ROBERT S. BRIGGS*
ELISHA ATKINS**

Department of Internal Medicine, Yale University School of Medicine

STUDIES IN CRYPTOCOCCAL FEVER
I. RESPONSES TO INTACT ORGANISMS AND TO A SOLUBLE AGENT DERIVED FROM CRYPTOCOCCI†

It is a long-established clinical observation that most microbial infections are associated with fever. With many of these agents, however, the pathogenesis of fever has been only recently elucidated and it is apparent that several different mechanisms may be implicated.[1] The following studies were undertaken to compare fevers induced in rabbits by a nonpathogenic fungus, *Cryptococcus albidus,* with those reported in similar models employing viruses[2-5] and both Gram-negative[6-12] and Gram-positive bacteria.[13-21]

In contrast to extensive investigations with other microbes, there are only a few previous studies demonstrating the pyrogenicity of various fungi.[22-28] Both the whole cells and soluble products of these microorganisms have been utilized to produce fever, but hitherto no systematic attempt has been made to determine if different pyrogenic mechanisms were involved; nor has there been any previous effort to establish the relationship of fevers induced by fungi to those caused by bacterial pyrogens, the co-called "endotoxins" of Gram-negative bacteria.

In the following studies, evidence is presented that both living and dead cells of this cryptococcal species are pyrogenic in normal rabbits, whereas its soluble products seem to cause fever only, or primarily, in specifically sensitized animals. Further, in their pyrogenic actions, the cells and soluble products of this fungus appear to be clearly separable from each other, as well as from Gram-negative bacterial endotoxins, and in this regard resemble fever-inducing factors associated with the cells and culture filtrates, respectively, of certain phage types of staphylococci.[14-17]

---

* Present address: Litchfield, Connecticut.
** Associate Professor of Medicine.
† Supported by a grant (A1-01564) from the U. S. Public Health Service.
Received for publication 9 November 1965.

MATERIALS AND METHODS

Source and culture of Cryptococcus albidus. A single strain of *C. albidus* was used.* Prior to these studies, the organism had been passed at 3-month intervals on Sabouraud's glucose agar. During the present studies, the yeast was transferred at least every 10 days and often twice a week. Cultures were maintained at room temperature since there was no growth at 37°C. Organisms subcultured to Sabouraud's agar plates were used in all experiments. The mucoid yeast cells were removed from the solid agar surface with glass rods and then suspended in various liquid media. Cell counts were determined in the standard manner with an A-O Spencer Bright-Line Improved Neubauer Hemocytometer.

Suspending media. Cryptococci were suspended in both physiologic saline (0.15M) and a phosphate buffer. The NaCl and phosphate salts were heated in a dry air oven at 100°C. for two hours and then added to autoclaved, doubly-distilled water. Phosphate buffer (pH 7.4, about 310 mOsM/liter) was prepared with 4 parts 1/15 M Na_2HPO_4 to 1 part 1/15 M NaH_2PO_4. All solutions, including reagent preparations of HCl and NaOH (titrated against each other to neutrality) and of 1 per cent formalin in physiologic saline, were determined to be nonpyrogenic before use by intravenous injection of 10 to 20 ml. in normal rabbits.

Preparation of killed cryptococci. Preparations of cryptococci, washed twice with saline, were killed by suspension in 1 per cent formalin in saline at 37°C. for 12 to 18 hours. After this, the dead cells were washed once again in saline and then resuspended in additional saline and refrigerated until use. In other experiments, cryptococci were suspended in saline and autoclaved (for 20 minutes at 120°C. and 15 lbs. pressure). The cells were then washed three times in saline and then resuspended in additional saline before use. All suspensions of killed cells were routinely cultured for sterility.

Partial decapsulation of cryptococcal cells. Cryptococcal cells were partially decapsulated when the live organism was suspended in 1/5 N HCl in a steam bath (maintained at 70 to 80°C.) for 1½ hours. The acid solution was then neutralized with NaOH and pH determined with pHydrion paper. The yeast cells were washed three times with saline before injection. The degree of decapsulation was determined by microscopic examination of India ink preparations.

Agitation of cryptococci in saline or phosphate buffer. Suspensions of live *C. albidus* were agitated in saline or phosphate buffer by either a magnetic mixer (for 24 to 72 hours) or on a horizontally oscillating shaking platform (for 1 to 6 days). The first method was generally used as it was simpler and more effective than the second.

Separation of organisms from suspending media. When the initial suspensions of either live or autoclaved cryptococcal cells were centrifuged at 4,000 r.p.m. for 30 to 40 minutes, supernates were obtained with less than 1,000 organisms per milliliter. Filtration of these supernates by gentle suction through fritted Corning Glass Filters** yielded cell-free filtrates, confirmed by culture or, in the case of dead cells, by microscopic examination.

---

* The culture was obtained from the late Rhoda Benham, Ph.D., College of Physicians and Surgeons, Columbia University, and was identified as *C. neoformans* var. *innocuous*. Studies of Leanor D. Haley, Ph.D. in the yeast laboratories at Delft and at Yale University led to its being reclassified as a strain of *C. albidus*.

** Ultra-fine fritted disc (pore size 0.9 to 1.4 microns).

Endotoxin. Typhoid vaccine (monovalent reference NRV-LS No. 1) from *Salmonella typhosa* V-58 was used as a source of Gram-negative bacterial endotoxin.[4] One and a half milliliters of a 1:10 dilution in saline was given both to establish pyrogen tolerance and as a challenge dose in cross-tolerance studies with the pyrogenic agents derived from cryptococci.

Equipment. Glassware, needles, cotton stoppers, and magnetic mixer blades were rendered pyrogen-free by heating in a dry-air oven for two hours at 170°C.

Pyrogen studies. Pyrogen studies were conducted upon 3 to 4 kg. albino rabbits of both sexes that were restrained in wooden boxes in an air-conditioned room maintained at 69°F. ± 1.5°F. Details about the housing and selection of rabbits have been

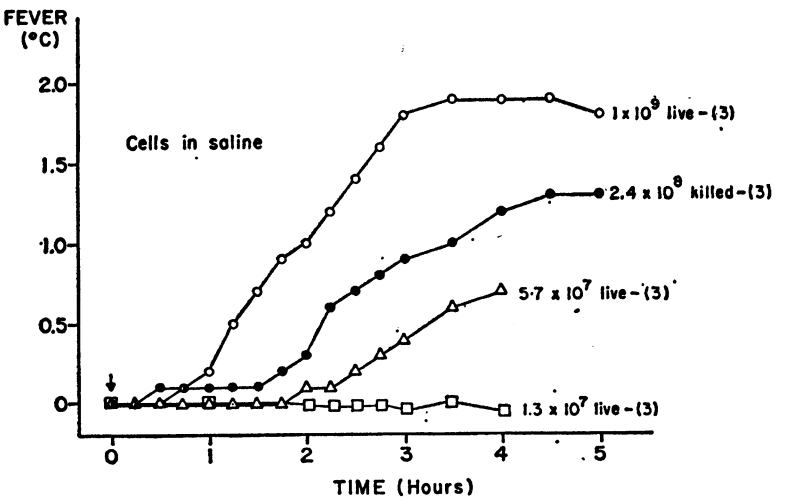

FIG. 1. Mean febrile responses of groups of normal rabbits to three different dosages of cryptococcal cells. In this and following figures, numbers in parentheses indicate number of rabbits in each group.

presented earlier.[3] Only trained and previously tested rabbits with normal body temperatures (below 40.5°C.) that varied less than 0.3°C. in the hour before inoculation were used in each experiment. Injections were made via a marginal ear vein. Temperatures were recorded every 15 minutes beginning at least 1½ hours before injection by means of indwelling rectal thermometers (Foxboro rabbit scanning switch and fever recorder).*

RESULTS

Pyrogenicity of intact cells

Figure 1 shows mean febrile responses of groups of rabbits to varying numbers of live and formalin-killed cryptococcal cells suspended in saline.

* Manufactured by Foxboro Co., Foxboro, Mass.

Groups of rabbits were given 1 x 10^9 or 5.7 x 10^7 unwashed, live yeast cells. In the larger dose, these cells caused an average maximal fever of 1.9°C., after a latent period of 45 to 60 minutes; 5.7 x 10^7 cells produced a mean fever of about 0.6°C. following a latency of approximately two hours. Dosages of 2.5 x 10^6 and 1.3 x 10^7 unwashed, live yeast cells were nonpyrogenic in four and three rabbits, respectively. Similar fevers were produced by both unwashed and washed live cells (in dosages of 1 x 10^9). Further, the saline in which this quantity of live organisms had been suspended was not pyrogenic. Intermediate fevers of about 1.3°C. were produced by 2.4 x 10^8 washed, formalin-killed organisms in three rabbits after latencies of 1 to 1½ hours.

Since 5.7 x 10^7 cells produced slight fevers after a prolonged latent period, the minimal number of organisms needed to produce a detectable response (0.3 to 0.5°C.) appears to lie between 1.3 x 10^7 and 5.7 x 10^7 live (and probably killed) *C. albidus*. From these data it is apparent that this fungus, live or killed, is pyrogenic in rabbits. The degree of fever is directly proportional to the number of injected organisms and the duration of the latent period before onset of fever varies inversely with the dosage.

Pyrogenicity of partially decapsulated cells

To determine the possible contribution of either the capsule or the size of *C. albidus* to fevers produced by the intact yeast cell, attempts were made to decapsulate cryptococci by exposure to hot acid solutions (see METHODS). This treatment resulted in an appreciable reduction in the size of the cell. Live intact *C. albidus* is about 3.7 microns x 4.3 microns, including the capsule, which has a thickness of about 1.0 micron. After acidification for 1½ hours, the capsule had an average thickness of 0.8 microns with over-all cellular dimensions of 2.2 microns x 3.0 microns. In a dosage of 1 x 10^9, these partially decapsulated cells produced in four animals essentially the same degree of fever as did an equal number of live organisms. Thus, pyrogenicity of the whole organism did not appear to depend either upon the specific size or upon the original capsular surface of the intact cell. Since it was not possible to remove the capsule entirely by this technique, the pyrogenic effects of a completely decapsulated cell could not be determined.

Comparison of the febrile responses to cryptococci and to Gram-negative bacterial endotoxin

The prolonged latent period (45 minutes or longer) before onset of fevers induced by inoculation of cryptococcal cells suggested that these agents do not contain substances similar to Gram-negative bacterial endo-

toxin. The responses to yeast cells may be further distinguished from those produced by endotoxin by the following evidence.

1. Daily injection of 1 x 10^9 autoclaved and washed cryptococcal cells (resuspended in saline) produced only a slight modification in fever (evident in some recipients as a reduction in the second fever peak) over a

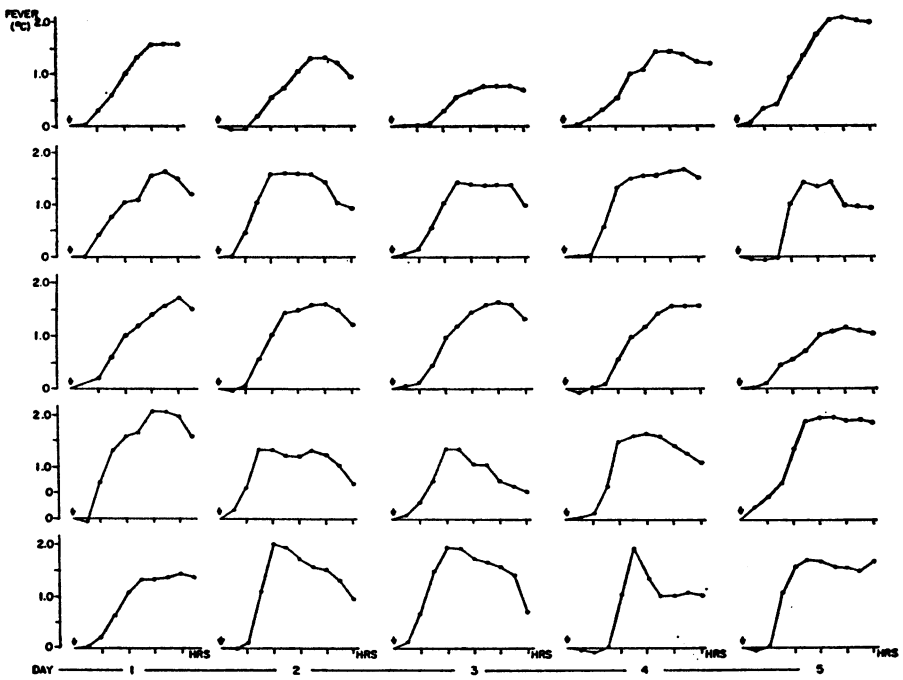

FIG. 2. Fevers induced in five rabbits given a course of five daily inoculations of 1 x 10^9 autoclaved cryptococci (washed and resuspended in saline).

5-day period (see Fig. 2), unlike the definite pyrogenic tolerance that appears with a similar course of endotoxin injections.[6]

2. Animals with pre-established tolerance to Gram-negative bacterial endotoxin showed no tolerance to cryptococcal cells. Two different dosages of cryptococci were employed, one 20-fold greater than the other, as shown in the following experiments.

(a) Seven rabbits were given an initial intravenous injection of 1 x 10^9 autoclaved cryptococcal cells (see METHODS). Four of the rabbits then received eight daily injections of 1.5 ml. of a 1:10 dilution of typhoid vaccine to establish tolerance to endotoxin. On the day following the last injection of endotoxin, all seven rabbits were given a second injection of 1 x 10^9

cryptococcal cells. As shown in Figures 3A and 3B, the febrile reaction to this dose of *C. albidus* was similar in the endotoxin-tolerant and control groups and, in fact, was somewhat greater than the fevers induced in both groups by the initial inoculation of cryptococci.

(b) When the same two groups of animals were later challenged with a minimal pyrogenic dose of autoclaved cryptococcal cells (5×10^7), their mean responses were again almost identical (see Figs. 3A and B).

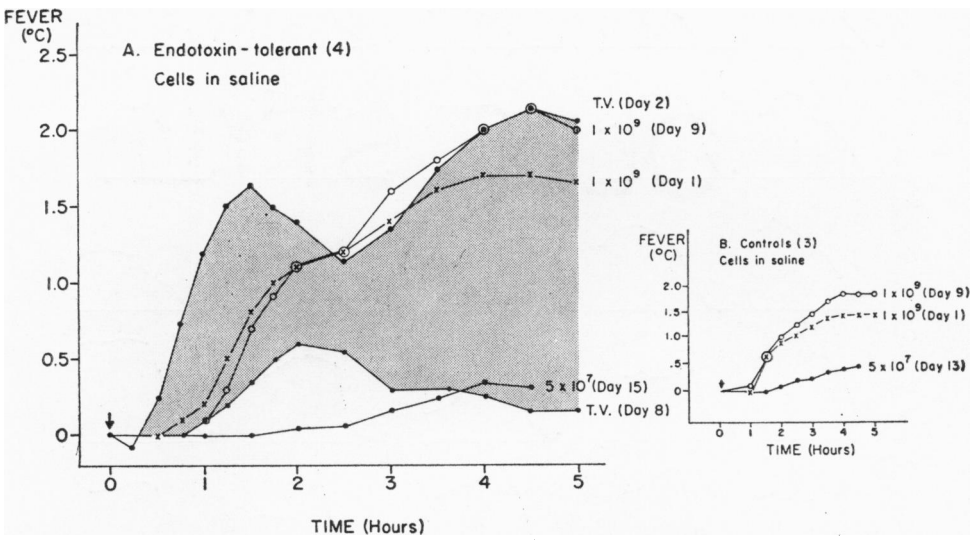

FIG. 3. A. Response of a single group of rabbits to 1×10^9 autoclaved cryptococci given both before and after, and to 5×10^7 cells given after establishment of tolerance to endotoxin by daily injections of typhoid vaccine (shown in shaded lines).
B. Responses of control rabbits to same dosages of cryptococci given at the same intervals as in A.
TV = typhoid vaccine.

On the other hand, cross-tolerance is readily produced by this technique to either the endotoxins[8] or the cells[14] of immunologically unrelated Gram-negative bacteria.

Pyrogenicity of a soluble product of cryptococcal cells agitated in saline or phosphate buffer

The possibility of extracting a soluble pyrogen from *C. albidus* was next investigated. Varying numbers of live yeast cells were suspended in saline or phosphate buffer and agitated for 1 to 6 days at room temperature on a shaker or with a magnetic mixer (see METHODS). Supernates of this

preparation were clear and pale olive in color and contained large amounts of both carbohydrate and protein.

Febrile responses to supernates (in which the total number of organisms never exceeded 5 x 10^4 per inoculum) and to cell-free filtrates of the same material are considered to result from the same factor (or factors) since previous studies showed that more than 1 x 10^7 organisms were necessary to produce fever (see Fig. 1). This agent (from either supernates or cell-free filtrates) is hereinafter referred to as "soluble" or "filtrable" agent.

The soluble factor was tested in 37 rabbits with varying backgrounds of exposure to C. albidus or its products. Sixteen animals responded with temperature elevations of 0.5°C. or greater, with onset of fever ranging from 45 minutes to 1½ hours. Since the pyrogenicity of any given supernate or filtrate appeared to vary with the method of preparation, the average response of all tested rabbits would have little meaning. The volume of solution injected into each recipient (generaly 10 to 25 ml.) was that in which 2 x 10^9 to 6 x 10^9 cryptococcal cells had been suspended and agitated prior to separation by centrifugation or filtration.

Of the 16 rabbits that developed febrile responses to the filtrable agent, 3 had never been exposed to the whole organism or its products and 2 had received filtrates seven days before the positive response. The remaining 11 had received one or more of the following preparations of cells within 20 days of testing: autoclaved, formalin-killed, partially decapsulated, or live *C. albidus*. In addition, one rabbit, known to have been previously unreactive to the filtrable agent, became reactive after a 14-day course of formalin-killed cells.

Of the 21 rabbits that were afebrile when first exposed to the filtrable agent, 16 had no history of exposure to this yeast or its products, and the other 5 had received no cryptococci within the preceding 28 days.

It is of note that six rabbits that had never received any form of cryptococcus were reactive to the filtrable agent after 14 daily injections of 2.5 x 10^8 formalin-killed, once washed organisms. From previous evidence, it can be assumed that most, if not all of these rabbits would have been unreactive to the filtrable agent before the series of injections, although this was confirmed in only one rabbit. Live organisms, therefore, do not appear to be required to sensitize the host to the soluble agent—a finding that is not surprising as the yeast is incapable of infecting rabbits, perhaps because of their high body temperature.

Examination of the previous histories of the 37 rabbits in the experiments with the soluble agent strongly indicates, therefore, that recent ex-

posure (within 20 days) to the whole organism (and, perhaps, to its products alone) had sensitized rabbits to react to this agent with fever.

Comparison of the responses of normal and previously exposed rabbits to a minimal pyrogenic dosage of cryptococcal cells

A group of four rabbits was rendered reactive to the soluble agent by two intravenous inoculations of 1 x 10^9 autoclaved cryptococci, spaced one week apart. The febrile responses of this group to a low dosage of

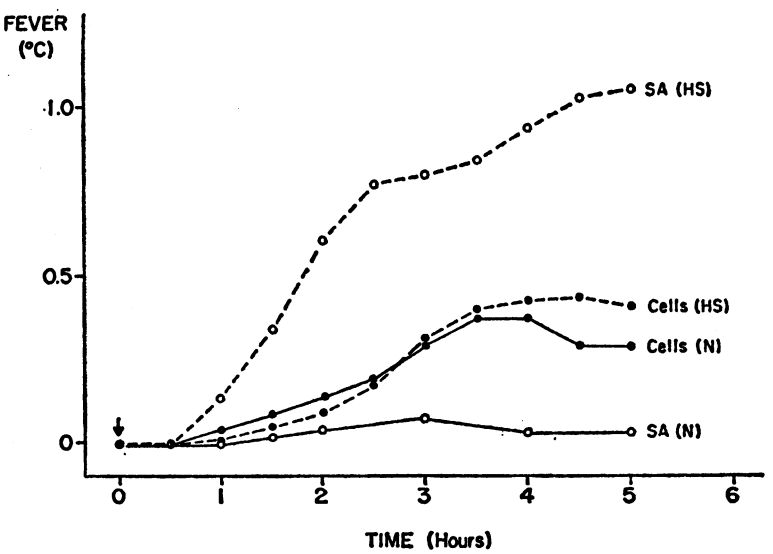

Fig. 4. Mean responses of two groups of rabbits (one normal and the other previously exposed to cryptococci) to 5 x 10^7 autoclaved cryptococcal cells and to the soluble agent (SA) derived from cryptococci.
N = Normal; HS = Hypersensitive.

autoclaved cryptococcal cells (5 x 10^7) were then determined and compared with the responses of a group of four normal rabbits that had not been previously exposed to cryptococci. As shown in Fig. 4, the mean responses of the two groups were nearly identical despite a striking difference in their responses to the soluble agent. Since sensitization to the filtrable product of cryptococci does not modify the response to a minimal pyrogenic dose of cryptococcal cells, the sensitizing agent would not appear to be located in significant amounts on the cell surface.*

* In another experiment, febrile responses of three sensitized rabbits were the same as those of three unexposed controls to 2.4 x 10^8 formalin-killed cells.

Desensitizing effect of the soluble agent

The results of the previous two experiments suggested that rabbits that responded to the soluble factor had been sensitized by previous exposure to cryptococci or to agents derived from this fungus.

To furnish further evidence for this hypothesis, an attempt was made to desensitize (or render "tolerant") rabbits that were reactive to the pyrogen contained in the cryptococcal filtrates. Six rabbits were rechallenged on the first day following a fever produced by the soluble agent, and four others

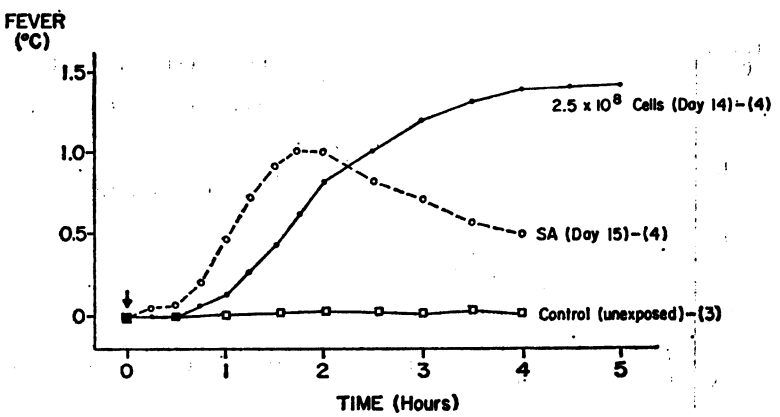

FIG. 5. Mean response of a group of previously exposed rabbits to the soluble agent (SA) derived from cryptococci given the day following a 14-day series of injections of formalin-killed cryptococcal cells (2.5 x 10^8). Average febrile response of this group to the last injection of cells is also shown. Mean response of three unsensitized controls to the soluble agent is indicated for comparison.

were rechallenged on the first and second day. All 10 rabbits remained unresponsive to these subsequent injections. The soluble agent retained its pyrogenicity for at least several weeks at 4°C., so that the lack of febrile response in these rabbits cannot be attributed to loss of activity of the agent during the brief period of the experiment.

Lack of cross-tolerance between fevers induced by the intact cell and by the soluble agent

Since the intact cell seemed capable of sensitizing rabbits to a soluble agent extracted from cryptococcal cells, the possibility was next investigated of desensitizing rabbits to the soluble material by means of repeated exposure to the whole organism. Four rabbits, previously exposed to *C. albidus,* received 14 daily injections of 2.5 x 10^8 washed, formalin-killed cells. On the last day of injections, these rabbits continued to react

with typical delayed fevers to this dosage of cells. On the fifteenth day, they also responded vigorously to the soluble agent, with a mean fever of 1.0°C. after a latency of one hour (Fig. 5). Thus, a prolonged series of inoculations of cells failed to abolish the response of these rabbits to the soluble agent, suggesting that in the dosage given the cells did not have enough readily available soluble factor to desensitize the rabbits to the test dose of this material.

In a reciprocal experiment of the same design, rabbits that were reactive to the soluble agent were given a 3-day course of inoculations with this

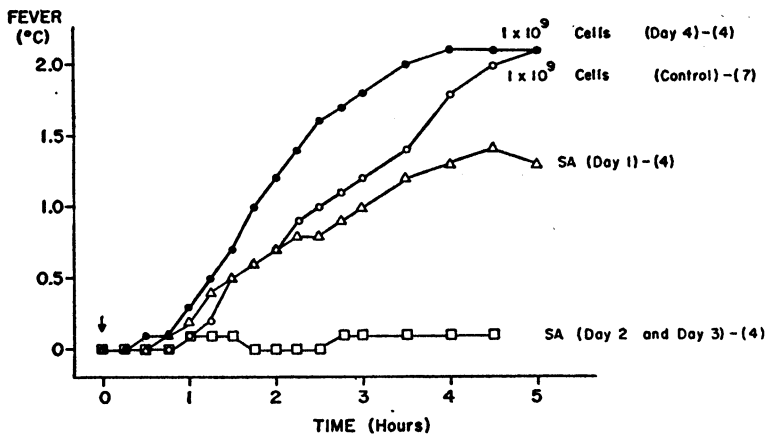

FIG. 6. Mean responses of a single group of rabbits to cryptococcal cells given the day following a 3-day series of inoculations of the soluble agent (SA) derived from cryptococci. The mean fever of a group of control rabbits to the same dosage of cells is shown for comparison.

material. As in the previous experiment, they were unresponsive on the second and third days. The day following the last injection of filtrate, they were then given 1 x 10^9 cryptococcal cells (derived from the same suspension as was the filtrate). The response to cells was unmodified by the preceding injections of filtrate so that there was again no evidence of cross-tolerance between the two agents (Fig. 6).

Characteristics of fevers induced by the cryptococcal soluble agent and by endotoxins of Gram-negative bacteria

Fevers induced by the soluble agent derived from cryptococcal cells have been shown thus far to differ from those caused by the endotoxins of Gram-negative bacteria in three respects. (1) The cryptococcal agent, like the intact cell, caused fever that was delayed in onset (60 minutes or

longer), as compared with the characteristic lag period of 15 to 30 minutes before endotoxin-induced fever. (2) The soluble agent caused fever almost exclusively in animals previously exposed to *C. albidus* or its products. Endotoxin, on the other hand, is pyrogenic in nearly all normal rabbits. (3) Tolerance rapidly developed to the pyrogenic effects of the soluble agent in rabbits previously exposed to cryptococci, presumably because of desensitization. Significant tolerance to the pyrogenic effects of endotoxin, on the other hand, does not appear until the 3rd or 4th day and is never complete to a constant dosage.[6, 9]

In order to examine further the relation of the filtrable cryptococcal agent to endotoxin, an attempt was made to induce cross-tolerance between

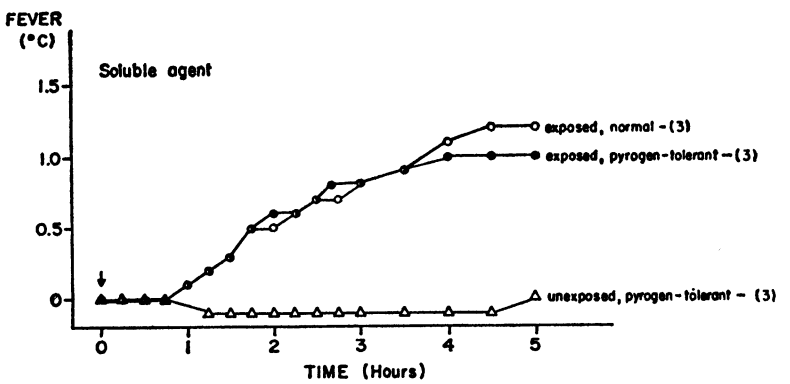

FIG. 7. Mean fevers of 2 groups of rabbits previously exposed to cryptococci and both given the soluble pyrogen derived from cells. One of these groups had been rendered pyrogen-tolerant by daily injection of typhoid vaccine. Responses of a group of unexposed, pyrogen-tolerant rabbits to the same material is shown for comparison.

these two agents. Three rabbits, reactive to cryptococcal filtrates, were given a 14-day course of typhoid vaccine, at the conclusion of which they had developed significant pyrogenic tolerance to the vaccine (similar to that shown in Fig. 3A). On the following (15th) day, these rabbits were given the filtrable cryptococcal agent. All three responded to this material with typical delayed fevers, similar to those of reactive rabbits that had not been made pyrogen-tolerant (Fig. 7). As a control, three additional endotoxin-tolerant rabbits that had not been exposed to cryptococci were given the soluble agent. Like normal, unexposed rabbits, they did not develop fever (compare Figs. 4 and 7).

From these data, it is evident that the soluble agent derived from cryptococci and the endotoxins of Gram-negative bacteria share the ability to cause fever and to induce a pyrogenic tolerance. However, the responses

to these materials differ in significant aspects that must surely reflect differences in the mechanisms by which the two agents induce fever, on the one hand, and tolerance on the other.

Pyrogenicity of a soluble product derived from autoclaved cryptococcal cells

In dosages derived from 1 x 10^9 cells and contained in 1 to 10 ml., supernates of cryptococci that had been suspended in saline and autoclaved for 20 minutes were also found to be pyrogenic in a group of five rabbits

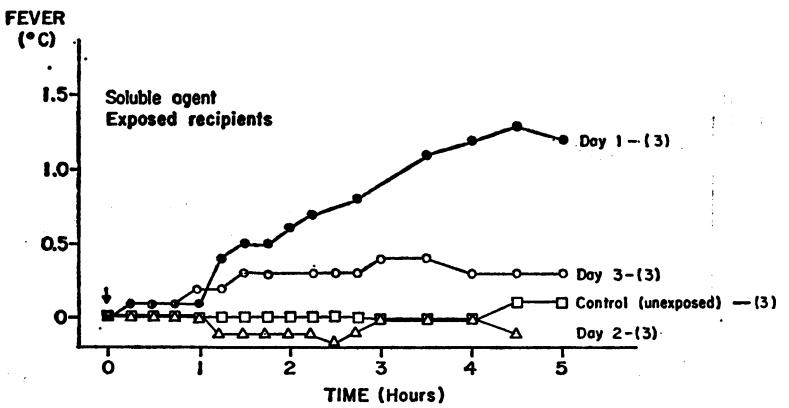

FIG. 8. Mean febrile responses of rabbits, previously exposed to cryptococci and injected on successive days with a soluble agent derived from supernates of autoclaved suspensions of cells. Responses of unexposed rabbits to the same material are shown for comparison.

previously given cryptococci, whereas none of five unexposed rabbits developed fever. The fevers of the animals with prior exposure to the fungus ranged from 0.9°C. to 2.0°C. following latent periods of 45 to 60 minutes. Figure 8 shows the response of three exposed and three unexposed rabbits. Complete pyrogenic tolerance to this material was produced in the three rabbits by a single previous injection, as with the soluble agent derived from cells by the techniques described earlier.

To determine whether autoclaving was necessary for the release of soluble pyrogen from cryptococcal cell suspensions, supernates of live cryptococci briefly suspended in saline were given in the same dosage to four rabbits that were reactive to the soluble product derived from autoclaved cells.* Supernates of the live cells had no pyrogenic effect in these

* Although as shown above, a single injection of the soluble pyrogen produces complete tolerance on the second day, this tolerance is transient and such rabbits recover their reactivity to the agent after a short rest (unpublished observations).

recipients so that the soluble agent does not appear to be released in detectable quantities by the suspension and centrifugation of the yeast cells alone.

DISCUSSION

These studies on the pyrogenicity of *C. albidus* have defined two different types of agents that produce fever after a similar delay in onset of 45 to 60 minutes.* The first of these agents is apparently associated with whole cells (live or killed) and the other with a water-soluble extract from the organism. Not only are these pyrogens distinguished by their particulate *vs.* soluble character, but they appear to differ as well in their action. On the one hand, the pyrogenicity of the whole organism is not dependent upon the recipient's previous exposure. Furthermore, the febrile response to the fungal cell does not seem to be significantly modified by previous or daily administration. On the other hand, extracts prepared from this organism appear to be pyrogenic only, or primarily, in animals previously injected with the whole organism (or its extracts). Unlike the cell itself, these extracts induce a rapid pyrogenic tolerance that is virtually complete on the day following a single intravenous injection.

The mechanism of fever produced by cryptococcal cells is still uncertain. Pyrogenicity is unaffected by partial removal of the capsule and it seems evident, therefore, that neither the original surface of the intact yeast nor its exact size are primarily responsible. Similar fevers, occurring after a delay of an hour or so, have been induced in normal rabbits injected intravenously with the cell bodies of various Gram-positive bacteria, both rods and cocci[14] as well as tubercle bacilli (unpublished observations). Since fever in these instances was preceded by a rapid, transient granulocytopenia,** it was postulated that phagocytosis of circulating bacteria by these cells of the host resulted in release of an intermediate pyrogen of endogenous origin—so-called "endogenous pyrogen" (EP). In partial confirmation of this hypothesis, a rapidly acting pyrogen was found to be present in passive transfer studies in which the serum of febrile donors given staphylococci was injected into normal recipient rabbits. Because of the long latent period before onset of fevers induced by staphylococci, the serum pyrogen was inferred to be the direct cause of fever. Phagocytosis of heat-killed pneumococci similarly leads to mobilization of EP from granulocytes *in vitro*.[27] Leukocytes are also capable of phagocyting

* Similar results have been obtained in preliminary experiments with *C. neoformans*.

** Suspensions of 1 x 10^9 live or autoclaved candida similarly evoke a sharp, transient fall in circulating granulocytes within 5 minutes after intravenous inoculation.[24]

cryptococci both *in vivo* and *in vitro*.[28] The similar latency of fevers induced by Gram-positive bacteria and by these cryptococcal cells, as well as the close correspondence between the number of injected organisms in both instances and the degree of induced fever, suggest a common mechanism via release of a circulating EP.

It is evident from these observations that if phagocytosis of these microorganisms is the initial event leading to the pathogenesis of fever, the size of the ingested particle may vary widely. Pneumococci have cellular dimensions of approximately 0.5 to 1.25 microns, whereas the cryptococci studied here have an average diameter of 4.0 microns, nearly half that of the polymorphonuclear leukocyte itself. Although it is tempting to ascribe all these fevers to the particulate nature of the stimulus,[29] factors other than phagocytosis *per se* must also operate in activating leukocytes to release EP. Polystyrene latex particles, although similar to cryptococci in size, do not induce fever when treated to remove contaminating bacterial pyrogens and injected intravenously,[25, 27, 30] and do not activate leukocytes to release EP when phagocyted by these cells *in vitro*.[27] Similarly, we have noted that suspensions of alumina particles (average size; 10-15 microns)* heated to inactivate bacterial endotoxins, are nonpyrogenic when suspended in saline and given in dosages of 1 x 10^9. Although the physico-chemical properties that are responsible for the pyrogenicity of these microbial cell suspensions are unknown, it seems unlikely that immunological reactions play a significant role. Aside from marked differences in surface antigens, cell bodies of both staphylococci and cryptococci are equally pyrogenic in rabbits without prior exposure to these agents and in rabbits that have been specifically sensitized or desensitized to the pyrogenic action of soluble agents derived from staphylococcal culture filtrates[15, 16] or autoclaved suspensions of cryptococci (see Fig. 4). Further, cell wall fragments of enzyme-treated yeast cells[26] and staphylococci** are not pyrogenic and it seems possible, therefore, that phagocytosis of the cell bodies may be a necessary initial step in activating leukocytes to release pyrogen and produce fever in these instances.

Cryptococcal cells[25] and a soluble material derived from these cells[24] have been noted previously to cause fever, but in these earlier studies no evidence was presented that these substances differ from each other or from the endotoxins of Gram-negative bacteria and, indeed, the fevers caused by these different classes of agents have been thought to be indistinguishable.[24, 25] In the experiments reported here, cryptococcal cells, whether

* Aluminum oxide flour (alundum) size 600, Norton Co., Worcester, Mass.

** Morse, S. I. and Atkins, E.: Studies in staphylococcal fever. VI. Febrile reactions induced by staphylococcal cell walls and soluble fractions. (To be published.)

live or formalin-killed, produced fever that differed in its relatively long latent period from fevers of comparable magnitude induced by Gram-negative bacterial endotoxins (Fig. 3A). Unlike endotoxin, daily injection of the yeast produced little or no pyrogenic tolerance, Moreover, rabbits tolerant to endotoxin reacted like normal animals to two markedly different dosages of cells—a finding that clearly distinguishes these two agents from each other.

The second pyrogenic factor associated with *C. albidus* in these experiments was water-soluble, active primarily in previously exposed rabbits, and produced nearly complete tolerance when given on two successive days. On the other hand, no cross-tolerance with endotoxin was demonstrable. Filtrates and supernates of cryptococcal cells agitated or autoclaved in pyrogen-free media had similar pyrogenic effects and presumably contained the same or a similar material. In its pyrogenic activity, which resembled that of cryptococcal cells, this soluble agent was also clearly separable from Gram-negative bacterial endotoxin.

Since animals can be sensitized to the filtrable material with formalin-killed organisms alone, sensitization is evidently not dependent upon infection or upon a metabolic product of the live fungus produced *in vivo*. In addition, several rabbits appear to have become reactive to the pyrogenic effect of this agent by prior injections of the filtrable product itself, indicating that it may be antigenic, an observation that has been confirmed by further work. Studies to be reported* suggest that the filtrable products of this fungus, obtained by these two different methods, produce reciprocal cross-tolerance. Further, they appear to be biochemically similar and to be associated with a somatic protein rather than with the capsular carbohydrate.

Although the cryptococcal cell must release these or similar antigenic substances *in vivo* in order to sensitize the host, the whole organism itself produces similar fevers when injected intravenously in rabbits either reactive or tolerant to the soluble agent as well as in normal, unsensitized rabbits (compare Figs. 4 and 6). It is evident, therefore, that the soluble agent does not contribute significantly to the fever produced by the intact cryptococcal cell. Preliminary evidence that the soluble agent is a protein (and hence primarily derived from the fungal cell proper rather than from its capsule) further suggests that the sensitizing material associated with the intact cell is released slowly in the host and becomes effective only

* Haley, L. D., Myer, R., and Atkins, E.: Studies in cryptococcal fever. II. Responses of sensitized and unsensitized rabbits to various substances isolated from cryptococcal cells. (To be published.)

after destruction of the cell *in vivo* or when released *in vitro* by the mechanical or thermal means employed in these studies.

The rapid development of complete tolerance (desensitization) on the second day to the pyrogenic effects of a soluble protein has been described by Uhr and Brandriss using antigens in guinea pigs with specific delayed hypersensitivity.[31] A similar desensitization has been observed in rabbits with both natural and acquired sensitivity to a staphylococcal protein antigen[16,17] as well as in specifically sensitized rabbits given several daily injections of old tuberculin[32] or bovine serum alumin.[33] In these last two systems, circulating antibodies have been demonstrated to play a role in producing the pyrogenic response.[34,35] The evident relationship between previous exposure to *C. albidus* and the ability of such rabbits to respond with fever to the fungal extracts, as well as the marked second-day tolerance to these agents, both suggest a specific acquired hypersensitivity, as has been demonstrated with other fungi.[24] In this connection, delayed hypersensitivity has been produced in rabbits and guinea pigs to both somatic and whole cell antigens of *C. neoformans*.[36] Further studies are in progress to determine whether sensitivity to the pyrogenic effects of the extracts is primarily of the delayed or immediate type.

SUMMARY

The pyrogenicity of whole cells and certain soluble extracts of a non-pathogenic fungus, *Cryptococcus albidus,* has been investigated.

Both live and formalin-killed cells were pyrogenic in animals regardless of previous exposure to the organism. Fever characteristically appeared one hour after injection; with smaller doses, the latent period before onset of fever was prolonged and the pyrogenic response correspondingly reduced. Only a minimal degree of tolerance occurred when cells were given daily. Washing and partial decapsulation of the organism did not detectably alter its capacity to induce fever.

Soluble pyrogens were obtained both when the organism was autoclaved or agitated in pyrogen-free media. These agents similarly produced fever after a latency of about one hour, but primarily in animals with previous exposure to the whole organism, live or killed. In contrast to the response to cells, tolerance to these soluble products was nearly complete on the second day. Since tolerance to filtrate did not produce tolerance to cells and, conversely, daily inoculations of cells did not suppress the response to filtrate, it appears that these two agents differ from each other both in the mechanisms by which they induce fever and by which they confer tolerance.

No cross-tolerance was evident between either the cells or soluble products of this fungus, on the one hand, and Gram-negative bacterial endotoxin on the other.

From these observations, it is inferred that this fungus does not contain substances similar to the endotoxins of Gram-negative bacteria but produces fever by two distinctly different mechanisms: one presumably dependent upon a factor associated with the intact yeast cell and the other, by means of a soluble material that requires a state of specific hypersensitivity in the recipient.

Similarities are pointed out between these two postulated pyrogenic mechanisms and those previously reported with certain phage types of *Staphylococcus aureus.*

ACKNOWLEDGMENT

The authors gratefully acknowledge the expert assistance of Leanor D. Haley, Ph.D. who supplied and characterized the cryptococcus and provided many suggestions for the techniques used in these studies. We also wish to thank Ruth Myer for technical help in several of the experiments.

REFERENCES

1. Atkins, E. and Snell, E. S.: Fever. In, *Biology of Inflammation,* edited by B. W. Zweifach, L. Grant, and R. T. McCluskey. New York, Academic Press, 1965, pp. 495-534.
2. Wagner, R. R., Bennett, I. L., Jr., and LeQuire, V. S.: The production of fever by influenzal viruses. I. Factors influencing the febrile response to single injections of virus. *J. exp. Med.,* 1949, *90,* 321-347.
3. Atkins, E. and Huang, W. C.: Studies on the pathogenesis of fever with influenzal viruses. I. The appearance of an endogenous pyrogen in the blood following intravenous injection of virus. *J. exp. Med.,* 1958, *107,* 383-401.
4. King, M. K.: Production of fever in rabbits with extracts of tissue culture cells infected with Coxsackie virus. *J. Lab. clin. Med.,* 1962, *59,* 986-992.
5. Siegert, R. and Braune, P.: The pyrogens of myxoviruses. I. Induction of hyperthermia and its tolerance. *Virology,* 1964, *24,* 209-217.
6. Beeson, P. B.: Tolerance to bacterial pyrogens. I. Factors influencing its development. *J. exp. Med.,* 1947, *86,* 29-38.
7. Wylie, D. W. and Todd, J. P.: An examination of pyrogen from various sources. *J. Pharm. Pharmacol.,* 1949, *1,* 818-835.
8. Dare, J. G.: Some quantitative studies on a bacterial pyrogen. *J. Pharm. Pharmacol.,* 1953, *5,* 528-546.
9. Farr, R. S., Clark, S. L., Jr., Proffitt, J. E., and Campbell, D. H.: Some humoral aspects of the development of tolerance to bacterial pyrogens in rabbits. *Amer. J. Physiol.,* 1954, *177,* 269-278.
10. Grant, R.: Nature of pyrogen fever: effect of environmental temperature on response to typhoid-paratyphoid vaccine. *Amer. J. Physiol.,* 1949, *159,* 511-524.
11. Atkins, E. and Wood, W. B., Jr.: Studies on the pathogenesis of fever. II. Identification of an endogenous pyrogen in the blood stream following the injection of typhoid vaccine. *J. exp. Med.,* 1955, *102,* 499-516.
12. Petersdorf, R. G., Keene, W. R., and Bennett, I. L., Jr.: Studies on the pathogenesis of fever. IX. Characteristics of endogenous serum pyrogen and mechanisms governing its release. *J. exp. Med.,* 1957, *106,* 787-809.

13. Probey, T. F. and Pittman, M.: Pyrogenicity of bacterial contaminants found in biologic products. *J. Bact.*, 1945, *50,* 397-411.
14. Atkins, E. and Freedman, L. R.: Studies in staphylococcal fever. I. Responses to bacterial cells. *Yale J. Biol. Med.*, 1963, *35,* 451-471.
15. Atkins, E.: Studies in staphylococcal fever. II. Responses to culture filtrates. *Yale J. Biol. Med.*, 1963, *35,* 472-488.
16. Atkins, E.: Studies in staphylococcal fever. III. Tolerance to culture filtrates. *Yale J. Biol. Med.*, 1963, *35,* 489-503.
17. Bodel, P. T. and Atkins, E.: Studies in staphylococcal fever. IV. Hypersensitivity to culture filtrates. *Yale J. Biol. Med.*, 1964, *37,* 130-144.
18. Watson, D. W.: Host-parasite factors in group A streptococcal infections. Pyrogenic and other effects of immunologic distinct exotoxins related to scarlet fever toxins. *J. exp. Med.*, 1960, *111,* 255-284.
19. Cremer, Natalie and Watson, D. W.: Host-parasite factors in group A streptococcal infections. A comparative study of streptococcal pyrogenic toxins and gram-negative bacterial endotoxin. *J. exp. Med.*, 1960, *112,* 1037-1053.
20. Roberson, B. S. and Schwab, J. H.: Endotoxic properties associated with cell walls of group A streptococci. *J. infect. Dis.*, 1961, *108,* 25-34.
21. Schuh, V.: The pyrogenic effect of scarlet fever toxin. I. Neutralization with antitoxin; the nature of tolerance. *Folia microbiol. (Praha)*, 1965, *10,* 156-162.
22. Harkness, W. D., Loving, W. L., and Hodges, F. A.: Pyrexia in rabbits following injection of filtrates of typical mold cultures. *J. Amer. pharm. Ass.*, (sci. Ed.), 1950, *39,* 502-504.
23. Wylie, D. W. and Todd, J. P.: An examination of the sources and the quantitative methods of testing pyrogen. *Quart. J. Pharm.*, 1948, *21,* 240-252.
24. Braude, A. I., McConnell, J., and Douglas, H.: Fever from pathogenic fungi. *J. clin. Invest.*, 1960, *39,* 1266-1276.
25. Kobayashi, G. S. and Friedman, Lorraine: Characterization of the pyrogenicity of *Candida albicans, Saccharomyces cerevisiae,* and *Cryptococcus neoformans.* *J. Bact.*, 1964, *88,* 660-666.
26. Kobayashi, G. S., Friedman, Lorraine, and Kofroth, J. F.: Some cytological and pathogenic properties of spheroplasts of *Candida albicans.* *J. Bact.*, 1964, *88,* 795-801.
27. Berlin, R. D. and Wood, W. B., Jr.: Studies on the pathogenesis of fever. XIII. The effect of phagocytosis on the release of endogenous pyrogen by polymorphonuclear leucocytes. *J. exp. Med.*, 1964, *119,* 715-726.
28. Gadebusch, H. H.: Passive immunization against *Cryptococcus neoformans.* *Proc. Soc. exp. Biol. (N. Y.)*, 1958, *98,* 611-614.
29. Eisler, R., Moeller, H. C., and Grossman, M. I.: Report on febrile response of rabbits to intravenous injection of colloidal substances. Army Medical Nutrition Lab. Report, Denver, Colorado, 99937 TU, 1955, Report No. 167.
30. Kobayashi, G. S. and Friedman, Lorraine: Falsely positive pyrogenic responses induced in rabbits by latex particles. *Proc. Soc. exp. Biol. (N. Y.)*, 1964, *116,* 716-718.
31. Uhr, J. W. and Brandriss, M. W.: Delayed hypersensitivity. IV. Systemic reactivity of guinea pigs sensitized to protein antigens. *J. exp. Med.*, 1958, *108,* 905-924.
32. Hall, C. H., Jr. and Atkins, E.: Studies on tuberculin fever. I. The mechanism of fever in tuberculin hypersensitivity. *J. exp. Med.*, 1959, *109,* 330-359.
33. Farr, R. S.: The febrile response upon injection of bovine albumin into previously sensitized rabbits. *J. clin. Invest.*, 1958, *37,* 894.
34. Atkins, E. and Heijn, C., Jr.: Studies on tuberculin fever. III. Mechanisms involved in the release of endogenous pyrogen *in vitro.* *J. exp. Med.*, 1965, *122,* 207-235.
35. Grey, H. M., Briggs, W., and Farr, R. S.: The passive transfer of sensitivity to antigen-induced fever. *J. clin. Invest.*, 1961, *40,* 703-706.
36. Lomanitz, R. and Hale, J. M.: Production of delayed hypersensitivity to *Cryptococcus neoformans* in experimental animals. *J. Bact.*, 1963, *86,* 505-509.