Separation of Contaminating Pyrogenic Material from Commercial Bovine Serum Albumin.* (31546)

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Bacterial endotoxins, with their multiple potent effects on biologic systems, represent a constant possible source of variability in experimental biology and pathology. In immunologic studies, endotoxin contamination is rarely considered important, although these materials have potent effects in enhancing antibody formation(1) and have been demonstrated recently to be effective in breaking immunologic tolerance(2).

In studies of the acute local inflammatory reaction produced by foreign proteins and polysaccharides, we were surprised that bovine serum albumin (BSA) caused a more intense inflammatory reaction than chemically similar human serum albumin (HSA) (3). Also, profound immunologic tolerance to HSA was readily produced in newborn rabbits by antigen loading during the first few days of life, using the method of Weigle(4). With BSA, however, almost no immunologic tolerance resulted.

That these divergent results might be explained on the basis of contamination of commercially available BSA with bacterial endotoxin was suggested when the studies of the possible causes of acute synovial inflammation in rabbits were extended to endotoxins. An acute inflammatory reaction was produced with doses of endotoxin approximately 1,000 times smaller than the dose causing fever in rabbits after intravenous injection(5). The samples of BSA used in the studies of synovial reactions for foreign proteins were sterile, but were not claimed by their manufacturers to be 'pyrogen-free.'

When several commercial samples of bovine serum albumin were tested intravenously in rabbits, all were found to contain a pyrogenic material, presumably bacterial endotoxin. Studies were undertaken on preparation of endotoxin-free bovine albumin, and on methods for chemical separation of the pyrogen from protein in commercial samples of bovine serum albumin by column chromatography with Sephadex G-100.

Methods and materials. 1. Samples of BSA. Lots of BSA were obtained from Armour and Co., Nutritional Biochemicals Corp., and Pentex Corp.

2. Testing for synovial inflammatory reaction. One-half milliliter of the material to be tested was injected into the suprapatellar bursae of 3-4 kg albino rabbits anesthesized with pentobarbital. Six hours later the animals were killed with pentobarbital, and the synovial exudate harvested by reentering the bursae with a syringe containing 2 ml of pyrogen-free saline and washing the bursae by repeated distension and withdrawal of the saline. Cell counts (Coulter Electronic Particle Counter) multiplied by the total volume of saline wash gave figures for the total cells in the synovial exudate. These methods have been described elsewhere(3,5).

3. Testing for pyrogenicity. The procedure used in selecting rabbits and in recording temperatures were similar to those previously reported(6). Precautions were taken to avoid pyrogenic contamination of syringes and glassware. Samples to be tested were cultured routinely in thioglycollate broth.

4. Isolation of non-pyrogenic BSA from sterile calf serum. Bovine serum albumin was separated from serum by the method of Kabat and Mayer(7). Blood from the jugular vein of calves was collected aseptically in 250 ml Red Cross blood serum bottles. It was permitted to clot overnight at 4°C before being spun at 3000 rpm for 1 hour in a refrigerated centrifuge. The serum was then transferred by aspiration into sterile, pyrogen-free bottles and mixed with an equal volume of 10% trichloracetic acid (TCA) at room temperature

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to precipitate the protein. The sediment was separated by centrifuging for 1 hour at 1250 rpm and washed 3 times with the TCA solution. The final washed precipitate, equivalent to 1000 ml of serum, was separated by centrifuging for 30 minutes at the same speed and then dissolved in 450 ml of sterile pyrogen-free distilled water mixed with 1000 ml of acetone. The latter component was removed by distributing the solution into several bags of Visking cellulose tubing and dialyzing against 10 liters of distilled water for 5 days at 4°C. The dialysis water was changed 3 times daily. The final protein solution was concentrated by blowing room temperature air across the dialysis bags and allowing the solvent to evaporate. The bag contents were pooled and centrifuged for 30 minutes at 15,000 rpm to remove precipitated material which was discarded. The final solution had a protein concentration of 1.3 gram% as measured by a standard biuret method and the total yield came to 17 grams. The protein gave a single peak in the albumin area on electrophoresis and also gave a reaction identical to that of commercial BSA in agar diffusion with hyperimmune serum.

5. Separation of endotoxin from commercial BSA by gel-filtration on Sephadex G-100 columns. Thirty-five grams of Sephadex G-100 (Pharmacia Fine Chemicals, Inc.) were allowed to swell while being stirred for 4 days in 2 liters of sterile pyrogen-free physiological saline. Small gel particles were removed by periodically interrupting the procedure and decanting the supernatant which was then replaced with fresh saline. The wet gel was autoclaved for 40 minutes at 121°C. This presumably sterilized the gel, but endotoxin would not have been inactivated by this procedure. The 2.5 × 120 cm column was autoclaved. All other components of the column were either soaked in benzalkonium chloride solution (1:750), autoclaved for 2 hours at 121°C, or heated for 2 hours at 168°C as practicable.

Every effort was made to maintain sterile technique while assembling and pouring the column. A sintered‡ glass disc was covered with a 5 cm layer of purified sand to prevent clogging of the pores with fine gel particles. The column was filled to ⅓ its height with saline solution before the gel suspension was introduced. The suspension was poured in slowly while being stirred. When the bed height reached 10 cm, the column outlet was partially opened. Slow addition of the gel suspension was continued until the column height reached 100 cm. A solvent reservoir containing non-pyrogenic sterile saline was then connected to the top of the column and the gel was washed with 3 liters of this material over a period of 5 days. At the end of this time, the column effluent was found to be both sterile and pyrogen-free (see *Results*).

Before addition of materials to be chromatographed, the reservoir was disconnected and the solvent level was allowed to drop until the gel was covered with a layer only a centimeter or two in depth. Since saline solutions of the proteins studied were more dense than the eluant, 1 or 2 ml of each containing 50 to 100 mg of protein were layered between the surface of the gel and the protein-free saline. Care was taken to avoid disruption of the gel or unnecessary mixing of the sample and the eluant. The protein solution was then allowed to flow into the gel before more eluant was added and the reservoir reconnected. Measurement of the effluent volume was begun as soon as the addition of the sample was completed. The flow rate was maintained at about 30 ml/hour. Five ml samples were collected in sterile pyrogen-free test tubes, immediately stoppered, and frozen until assayed. For the protein determinations, 0.6 ml samples were taken from the tubes with sterile pipettes and the optical density at 280 mμ was measured in microcuvettes having a 1 cm light path in a Beckman DU spectrophotometer.

Results. A. Pyrogenicity of commercial BSA. Fig. 1 is a typical pyrogenic response characteristic of those obtained with all 5 commercial BSA preparations tested. The recipient rabbit became febrile within 30 minutes after injection, and usually the fever curve was bi-phasic as illustrated in this example. The dark area of such curves was used to calculate the fever index, a standard technique for quantitation of febrile responses in rabbits(8).

‡ Morton bacteriological filter apparatus with ultra fine disc (Corning).

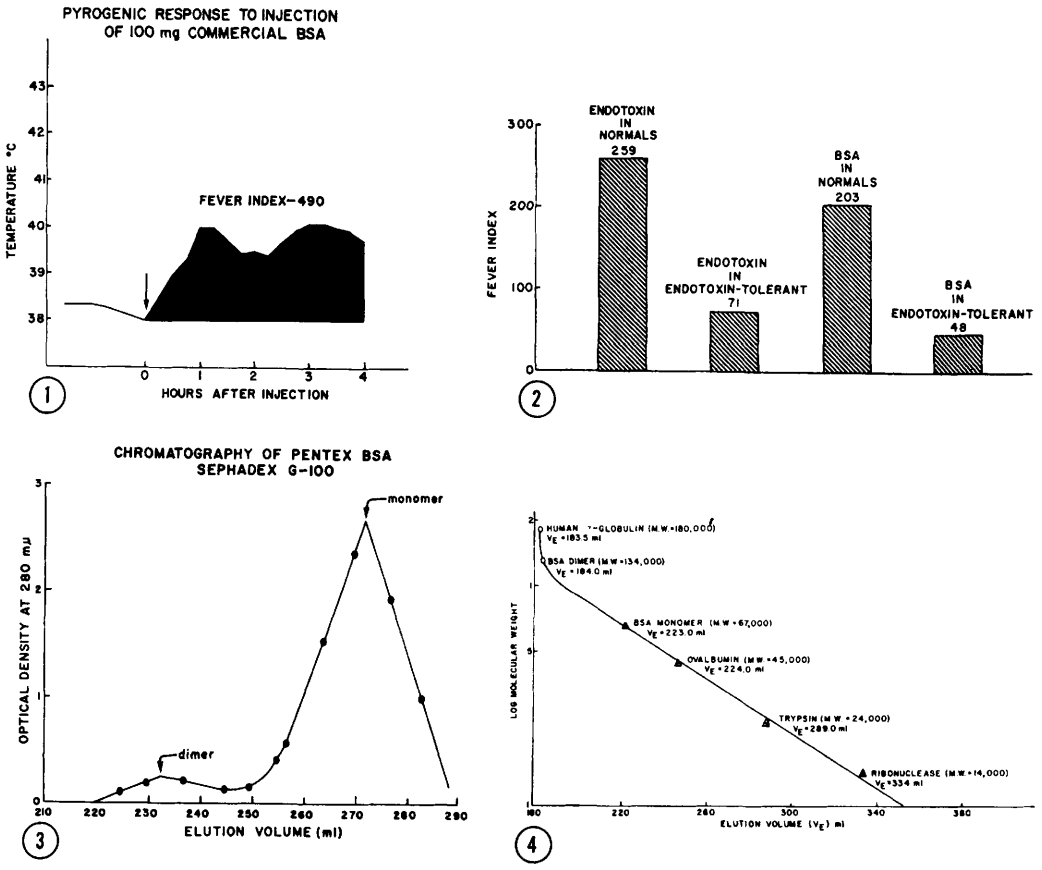

FIG. 1. Fever curve of a rabbit after intranvenous injection of a commercial BSA preparation. The black area of fever may be used to calculate fever index.
FIG. 2. Fever indices produced by BSA in normal and endotoxin-tolerant rabbits.
FIG. 3. Chromatographic separation of monomer and dimer BSA.
FIG. 4. Calibration of Sephadex G-100 column.

Crystalline BSA (Armour & Co., Lot #B22702), a material commonly used in immunologic experiments, produced fever in amounts as low as 100 mg (fever index 101). A sample of BSA from another source (Pentex Corp. Lot #19) was even more pyrogenic. One milligram caused a fexer index of 72; 5 mg, 234; 10 mg, 340; 100 mg, 490. This BSA sample was used in later purification studies with the Sephadex G-100 column.

Repeated daily injections of endotoxin cause progressively less fever in rabbits, a phenomenon that has been termed endotoxin tolerance. Such rabbits, hyporeactive to endotoxin, have been found to produce normal fever responses to other pyrogenic stimuli. The first and second columns of Figure 2 show the mean fever indices obtained in 4 normal rabbits with an initial injection of typhoid vaccine, and the response of the same rabbits after 7 days of repeated vaccine injection. A comparison of the mean fever index of BSA in 4 normal rabbits and in 4 rabbits made endotoxin-tolerant by repeated injections of typhoid vaccine is shown in the other two columns of Fig. 2.

B. Pyrogenicity and synovial inflammatory reaction with BSA prepared from sterile calf serum. The BSA prepared in the laboratory from sterile calf serum, using care to avoid bacterial contamination, was tested for pyrogenicity and for its ability to produce synovial inflammatory reactions in rabbits. No fever was produced by 120 mg given intravenously, and 5 mg injected intrasynovially into 6 rabbit knee joints produced only a minimal

TABLE I. Synovial Exudate 6 Hours After Injection of Sterile Pyrogen Free Saline (PFS) and Saline from the Sephadex G-100 Column.

	Total exudate cells (millions)	
	PFS	Column eluate
	1.6	6.0
	.5	3.6
	.5	4.3
	.5	5.1
	.6	2.6
	1.0	1.2
Mean	.8	3.8

inflammatory response (2.3 to 5.1 million exudate cells 6 hours after injection).

C. Pyrogenic and synovial inflammatory reaction of materials from the Sephadex G-100 column. BSA came off the column separated into 2 peaks, the first representing a small amount of dimer and the second, a much larger amount of monomer (Fig. 3). The identities were verified by the method of Andrews(9) by chromatographing a variety of proteins having different molecular weights as shown in Fig. 4.

The lack of resolution shown in Fig. 3 as evidenced by the spread in the protein peaks may have been caused by mixing of droplets between the bottom of the gel and the outlet valve of the column. Even though the volume in this region was reduced by incorporating a teflon core with a narrow channel down its center, better results could probably be obtained with columns originally designed to include a minimum of such space.

The pyrogen-free saline that was collected from the column before BSA samples were applied remained pyrogen-free in amounts of 10 ml injected intravenously into rabbits. Using the more sensitive method, synovial exudation, the saline that had passed through the column was slightly more reactive than the saline that was used as the eluant (Table I). This inflammatory response is within the general range that we have found with saline injection alone, in more extensive experiments (3).

When the heavily contaminated commercial sample of BSA was put through the Sephadex column, the pyrogenic activity was found primarily in the fractions preceding the protein (Fig. 5). Eluate from the monomer peak of BSA, representing 34 mg of the synovial protein, was non-pyrogenic (Fig. 5).

Discussion. From these studies it is clear that commercial supplies of bovine serum albumin (BSA) contain pyrogenic material that undoubtedly is endotoxin from Gram-negative bacteria. Probably this contamination begins with collection of non-sterile blood in slaughter houses, and may well be augmented during the various separation and purification procedures.

Several observations indicated that the fever-producing material is indeed endotoxin,

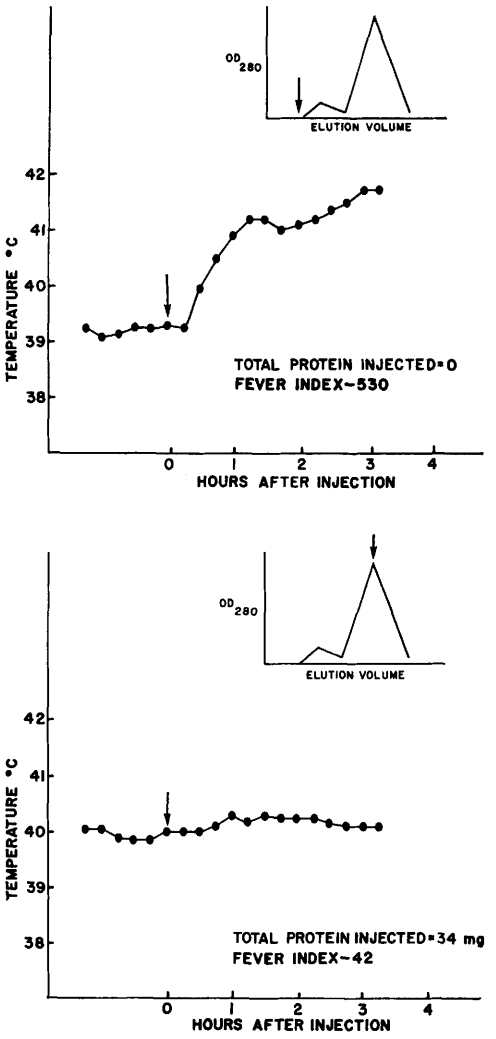

FIG. 5. In the supper section, a non-protein containing exudate contained the pyrogen, while the protein peak (below) was almost nonpyrogenic.

and not some pyrogenic reaction in rabbits from the injection of foreign protein. Rabbits made tolerant or hyporeactive to the pyrogenic action of typhoid vaccine were similarly hyporeactive to the pyrogen in BSA. Using sterile calf serum, we were able to prepare non-pyrogenic BSA. Finally, by chromatography of commercial samples of BSA on Sephadex G-100 gel the pyrogenic material could be separated cleanly from the monomer peak of BSA, a protein with a molecular weight of 67,000.

When very pyrogenic samples of BSA were chromatographed, pyrogen was separated completely from monomer BSA, but not from the dimer. Since such small amounts of endotoxin can exhibit pyrogenic behavior, contamination in the dimer is probably a reflection of the resolution problem mentioned above. Therefore, we must conclude that this method of separating endotoxin from protein works best for those proteins having molecular weights lower than or equal to that of monomer BSA. Separation of pyrogen from higher molecular weight compounds could probably still be accomplished with either a better designed column or by using Sephadex G-200. However, a full separation may prove difficult because, although the molecular weight of endotoxins obtained by chemical treatment of bacterial cell walls is thought to range from 1 to 10 million, smaller fragments in naturally occurring endotoxin contamination could be biologically active.

The biologic implications of the occurrence of significant endotoxin contamination in commercial BSA samples may be far-reaching. The protein is undoubtedly the most commonly used antigen in immunologic work, and interpretation of data after intravenous use of BSA as an antigen may well be in error because of the simultaneous injection of a potent immunologic adjuvant, endotoxin(1). Biochemists also use BSA to hold materials in solution, again without consideration of endotoxin effects. Simply reviewing the titles of a recent symposium on bacterial endotoxins(10) indicates the wide scope of biologic actions of endotoxin, and points to areas of research where endotoxin contamination of BSA could have altered results or led to their misinterpretation.

Summary. A pyrogenic material in commercial supplies of bovine serum albumin (BSA) has been identified as bacterial endotoxin by its hyporeactivity in endotoxin-tolerant rabbits. Non-pyrogenic BSA was prepared from sterile calf serum. Using Sephadex G-100 column chromatography, the pyrogen could be separated from the protein in commercial BSA samples. Because of the widespread use of commercial BSA in immunologic and other types of biologic research, the contaminating endotoxin could modify results or lead to misinterpretation of experimental observations.

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