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COLUMN LIQUID CHROMATOGRAPHY OF ENDOTOXINS

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SUMMARY

A new, fast and highly reproducible column liquid chromatographic method was elaborated for the analysis and small-scale preparative isolation of endotoxin from *Serratia marcescens* Bizio (ATCC No. 264). This procedure detects contaminants of such preparations with high sensitivity and it is capable of separating them from endotoxic components. Extensive heterogeneity of both 5% trichloroacetic acid and phenol–water-extracted endotoxin preparations was recorded. Heterogeneity among the endotoxic components of purified preparations could also be detected by this method. Measurements of biological activities, such as *Limulus amoebocyte* lysate activation, lymphoblastogenesis (mitogenicity) and tumor necrosis factor (TNF) liberation were carried out on the chromatographically separated fractions. During these studies, non-toxic but in vitro TNF-generating components of crude endotoxin extracts were also detected.

INTRODUCTION

The heterogeneity of endotoxin preparations was first reported in 1966 [1]. In this work, a weak anion-exchange resin, Amberlite XE220, was used which was kindly provided for our study by the Rohm and Haas Company (Philadelphia, PA, U.S.A.) as a ground powder of their Amberlite IR68. We used flotation to obtain a batch of rather uniform fragments from XE220, where the particle size varied between 200 and 400 mesh. The column size was 200 mm × 20 mm I.D. The collected tubes were manually developed for carbohydrate and nucleic acid content [1,2]. Considerable improvement was achieved by adapting the continuous-monitoring system for carbohydrates using an automatic analyzer [3,4], but the entire procedure remained rather complex, and the reproducibility of the chromatographic patterns depended on uniform fill-

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ing of the columns with the freshly regenerated resin and on the flow-rates of these repacked columns. The resolution given by this resin was not ideal either, in spite of all efforts to obtain a more homogenous particle size of the ground ion exchanger.

The study of methods for the isolation of chromatographically pure endotoxin preparations was renewed recently in our laboratories, using the Fast Protein Liquid Chromatography (FPLC) system of Pharmacia (Piscataway, NJ, U.S.A.).

We adapted the Mono Q column and the FPLC system of Pharmacia for the analysis of trichloroacetic acid (TCA) and 45% phenol-extracted endotoxin (PHW) preparations from *Serratia marcescens* Bizio (ATCC No. 264) bacterial strain. The methodology and the obtained results are reported here.

EXPERIMENTAL

Bacteria

Serratia marcescens Bizio No. 264 (non-pigmented) strain was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown in M3 media containing 1.5% tryptone, 0.3% NaCl, 0.23% Na_2HPO_4, 0.5% yeast extract, 0.5% beef extract and 0.3% glucose. A 20% glucose solution was prepared separately with sterile filtration and added to the media after the media were autoclaved to reach a 0.3% final glucose concentration.

Extraction of endotoxin

The 5% TCA procedure of Boivin et al. [5] was modified to obtain crude endotoxin. The preparation was further purified by precipitation with two volumes of ethanol containing 0.2% $MgCl_2$ and by sedimentation at 110 000 *g* for 3 h. [6]. The PHW procedure of Westphal and Lüderitz [7] was also applied to obtain crude and purified endotoxin preparations. The crude sample was obtained by the lyophilization of the dialyzed water phase of the extraction mixture. Purified endotoxin was obtained from this preparation by precipitation with ethanol and ultracentrifugation, as described by Westphal and Lüderitz [7].

Chromatography on Mono Q

A 0.5-mg sample of endotoxin, dissolved in 100 μl of 0.02 *M* ethanolamine, was injected to the top of the column. The endotoxin sample, 5 mg/ml, was filtered through a 0.22-μm Millipore membrane (Bedford, MA, U.S.A.). Eluent in pump A was 0.02 *M* ethanolamine (pH 9.6) containing 2% (v/v) eluent B, which was 0.5 *M* sodium perchlorate. Pump B contained eluent B. Elution started with 10 ml solvent A, followed by a linear gradient of increasing solvent B. The gradient reached a composition of 60% B in 60 min. At this point, 100% B was pumped through the system for 20 min. The flow-rate was 1.0 ml/min.

The effluent was sent through a variable-wavelength monitor (Isco Model 1840, Lincoln, NE, U.S.A.) at 230 nm.

Endotoxins adhere quite strongly to basic anion exchangers, as we observed earlier [2,3]. Therefore, it was essential to check the cleanliness of the column before using it again. This could be done by washing the column with solvent A and repeating the gradient elution with solvent B, while monitoring the effluent at 230 nm, as above. If all the endotoxin was eluted from the Mono Q column (Pharmacia), a rather clean baseline could be obtained. If endotoxin was not completely removed the column was purged by the injection of 500 μl pure pyridine followed by extensive washing with solvent A. This process was repeated until a clean baseline was achieved.

Determination of biological activities

Fractions (2 ml) were collected after the UV monitor and 0.5-ml aliquots of the fractions were dialyzed in a BRL MD1200 instrument (Gaithersburg, MD, U.S.A.) against distilled water to remove solvents A and B. The samples were tested for *Limulus amoebocyte* lysate (LAL) reactivity, lymphoblast induction and tumor necrosis factor (TNF) generation.

The LAL test was used to determine the endotoxin content of the fractions: 10^3, 10^6, 10^9, 10^{12}, 10^{15} and 10^{18} dilutions were made for this purpose in pyrogen-free saline. The chromogenic LAL test was applied by using the kit marketed by the M.A. Whitaker Corporation (Baltimore, MD, U.S.A.) and by following their procedure. The values were expressed as femtogram (10^{-15} g) endotoxin per ml. A purified endotoxin sample from the same strain served as a calibration standard, assuming that it consists of 100% endotoxin, but for the sake of simplicity, in this publication, the percentage light absorption measured at 405 nm has been plotted on Figs. 1–4. The LAL activity levels are indicated by large dots on these figures. Due to the very high dilutions of the samples, the removal of solvent A and solvent B by dialysis was not necessary for the LAL test. The chemicals in these solvents at 10^3 or higher dilutions showed no interference with the LAL test.

For the lymphoblast assay, dialyzed samples were used without further dilution. Five ICR mice (Skin and Cancer Hospital, Philadelphia, PA, U.S.A.) were sacrificed and single cell suspensions were prepared from their pooled spleens. Samples (0.1 ml) were added to $2 \cdot 10^6$ cells in 1 ml of RPMI 1640 medium and incubated at 37°C in a 5% CO_2-containing atmosphere for 68 h. At this point 1 μCi [^{3}H]-thymidine in 100 ml medium was added to each sample and incubation continued for four more hours. The cells were sedimented by centrifugation (1000 *g*, 5 min) mixed with 10% TCA and filtered, and the incorporation of [^{3}H]thymidine was determined by liquid scintillation counting. Stimulation index was calculated by taking the non-stimulated control's uptake as 1.00. Details of the procedure were described elsewhere [6].

The TNF-inducing potential of the collected fractions was determined by

the same procedure as described earlier. Briefly, dialyzed fractions were diluted 1:10 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% serum. Samples or controls (0.5 ml) were added in duplicate to $5 \cdot 10^5$ RAW 264.7 cells in 24-well tissue culture plates. After 18 h incubation, the media were collected, centrifuged, and the supernatants were frozen until they were tested for TNF. To test for TNF activity, L929 cells were preloaded with ^{51}Cr (0.3 mCi, Amersham, Arlington Heights, IL, U.S.A.) for 2 h before plating into 96-well plates at $4 \cdot 10^4$ cells/well in 100 μl of DMEM containing 10% serum. The cells were allowed to adhere for 2 h before addition of samples diluted in DMEM containing 2 μg/ml Actinomycin D (Sigma, St. Louis, MO, U.S.A.). Percentage lysis of L929 cells was determined by measuring ^{51}Cr released by control and samples with the following calculation:

$$\text{Lysis} = \frac{(\text{experimental release}) - (\text{spontaneous release})}{(\text{freeze–thaw release}) - (\text{spontaneous release})} \times 100\%$$

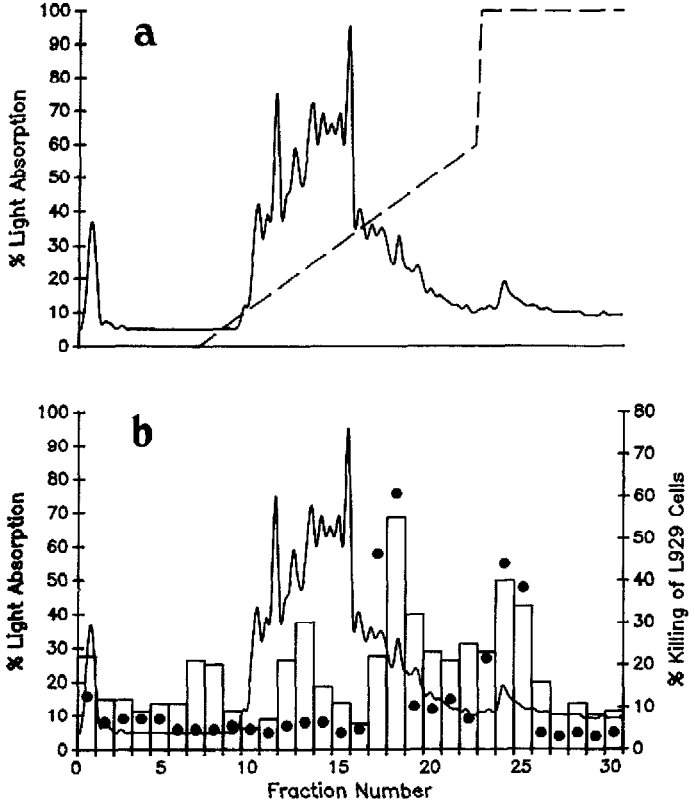

Fig. 1. (a) FPLC profile of TCA-extracted crude endotoxin. Solid line: eluted component monitored at 230 nm, using an ISCO Model 1840, at a sensitivity of 0.2. Broken line: solvent B gradient (b) The same chromatogram as in (a), but the elution profile is overlaid with LAL activity measurements (dots) and by the TNF-inducing activity of the fractions (bars).

Each supernatant was assayed in triplicate at three dilutions.

RESULTS

Extensive heterogeneity of the crude TCA or PHW extracts was observed at 230 nm as shown in Figs. 1a and 2a. Two further notable observations were made during these analyses. The first was the high reproducibility of the many peaks displaced from the column by the linearly increasing concentration of the perchlorate. These left the column between 10 and 40% solvent B content of the elution mixture in the case of the crude PHW extract, and between 10 and 30% solvent B content if crude TCA endotoxin was applied. The two patterns in this range were similar but clearly not identical. The other interesting observation was that the absorption of the same concentration of crude PHW endotoxin at 230 nm was much higher than the absorption of the crude TCA-extracted preparation.

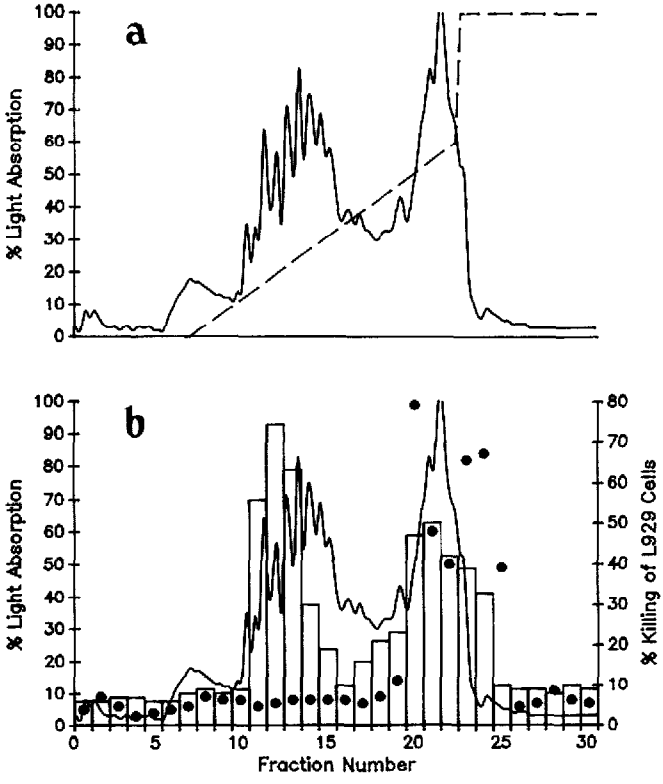

Fig. 2. (a) PHW-extracted crude endotoxin chromatographed on the FPLC system. Solid line: eluted component monitored at 230 nm, at a sensitivity of 0.2. Broken line: solvent B gradient. (b) The same chromatogram as in (a), but with indications of LAL activity (dots) and TNF activity (bars).

Determining the biological activities of the fraction, it became immediately evident that both preparations contain a very large percentage of LAL- and lymphoblast-negative components. Fig. 1b shows the LAL activity of the crude TCA fractions, indicated by dots. The solid line is a percentage transmission recorded by the instrument monitoring the effluent at 230 nm. Fig. 2b shows the LAL activity of the crude PHW fractions. Monitoring at that wavelength and detector sensitivity made the endotoxic components barely detectable. Purification by ethanol precipitation and centrifugation, as described in Experimental, removed most the non-endotoxic components and allowed us to use a five times higher sensitivity for the analysis of the endotoxin-containing zones. The elution profile of purified endotoxins is shown by the solid lines in Figs. 3a and 4a. Again, multiple peaks could be detected in both preparations. Superimposed on these elution profiles, the LAL activity is shown by large dots in Figs. 3b and 4b. It is evident from these figures that several chromatographically distinct components, with comparable biological activities, are present

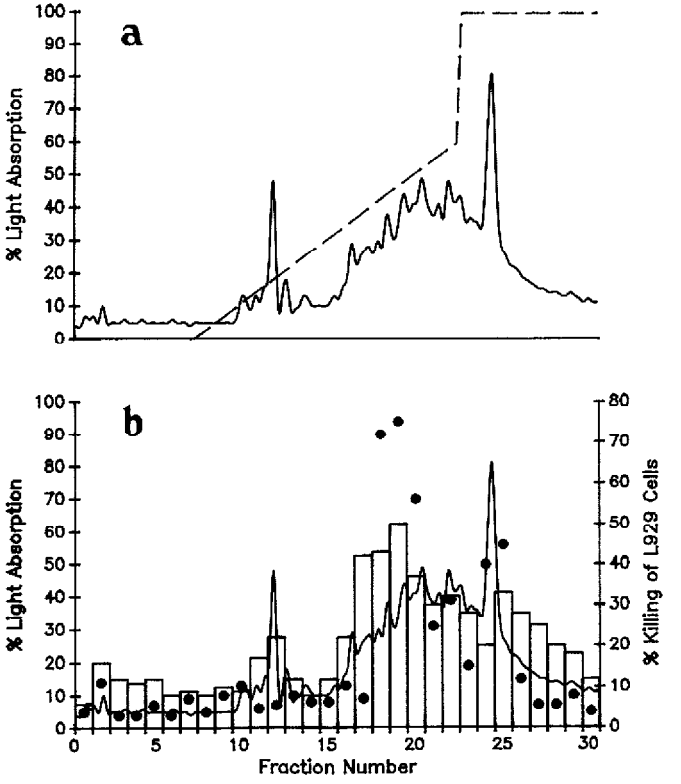

Fig. 3 (a) Chromatogram of purified TCA-extracted endotoxin Solid line: eluted component monitored at 230 nm, at a sensitivity of 1.0. Broken line: solvent B gradient. (b) The same chromatogram as in (a), but with indications of LAL activity (dots) and TNF activity (bars) of the collected fractions

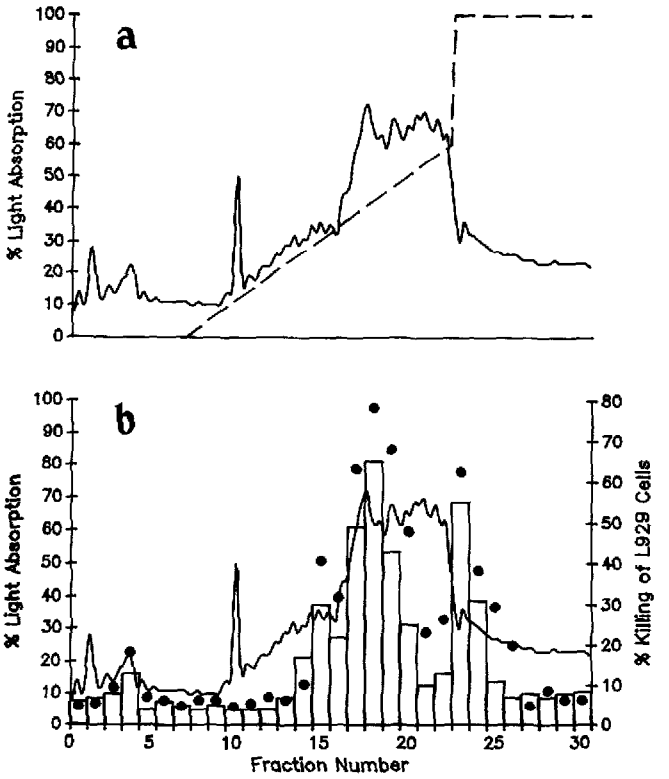

Fig. 4. (a) PHW-extracted purified endotoxin subjected to the same chromatographic process. Solid line: eluted component at a sensitivity of the monitor of 1.0. Broken line: solvent B gradient increase. (b) Purified PHW-endotoxin chromatographed as in (a). The elution profile is overlaid by LAL activities (dots) and by the TNF-inducing potential of the collected fractions (bars).

in the purified endotoxin preparations. It is also evident that these purified endotoxin samples are still contaminated with non-endotoxic LAL- or TNF-negative components (see fractions 12 and 17 in Fig. 3b and fractions 2, 10, 21 and 22 in Fig. 4b).

The results of the lymphoblast assays showed good correlation with the LAL test. All samples which were active in LAL were also quite potent in the induction of lymphocyte mitogenesis, although one zone was found in the TCA-extracted samples (Fig. 1a and b) which was lymphoblast-active and LAL-negative. The numbers of these fractions were 8, 9 and 10. The stimulation indices (not shown on the figures) of these mitogenic fractions were 2.6, 6.3 and 4.1. Most likely these are the fractions which contain the mitogenic 'endotoxin protein' components which are known to be present in the TCA-extracted but not in the PHW-extracted endotoxin preparations.

The pattern resulting from TNF activity measurements was quite different. The above fractions which were active in the LAL and mitogenicity assays

were also potent in vitro inducers of TNF, but TNF induction was also obtained by using fractions which were negative in LAL or in mitogenicity. Fig. 2b shows these findings, superimposed on the absorbance recording charts of crude PHW preparations (see fractions 11–13). The level of the TNF activity is indicated by vertical bars.

DISCUSSION

Sodium dodecyl sulfate polyacrylamide gel electrophoretic (SDS-PAGE) separation of endotoxin samples quite often reveals excessive heterogeneity, which is due to size heterogeneity of the endotoxic macromolecules, as has been assumed by a number of laboratories. That this might be true was shown by our efforts to recover three major zones of electrophoretically separated endotoxin bands such as the slowest moving top, the middle and the fastest moving bottom fraction [8]. These zones were tested in local Shwartzman, immune adjuvant, polyclonal B cell activation, macrophage cytotoxicity, colony-stimulating factor release and gel diffusion assays. No difference in these activities could be seen between the three zones as determined on the weight basis, with the exception of the polyclonal B cell activation, in which case the bottom zone (smallest molecular mass) was much more active than the others [8]. These results implied that, irrespective of the size of the endotoxin molecules, on weight basis their activity is the same. Serological reactivity, though, appears to be an exception. Immunoblotting of SDS-PAGE-separated endotoxin samples shows uneven reactivity with hyperimmune polyclonal antisera, the higher-molecular-mass bands in the slowest migrating zone being much more reactive than the faster-moving components [9].

SDS-PAGE separation is based primarily on molecular size, but ion exchange or thin-layer chromatography (TLC) demonstrates structural differences between the separated components. Such structural differences may be quite profound, but in the case of similar structures they are caused mainly by dissimilar distribution of charged (ion exchange) or polar versus non-polar (TLC) functional groups in the molecule. Earlier we observed the existence of chromatographically distinct but biologically indistinguishable bands in mutant endotoxins [10–15]. More recently we successfully separated smooth endotoxin samples on TLC into several components and observed similar heterogeneity among those bands which could be reacted on TLC directly with monoclonal anti-lipid A antibodies [15]. The results presented here revealed the existence of several endotoxin peaks in the ion-exchange chromatographic profile.

All these data, old and new, clearly show that endotoxins of a single bacterial strain are not represented by one molecular species, but by a family of molecules. The structural difference between the members of this family may be

subtle or more profound but they share a common structural subunit or a set of functional groups in a critical arrangement.

This arrangement seems to be a critical requirement for those biological activities we tested, such as toxic manifestations. We called such arrangements "toxic conformations" and assumed that long-chain carboxylic acids play a significant role in their formation [16]. Very elegant physicochemical studies of Rietschel et al. [17] put this assumption on solid foundation of experimental evidence. The molecules with similar biological potentials share this active site, but it is the rest of the molecule which is sufficiently individualistic to cause different behaviors in a variety of chromatographic systems.

How all these apply for other effects elicited by endotoxins, which are not obviously toxic, is still subject to some debate. LAL and even mitogenic activity seem to go parallel with conventional toxic activities, but there is increasing evidence that some beneficial endotoxic activities, such as immune adjuvancy, natural killer cell (NK) activation and colony stimulating factor (CSF) generation, do not require the presence of an intact 'toxic conformation'. The most likely explanations can be that disturbed or incomplete conformations, which are no longer toxic, are still able to elicit beneficial reactions and/or that non-endotoxic components of a crude endotoxin extract can also elicit beneficial reactions. These may be non-toxic members of the family of endotoxin molecules, or just co-extracted, quite unrelated other components of the bacterial cell. All above possibilities were discussed by us in a recent review [18].

Probably the most unexpected finding in this report is the existence of in vitro TNF-liberating components among the non-endotoxic substances separated by FPLC. TNF or cachectin liberated in vivo cause severe symptoms of endotoxemia. Passive transfer of TNF- or cachectin-containing sera reproduces the same phenomena. It was expected therefore that only the toxic components of endotoxin preparations would be able to liberate TNF. However, non-endotoxic components did so too. Explanations of this result obviously require more experimentation. Our TNF was produced in vitro by RAW 264.7 cells and its activity was tested only on L929 target cells, also in vitro. Our continuing studies will first test whether the TNF we liberated with non-toxic components has the same in vivo and in vitro effects as TNF generated by toxic endotoxin.

In this report we described our findings on the chromatography of endotoxins from one *Serratia* strain. The same procedure was eminently applicable for the separations of endotoxin from *Salmonella minnesota* 1114 and *Actinobacillus actinomycetemcomitans* strain Y4. Several chromatographically distinct but comparably potent in vitro bone resorption-inducing components were isolated from the Y4 strain, a suspected oral pathogen in juvenile periodontitis [19].

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