Preparation and Antitumor Activity of Nontoxic Lipid A*

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Summary. Highly refined, disaggregated endotoxic glycolipids (B5) from heptose-less (Re) mutant Salmonella typhimurium quantitatively converted to nontoxic (lethality for chick embryos) and nonpyrogenic (fever in rabbits) lipid A by treatment with boiling 0.1 N HCl (B5-HCl). Nontoxic B5-HCl, like toxic B5, caused regression of line-10 tumors and elimination of lymph node metastasis in 27 of 32 (84%) syngeneic strain 2 guinea pigs at a dosage of 150 µg. At this dosage, toxic B5 led to a cure in 54 of 67 (81%) tumor-bearing animals. All cured animals rejected a second line-10 tumor cell transplant. This activity depended on combining the toxic or nontoxic endotoxins with mycobacterial trehalose mycolate (P3) and an essentially nontoxic peptide-containing side-fraction (ACP) recovered during the isolation of B5. In contrast to toxic B5 or endotoxins in general, nontoxic B5-HCl did not cause endotoxic shock when combined with adjuvant dipeptide (MDP) and injected IV into guinea pigs. Chemical analysis showed that the phosphate content of nontoxic B5-HCl was about one-half that observed in toxic B5 or in toxic KDO-free lipid A, which was obtained by treating toxic B5 with sodium acetate at pH 4.5 at 100°C (B5-pH 4.5). The molar ratio of glucosamine : phosphorus : fatty acids was 2 : 1 : 4 for nontoxic B5-HCl and was 2 : 2 : 4 for toxic B5-pH 4.5. These results demonstrate that endotoxic extracts could be selectively detoxified while retaining antitumor properties. Thus, nontoxic B5-HCl may be a potential candidate for immunotherapy of human cancer.

Introduction

We have described the high potency of endotoxic, glycolipid-containing extracts from polysaccharide-deficient Re mutant strains of Salmonella typhimurium (ReGl) in regressing line-10 tumors in syngeneic strain 2 guinea pigs [15]. This activity depended on combining the endotoxic extract with purified mycobacterial trehalose dimycolate (P3). The bacterial components were incorporated into small amounts of oil and dispersed in a relatively large amount of saline. The resulting emulsion was directly inoculated into 1-week-old tumors. This treatment resulted in regression of tumors and elimination of metastases in the draining lymph node in at least 90% of the animals. Moreover, all cured animals rejected a second line-10 tumor cell transplant. When ReGl was freed of peptides, the resulting fraction of disaggregated glycolipids, designated B4, was endotoxic, but lacked tumor-regressive potency when administered either alone or in combination with P3 [18]. The tumor-regressive potency could, however, be restored to the B4-P3 mixture by the addition of synthetic muramyl dipeptide (MDP) or of an essentially nontoxic peptide-containing lipoid side-fraction (ACP) recovered during isolation of ReGl. We now report that further fractionation of B4 led to a toxic product, designated B5, that could quantitatively be
rendered essentially nontoxic (lethality for chick embryos) and nonpyrogenic (fever in rabbits) without loss of tumor-regressive potency by treatment with 0.1 N CHI at 100° C for 30 min. This procedure was previously used to prepare toxic and pyrogenic lipid A from standard endotoxic extracts [23].

**Materials and Methods**

*Endotoxic Extract (ReGl-PCP) from S. typhimurium, Strain G30/C21.* Strain G30/C21, a heptose-less Re mutant organism, was obtained from the late Werner Braun. The organisms were grown, and cell walls prepared from them, as previously described [22]. Endotoxic glycolipids (ReGl-PCP) were liberated from the cell walls with the aid of a monophasic solvent consisting of phenol, chloroform, and petroleum ether (PCP), according to the method of Galanos et al. [2]. ReGl-PCP isolated from cell walls, in contrast to ReGl-PCP isolated from whole cells, was ‘soluble’ in aqueous medium as well as in certain organic solvents. Thus, the solubility of ReGl-PCP facilitated its use for biological work and for fractionation by chromatographic techniques.

*Isolation of B5 from ReGl-PCP.* ReGl-PCP was fractionated by preparative microparticulate gel chromatography as previously described [14, 18]. Briefly, preparative columns 18 mm in diameter were packed with a slurry of microparticulate silica (water content of 6.5%) in methylene chloride-methanol (7: 3) by centrifugation for 90 min at 2,400 g in a Sorvall RC-3 centrifuge equipped with a HG-4 rotor. Samples (20 mg) of ReGl-PCP were applied to the column and then eluted with chloroform-methanol-water-diethylamine (50: 40: 5: 1) at 90 psi hydrostatic pressure. Fractions (5 ml) collected at 5-min intervals were checked for composition by analytical centrifugal chromatography. Chromatograms were developed with chloroform-methanol-water-diethylamine (50: 40: 5: 1) for 5 min. The major fraction, designated B4, was in tubes 25–32. Microparticulate silica and unidentified material of lower polarity was removed following chromatography through a Sephadex LH-20 column. For this purpose, B4 was dissolved in 1 ml chloroform-methanol-water (85: 15: 1) and applied to a 1.5 x 40 cm Sephadex LH-20 column (Pharmacia Fine Chemicals, Piscataway, NJ, USA) equilibrated with chloroform-methanol (2: 1). The column was eluted with chloroform-methanol (4: 1) and 2-ml fractions were collected. The fractions were examined by analytical centrifugal chromatography utilizing the chloroform-methanol-water-diethylamine solvent described above. The single-band toxic fraction, designated B5, was in tubes 4–7. Higher-numbered fractions contained nontoxic substances, chromatograms of which showed several components whose polarity was lower than that of B5. These nontoxic, lower polar fractions alone or in combination with ACP and P3 did not cause regression of line-10 tumors.

*Preparation of Lipid A from B5*

**Method 1.** A 30-mg sample of B5 was suspended in 48 ml 20 mM sodium acetate at pH 4.5, and incubated at 100° C for 30 min [20]. The reaction mixture was dialyzed against deionized distilled water, centrifuged at 100,000 x g for 60 min, and the residue was lyophilized. A second cycle of this treatment was performed and the yield was 12.3 mg (B5-pH 4.5).

**Method 2.** A 40-mg sample of B5 was hydrolyzed with 9 ml 0.1 N hydrochloric acid in a sealed container placed in a boiling water bath for 30 min. The precipitate (B5-HCI) was collected by centrifugation (2,000 g for 10 min), washed with distilled water three times and lyophilized. B5-HCI was soluble (opaque) in 0.5% aqueous triethylamine and the yield was 21.4 mg.

**Analytical Methods.** Total phosphorus was determined by the method of Lowry et al. [5]; 2-keto-3-deoxyoctonate (KDO) content by the method of Osborn [10]. Glucosamine, amino acids, ethanolamine, and their phosphate derivatives were analyzed in a Beckman 118 CL Analyzer (Beckman Instruments, Inc., Irvine, CA) with samples (1 mg) hydrolyzed in 6 N HCl at 100° C for 4 or 12 h in sealed tubes. Fatty acids were analyzed as methyl esters in a Perkin-Elmer 900 Gas Chromatograph (Perkin-Elmer, Norwalk, CT) with a stainless steel column 6 ft. x ½ in. inner diameter, packed with 10% ethylene glycol succinate on 100–200 mesh Gas-Chrom P support (Applied Science Laboratories, Park Ridge, IL). Samples (about 1 mg) were hydrolyzed in 4 N HCl at 100° C for 4 h in sealed tubes and then esterified by treatment with boron trichloride-methanol (Applied Science Laboratories, Park Ridge, IL).

**Materials.** Trehalose dimycolate (P3) was isolated from *Mycobacterium bovis* strain AN5 and purified by pressure elution chromatography through microparticulate silica gel [1]. The nontoxic ACP fraction was prepared from a crude endotoxin preparation according to the method of Ribi et al. [18]. Synthetic N-acetylmuramyl-t-tyrosyl-d-isoglutamine (MDP) was prepared according to the method of Schwartzman and Ribi [21]. Glucosamine and KDO standards were purchased from Sigma Chemical Co., St Louis, MO. Methyl esters of various fatty acids were purchased from Applied Science Laboratories, Park Ridge, IL.

**Determination of Biological Properties.** Stock solutions, containing 1 mg of the fraction to be tested in each case, were dissolved in 1 ml 0.5% triethylamine in saline. Appropriate dilutions were made with saline or phosphate-buffered saline. The endotoxicity of fractions was estimated as described by Milner and Finkelstein [7]. The data are reported as the medium lethal dose for 11-day-old chick embryos inoculated IV (CELD50 in μg) and the dose estimated to give a fever index (area under a fever curve) of 40 cm² in rabbits when 1 h and 1 ° C are plotted as 1 in. (F140 in μg). To determine whether MDP enhances the susceptibility of guinea pigs to endotoxic shock, groups of five strain 2 guinea pigs were inoculated IV (CELD50 in μg). To determine whether MDP enhances the susceptibility of guinea pigs to endotoxic shock, groups of five strain 2 guinea pigs were inoculated IV (CELD50 in μg) and the dose estimated to give a fever index (area under a fever curve) of 40 cm² in rabbits when 1 h and 1 ° C are plotted as 1 in. (F140 in μg).

**Tumors and Preparation of Emulsions for Injection.** The tumor model was developed at the National Cancer Institute [11]. The carcinogen-induced line-10 hepatocellular carcinoma was maintained in the ascites form and transplanted (10⁶ tumor cells) ID into 400- to 50-g syngeneic strain 2 guinea pigs. Untreated animals died in 60–90 days. Tumors were treated 6 days after transplantation, when they were 9–10 mm in diameter. At this time, tumor cells have metastasized into the regional lymph nodes. Test materials were incorporated into minute oil droplets suspended in 0.9% NaCl solution containing 0.2%. Tween 80 as described previously [16]. The concentration of light mineral oil in the resulting emulsion was about 1% (10–20 μl per mg material). The emulsions were injected in single 0.4-ml volumes directly into the tumors [6]. Treated animals were observed for at least 3 months. As used in this report, the terms 'regression' or 'cure' mean complete disappearance of primary dermal tumor, no clinical evidence of metastatic disease, and rejection of contralateral challenge with 10⁶ line-10 cells 2 months after the original tumor was treated.