). A broad band of absorption occurred between 250 and 270 mμ., and absorption below 230 mμ. This absorption band suggests the presence of nucleic acids, although aromatic amino acids may also absorb in this region. The ratio of optical density at 260/280 is 1.75. This concentrated material was strongly pyrogenic. Unfortunately, insufficient material was available to attempt further characterization of the pyrogen.

Alpha hemolysin

The relation of the pyrogen to Giorgio filtrate hemolysin was first examined. Giorgio filtrate was found to lyse sheep and rabbit red cells but not human red cells, thus suggesting the presence of alpha hemolysin.

* Kindly performed by Dr. Yash Myer, Department of Biochemistry.

Its action against sheep cells was not augmented by incubation at zero degrees, and no lysin was obtained from anaerobic cultures, thus giving no evidence for the presence of beta hemolysin. The lack of hemolytic activity against human red cells indicated that delta hemolysin was also not present. The heat stability of the Giorgio filtrate lysin was greater than that usually described for crude alpha toxin. Only one quarter to one

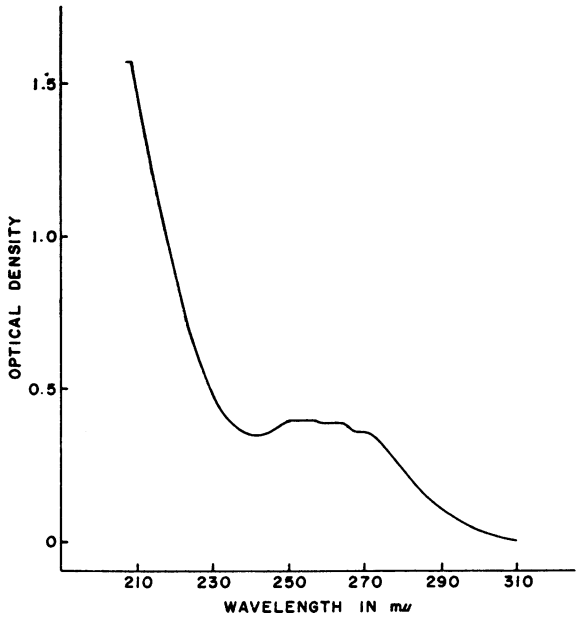

FIG. 4. Ultraviolet absorption of dialyzed, 10x concentrated, 80-81 amino-acid media filtrate.

third of the activity was lost by heating at 56° for 30 minutes. However, it has been reported that different preparations of alpha hemolysin vary considerably in their heat stability.[4] The presence of a small amount (titer < 8) of alpha toxin in Giorgio filtrate was confirmed in another laboratory;* none could be detected in 80-81 filtrate.

The similar heat stability of the pyrogen and of the hemolysin in Giorgio filtrate, and the presence of larger amounts of both pyrogenic and hemolytic activity in Giorgio filtrate as compared to 80-81 filtrate suggested that the pyrogen might be alpha hemolysin. However, considerable

* Through the kindness of Dr. Keiichi Goshi, Department of Medicine, Medical College of Virginia, Richmond.

evidence against this hypothesis was obtained: 1.) Pyrogen was present without detectable hemolysin in anaerobic cultures. 2.) Giorgio filtrate pyrogen was not inactivated when incubated with excess alpha hemolysin antitoxin.* 3.) A purified sample of staphylococcal alpha toxin** was found to be completely nonpyrogenic. Three rabbits that received an injection of alpha toxin containing the equivalent hemolytic activity of eight milliliters of Giorgio filtrate had an average fever index of 1.9.

Leucocidin

The possibility that leucocidin was the pyrogen was attractive, since this substance is known to injure rabbit white cells and thus might produce fever by stimulating release of endogenous pyrogen. However, staphylococcal leucocidin in culture filtrates is inactivated by heating at 56° C. for 30 minutes,[13] unlike our pyrogen. Furthermore, assays of leucocidin carried out according to the method of Gladstone[13] failed to reveal more than a trace of material damaging to human white blood cells in either Giorgio or 80-81 filtrates, and the observed changes were not those reported to be characteristic of leucocidin.*** This was expected, since staphylococci growing in stationary broth cultures reportedly produce very little leucocidin.[12]

Hyaluronidase

Staphylococcal hyaluronidase has been shown to be irreversibly inactivated by treatment with acetate buffer at pH 4.5.[13,14] To one volume of Giorgio filtrate was added two volumes of acetate buffer pH 4.5, and the tube was incubated at 37° C. for half an hour. The pH was then adjusted to 7.0, and 1.5 ml. given to each rabbit (containing 0.5 ml. Giorgio filtrate). No loss of pyrogenicity occurred.

Coagulase

Many properties of the filtrate pyrogen are similar to those that have been described for staphylococcal coagulase, including heat stability in crude preparations, loss of activity at alkaline pH, and precipitation with

* Giorgio filtrate was incubated with an equal volume of 1:10, 1:50, 1:100, or 1:250 dilution of alpha toxin antiserum (anti-hemolysin titer $>$ 1,000 against 0.5 ml. Giorgio filtrate) and tested for pyrogenicity. After variable incubation (½-2 hrs.) with 1:100 or 1:250 antiserum, centrifugation at 20,000 g. for one hour did not remove the pyrogen.

** Kindly provided by Dr. Alan W. Bernheimer, Department of Microbiology, New York University.

*** Fifteen to 30 minutes after addition of broth filtrate in dilution $<$1:10, rounding of cells and cytoplasmic extrusions were noted. No granule lysis was ever observed.

trichloracetic acid and alcohol.[15] However, coagulases from different or even single staphylococcal strains vary antigenically,[16,17] whereas by cross-tolerance experiments, staphylococcal pyrogen from three different strains appeared to be antigenically the same.[2] In many studies, pyrogenic activity of filtrate was very difficult to separate from coagulase activity. For example, filtrates with higher titers of coagulase appeared also to have greater amounts of pyrogen. Inactivation of pyrogen by heat or by incuba-

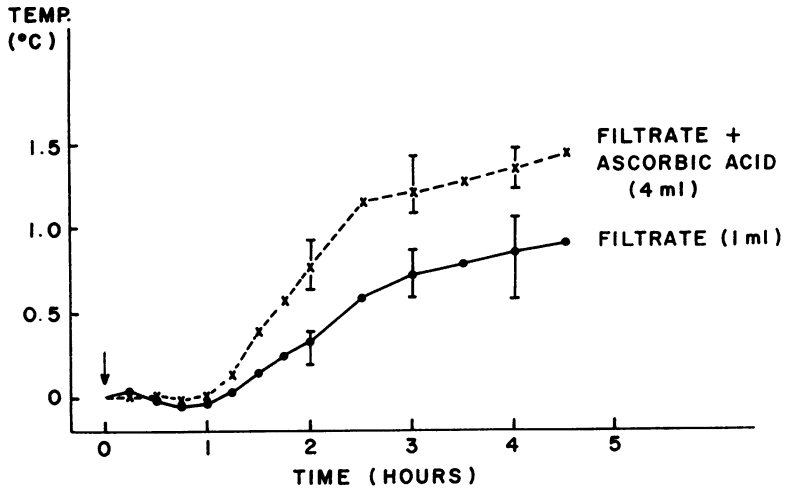

FIG. 5. Average fevers of a group of four rabbits to injection of two preparations of the same 80-81 broth culture filtrate. The solid line curve indicates the responses to injection of 1 ml. of filtrate with a coagulase titer of 16. The dotted line curve indicates the responses to 4 ml. of ascorbic acid-treated filtrate with a coagulase titer of 4. The bars indicate the total variation of responses at each given time.

tion at alkaline pH produced quantitatively similar loss of coagulase activity. Nevertheless, the two activities appear to be separate for the following reasons:

1. Despite the presence of pyrogen in amino-acid media filtrate, no coagulase activity was detectable. The medium itself had no inhibitory effect on clotting of rabbit plasma when coagulase-containing materials were added. Our observation confirms the original observation of Gladstone[11] that coagulase is not produced in this medium.

2. In several experiments, filtrates from either 80-81 or Giorgio strains were selected that differed as much as six to eightfold in coagulase titer. When two filtrates of the same strain were tested in a group of rabbits, in dosages containing equal amounts of coagulase, more pyrogen was

consistently present in the larger volume of filtrate. However, the difference in pyrogenicity was small.

3. Inactivation of coagulase by ascorbic acid[15] proved to be the most successful method for separating pyrogenic activity from coagulase activity (Fig. 5). In this experiment, 80-81 broth filtrate was obtained with a coagulase titer of 16. When 75 mg. of ascorbic acid* was added to 50 milliliters of filtrate, and the filtrate incubated at 37° for 36 hours, coagulase titer was reduced to four. A group of rabbits then received one milliliter of original filtrate, and two days later four milliliters of ascorbic acid-inactivated filtrate. Repeat coagulase titers at the time of injection showed the same fourfold difference in coagulase activity. As seen in Figure 5, more pyrogen was present in the larger volume of ascorbic acid-inactivated filtrate, although it contained the same coagulase activity as the smaller volume of untreated filtrate. From previous studies** it can be estimated that this difference in pyrogenicity is about fivefold.

DISCUSSION

Certain physical and chemical properties of the pyrogen found in staphylococcal broth filtrate have been described. The pyrogen is quite heat stable, but appears to be inactivated more by heating for 30 minutes than for one hour. Similar paradoxical behavior of crude preparations of alpha toxin has been attributed to the presence of a relatively heat stable inactivator. Such a factor could be operating in our preparations. The pyrogen is not destroyed by incubation at acid pH, but is partially inactivated at alkaline pH. The pyrogen behaves like a protein rather than a carbohydrate since it is found in trichloracetic-acid precipitate but not in the supernatant fluid, is soluble in phenol, and is inactivated by incubation with trypsin but not potassium periodate.

Although different stapylococcal strains release a variety of products into the culture medium during growth, evidence from cross-tolerance experiments suggests that pyrogen produced by three different staphylococcal strains is antigenically the same, although produced in varying amounts.[2] During studies of the physical and chemical behavior of the broth-filtrate pyrogen, filtrates from both 80-81 and Giorgio strains were used in most experiments, and both pyrogens were found to have the same properties. Two other pyrogenic substances from the staphylococcus were also investigated, one found in culture filtrate of 80-81 stapylococci grown

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* U.S. Vitamin and Pharmaceutical Corp., New York, N. Y., ampul for I.V. injection.

** See Fig. 3, ref. 1.

in an amino-acid medium, and one derived from disrupted washed 80-81 staphylococcal cells. Nearly complete cross-tolerance was observed between both these pyrogens and 80-81 broth culture filtrate pyrogen. Other studies* with the Giorgio strain have similarly shown nearly complete cross-tolerance between pyrogen from broth-culture filtrates and filtrates of Mickle-disrupted cells. Thus, there appears to be an antigenic similarity between pyrogens from different preparations of the same strain. However, the pyrogen from disrupted staphylococcal cells precipitated mainly with the nucleoprotein fraction at pH 3.5, and the slope of the fever curve differed from that of broth-filtrate pyrogen, suggesting a different action *in vivo*. This evidence, although obviously inconclusive, suggests that an antigen similar to that released into broth during growth is also present in the staphylococcal cell, perhaps associated with nucleoprotein.

The possibility that the pyrogen was one of the commonly described toxins of the staphylococcus was investigated. No evidence could be found to identify it with six other staphylococcal proteins—alpha, beta, or delta hemolysins, leucocidin, hyaluronidase, or coagulase. In many respects, however, the pyrogen appeared to have properties similar to those of the protein, coagulase. Recently, enterotoxin was reported to be pyrogenic in cats.[18] The preparation used was a highly purified sample containing only a single antigenic substance. The enterotoxin pyrogen appeared to be distinct from *Salmonella typhosa* endotoxin. We do not know if our staphylococcal strains produce enterotoxin, but, since enterotoxin is trypsin-resistant,[19] it is unlikely that this substance is identical with the pyrogen reported here. It would be of interest to know whether the febrile reaction in cats was similarly mediated through a delayed hypersensitivity reaction, and if so, whether the responsible antigen was strain-specific.

A number of different staphylococcal antigens have been isolated and characterized. A surface antigen, primarily carbohydrate, that can be detected only in mucoid strains[20] has been described by Morse.[21] Other materials that seem clearly distinct from our pyrogen on the basis of chemical properties that characterize them as carbohydrates are polysaccharide A,[22] erythrocyte-coating antigen,[23] and the Rantz antigen.[24]

Verwey described a staphylococcal cell protein which was antigenic, with broad group specificity among human pathogenic strains.[7] Recently, several investigators have further characterized a staphylococcal protein antigen that appears to be the same as Verwey's.[25-28] Stamp[29] reported that in rabbits this protein produces protective antibodies against lethal infection with

* Studies made by the authors, and not yet published.

certain staphylococcal strains. Similar studies in mice were reported by Ekstedt[80] using a protein extract of a mucoid strain. Antibodies to Verwey's protein have been reported in normal human sera.[81]

There are many similarities between Verwey's antigen and our pyrogen.

1. Chemical methods of isolation are almost identical, except that the starting material for the protein antigen was ground staphylococcal cells.

2. The proteins isolated by Verwey and others have broad group specificity; our pyrogen has only been tested in three strains, but appears to have similar antigenic cross-reactivity.

3. Verwey[7] and Stamp[28] both reported antigenic similarities between the protein and nucleoprotein fractions in their preparations; similar results were obtained with the pyrogens we derived from different staphylococcal preparations.

4. The protein antigen, like our pyrogen, is inactivated by trypsin.[7, 35]

Grov, *et al.*[35] have reported that pure protein antigen (antigen A) does not adsorb to normal sheep red cells, whereas a nontrypsin-sensitive antigen, similar to the Rantz[34] antigen, will do so. Both antigen A and another trypsin-sensitive antigen, however, sensitize tanned sheep cells. We have been able to remove pyrogen from 80-81 broth culture filtrate by absorption with normal sheep cells. Further studies will be necessary to clarify both the numbers and types of staphylococcal antigens, and the relationship of our pyrogen to them.

The staphylococcal filtrate pyrogen is most likely a protein antigen which is released into the culture medium during growth, either by autolysis of staphylococcal cells or by actual production and release by growing cells. In rabbits, the febrile response to this pyrogen appears to be mediated by hypersensitivity of the delayed (cellular) type.[3] The exact mechanism by which such a reaction produces fever is not known. Presumably, injected antigen reacts with cell-associated antibody of the host as a preliminary step. This reaction, in turn, may activate the polymorphonuclear leukocyte, causing it to release an endogenous pyrogen, which has been detected in the circulation of rabbits with fever induced by culture filtrates.[1] We were unable to demonstrate any role of serum antibody in producing fever, either by passive transfer experiments or by correlation of circulating antibody titers with height of fever.[3] In preliminary experiments, the pyrogenicity of filtrate was not altered by incubation with immune serum followed by high speed centrifugation. It is possible that both circulating and cell-associated antibody are induced to the pyrogen during staphylococcal infection, but that only cell-associated

antibody and antigen are capable of activating the polymorphonuclear leukocyte to release endogenous pyrogen.

Other work in this laboratory[82] indicates that serum antibody does participate in the production of tuberculin fever in rabbits previously infected intravenously with BCG. The role of circulating antibody has been shown both by serum transfer and by experiments in which normal blood cells, incubated with old tuberculin in hypersensitive plasma, released endogenous pyrogen *in vitro*.

The importance of staphylococcal "toxins" in the production of staphylococcal disease is not clear. Delayed hypersensitivity to staphylococcal products occurs in normal rabbits[2] and in man,[33] presumably as a result of natural infection. Old tuberculin, a protein antigen of the tubercle bacillus, produces delayed hypersensitivity and also alters the progress of tuberculous disease.[34, 35] Both the staphylococcus and the tubercle bacillus tend to produce chronic infections, which are known to be particularly effective in inducing states of delayed hypersensitivity. Organisms that are either nonpathogenic or that produce acute limited infections in rabbits do not appear to induce states of delayed hypersensitivity under natural conditions.*

An attractive hypothesis is that the staphylococcal pyrogen described here is an antigen which, like old tuberculin, is produced and released during infection. Antigen-antibody reactions in the host may then induce responses that include fever and perhaps other manifestations of staphylococcal disease.

SUMMARY

The pyrogen present in broth culture filtrates of two staphylococcal strains appears to be a protein, since it precipitates with trichloracetic acid, is soluble in phenol, and is inactivated by trypsin. Other pyrogens, which appear to be antigenically similar by cross-tolerance experiments, were obtained from disintegrated staphylococcal cells and from filtrates of staphylococcal cultures grown in an amino acid medium. The pyrogen appears to be distinct from alpha, beta, or delta hemolysins, leucocidin, hyaluronidase, and coagulase. The similarities between this pyrogen and a previously described protein antigen of the staphylococcus suggest that a group-specific substance, released during growth, may induce antigen-antibody reactions that result in fever in the sensitized host.

* Unpublished observations.

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