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STUDIES IN STAPHYLOCOCCAL FEVER
III. Tolerance to Culture Filtrates**

In the preceding paper, certain characteristics of a filterable pyrogen produced by the Giorgio strain of *Staphylococcus aureus* were defined.[3] It was shown that this pyrogenic substance differs in a number of respects from the endotoxins of Gram-negative bacteria, notably in its greater heat lability and in its apparent association only with actively multiplying cells. Staphylococcal pyrogen could also be distinguished from Gram-negative endotoxin by certain characteristics of its pyrogenic activity, especially the greater length of the latent period before onset of fever.

Since rabbits given an infecting dose of staphylococci developed significant febrile responses to previously nonpyrogenic doses of staphylococcal culture filtrate, it was postulated that the response of normal rabbits to this agent is due to a naturally acquired, specific hypersensitivity.

In the present paper, certain features of tolerance to staphylococcal culture filtrate are presented and compared with tolerance occurring in other models of experimental fever.

METHODS

All materials and techniques were similar to those employed earlier.[1,2]

Cultures. Bacterial cells and filtrates of 18-hour broth cultures of *Staphylococcus aureus,* Giorgio strain, were employed. Characteristics of this organism, techniques for its culture and preparation of the culture filtrate have been described.[1,2]

Typhoid vaccine. Source of vaccine and technique of producing tolerance to this agent in rabbits were identical to those presented earlier.[2]

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RESULTS

Development of tolerance to staphylococcal culture filtrate and recovery

In a previous paper[3] it was shown that rabbits given daily injections of autoclaved staphylococcal cells respond with slight diminution in fever affecting the second peak of fever only. To determine whether there would be a similar reaction to pyrogen in culture filtrates of this organism, the following experiment was conducted.

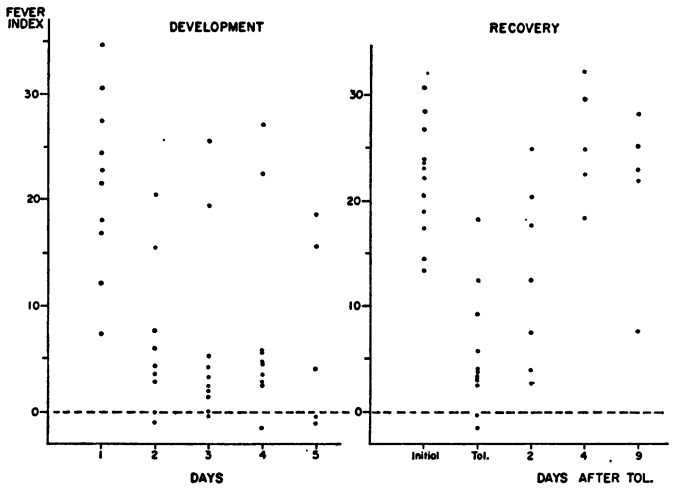

FIG. 1. Left: Fever indices of 10 rabbits given four daily injections of staphylococcal (Giorgio) culture filtrate. Only one-half the original group was injected on the fifth day. Right: Fever indices for initial and tolerant responses of a group of 12 rabbits to culture filtrate. Recovery values for seven rabbits are shown on the second day after cessation of injections and compared with responses of another group of five rabbits reinjected on days 4 and 9. Culture filtrate was regularly 1 ml. of 18-hour broth culture in this and following Figures except where indicated.

New rabbits were given a daily series of four or five injections of filtrate from a single pool of 18-hour broth culture. The fever curves of each animal were plotted for the entire series and the areas under these curves expressed numerically as fever indices.[3]

The results are presented in Figure 1. There was considerable individual variation in the initial response. Thereafter, 8 of the 10 animals developed a marked tolerance which appeared as early as the second day and persisted throughout the course of inoculations. The remaining two recipients continued to have considerable residual fevers after an initial fall of 40-50 per cent in fever index on the second day.

Under "Recovery" in Figure 1 are the combined results of several experiments showing recovery from tolerance in rabbits rechallenged once or twice with filtrate after being rested for varying intervals. Initial and tolerant responses are shown for comparison. Tolerance was induced in most animals by four daily injections (with a range of 3 to 10). It is evident that many rabbits recovered significant sensitivity by the second day, after only one day's rest. Five tolerant recipients responded normally when tested 4 and 9 days later. Two animals given a 10-day course of

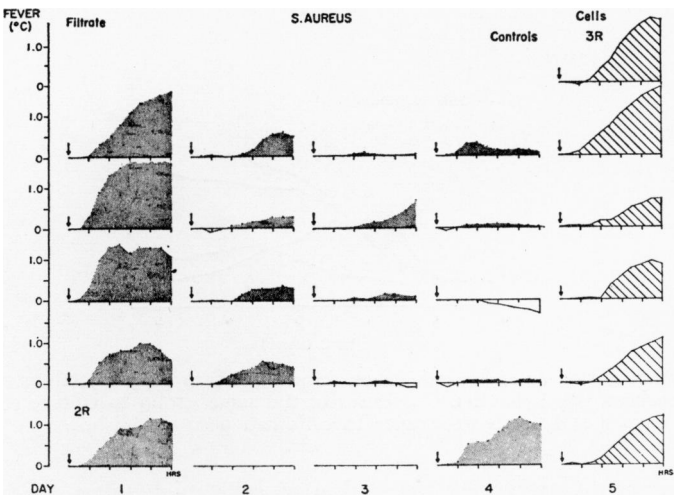

FIG. 2. Fever curves of four rabbits given daily injections of staphylococcal culture filtrate (Giorgio) followed on day 5 by inoculation of 6 x 10^8 washed live staphylococci. Mean responses of two controls given two spaced injections of filtrate only are shown on the bottom line. Average fever of three other rabbits given the same dose of cells without preceding inoculation of culture filtrate is shown at the upper right.

injections had the lowest responses on the second day, but had almost completely recovered on the 12th day.

Response of filtrate-tolerant recipients to staphylococci

The nearly complete tolerance which developed to staphylococcal culture filtrate on the second day contrasted with the persistent fevers produced by daily injections of cells and suggested that these pyrogenic factors were distinct. Accordingly, an experiment was devised to determine if cross-tolerance could be demonstrated between filtrates and cells of staphylococci.

Four rabbits were made tolerant to filtrate with a series of four daily inoculations. On the fifth day, after tolerance to filtrate had been con-

firmed, the rabbits were given a low dose of washed live staphylococci, as were two control animals which received the two spaced inoculations of filtrate only.

The results are shown in Figure 2. The responses of the four filtrate-tolerant rabbits to cells were comparable to those of the two controls as well as of three rabbits not previously injected with filtrate.

Response to filtrate after repeated inoculations of staphylococci

Although tolerance to staphylococcal filtrate did not confer tolerance to cells, it was thought advisable to reverse the order of injections to

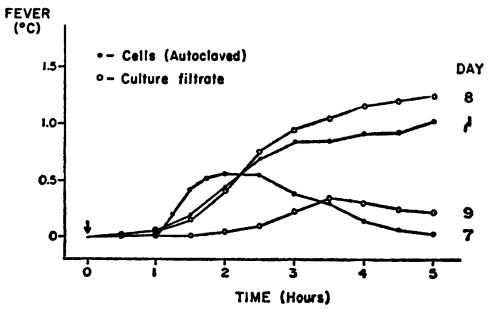

FIG. 3. Mean responses of five rabbits on days 1 and 7 to daily injections of 4 x 10^8 washed autoclaved staphylococci. Responses of the same group to culture filtrate are shown for days 8 and 9 after tolerance to cells had been established.

determine whether repeated inoculation of cells might alter the response to filtrate. A new group of recipients was therefore given a series of daily injections of autoclaved cells. The mean responses of these animals on the first and seventh days are shown in Figure 3. The group developed monophasic fevers on the second through the seventh day, characteristic of tolerance to cells. The mean response to filtrate given on the eighth day was unaffected by the preceding course of cells, although, in conformity with previous results, nearly complete tolerance developed to a second injection of filtrate the following day.

Lack of cross-tolerance between staphylococcal culture filtrate and Gram-negative bacterial endotoxin

Repeated inoculation of Gram-negative endotoxin also produces tolerance to its physiological effects, including fever, as well as to those of endotoxins from immunologically unrelated Gram-negative bacteria.[8] This form of tolerance appears to have a certain degree of specificity, however, in that

responses to most other pyrogenic agents are not modified by pre-existing tolerance to endotoxins.[4]

Endotoxin-tolerant recipients, however, have been shown to have moderately depressed pyrogenic reactions to a low dose of autoclaved

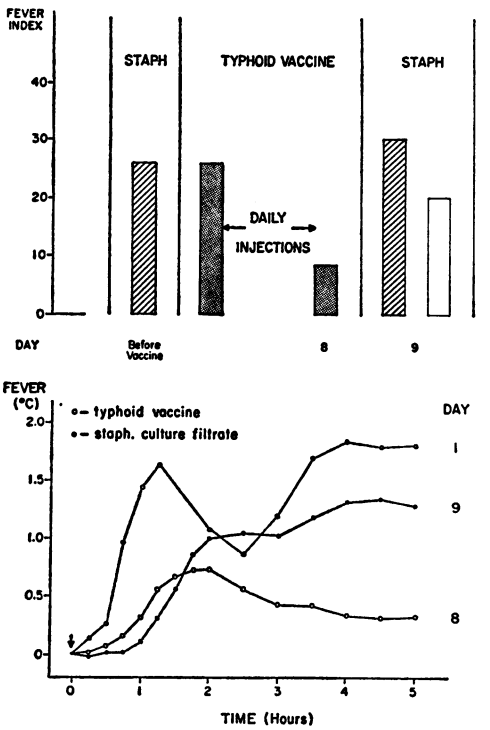

FIG. 4. Top (bar graph): Mean fever indices of five rabbits to 2 ml. staphylococcal serum culture supernate before and the day after tolerance had been established by eight daily injections of 1.5 ml. 1:10 dilution of typhoid vaccine (cross-hatched bars in center of chart). The plain bar at right represents average fever index of four controls on day 9. Bottom: Mean febrile responses of another group of five rabbits given eight daily inoculations of vaccine and injected the following day with 1 ml. staphylococcal broth culture filtrate.

staphylococcal cells[2] presumably by enhancing the removal of circulating staphylococci by the reticulo-endothelial system (RES).

In view of the slight but definite tolerance induced by endotoxins to staphylococcal cells, it seemed appropriate to test the response of normal and endotoxin-tolerant recipients to a dose of staphylococcal culture filtrate. Accordingly, a group of rabbits was rendered tolerant by daily inoculation of typhoid vaccine (see METHODS). Although the prolonged

latencies and low monophasic fevers of the group on the eighth day indicated a significant degree of tolerance to vaccine (see lower half of Figure 4), these rabbits had normal responses to staphylococcal filtrate on the following day. Similar results were obtained in another experiment in which supernate from staphylococcal cultures were used. As shown in the bar graph at the top of the same Figure, the mean fever index of this group of rabbits to staphylococcal culture supernates remained unchanged when compared before and after induction of tolerance to typhoid vaccine.

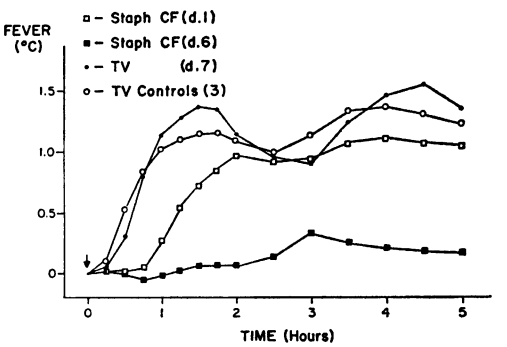

FIG. 5. Initial and tolerant responses of five rabbits given six daily inoculations of staphylococcal culture filtrate. On day 7, the group received 1.5 ml. of a 1 : 10 dilution of typhoid vaccine. The average response of three controls to the same dose of vaccine is shown for comparison. CF = culture filtrate. TV = typhoid vaccine.

In a further experiment, the pyrogenic responses of rabbits to a small dose of typhoid vaccine were measured before and after tolerance had been produced to staphylococcal filtrate. The results are shown in Figure 5. It is apparent that the response to this dose of vaccine was uninfluenced by the intervening tolerance induced to staphylococcal filtrate.

Failure of Thorotrast to abolish tolerance to staphylococcal culture filtrate

Tolerance to endotoxins is characteristically abolished by injection of a number of RES blockading agents such as thorium dioxide (Thorotrast).[5] To test the effect of RES blockade on tolerance to staphylococcal filtrate a group of four rabbits was made tolerant by a series of nine daily inoculations and their fevers recorded on the initial and last days. On the ninth day, there was virtually no response to this dosage of filtrate. Each animal was then given 5 ml. of Thorotrast intravenously. On the following day none of the recipients had fevers when reinjected with

staphylococcal filtrate. Tolerance to other pyrogenic agents such as myxoviruses, and tuberculin in sensitized recipients is similarly unaffected by blockade of the RES by Thorotrast.[4]

Effect of repeated intermittent injections of culture filtrate

The preceding experiments have demonstrated that tolerance is rapidly induced and lost in most rabbits given daily inoculations of staphylococcal filtrate. To determine the effect of intermittent injections of filtrate, a group of rabbits was given a series of five bi-weekly inoculations. The average

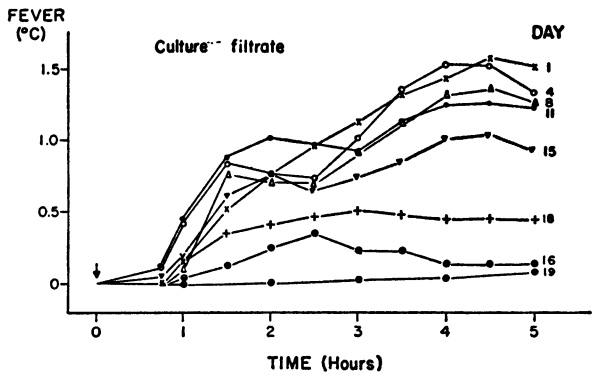

FIG. 6. Average fevers of a single group of four rabbits given bi-weekly inoculations of staphylococcal broth culture filtrate from a single pool. Tolerance only developed with later daily injections and was partially lost on day 18, after only one day's rest.

pyrogenic response of the group remained virtually unchanged at the end of this period on the 15th day (see Figure 6). There was nearly complete tolerance to reinjected filtrate the following day but reactivity was partially restored on the 18th day after a day's rest. It appears, then, that despite a series of injections over a two-week period which would be expected to increase the level of circulating antibodies, the febrile response to this agent was unaffected as long as injections were spaced at intervals to allow for recovery from tolerance. These results supply further evidence that tolerance is unlikely to be due to acquisition of humoral immunity to the pyrogenic agent present in staphylococcal culture filtrates.

Failure of serum from tolerant animals to modify response to staphylococcal culture filtrate

Although the evidence presented so far suggests that tolerance to filtrate was not due to the development of serum antibodies to staphylococcal

pyrogen, it seemed desirable to test this inference directly by *in vitro* incubation studies. Accordingly, a small amount of filtrate was added to serum from rabbits with established tolerance to filtrate produced by a 6 to 10-day course of daily injections. The mixture was incubated for a period of one hour at 37° C. and injected in doses containing 0.5 ml. filtrate in 5 ml. sera. Control animals received a similar amount of filtrate incubated in serum of normal rabbits. The results of *in vitro* incubation of filtrate with sera of normal and of tolerant donor rabbits are shown in Figure 7.

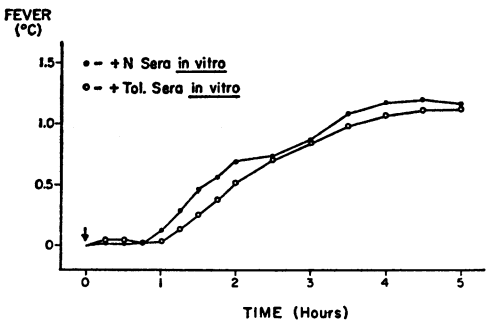

FIG. 7. Mean febrile responses of two groups of nine rabbits given individual doses of 0.5 ml. staphylococcal culture filtrate which had been incubated for 1 hr. with 5 ml. of either normal sera or sera from filtrate-tolerant animals.

Since the responses of the two groups are nearly identical, it seems improbable that serum inhibitors play a significant role in producing tolerance to staphylococcal filtrate.

Large infusions of sera of recipients tolerant to culture filtrate also failed to produce tolerance in normal animals, although similar techniques have been said to be successful in transferring partial tolerance to endotoxins, presumably by accelerating the clearance of these agents by the RES.[6-8]

Leukocyte response of normal and tolerant recipients to culture filtrate

Intravenous inoculation of antigen in a variety of specifically sensitized hosts, including man, produced marked changes in circulating leukocytes.[9] Profound and sustained lymphopenias as well as transient falls in granulocytes have been reported in both guinea pigs[10] and rabbits.[11]

Since many of the features of the pyrogenic response to staphylococcal filtrate resemble a naturally occurring reaction of hypersensitivity, the

response of circulating leukocytes to this agent was studied in a small group of animals.

After a period for stabilization of temperature and baseline leukocyte counts, new rabbits were given 1 ml. dosages of staphylococcal filtrate. Temperatures were recorded at the usual intervals and both total and

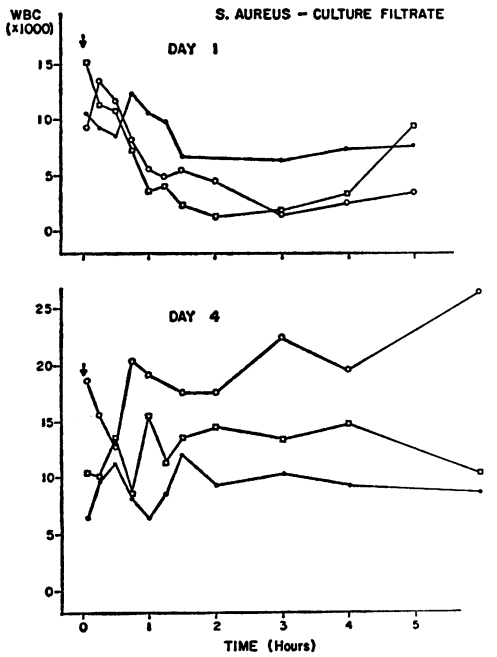

FIG. 8. Top: Total leukocyte counts of three rabbits to an injection of 1 ml. staphylococcal culture filtrate. Bottom: Leukocyte responses of three rabbits on the fourth day of injections after tolerance had been established. WBC = white blood cell.

differential leukocyte counts performed by techniques reported previously.[11] On the fourth day, when virtually complete tolerance had been established by daily injections, repeat total and differential leukocyte determinations were made in the same manner as on the first day.

The results are presented in Figure 8 and Table 1. In all three rabbits, the first injection of staphylococcal culture filtrate produced a significant leukopenia, involving both granulocytes and mononuclear cells, which was slow in onset and became maximal at 2-4 hrs. The mean maximal decrease (at 3 hrs.) was greater in mononuclear forms, which fell to less than 10 per cent of the initial value, than in granulocytes, where a

TABLE I. CHANGES INDUCED IN TOTAL POLYMORPHONUCLEAR (PMN) AND MONONUCLEAR (M) CELLS ON THE FIRST AND FOURTH DAY OF STAPHYLOCOCCAL CULTURE FILTRATE GIVEN INTRAVENOUSLY

		Day 1							
		Minutes after inoculation							
R	*Cell*	*0*	*5*	*15*	*30*	*60*	*90*	*180*	*300*
1	PMN	7000	7700	7300	7400	8600	6200	6000	6700
	M	3000	2900	2000	1200	2000	700	300	1100
2	PMN	7400	6500	8800	8800	3800	4000	1200	2600
	M	3600	2900	4800	2900	1800	1600	400	700
3	PMN	7900	13500	9600	8900	3200	1900	1700	8900
	M	5300	1700	1600	2000	700	400	200	400
Av	PMN	7500	9200	8600	8400	5200	4000	3000	6100
	M	4000	2500	2800	2000	1500	900	300	700

		Day 4							
		Minutes after inoculation							
R	*Cell*	*0*	*5*	*15*	*30*	*60*	*90*	*180*	*300*
1	PMN	3300	5200	5500	5500	3700	7300	7100	5900
	M	5000	1800	4200	5500	2800	4700	3100	2900
2	PMN	7600	11300	11600	8600	13200	11500	15100	—
	M	9700	7600	4100	4400	5900	6200	7400	—
3	PMN	5800	5700	5700	7900	11000	8800	8800	7800
	M	4600	4800	4600	5900	4700	5100	4800	2800
Av	PMN	5600	7400	7600	7300	9300	9200	10300	6800
	M	6400	4700	4300	5300	4500	5300	5100	2900

mean fall to about one-third of the original was recorded. On the fourth day, when tolerance to the filtrate had developed, there were no significant changes in circulating cells.

The marked and prolonged fall in lymphocytes, as well as granulocytes, which followed initial injection of culture filtrate is unlike the immediate but transient granulocytopenias produced by staphylococcal cells[2] and is characteristic of many immunologic reactions.[9]

Results of skin tests

Several groups of animals were skin-tested at various abdominal sites. The groups included: (a) new rabbits, which had not received any previous inoculations of staphylococcoci or their products; (b) rabbits sensitized by intravenous inoculation of an infecting dose of organisms one to two weeks earlier, and (c) normal rabbits "desensitized" (made tolerant) by a series of daily injections of staphylococcal filtrate. Both 18-hour broth culture filtrate and whole culture (autoclaved) were given intradermally in a dose of 0.5 ml. The tests were read at 24 and 48 hours. In no instance were the characteristic reactions of delayed hypersensitivity seen. Most animals developed pale, poorly circumscribed areas of erythema which were not indurated and were about equally prominent at 24 and 48 hours.

Attempts to restore reactivity to culture filtrate in tolerant animals by means of spleen cells or large volumes of sera from infected (hypersensitive) donors

Attempts were made to transfer reactivity to staphylococcal culture filtrate from previously infected (hypersensitive) donors to recipients rendered tolerant by several daily injections of culture filtrate. Donor animals were exsanguinated and the spleen removed under aseptic conditions. The organ was then cut into fine pieces, placed in a small volume of pyrogen-free saline and pressed through a fine wire screen (40 mesh). Counts revealed approximately 2-4 x 10^9 cells per spleen (> 95% lymphocytes) virutally all of which were viable by supravital staining. All procedures after removal of spleen from donor animals were carried out in an ice-water bath to ensure maximal viability. The emulsified material from one or more spleens was then given intravenously to tolerant recipients in individual dosages of about 1 x 10^9 cells (suspended in 30-40 ml. saline). One and one-half to three hours later each recipient received 1 ml. staphylococcal filtrate. No consistent recovery from tolerance was demonstrated by this technique. Unfortunately, longer and probably more appropriate intervals of one to two days between transfer of cells and

injection of filtrate could not be employed because of the rapid spontaneous recovery from tolerance to staphylococcal culture filtrate (see Figure 1).

Similar experiments involving transfer of large volumes (25-60 ml.) of sera from similarly infected donors to tolerant recipients were also carried out. Sera were usually given in two divided doses one to three hours before injection of staphylococcal culture filtrate. Since serum transfer was also ineffective in restoring responsiveness of tolerant animals to culture filtrate, tolerance would not appear to be due to lack of factors present in the fresh serum of the reactive donor animals.

DISCUSSION

In most experimental fevers, including those produced by Gram-negative bacterial endotoxin, myxoviruses, and antigen in specifically sensitized recipients, tolerance develops to repeated intravenous injections.[4] In the case of the strain of *Staphylococcus aureus* studied here, daily inoculation of washed, autoclaved cells caused only minimal reduction in fever whereas tolerance was nearly complete in most recipients reinjected with culture filtrate on the second day. Other evidence that the two pyrogens in staphylococcal cultures are distinct is the lack of cross-tolerance between them and the different leukocytic responses which each evokes when injected intravenously.

Both in rapid onset and recovery from tolerance, responses to staphylococcal culture filtrate resemble those seen in a number of models of hypersensitivity of both immediate and delayed types. Repeated inoculation of either tuberculin[11] or diphtheria toxoid[10] in specifically sensitized recipients results in a state of nearly complete tolerance to their pyrogenic effects. In guinea pigs sensitized to diphtheria toxoid, tolerance to specific antigen is complete on the second day. Tolerance to tuberculin, on the other hand, is usually progressive and reaches its maximum after five or six daily injections in BCG-vaccinated rabbits.[11, 12] Daily inoculations of bovine serum albumin (BSA) also produce tolerance in specifically sensitized rabbits.[13] Hypersensitivity to BSA, as opposed to that induced to diphtheria toxoid and tuberculin, appears to depend critically upon humoral rather than upon cellular antibody. Passive transfer of sensitivity to the systemic effects of tuberculin (including fever) has been accomplished with both lymphocytes and spleen cells of vaccinated donor animals,[14-16] whereas responsiveness to the pyrogenic action of BSA has only been transferred with serum of sensitized rabbits.[17]

From results reported here, it seems unlikely that humoral antibodies play a significant role in either fever or tolerance produced by staphylococcal culture filtrate. In contrast to the findings in the case of hypersensitivity to BSA, transfusion of large amounts of serum from donors presumably sensitized by a prior infecting dose of staphylococci did not restore reactivity of recipients rendered tolerant to culture filtrate—a result which suggests, in addition, that tolerance is not due to transient depletion of a normal serum factor such as complement. On the other hand, tolerance to staphylococcal culture filtrate does not seem to be produced by specific humoral immunity, since recovery is rapid and febrile responses to intermittent injections are undiminished two weeks later when serum antibodies should be elevated. Most significantly, sera from tolerant animals failed to modify the pyrogenicity of filtrate when incubated with it *in vitro,* as would be expected if tolerance were due to acquisition of specific humoral antibodies. These results may be contrasted with tolerance to certain streptococcal exotoxins in which development of a strain-specific humoral immunity appears to be important, since the streptococcal pyrogen is inactivated when incubated with sera of immunized rabbits *in vitro.*[18]

Recently, Freedman has shown that serum factors may play another, less specific role in tolerance to Gram-negative bacterial endotoxin.[8] Prior injection of large volumes of sera from tolerant donors caused some suppression of the febrile response of normal rabbits to purified endotoxin. Since tolerance to fever was associated in the recipients with accelerated clearance of various colloidal agents from the blood stream, Freedman has postulated that serum of tolerant animals increases the ability of the RES to remove circulating endotoxin, presumably by the opsonic effect of such serum[19, 20] rather than by direct inactivation of toxin which was not demonstrable *in vitro.* Large intravenous doses of serum from donors tolerant to staphylococcal filtrate failed, however, to confer tolerance to subsequently administered filtrate. In addition, cross-tolerance between staphylococcal filtrate and Gram-negative bacterial endotoxins was not demonstrable and tolerance to filtrate was not abolished by RES blockade with Thorotrast.

Since both pyrogenic and leukocytic reactions of rabbits to staphylococcal culture filtrate closely resembled responses which occur in models of delayed hypersensitivity, it seems reasonable to infer that tolerance is due to specific desensitization of a naturally acquired, cellular type of hypersensitivity to a filterable product of *Staphylococcus aureus.* As yet, however, conclusive evidence to support this hypothesis is lacking. Normal

rabbits which were responsive to the pyrogenic activity of filtrate did not develop clear-cut delayed cutaneous reactions to either staphylococcal filtrate or autoclaved whole broth culture. Furthermore, passive transfer of spleen cells from donors hypersensitized by staphylococcal infection did not enable tolerant recipients to respond to culture filtrate. However, the interval between inoculation of donor spleen cells and challenge of the recipients was purposely designed to be short (less than six hours) because of the normally rapid recovery from tolerance which takes place in rested animals (see Figure 1). This interval may be too brief to allow for passive sensitization of the cellular type which, in any case, is difficult to demonstrate in rabbits. Studies are now under way using other strains of staphylococci to which rabbits are not normally responsive in order to investigate further the possible participation of delayed hypersensitivity in both fever and tolerance to this organism.

SUMMARY

Certain characteristics of tolerance which develops to a pyrogenic agent in staphylococcal broth culture filtrates have been described.

Tolerance was rapidly acquired but transient. It appeared to be unrelated to levels of circulating antibody and was not abolished by blockade of the reticulo-endothelial system with Thorotrast. These features suggest that this phenomenon is not due to acquisition of specific humoral immunity and is unlike the tolerance produced by repeated inoculation of Gram-negative bacterial endotoxin.

Since there was no cross-tolerance between cells and filtrates of the same culture of *Staphylococcus aureus,* it may be inferred that these two pyrogenic factors from staphylococcal cultures are distinct and that they differ in the mechanisms by which they produce tolerance.

Similarities between the febrile and leukocytic responses obtained to staphylococcal culture filtrates and those occurring in certain forms of delayed (cellular) hypersensitivity suggest that fever and tolerance are due, respectively, to a naturally acquired hypersensitivity and specific desensitization to a product of this strain of *Staphylococcus aureus.*

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