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STUDIES IN CRYPTOCOCCAL FEVER. II. RESPONSES OF SENSITIZED AND UNSENSITIZED RABBITS TO VARIOUS SUBSTANCES DERIVED FROM CRYPTOCOCCAL CELLS†

The cause of fevers induced in rabbits by intravenous injection of cryptococcal cells and soluble extracts was investigated in a previous paper in this series.[1] Two distinct types of pyrogenic responses were found to be produced by this organism. Intact cells, whether live or killed, caused fevers in normal rabbits, whereas the soluble material induced responses primarily in animals that had been previously exposed to cryptococci or its products. Since some unsensitized rabbits were also found to react to the soluble pyrogen with fever, the present studies were undertaken to investigate further the nature of this material. Evidence is presented that the soluble material from cryptococci contains two different pyrogenic agents, a polysaccharide and a protein, which appear to act by different mechanisms to produce fever.

MATERIALS AND METHODS

Animals. White, New Zealand female rabbits weighing 8 to 9 pounds were used both as donors and as recipients. They were housed in air-conditioned quarters and received a standard diet of pellets and water *ad libitum.* All fever studies were made in an adjacent air-conditioned room maintained at 20° C. ± 1.0° C. Animals were boxed and temperatures recorded for two or three days before they were used as recipients.

Temperature recording. Temperatures were obtained with indwelling rectal thermocouples‡ and taken at intervals of 15 to 30 minutes for one to two hours before the beginning of an experiment. After inoculation, temperatures were recorded every 15 minutes for a minimum of five hours. No rabbit was used if its temperature varied

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more than 0.3° C. in the period before inoculation. As in previous studies,[2] responses of 0.5° C. or more above baseline were considered significant.

Fever indices were computed according to the method of Hall and Atkins.[2] For these studies any fever index below 11 (representing a febrile response of $< 0.5°$ C. in five hours) was not considered significant. The fever curves were plotted on 1/6-inch graph paper, with five lines on the ordinate representing 1° C. and six lines on the abscissa for each hour. The area under the 5-hour fever curve was measured by means of a compensating planimeter and the value read directly from the vernier.

Preparation of pyrogen-free equipment. Glassware and reagents were rendered pyrogen-free by standard methods.[1] Solutions were tested at intervals for contaminating pyrogens by intravenous injection of 10 to 20 ml. in rabbits. The stainless steel centrifuge cups used with the Servall RC-2 centrifuge were covered with heavy duty tin foil and sterilized in the hot air oven at 180° C. for three hours. The stainless steel caps, which contained neoprene washers, were placed in 1:750 tincture of zephiran overnight; they were then rinsed three times with pyrogen-free saline before being inserted into the centrifuge tubes.

Organism. The strain of *C. albidus* used in this study was from the collection of Dr. Rhoda Benham and was classified as *C. neoformans* var. *innocuous;* however, our studies indicate it to be *C. albidus.* It has been maintained in our stock collection on Sabouraud's glucose agar and on yeast malt extract agar under sterile mineral oil. During these studies the organism was maintained on Sabouraud's glucose agar at 19° C. and was subcultured every 10 to 14 days. It was frequently checked to make certain that no change had occurred in its gross and biochemical properties.

Preparation of vaccine. Plates of Sabouraud's glucose agar were streaked with a sterile cotton applicator that had been dipped into a 48-hour-old stock culture of *C. albidus.* After incubation at 19° C. for four days, the fungus was removed with a glass spreader and suspended in physiological saline. The cell suspension was then diluted to give a concentration of 8×10^8 cells per milliliter and autoclaved (120° C. at 15 pounds pressure for 15 minutes). Merthiolate was added to a concentration of 0.01 per cent and the vaccine placed in small bottles and refrigerated.

Preparation of soluble extract (SE). Autoclaved cell suspensions of *C. albidus* (prepared as in the preceding paragraph) were centrifuged at 16,500 r.p.m. at 2 to 4° C. in a Servall RC-2 superspeed refrigerated centrifuge for two hours. The clear, viscous supernatant was transferred with 50 ml. volumetric pipettes to glass flasks, and stored at 4° C. Both microscopic examination and cultures failed to reveal organisms in this material. Unless otherwise indicated, intravenous dose of SE was 5 or 10 ml.

Analysis of the soluble extract included ultraviolet absorption spectra, amino acid analysis, and estimation of protein as well as polysaccharide content. Ultraviolet absorption studies* were made at pH 7.0 in 0.1M phosphate-HCl buffer and showed the presence of a maximum absorption at approximately 260 mμ, indicating presence of nucleic acids or similar substances. The amino acid study* was done following acid hydrolysis of SE in vacuum-sealed tubes according to the method of Hirs, Stein and Moore.[3] The analysis was conducted on a Beckman Recording Amino Acid Analyzer. The relative amounts of amino acids present are shown in Table 1.

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

Preparation of polysaccharide. Purified polysaccharide was obtained from SE using a modification of the method of Evans and Kessel.[4] The pH of SE was adjusted to 7.0 with 1N NaOH, and sodium acetate added to a final concentration of 10 per cent. Following addition of 2.5 volumes of cold 95 per cent ethanol, the mixture was refrigerated overnight. After centrifugation at 2,000 r.p.m. for 30 minutes at 4° C., the polysaccharide precipitate was dissolved in a 10 per cent solution of sodium acetate and reprecipitated with ethanol. The precipitate was then redissolved in a 1 per cent solution of sodium acetate, and 0.25 volume of chloroform and 0.1 volume of n-butanol were added. The mixture was shaken for 60 minutes, centrifuged and the

TABLE 1. AMINO ACID ANALYSIS OF SOLUBLE EXTRACT (CONTAINING CRYPTOCOCCAL ANTIGEN)

Amino acid	*MicroMole/ml. of SE*
1. Lysine	7.9
2. Histidine	2.3
3. Arginine	8.3
4. Aspartic acid	10.5
5. Threonine*	4.9
6. Serine*	8.3
7. Glutamic acid	18.8
8. Proline	19.1
9. Glycine	33.3
10. Alanine	31.2
11. Valine	6.0
12. Methionine	2.3
13. Isoleucine	4.1
14. Leucine	7.5
15. Tyrosine*	1.5
16. Phenylalanine	3.8

* Not corrected for losses on hydrolysis.
Cysteine, cystine, and tryptophan were not looked for in this analysis.

aqueous layer containing polysaccharide was removed. After being washed in this manner seven times, the polysaccharide was reprecipitated with 95 per cent ethanol, dried on a freeze-dry apparatus and stored under vacuum over $CaSO_4$. Approximately 1.5 mg. was present per milliliter of the unfractionated SE.* One milligram of the purified polysaccharide contained 9.6 μg of protein. When made from extracts obtained by shaking rather than autoclaving cell suspensions, however, the purified polysaccharide contained only 1 μg or less of protein per milligram.

Preparation of protein fraction. The protein fraction was that portion of SE remaining after the first precipitation of polysaccharide with absolute ethanol. It was

* The specific carbohydrates in the polysaccharide fraction were not determined but the polysaccharide capsule of a strain of the same organism used here (*C. neoformans* var. *innocuous*) has been reported to contain 6.7 per cent hexuronic acid (as glucuronic acid), 18.1 per cent hexose (as glucose) and 31 per cent pentose (as arabinose).[5]

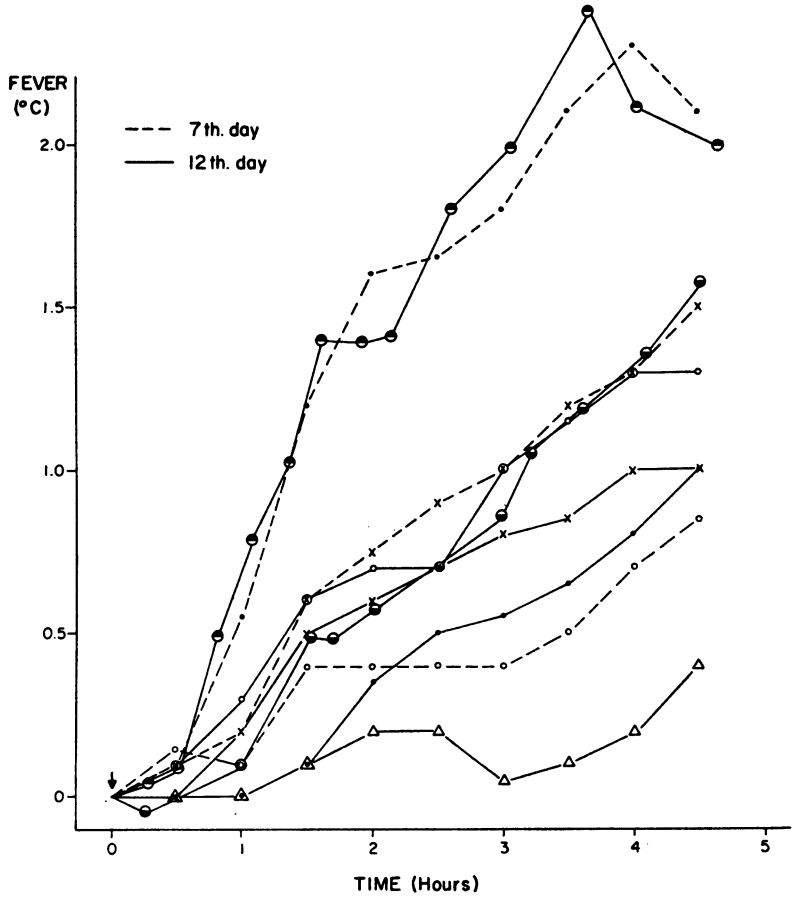

FIG. 1. Individual fever curves of Group I rabbits (sensitized by subcutaneous route) challenged with 5 ml. of the soluble extract (SE) of cryptococcal cells on 7th or 12th day following completion of sensitization.

dried on the flash evaporator at 40° C. and dissolved in water to a concentration of 960 μg of protein per milliliter. No attempt was made to purify the protein further. Protein was determined by the Lowry method[6] and indicated 2.2 mg. per milliliter of unfractionated SE.

Skin tests. The same vaccine used to sensitize rabbits was employed as the skin test antigen. One-tenth milliliter of antigen was given intradermally on the left side of the shaven abdomen. When additional skin tests were performed, the antigen was injected either on the opposite side of the abdomen or approximately three inches below the previous skin test site. Skin tests were read in 6, 24, 48, and 72 hours; maximal reactions were seen in 48 hours. Reactions with 5 mm. induration or more were considered positive.

Endotoxin tolerance. Typhoid vaccine (monovalent reference standard NRV-LS No. 1) made from *Salmonella typhosa* V-58 was used to establish endotoxin tolerance, as in the previous study.[1]

RESULTS

Sensitization of animals

In order to determine the best procedure to sensitize rabbits* to react to the soluble extracts from cryptococcal cells, the following protocol was devised.

Seven rabbits in Group I were given 0.2 ml. of whole cell vaccine in each front foot pad. One week later, an additional 0.4 ml. antigen was given by subcutaneous injection. Three of the rabbits were challenged with 5 ml. SE on days one and seven following the last exposure to vaccine. Two of these three rabbits and the remaining four were injected on the 12th day. Figure 1 shows the individual fever curves for animals challenged on the 7th or 12th day. Except for one unreactive rabbit, responses to the SE varied from 0.85° C. to 2.3° C. and were not significantly different on the two days. When the rabbits were again challenged on the 19th and 28th days they had essentially unchanged febrile responses.

These data indicate that rabbits were sensitized by the subcutaneous route and attained their maximal hypersensitivity by the 7th day following completion of sensitization regimen. Their degree of sensitivity appeared to persist almost undiminished up to the 28th day.

Six rabbits (Group II) were each given 1 ml. of vaccine intravenously every other day for a total of three inoculations. Two rabbits were challenged on the 8th day and the remaining four on the 11th day following completion of the sensitizing regimen. Figure 2 indicates the responses to intravenous inoculation of 5 ml. SE. The fevers ranged between 1.1°C. and 1.7° C. (except in one unresponsive rabbit) and were comparable to those induced by SE in rabbits sensitized subcutaneously.

When animals in Group II were again challenged on the 16th and 23rd days, the average fever indices were similar to those produced earlier (see Fig. 3). According to these data, maximal sensitivity, as evidenced by fever, appeared to be reached by the 8th day.

These findings suggest that the route used to sensitize rabbits to *C. albidus* does not significantly affect either the degree or the period of acquired hypersensitivity as evidenced by the febrile response to intravenous

* Rabbits in our colony did not carry cryptococci in their intestinal tracts and were presumably not "naturally" sensitized to react to products of this organism.

inoculation of SE. Because of its technical simplicity, the intravenous route was subsequently used for sensitization.

To determine when sensitized animals ceased to have significant febrile responses when challenged with SE, both subcutaneously and intravenously sensitized animals were injected at 7 to 10 day intervals for a total of 6 to 7 weeks after completion of the sensitization regimens. Figure 3 presents the average fever indices for the animals in both groups. Most rabbits continued to have significant febrile responses when tested on the 28th or 35th day. Thereafter, Group I rabbits (injected by subcutaneous route) were markedly less reactive to SE, with an average fever index of 10.5 on the 44th day. Similarly, Group II (intravenously injected rabbits) had an average of only 11.7 when challenged on the 49th day. Thus most

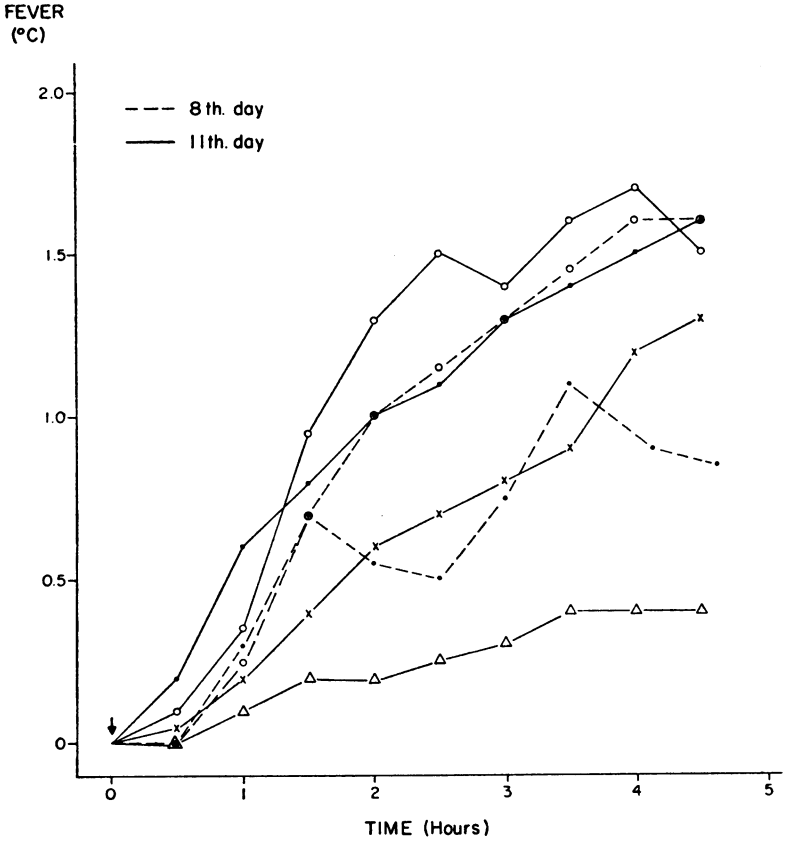

FIG. 2. Individual fever curves of Group II rabbits (sensitized by intravenous route) challenged with 5 ml. of the soluble extract (SE) of cryptococci on 8th or 11th day following completion of sensitization.

animals are responsive for at least 4 to 5 weeks after sensitization by either route, with some individuals remaining sensitized for longer periods.

Sensitizing properties of C. albidus soluble extract (SE)

To determine whether SE could sensitize by itself, rabbits were injected repeatedly with 5 or 10 ml. dosages (totalling 25-35 ml.) over a 2-week period.

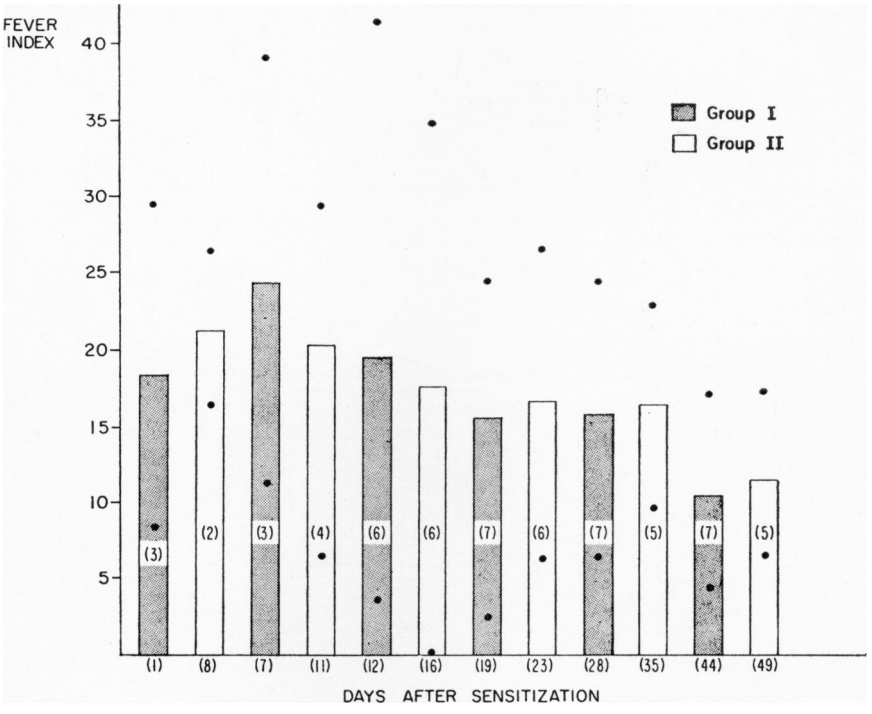

FIG. 3. Average fever indices of rabbits (Groups I and II) given repeated injections of SE over a 7-week period. The number in brackets indicates the interval in days after completion of sensitization. The dots represent the maximum and minimum fever indices.

No rabbits responded with fever until a total dose of at least 20 ml. SE had been given in several spaced injections over a period of one or more weeks. Subsequent inoculation of 5 or 10 ml. produced characteristic fevers in about half the recipients. However, repeated inoculations of 5 ml. SE at weekly intervals failed either to augment or maintain the febrile responses of a group of seven rabbits that had been originally sensitized with vaccine and whose reactivity to SE was waning.

Tolerance and skin tests

Five sensitized rabbits were given intravenous inoculations of 5 ml. SE on three consecutive days. The average febrile response of 1.3° C. on the first day was reduced to less than 0.3° C. on the third day. Shadowgraphs of the daily fever curves for three of the rabbits are shown in Figure 4. As indicated in Figure 5, the rabbits gradually recovered their responsiveness when challenged at intervals after development of tolerance.

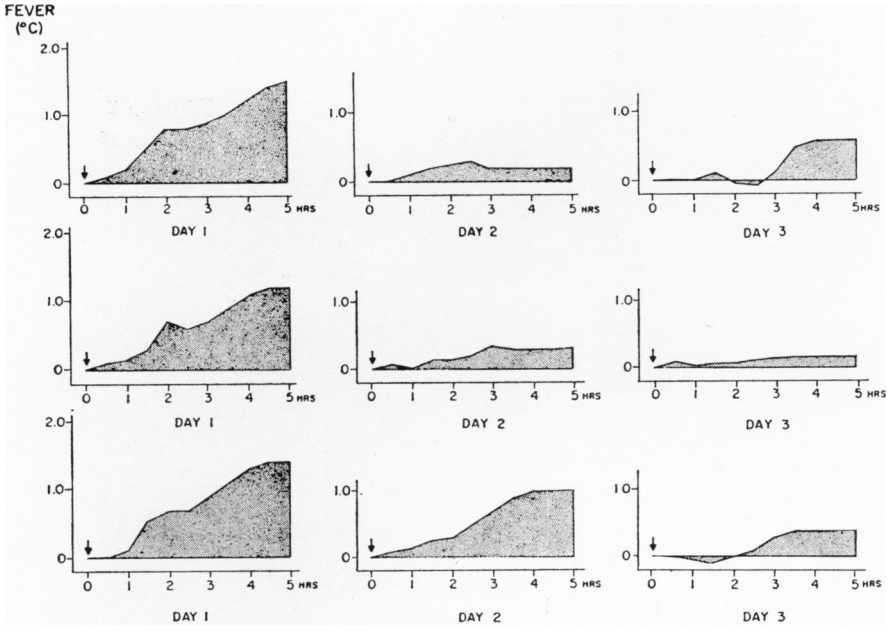

FIG. 4. Representative febrile responses of three sensitized rabbits showing the development of tolerance to 5 ml. soluble extract (derived from *C. albidus* cells) given on three successive days.

To determine the correlation of positive skin tests with fever as evidence of hypersensitivity to the soluble extract of *C. albidus,* four rabbits were sensitized by the intravenous route (see METHODS). All rabbits developed significant febrile responses to intravenous injection of SE. Four or five days later when these animals were skin-tested, two had strongly positive skin reactions, one was slightly positive, and the fourth was negative. There was no correlation between the magnitude of the febrile response and the intradermal reaction to the vaccine. However, after tolerance to the pyrogenic action of SE had been established by three

TABLE 2. SKIN TEST REACTIONS (AT 48 HOURS) OF FOUR SENSITIZED RABBITS BEFORE AND AFTER INDUCTION OF TOLERANCE TO THE SOLUBLE EXTRACT (SE) DERIVED FROM C. ALBIDUS CELLS

	Rabbit no.	*1*	*2*	*3*	*4*
Sensitized	Skin test	15 mm I 17 mm E	5 mm I 10 mm E	10 mm I 11 mm E	4 mm I 1 mm E
	Max. fever	1.2	1.2	0.8	1.5
Tolerant	Skin test	4 mm I 6 mm E	4 mm I 1 mm E	1 mm I 1 mm E	2 mm I 1 mm E
	Max. fever	0.2	0.2	0.2	0.1

I = induration. E = erythema.

Four normal rabbits had negative skin tests and were unresponsive to intravenous inoculation of SE.

daily inoculations, the three rabbits with initially positive skin tests became negative. Table 2 summarizes these data.

Therefore, although there was no good correlation between fever and skin reactivity as measures of hypersensitivity to *C. albidus,* tolerance to fever induced by daily injection of SE was associated with conversion of

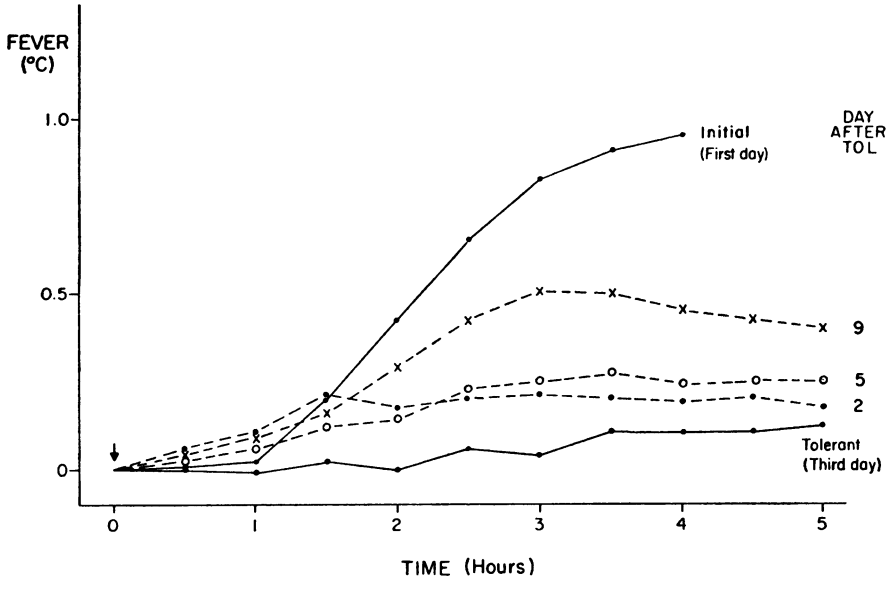

FIG. 5. Mean responses of four hypersensitive rabbits to soluble extract (SE) given initially and on days 2, 5, and 9 after induction of tolerance.

initially positive skin tests to negative ones. Similar findings have been reported in rabbits sensitized by BCG and tested with tuberculin.[2]

Febrile responses to purified polysaccharide of C. albidus

Purified polysaccharide was obtained from the extract (see METHODS) and tested in both sensitized and normal rabbits to see if this material contained the pyrogenic activity of the crude extract.

TABLE 3. RESPONSES OF NORMAL AND SENSITIZED RABBITS TO VARYING AMOUNTS OF PROTEIN AND POLYSACCHARIDE DERIVED FROM A SOLUBLE EXTRACT (SE) OF C. ALBIDUS CELLS

Material given	*Quantity in material injected*		*Average febrile response of rabbits (°C.)*	
	Protein	*Polysaccharide*	*Sensitized*	*Normal*
	mg.	*mg.*		
SE (5 ml.)	11	7.5	1.5 (3)	0 (3)
Protein	9.6		1.8 (3)	0 (3)
	4.8		0.7* (3)	0 (3)
	2.0		0.3† (6)	0 (3)
	1.0		0 (2)	0 (3)
	0.5		0 (2)	0 (3)
Polysaccharide	0.1	13	0.3‡ (3)	—
	0.2	20	0.9 (5)	0.9 (5)

* 1 of 3 rabbits unreactive.
† 3 of 6 rabbits unreactive.
‡ 2 of 3 rabbits unreactive. (Single response of 0.7° C.)

Six vaccine-sensitized rabbits were divided into three groups of two each. The first group was given 10 μg of purified polysaccharide intravenously; the second, 50 μg and the third group received 100 μg. None of the six animals had a significant febrile response.

A single injection of 300, 600, or 900 μg was given to three additional sensitized animals. None developed fever although all had febrile responses of 0.8° C. to 1.2° C. when given 5 ml. of SE 2½ hours later.

Thirteen milligrams of purified polysaccharide produced a fever of 0.7° C. in one of three sensitized rabbits (see Table 3).

Fourteen sensitized rabbits and seven normal rabbits were each given 20 mg. of purified polysaccharide intravenously. Thirteen of the fourteen hypersensitive rabbits and all the normal rabbits had febrile responses of 0.5° C. to 1.9° C. which were similar in both groups. Figure 6 indicates

the average fever curve for five representative rabbits from the hypersensitive and normal groups.

It is apparent from these results that small doses of polysaccharide were nonpyrogenic in both normal and sensitized rabbits. In larger amounts, this material caused fever of equal magnitude in both groups of animals and,

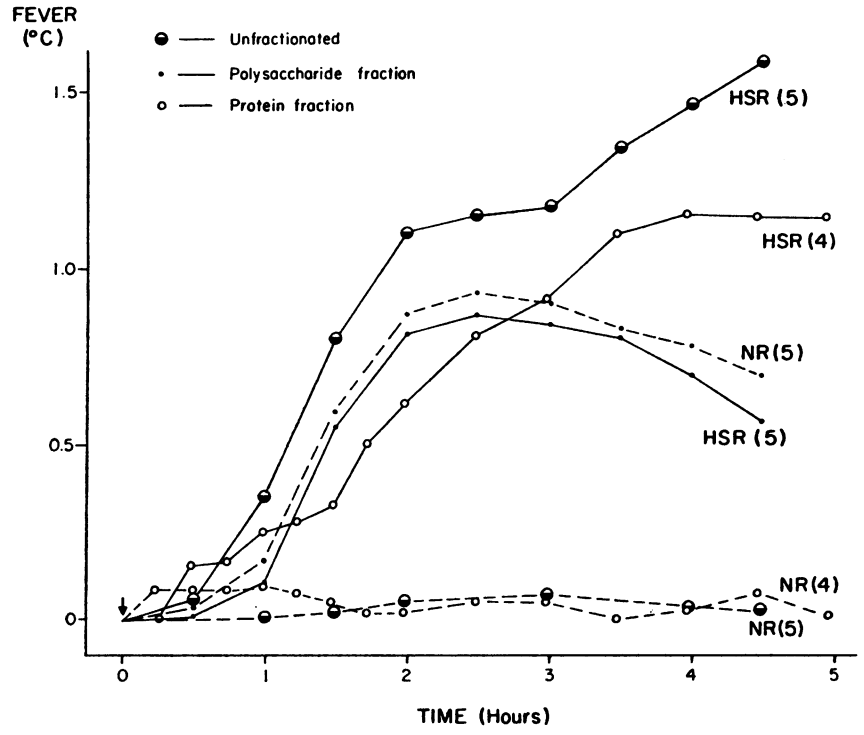

FIG. 6. Mean febrile responses of two groups of rabbits (one normal and the other sensitized) to 5 ml. of the unfractionated soluble extract (SE) and to 20 mg. of the polysaccharide and 9.6 mg. of the protein fractions derived from SE.
HSR = hypersensitive recipient; NR = normal recipient. Numbers in parentheses indicate number of rabbits in each group.

therefore, did not appear to require a previous state of sensitivity to produce its pyrogenic effects.

Although the delay in onset of fever following intravenous inoculation of polysaccharide indicated that this material differed in its action from the endotoxins of Gram-negative bacteria, further cross-tolerance experiments were carried out to confirm that the two agents were unrelated. Three rabbits were injected with 20 mg. of polysaccharide and several days

later received an initial injection of an endotoxin contained in typhoid vaccine. The animals were then made tolerant to the endotoxin by a series of six daily injections (see METHODS). On the day following the last injection of endotoxin they were given a second injection of polysaccharide. Figure 7 shows the average febrile responses of these rabbits to the two injections of polysaccharide as well as their initial and tolerant responses to endotoxin. Although endotoxin-tolerant animals responded to the poly-

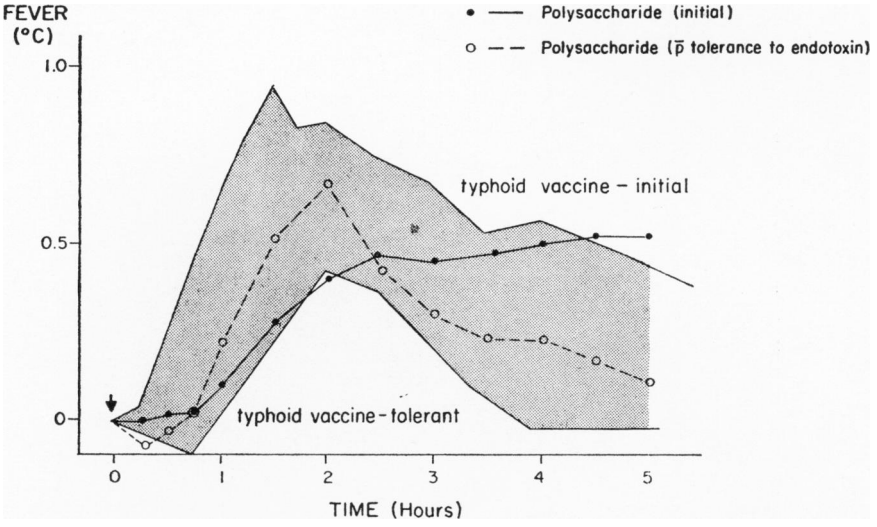

FIG. 7. Average febrile responses of three rabbits to 20 mg. polysaccharide (derived from *C. albidus*) given before and the day after tolerance to a bacterial endotoxin had been established by six daily injections of typhoid vaccine. The shaded outlines indicate the mean initial and tolerant responses to typhoid vaccine.

saccharide with a slightly different type of fever, the average fever indices for these responses were the same (see Fig. 8). The differences in the febrile response to polysaccharide as well as the ability of endotoxin-tolerant rabbits to respond fully to this material suggest that the cryptococcal polysaccharide is not identical with the endotoxins of Gram-negative bacteria and differs in its mechanism of inducing fever.

Response to the crude protein fraction

Since polysaccharide did not appear to be the fraction of SE that induced fever in specifically sensitized rabbits only, further experiments were conducted with the protein fraction.

Table 3 contains the data obtained when vaccine-sensitized and normal rabbits were challenged with different concentrations of the protein fraction

recovered from the soluble extract (SE). It is apparent that the protein fraction caused fever only in sensitized animals, whereas in dosage of 20 mg. the polysaccharide produced equal fevers in both sensitized and normal animals (see Fig. 6 also). Three of six sensitized rabbits responded with small fevers when challenged with 2.0 mg. of protein, indicating

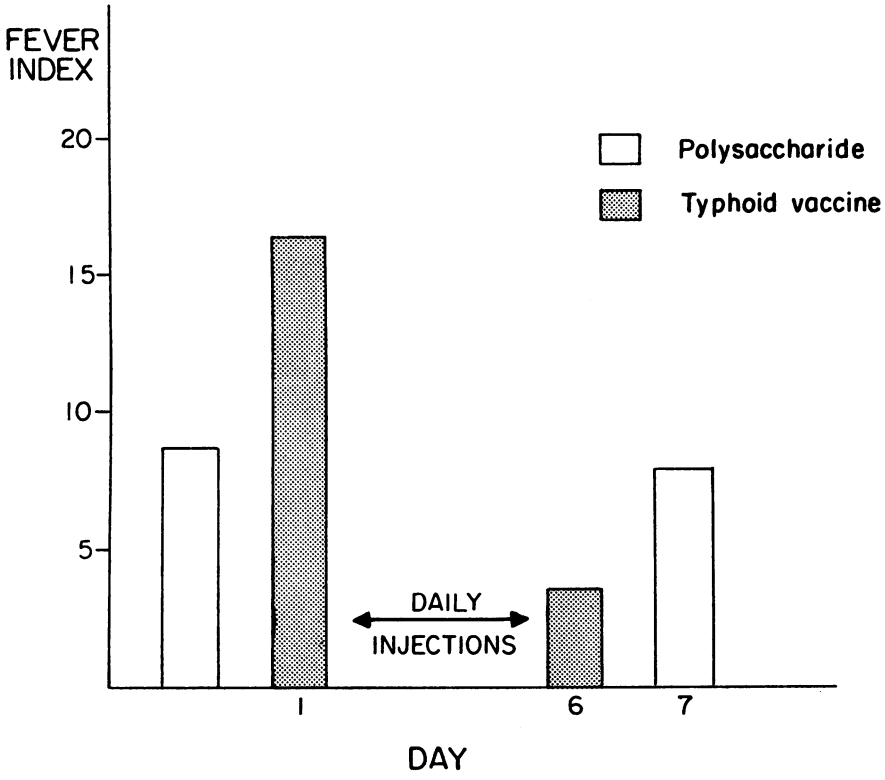

FIG. 8. Mean fever indices for the responses of the three rabbits shown in Fig. 7.

that this was about the minimal pyrogenic dose. In doses of less than 2 mg. the protein consistently failed to produce fever.

These results clearly indicate that the protein fraction is responsible for the febrile responses that develop only in sensitized rabbits.

Passive transfer of reactivity to SE

Since there was evidence that the protein fraction of SE was acting as an antigen in specifically sensitized rabbits, an attempt was made by passive transfer experiments to determine whether hypersensitivity, as evidenced

by febrile reactivity to this cryptococcal product, was of the immediate or delayed type, and hence might be transferred by serum or mononuclear cells, respectively, from sensitized donor rabbits to unsensitized recipients.

Processing of donor cells and plasma for passive transfer

1. *Tissue and blood cells.* Three to five days before a passive transfer experiment, several sensitized rabbits were challenged with 5 ml. of *C. albidus* SE and their fevers recorded. Only rabbits with fevers of 0.9° C. or greater were selected as donors. On the day of the experiment, the donor was given 84 mg. of pentobarbital sodium* and 2,000 USP units of sodium heparin (1,000 USP units per ml.) intravenously. After the chest and abdominal wall had been shaved, the rabbit was exsanguinated by cardiac puncture. The abdominal cavity was then opened and the spleen removed and placed in a petri dish containing approximately 15 ml. of saline. The lymph nodes were dissected from the root of the mesentery and placed in a second petri dish with saline.

The blood was centrifuged at 2,000 r.p.m. at 4° C. for 30 minutes, the plasma removed, and stored at 4° C. The blood cells were washed twice in approximately equal volumes of physiological saline and after final suspension in saline the total white blood cell count was determined.

The spleen was stripped of its capsule, cut into small fragments and gently forced with pestle through a No. 40-gauge copper and mesh screen placed over the mouth of a glass beaker. The lymph nodes were processed in the same manner. Total cell counts were determined for each preparation.

The blood cells, spleen, and node cell suspensions were given intraperitoneally, each to a single unsensitized rabbit. Forty-eight hours later, the three recipients were injected intravenously with 10 ml. of SE. On the day of challenge with SE, a fourth recipient received an injection of plasma from the same donor followed in two hours by 10 ml. of SE.

2. *Chronic peritoneal exudates.* Each of five sensitized rabbits were given 100 ml. of sterile, heavy mineral oil intraperitoneally. Three days later, each animal was given 500 ml. of physiological saline intraperitoneally, and the peritoneal fluid immediately withdrawn, placed in 250 ml. glass centrifuge bottles, and centrifuged at 2,000 r.p.m. for 30 minutes at 4° C. The supernatant of each exudate was discarded and the cells washed three times with pyrogen-free saline. After the third washing, the cells were suspended in 30 ml. of pyrogen-free saline and total cell differential and viability counts were done. In two experiments (Nos. 4 and 5) two exudates were pooled; a single exudate was used for the third experiment (No. 6).

* Diabutal® (60 mg./ml.), Diamond Laboratories, Inc., Des Moines, Iowa.

Each exudate was given intravenously to a normal recipient; 24 and 96 hours later, two of the recipients were injected with 5 ml. SE; the third recipient was challenged at 48 hours only.

Results of passive transfer studies

Table 4 presents data obtained from seven passive transfer experiments. Recipients of spleen cells in experiment No. 1 and of white blood cells in experiment No. 2 had weak but characteristic febrile responses when challenged 48 hours after receiving these materials. The two rabbits receiving peritoneal cell exudates containing a high percentage of mononuclear cells developed fever when challenged with SE. None of the recipients of plasma from sensitized donors responded to SE with fever.

These preliminary results suggest that sensitization to *C. albidus,* as measured by febrile response to intravenous inoculation of SE, can be passively transferred within 48 hours by certain mononuclear cells but not by plasma, a feature characteristic of hypersensitivity of the delayed type.

DISCUSSION

In an earlier paper of this series, Briggs and Atkins[1] reported that intravenous inoculation of live or autoclaved cells of *C. albidus* induced fevers in normal rabbits. The degree of fever was directly related to the dosage of cells above a minimal pyrogenic dose of 5×10^7.

These authors also obtained a soluble substance by either autoclaving or mechanically agitating a saline suspension of cells. This agent produced fever primarily in rabbits with previous exposure to the fungal cell or its extracts and when given daily, rapidly induced a state of complete refractoriness (tolerance) to its pyrogenic activity. Animals rendered tolerant to the soluble substance had undiminished febrile responses, however, to the cells of *C. albidus.* The pyrogenic factors in the intact cell and soluble extract could both be differentiated from the endotoxins of Gram-negative bacteria by the fact that rabbits with preestablished tolerance to endotoxin had unaltered febrile responses to these materials.

On the basis of these findings, it was concluded that *C. albidus* contained two separate factors that could cause fever. One was associated with the whole cell and behaved as a particulate substance. The particulate nature of the cells did not in itself appear to be critical since in other studies endotoxin-free polystyrene latex particles of the same size and number failed to cause fever.[7,8] The second factor, present in saline extracts of the encapsulated cell, was soluble and presumed to be an antigen, since it caused fever almost exclusively in specifically sensitized animals.

TABLE 4. DATA FROM SEVEN PASSIVE TRANSFER EXPERIMENTS, FOUR WITH PLASMA, TISSUE AND BLOOD CELLS AND THREE WITH PERITONEAL EXUDATES. (SE = SOLUBLE EXTRACT, DERIVED FROM CRYPTOCOCCI.)

Donor			*Recipient*				
			Febrile response (° C.) to I.V. inoculation of SE (5-10 ml.) after receiving:				
Exp. no.	*Fever (° C.)*	*Hours between transfer and challenge with SE*	*Blood cells* (6.5-14 x 10^8)	*Plasma* (60-75 ml.)	*Spleen* (1-3 x 10^9)	*Lymph nodes* (2-3 x 10^9)	*Peritoneal exudate* (2.5-3 x 10^8)
1	1.4	48	neg.		0.4	neg.	
		96	neg.**		neg.**	neg.**	
2	1.2	2		neg.			
		48	0.5	neg.**	neg.	Δ	
3	1.1	2		neg.			
		48	neg.	neg.**	neg.	neg.	
4	1.0	24					1.0†
	1.1*	96					neg.**
5	1.3	24					neg.‡
	0.9*	96					neg.**
6	0.9	48					2.0§
7	neg.	2		neg.			
	(control)	48	neg.	neg.**	neg.	neg.	

* Second donor (see Text).
** Retested.
Δ Recipient died before injection of SE.
† 89 per cent mononuclear cells.
‡ 43 per cent mononuclear cells.
§ 89 per cent mononuclear cells.

In view of the observation that normal rabbits occasionally responded to the soluble extract (SE) with fever,[1] the studies reported here were designed to determine the nature of this material and the factors present in it that might induce fever.

When initially prepared, the soluble extract (SE) contained approximately 2.2 mg. of protein per ml. and 1.5 mg. of polysaccharide per ml. Protein and polysaccharide fractions were subsequently recovered from this material; the protein fraction was used in its crude form, but the polysaccharide was purified. The protein caused fever only in sensitized rabbits; no rabbit had a febrile response to less than 2.0 mg.; all sensitized animals, however, developed fever when challenged with 9.6 mg. of the protein fraction (see Table 3). Indirect evidence to indicate the role of the protein in producing the fevers caused by the unfractionated extract (which was also active primarily in sensitized rabbits) was obtained when the extract was prepared by shaking rather than by autoclaving the cell suspensions. The protein content of this material was approximately 1.5 mg. per ml., two thirds the amount of protein present in the material prepared by autoclaving. It was necessary to give about twice the volume of the "shaken preparation" (10 ml. containing 15 mg. protein) to produce febrile responses in sensitized rabbits equivalent to those obtained when 5 ml. of the autoclaved preparation (11 mg. protein) was used.* Because of the greater ease of preparation as well as its greater effectiveness per volume, autoclaved rather than shaken material was used in the studies reported here.

In sufficient amounts, the purified polysaccharide fraction, containing only 9.6 μg of protein per ml. of polysaccharide, caused delayed but brisk fevers that were equal in normal and sensitized rabbits. Dosages of 1 mg. or less were nonpyrogenic, but one of the three recipients given 13 mg. had fever and all animals developed fever when given 20 mg. (containing 192 μg of protein). It seems unlikely that fever in the sensitized animals was due to the small amount of protein present in the preparation since at least ten times this amount of protein (2.0 mg.) was required to obtain febrile responses in hypersensitive animals (see Table 3). Certainly the protein present did not cause fever in normal rabbits. Since 10 ml. of unfractionated SE contain about 15 mg. of polysaccharide, it now seems likely that the occasional febrile response previously induced by this dose of extract in unsensitized animals[1] may be attributable to the polysaccharide present in the whole extract.

* Similar results were obtained in the earlier study in which saline suspensions of cryptococci were agitated in a magnetic mixer for 1 to 3 days.[1] The volume of material necessary to produce comparable fevers was 10 or more ml., as in the shaken preparations used here.

Other investigators have observed that complex polysaccharides can cause fever in normal rabbits. Certain preparations of glycogen[9] and methyl cellulose (with molecular weight in excess of 200,000)[10] are reported to be pyrogenic. Bennett[11] inoculated rabbits with crude dextran (M.W. 100-200,000) in concentrations varying from 10 to 200 mg. He obtained slight fevers with 10 mg. and significant febrile responses when 20 to 30 mg. were injected. Dosages of 100 to 200 mg. produced rapidly rising fevers within 30 to 60 minutes. To exclude the possibility of pyrogen contamination of the various dextrans used, Bennett deliberately contaminated some samples with endotoxin (typhoid vaccine or Pyromen, a Pseudomonas concentrate); he then hydrolyzed the dextrans by boiling at an acid pH followed by re-precipitation with alcohol. The contaminated dextrans retained pyrogenic activity while uncontaminated dextrans, treated in a similar manner, lost their fever-inducing properties. Unfortunately, information was not given as to the amount of endotoxin added to the dextrans or whether the endotoxins added to the contaminated preparations lost any of their pyrogenic activity after this treatment. A small amount of endotoxin initially present as an inadvertent contaminant in the dextran might be inactivated by this technique; development of equal fevers in normal and endotoxin-tolerant rabbits given dextran would have been more conclusive evidence that such materials were not detectably contaminated with bacterial pyrogens.

In the studies reported here, rabbits rendered endotoxin-tolerant had nearly unchanged febrile responses when given 20 mg. of the purified polysaccharide of *C. albidus*. This finding, together with the longer latent period before onset of fever suggests that the fever-inducing activity of the fungal polysaccharide was not related to incidental contamination with Gram-negative bacterial endotoxins.

The present study indicates that *C. albidus* has at least three factors that induce fever in rabbits. The first is the whole cell which is equally pyrogenic in normal and hypersensitive rabbits. The second agent is associated with the protein fraction derived from soluble extracts (SE) of cell suspensions that have been autoclaved or shaken. Since this material is active only in specifically sensitized rabbits and since sensitivity was passively transferred to normal rabbits with mononuclear cells rather than with plasma (see Table 4), the protein in SE appears to cause fever by means of an immunological reaction of the delayed type, presumably analogous to those produced by injection of tuberculin or staphylococcal protein antigen into hypersensitive rabbits.[12]

The third pyrogenic factor associated with *C. albidus* is the capsular polysaccharide. This material produced equal febrile responses in normal rabbits and in those sensitized by injections of heat-killed cells; it appears

unlikely, therefore, that the polysaccharide is acting as an antigen. For reasons that are unclear, pyrogenic tolerance rapidly develops to daily injections of the polysaccharide (unpublished observations) as well as the protein (see Fig. 4), a finding that suggests that these agents differ from the intact cryptococcal cell in the mechanism by which they cause fever.

In view of the number of pyrogenic factors present in cryptococci the question may be raised why disseminated cryptococcosis is so often associated with little or no fever clinically. It is tempting to speculate that the relative nontoxicity of this yeast may be in part related to its large polysaccharide capsule. On a weight basis, it is apparent that this material is far less pyrogenic than are the endotoxins forming the cell walls of Gram-negative bacteria. The minimal pyrogenic dose of the yeast polysaccharide, as determined by this study, appears to be about 10 mg., whereas that of various purified lipopolysaccharides from Gram-negative bacteria has been estimated between 0.0003 μg[13] and 0.03 μg[14] for rabbits of the same size. The difference between the toxicity of these two agents as expressed by fever-inducing capacity is, therefore, at least 3×10^5. In terms of the number of organisms involved, a single pyrogenic dose of polysaccharide is derived from $1\text{-}2 \times 10^9$ organisms, whereas as few as 3×10^4 Gram-negative bacteria of the most pyrogenic species are capable of causing fever.[15]

Since this capsular polysaccharide is not only relatively nontoxic but appears to be metabolized slowly if at all by the host's tissues,[16] it seems possible that the somatic protein of the yeast may be partially prevented from exerting its effect as an antigen in an infected host. The fungal cell may thereby be less able to cause the kind of responses (such as fever) that arise from immunological reactions involving tissue damage and that are characteristically induced by organisms like the tubercle bacillus or staphylococcus in specifically sensitized animals.

SUMMARY

The cause of fever following intravenous injection of a soluble extract (SE) obtained from a nonpathogenic fungus, *Cryptococcus albidus,* has been investigated. Polysaccharide and protein fractions were derived from this material and their fever-inducing properties compared. The polysaccharide was regularly pyrogenic only when given in relatively large doses of 20 mg. and induced similar fevers in both normal and specifically sensitized rabbits. The protein fraction, on the other hand, was pyrogenic in much lower dosages (2 mg.) but like the unfractionated extract caused fever only in sensitized animals and, hence, appeared to act as an antigen.

Since the capacity to react to the soluble extract with fever was passively transferred with mononuclear cells but not with serum from sensitized donor rabbits to unsensitized recipients, the sensitivity appears to be of the delayed type.

Two regimens of sensitization with a cryptococcal vaccine were compared. As measured by the degree of fever induced by the extract, sensitization was nearly maximal by the end of the first week and persisted without significant change for 4 or 5 weeks after completion of the series of injections, whether given by the subcutaneous or intravenous route.

Although skin tests and fever were not well correlated as indices of hypersensitivity, animals desensitized by daily injection of the cryptococcal extract became unresponsive to the fever-inducing effect of the antigen present in the extract and developed negative intradermal reactions.

The possible significance of these findings to the manifestations of clinical cryptococcosis has been discussed and compared with responses to other bacterial infections.

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