atol itself, and/or the ratio of (**6**) versus creatinine or another creatinine metabolite, provide a useful diagnostic tool for judging the severity of uremia in patients. Moreover, creatol (**6**) should now be considered as a candidate uremic toxin, mainly because it is a pro-toxin as the precursor of methylguanidine (**8**), which is already accepted as a toxin, but also because creatol (**6**) itself contains an intrinsically toxic guanidino-structure. Studies on the toxicity of creatol (**6**) using animals, including rats with chronical renal failure [7, 12], are now in progress. Each of the two catabolic pathways for creatinine (**1**) via hydroxylated heterocycles, (**3**) or (**6**), in creatininemia might be characteristic enough to be classified, respectively, as a detoxification route and a toxin-producing process.

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The effect of endotoxin on membrane fatty acid composition in BCG-sensitized mice

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Summary. The effects of endotoxin on mouse liver phospholipid fatty acid composition have been investigated. Administration of endotoxin from *Salmonella abortus equi* led to a decrease in the polyunsaturated fatty acid content of livers from mice sensitized with Bacille Calmette Guérin (BCG). The content of arachidonic acid fell significantly in both the phosphatidylcholine and phosphatidylinositol fractions whereas in the phosphatidylethanolamine fraction the linoleic acid content was significantly reduced. The polyunsaturated fatty acids were replaced by increased amounts of oleic acid and palmitic acid, leading to a reduction in the polyunsaturated to saturated fatty acid ratio.
Key words. Endotoxin; fatty acid; phospholipid; BCG.

Mice can be made highly sensitive to bacterial endotoxin by the intravenous injection fourteen days beforehand of attenuated live mycobacteria (BCG, Bacille Calmette Guérin) [1]. The mechanisms responsible for this enhanced susceptibility are not fully defined. Recently we have shown that mice infected with BCG exhibit changes in their phospholipid fatty acid composition and triacylglycerol content [2]. In particular, we found that BCG infection resulted in an increased polyunsaturated to saturated fatty acid ratio in the phospholipids of the liver, spleen and peritoneal macrophages. This change in membrane fatty acid composition might form the basis of the increased sensitivity to endotoxin.

BCG infection is associated with a state of profound macrophage hyperactivity [3]. Such 'primed' macrophages may be stimulated into a fully 'activated' state by endotoxin in which they produce increased amounts of tumour necrosis factor, prostaglandins, leukotrienes and superoxide anion [4, 5].

After administration of endotoxin, the polyenoic content might be expected to fall due to the conversion of these fatty acids to eicosanoid derivatives such as prostaglandins and leukotrienes [5, 6].

This paper details the changes in membrane fatty acid composition in livers from sensitized mice after injection with bacterial endotoxin.

Materials and methods

Mice: Adult TO strain mice (25 g) were given i.v. approximately 10^7 live organisms of BCG (Glaxo) suspended in 0.4 ml of phosphate buffered saline (PBS) to establish the

systemic infection. (The animals do not appear ill during this period but will usually die within 6–24 h if they are given a 10-μg dose of endotoxin). Fourteen days later 10μg endotoxin from *Salmonella abortus equi* (Sigma) was given in 0.25 ml PBS i.p. and 6 h later the animals were sacrificed by cervical dislocation.

Three groups of 10 livers were examined from control animals, from animals given BCG only, and from animals given both BCG and endotoxin. For comparison, livers from control (non-BCG) animals given endotoxin were also examined.

Extraction of lipids. Total lipids were extracted by the method of Garbus et al.[7], as follows: to 1 g of tissue 3.75 ml chloroform/methanol (1:2 v/v) were added, and the tissue homogenized on ice and left for 30 min at room temperature. Then chloroform (2.5 ml) and 2 M KCl in 0.5 M KPO_4 buffer, pH 7.4 (2.5 ml) were added and the mixture shaken thoroughly.

After brief centrifugation, the lower (lipid-containing) layer was taken to dryness with N_2. The lipid extract could be stored at this stage at − 60 °C after addition of butylated hydroxytoluene (BHT) (Sigma) at 0.5 mg/ml in chloroform. All solvents were of chromatography grade and were obtained from BDH.

Separation of phospholipids. Phospholipids were separated from neutral lipids by thin layer chromatography (TLC) on silica gel-G plates (BDH) with chloroform/methanol/acetic acid/isotonic saline (50:25:8:4 by vol.) as solvent. Phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol were separated from other phospholipid classes by TLC with chloroform/methanol/28% ammonia (65:25:4 by vol.). The phospholipids were visualized under UV light after staining with 8-anilino-1-naphthalene-sulphonic acid (Sigma) and scraped off into sealable tubes.

Analysis of lipid fatty acid content by gas-liquid chromatography. To the lipids scraped from TLC plates, 1 ml 2.5% H_2SO_4 in anhydrous methanol was added. As an internal standard, 50 μg heneicosanoic acid (21:0) was added. After sealing, the tubes were heated for 2 h at 70 °C. The tubes were then cooled, and 2.5 ml 5% NaCl was added. The methyl esters were extracted three times with 3 ml petroleum ether (60–80°) and placed into conical glass tubes. This procedure has been shown to give quantitative derivation of fatty acids of all classes of lipids containing esters. The extracts were then taken to dryness with N_2 and redissolved in chromatographically pure petroleum ether.

The methyl esters were analysed on a Perkin Elmer 8310 chromatograph equipped with a flame ionization detector. A glass column packed with 15% ethylene glycol succinate methyl silicone polymer (EGSS-X) coated on Gas-chrom. P (100–120 mesh) was used. The column temperature was 170 °C, injector and detector temperatures were 210 °C. Carrier gas (N_2) flow rates were 30–34 ml/min. Peak areas, retention times and response factors were automatically computed, the yields being calculated from the 21:0 internal standard. Peaks due to BHT (retention time similar to myristate) and its oxidation products (between palmitate and stearate) were omitted.

Statistical comparisons. Comparisons between different experimental groups were made by Student's t-test.

Results

The fatty acid composition of liver phospholipids in unstimulated mice was in agreement with our previous results and literature values[8]. The table gives the phospholipid fatty acid composition of livers from normal and BCG-primed mice before and after the administration of 10 μg endotoxin. In mice not infected with BCG, admin-

Changes in fatty acid composition of phospholipids in livers of BCG-sensitized mice after endotoxin administration (% fatty acid by weight)

Liver phospholipid examinated	Mouse group (n = 10)	Palmitic acid (16.0)	Stearic acid (18.0)	Oleic acid (18.1)	Linoleic acid (18.2)	Arachidonic acid (20:4)	Ratio polyunsaturated/saturated
Phosphatidylcholine	Normal liver	36.6 ± 6.0	18.2 ± 3.9	12.4 ± 4.6	14.8 ± 3.6	18.0 ± 4.0	0.60 ± 0.32
	[a] Normal liver + endotoxin	36.9 ± 4.1	16.0 ± 3.9	12.5 ± 4.8	20.6 ± 5.0	14.0 ± 4.0	0.65 ± 0.1
	BCG-sensitised	37.2 ± 4.9	16.3 ± 5.0	12.7 ± 6.2	16.5 ± 4.9	18.3 ± 3.8	0.66 ± 0.41
	BCG-sensitised + endotoxin	40.4 ± 4.5	13.1 ± 3.2	17.9 ± 1.8	19.0 ± 3.0	9.6 ± 1.44**	0.54 ± 0.07**
Phosphatidylethanolamine	Normal liver	28.5 ± 1.0	22.5 ± 3.6	14.4 ± 3.9	18.0 ± 1.5	17.0 ± 3.4	0.69 ± 0.15
	[a] Normal liver + endotoxin	34.8 ± 3.8	23.3 ± 2.7	10.7 ± 2.4	11.1 ± 3.7	20.1 ± 4.2	0.57 ± 0.2
	BCG-sensitised	23.2 ± 3.1*	21.6 ± 2.2	18.5 ± 3.6	17.8 ± 2.1	22.6 ± 4.3*	0.90 ± 0.18
	BCG-sensitised + endotoxin	24.0 ± 4.9	27.6 ± 3.1	12.8 ± 2.5	12.3 ± 2.9*	23.2 ± 6.3	0.77 ± 0.23
Phosphatidylinositol	Normal liver	10.3 ± 0.7	47.0 ± 2.9	3.8 ± 1.2	2.5 ± 0.7	36.1 ± 2.9	0.71 ± 0.25
	[a] Normal liver + endotoxin	11.4 ± 1.5	46.5 ± 3.3	3.8 ± 1.5	2.4 ± 0.2	35.9 ± 5.4	0.64 ± 0.1
	BCG-sensitised	11.5 ± 3.1	41.9 ± 3.2	5.3 ± 1.2	10.3 ± 4.1*	30.9 ± 5.0	0.80 ± 0.31
	BCG-sensitised + endotoxin	10.6 ± 0.9	47.0 ± 3.1*	10.2 ± 3.6*	7.3 ± 1.9	25.0 ± 3.6*	0.58 ± 0.07**

[a] n = 6; *p = < 0.05; **p = < 0.01; vs BCG-sensitized animal.

istration of endotoxin resulted in a decrease of polyunsaturated fatty acids in liver phospholipids with a corresponding decrease in the ratio of polyunsaturated to saturated fatty acids. However the changes were outside the levels of significance. In BCG-primed mice, however, the content of arachidonic acid (20:4) fell significantly in both the phosphatidylcholine and phosphatidylinositol fractions after administration of endotoxin. In the phosphatidylethanolamine fraction, the linoleic acid (18:2) but not the arachidonic acid content fell significantly after endotoxin. In the phosphatidylinositol and phosphatidylethanolamine fractions, the content of oleic acid (18:0) was increased following endotoxin, while the palmitic acid (16:0) content was increased in phosphatidyl choline.

The changes in fatty acid content have the result of decreasing the polyunsaturated/saturated fatty acid ratio in each of the phospholipids after endotoxin administration. In both the phosphatidylcholine and phosphatidylinositol fractions this ratio is decreased below that found in normal, unsensitized mice (table).

Discussion

The effect of endotoxin in the present experiments is to cause a significant reduction in the polyunsaturated fatty acid content of the liver phospholipids of BCG-primed mice. This is a reversal of the effects of BCG stimulation which was to increase the polyunsaturated fatty acid content[2].

When cells are activated by external stimuli (cytokines, hormones, endotoxin) one of the early changes commonly observed is increased turnover and metabolism of the fatty acyl moieties of membrane phospholipids. The compositional changes of the membrane lipids mediate alterations in membrane permeability and activation of membrane-bound enzymes[9].

Endotoxin administration was shown to lead to a decrease in either arachidonic or linoleic acid in all three phospholipids analysed. These fatty acids are the precursors of prostaglandins and leukotrienes, and endotoxin is known to stimulate the production of these biologically active molecules[10,11]. Thus the interaction of endotoxin with membranes rich in polyunsaturated fatty acids may lead to the production of pharmacologically active mediators of the endotoxic crisis.

Endotoxin, both directly and indirectly by cytokine production, may stimulate phospholipase A_2 activation leading to the removal of arachidonic acid from membrane phospholipids[12]. In order to replace fatty acids removed in this way, the lysophospholipids, generated by cleavage of the fatty acyl moiety from the glycerol backbone, are then reacylated. This provides a mechanism for altering the fatty acid composition of membrane phospholipids without the necessity of de novo phospholipid synthesis.

Phospholipase A_2 also takes part in the synthesis of platelet-activating factor (PAF). PAF has been shown to mimic many of the effects of endotoxin in vivo[13].

Macrophages are among the most important cells with which endotoxin interacts: they mediate many of the reactions in the pathophysiology of this agent[14]. The animal host with a major systemic infection with BCG has greatly enlarged lymphoreticular organs inflamed and modified by the presence of innumerable granulomata. There is thus an increased and stimulated population of macrophages within the livers and spleens of these animals and the endotoxin sensitive state is likely to be due to the presence of these primed cells. In addition, the Kupffer cells of the liver represent the largest population of tissue macrophages in the body and are also the primary site for the clearance of most foreign material from the blood stream. Furthermore, they mediate alterations in hepatocyte function, especially during uncontrolled infection[15]. The changes in phospholipid fatty acid composition in the present paper are thus thought to reflect changes in fatty acid metabolism in Kupffer cells and the cells of liver granulomata in responding to endotoxin.

The results of the present experiments lend weight to the hypothesis that increased sensitivity to endotoxin, and possibly all acute infections is due at least, in part, to alteration of phospholipid fatty acid composition in active phagocytes such as macrophages[2]. Thus, a microbially derived stimulus may trigger the release of unsaturated fatty acids from modified phospholipids to produce the mediators of a crisis.

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