

## **Synthesis of hydroxyeicosatetraenoic acids and leukotrienes in rat nephrotoxic serum glomerulonephritis. Role of anti-glomerular basement membrane antibody dose, complement, and neutrophiles.**

E A Lianos

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### **Research Article**

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# Synthesis of Hydroxyeicosatetraenoic Acids and Leukotrienes in Rat Nephrotoxic Serum Glomerulonephritis

## Role of Anti-Glomerular Basement Membrane Antibody Dose, Complement, and Neutrophiles

Elias A. Lianos

Department of Medicine, Nephrology Section, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

### Abstract

The basal and stimulated synthesis of immunoassayable 12- and 5-monohydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT) B<sub>4</sub> and C<sub>4</sub> was studied in glomeruli isolated from rats with nephrotoxic serum glomerulonephritis (NSGN) induced by low (30 µg/g body weight) or high (105 µg/g) doses of anti-rat glomerular basement membrane (GBM) immunoglobulin (Ig). In the early heterologous phase of the disease, low doses of anti-GBM Ig enhanced the basal synthesis of 12-HETE but not that of 5-HETE or LT. High anti-GBM Ig doses enhanced the basal synthesis of 5-HETE and LTB<sub>4</sub> as well. Under stimulated conditions, enhanced glomerular production of 5-HETE and LTB<sub>4</sub> occurred at 15 min after infusion of anti-GBM Ig, peaked at 1 h, and returned toward control levels by 24 h. At 48 h, 72 h, and on day 12, the synthesis of these eicosanoids was impaired. Neutrophile depletion only partially reduced glomerular eicosanoid synthesis after induction of NSGN whereas complement depletion significantly reduced 5-HETE, 12-HETE, and LTB<sub>4</sub>. These observations indicate that in the heterologous phase of NSGN there is enhanced but short-lived glomerular 5-HETE and LTB<sub>4</sub> synthesis. This phenomenon is mediated by complement activation and may be an important proinflammatory event leading to capillary wall injury in the early stages of the disease.

### Introduction

The lipoxygenation of arachidonic acid in various cell types generates diverse mediators of inflammation (1, 2). Arachidonate 5-lipoxygenation exhibits cellular specificity and the various end products, monohydroxyeicosatetraenoic acids (HETE)<sup>1</sup> and leukotrienes (LT), have been identified and quantified primarily in cell types involved in inflammatory

processes, i.e., neutrophiles, monocytes, alveolar macrophages, and mast cells. Arachidonate lipoxygenation has also been demonstrated in isolated rat glomeruli which synthesize primarily 12-HETE when stimulated by the phospholipase activator, cationophore A23187 (3). Enhanced lipoxygenation of exogenous arachidonate was observed in experimental anti-glomerular basement membrane (GBM) antibody-induced glomerulonephritis in the rat with increased levels of immunoassayable 12-HETE detected in isolated glomeruli over a 14-d period (4). In these studies proteinuric doses of anti-GBM immune serum were employed and glomerular 12-HETE synthesis was found to be independent of glomerular platelet infiltration (4). The glomerular pathology in this model of experimental glomerulonephritis presents a spectrum of lesions including mild to severe proliferation of endogenous glomerular cells (mesangial, resident macrophages) and injury and exfoliation of endothelial cells (5). It is possible, therefore, that the type of eicosanoid synthesized in immunologically injured glomeruli may depend on the degree and stage of glomerular injury. This study explores the synthesis of the glomeruli-derived arachidonate lipoxygenation products 12-HETE, 5-HETE, and LTB<sub>4</sub> and LTC<sub>4</sub> in rats with nephrotoxic serum glomerulonephritis (NSGN) using low and high doses of anti-GBM immunoglobulin in order to assess the extent of glomerular injury on the synthesis of these eicosanoids. Since this model of glomerular injury is complement dependent and leukocyte mediated, the role of glomerular complement deposition and polymorphonuclear leukocyte infiltration in mediating changes in glomerular HETE and LT synthesis was also assessed.

### Methods

**Induction of NSGN and histopathologic studies.** NSGN was induced in male Sprague-Dawley rats, 250–275 g, by a single intravenous injection of proteinuric doses of rabbit immune serum raised against particulate rat GBM as previously described (6). The immunoglobulin concentration in the immune serum employed was determined as in previous studies (4) by the single radial immunodiffusion method of Mancini et al. (7) using goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) and rabbit IgG (Sigma Chemical Co., St. Louis, MO). Two doses of immunoglobulin were employed: a low, 30-µg, and a high, 105-µg Ig/g, of rat body weight. Both doses induced proteinuria (18-h urine protein excretion range: 63–85 mg in the low-dose group and 120–205 mg in the high-dose group) and glomerular lesions which were assessed by routine methods of light, electron, and immunofluorescence microscopy. The degree of glomerular PMN infiltration was assessed by light microscopy in renal cortical sections and was expressed as mean±SEM of PMN per glomerulus based on examination of 20 glomeruli in cortical biopsies obtained from each individual rat before sacrifice. Glomerular leukocyte infiltration was also assessed by measuring myeloperoxidase (MPO) activity (8) in preparations of iso-

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Address reprint requests to Dr. Lianos, Froedtert Memorial Lutheran Hospital, 9200 West Wisconsin Avenue, Milwaukee, WI 53226.

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1. **Abbreviations used in this paper:** CVF, cobra venom factor; FTIR, Fourier-transform infrared spectroscopy; HETE, hydroxyeicosatetraenoic acid; MPO, myeloperoxidase; NSGN, nephrotoxic serum glomerulonephritis.

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lated glomeruli at various time points after induction of NSGN. Briefly, glomeruli isolated by differential sieving were suspended in 3 ml of 0.5% hexadecyltrimethylammonium bromide (Sigma Chemical Co.) in 50 mM potassium phosphate buffer, pH 6.0, and the suspension was sonicated in an ice bath for two 30-s periods. Preparations were subsequently freeze-thawed three times after which sonication was repeated. Preparations were then centrifuged at 40,000 *g* for 15 min, and the resulting supernates were assayed spectrophotometrically for presence of MPO. 0.1 ml of sample was mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml of *O*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide (Mallinckrodt Inc., Paris, KY). The change in absorbance at 460 nm was measured using a DU-7 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). One unit of MPO activity was defined as that degrading 1  $\mu$ mol of peroxide per minute.

Control rats received identical doses of nonimmune rabbit serum obtained from rabbits immunized with complete Freund's adjuvant but not rat GBM.

**Decomplementation studies.** To assess the effect of complement depletion on glomerular HETE and LT synthesis, NSGN was induced in a group of rats ( $n = 10$ ) after complement depletion using purified cobra venom factor (CVF), and results were compared to those obtained in rats in which NSGN was induced without prior decomplementation. Controls received CVF alone ( $n = 9$ ) followed by nonimmune rabbit serum or CVF vehicle ( $n = 4$ ). Before use, CVF (*Naja naja*, Cordis Laboratories, Miami, FL) was purified to remove contaminating phospholipases by ion exchange chromatography followed by gel filtration as described elsewhere (8, 9). To induce complement depletion, rats received a single intraperitoneal injection of CVF 40 U/100 g of body weight. Pilot experiments established this dose to be sufficient to induce undetectable circulating complement levels within 20–30 min after injection. Decomplementation was monitored in serum samples (75  $\mu$ l) obtained from the tail vein at 10-min intervals after CVF injection by a hemolysis inhibition assay ( $CH_{50}$ ) and by a single radial-immunodiffusion method for rat C3 (7). At 30 min after CVF administration, NSGN was induced as described above and rats were killed 3 h later for glomerular biochemical and histopathologic studies.

In order to assess the effect of complement activation induced by CVF administration on glomerular eicosanoid synthesis an *in vitro* system was employed. In these experiments, glomeruli isolated from normal rats were incubated in 2 ml of normal rat serum/RPMI-1640 (1:10). Purified CVF 2 U or 10 U ( $n = 4$  and 6, respectively) was subsequently introduced in glomerular incubation media and incubations were allowed to proceed for 1 h at 37°C. Glomerular suspensions were subsequently extracted and analyzed for LTB<sub>4</sub> and PGE<sub>2</sub> levels. Before extraction, aliquots of media were obtained for testing by a hemolysis inhibition assay, as mentioned above, in order to document *in vitro* complement activation as a result of the introduction of CVF (cobra C3b) in glomerular preparations. Controls ( $n = 4$ –6) were glomeruli incubated in normal rat serum/RPMI-1640, RPMI alone and RPMI with CVF (10 U).

**PMN depletion studies.** To assess the extent to which infiltrating PMN contribute to glomerular HETE and leukotriene synthesis after induction of NSGN, PMN depletion was induced in a group of rats ( $n = 12$ ) before administration of anti-GBM immune serum. To deplete rat PMN, a specific rabbit anti-rat PMN immune serum, generously provided by Drs. K. Johnson and A. Rehan (University of Michigan, Ann Arbor, MI) was employed. Pilot experiments established a dose of 400  $\mu$ l/100 g of body weight of anti-PMN serum administered intraperitoneally to be capable of reducing absolute whole-blood PMN counts < 200/mm<sup>3</sup> within 12–18 h without a significant change in lymphocyte counts. PMN counts were determined in whole-blood samples (75  $\mu$ l) obtained from the tail vein in EDTA-containing polypropylene tubes using an automated hematology analyzer (Coulter Electronics, Inc., Hialeah, FL) and a Fuchs-Rosenthal type of hemocytometer. When absolute peripheral PMN counts < 200/mm<sup>3</sup> were

documented, NSGN was induced as described above and rats were killed at 3 h for glomerular biochemical and histopathologic studies.

**Study groups.** The following groups of animals were employed for glomerular biochemical studies: (a) a low-dose group studied at 3 h after induction of NSGN using 30  $\mu$ g of anti-GBM Ig/g of body weight; (b) a high-dose group also studied at 3 h after induction of NSGN using 105  $\mu$ g of anti-GBM Ig/g of body weight. In these two groups, isolated glomeruli were incubated in 2 ml of RPMI-medium 1640 at 37°C for 45 min in the presence (stimulated synthesis,  $n = 6$ ) or absence (basal synthesis,  $n = 6$ ) of the phospholipase activator, cationophore A23187 (2  $\mu$ M). (c) Rats with NSGN using high doses of anti-GBM Ig and killed at 5 min ( $n = 7$ ), 15 min ( $n = 6$ ), 30 min ( $n = 4$ ), 1 h ( $n = 8$ ), 3 h ( $n = 9$ ), 24 h ( $n = 4$ ), 48 h ( $n = 7$ ), 7 d ( $n = 4$ ), and 12 d ( $n = 6$ ) after induction of the disease. Controls for all NSGN groups received identical doses of nonimmune rabbit serum. (d) A complement-depleted group ( $n = 10$ ) and (e) a PMN-depleted group ( $n = 12$ ) both studied at 3 h after induction of NSGN. Controls received purified CVF ( $n = 9$ ), 40 U/100 g body weight intraperitoneally, or anti-rat PMN serum ( $n = 5$ ). Results obtained in groups *d* and *e* were compared to those in complement and PMN replete rats (group *b*). In groups *c*, *d*, and *e*, high doses of anti-GBM immune serum were employed (identical to those in group *b*) and glomerular eicosanoid synthesis was studied in the presence of A23187.

**Glomerular biochemical studies.** Each animal was studied individually using ether anesthesia. At the various time points of study, rats were nephrectomized and kidneys were placed in ice-cold RPMI. Cortices were subsequently dissected, minced to a pastelike consistency and glomeruli were isolated by differential sieving as previously described (4) and suspended in 2 ml of RPMI medium 1640. The purity of glomerular preparations assessed in a group of six rats with NSGN at 3 h and a group of six control rats, was  $90.5 \pm 1.6\%$  and  $87.5 \pm 2.0\%$ , respectively. In incubation experiments using A23187, working solutions of A23187 (Sigma Chemical Co.) were made in dimethylsulfoxide and used at a final volume of 0.05% of the total glomerular suspension. All glomerular incubations and extractions were performed in polypropylene tubes. Before eicosanoid extractions, glomerular suspensions were "spiked" with [<sup>3</sup>H]5-HETE, [<sup>3</sup>H]LTB<sub>4</sub>, and [<sup>3</sup>H]LTC<sub>4</sub> solutions made in phosphate-buffered saline (0.5% gelatin) and containing 3,500–4,000 cpm of each eicosanoid (New England Nuclear, Boston, MA; specific activities: 30–60 Ci/mmol). Glomerular suspensions were subsequently extracted with three volumes of ice-cold absolute ethanol at 4°C under vigorous shaking for 45 min to precipitate protein and centrifuged at 4°C at 10,000 *g*. Pellets were dissolved in 1 ml NaOH, 2 N, to dissolve tissue protein, and aliquots were used for protein determination by the method of Bradford (10). Supernates were dried under vacuum (Rotavap, Savant Instruments, Inc., Farmingdale, NY). To extract HETE and LT, dried ethanolic extracts were reconstituted in 1 ml of distilled deionized water, mixed with 0.5 ml of isopropanol, and acidified with formic acid 0.5 M to pH 3.0–3.5. A second extraction was subsequently performed using 2.5 ml of diethyl ether. This two-step approach optimizes tissue extraction of LTB<sub>4</sub> (11) and provides LT-enriched extracts appropriate for identification studies by eliminating other tissue-derived substances such as vasoactive amines and platelet-activating factors. The organic phases (isopropanol/diethyl ether) were pooled, dried under vacuum, and reconstituted in 1 ml of methanol/water (3:7). This final solution was filtered through 0.2- $\mu$ m filters (Spartan-3, Schleicher & Schnell, Inc., Keene, NH) and injected in a high pressure liquid chromatograph consisting of two solvent delivery modules (Beckman 112), a gradient controller (Axxiom 710 HPLC controller, Anspec Co., Inc., Ann Arbor, MI), and a reverse-phase C<sub>18</sub>, 5- $\mu$ m cartridge (Nova Pak, Waters Associates, Milford, MA) placed in a radial compression module (RCM-100, Waters Associates). Elutions were performed at flow rates of 1 ml/min. An initial isocratic phase (15 min) using methanol/water/acetic acid (65:35:0.02 vol/vol/vol), pH 5.7 adjusted with concentrated ammonium hydroxide (solvent B), was followed by a progressive gradient to 100% methanol over a 30-min period as previously described (12).

Leukotrienes were detected at  $\lambda = 280$  nm using an in-line variable-wavelength absorbance detector (Spectroflow 773, Kratos Analytical, Ramsey, NJ) interfaced with an integrator/printer plotter (SP 4270, Spectra-Physics, Inc., Mountain View, CA). HETE were monitored at  $\lambda = 232$  nm. LTC<sub>4</sub> eluted during the isocratic phase whereas LTB<sub>4</sub> and HETE eluted during the gradient phase, upon completion of which the column was reconstituted with solvent B for 15 min. Elution fractions (1 ml) were collected at 1-min intervals and those corresponding to retention times of LTC<sub>4</sub>, LTB<sub>4</sub>, 12-HETE, and 5-HETE standards were dried under vacuum and reconstituted in 300 ml of 0.01 M phosphate buffer containing 0.1% bovine  $\gamma$ -globulin for subsequent quantification by RIA. LTC<sub>4</sub>, LTB<sub>4</sub>, 12-HETE, and 5-HETE standards were generously supplied by Dr. J. Rokach, Merck-Frosst Laboratories, Dorval, Quebec, Canada. Retention times ( $n = 4$ ) were:  $14.2 \pm 0.2$ ,  $27.7 \pm 0.12$ ,  $37.5 \pm 0.2$ , and  $39 \pm 0.15$  min, respectively. Recovery range of [<sup>3</sup>H]LTC<sub>4</sub>, [<sup>3</sup>H]LTB<sub>4</sub>, and [<sup>3</sup>H]5 HETE added in glomerular suspensions before extractions and chromatographic separations was 65–72% in control and 58–62% in nephritic glomeruli. RIAs employed commercially available antibodies for LTC<sub>4</sub> (New England Nuclear), LTB<sub>4</sub> (Amersham Corp., Arlington Heights, IL), 5-HETE, and 12-HETE (Advanced Magnetics Inc., Cambridge, MA). The cross-reactivity of antisera employed to measure 5-HETE and 12-HETE was determined before RIA of HPLC eluates. Briefly, a standard curve for 12-HETE was constructed (concentration range 8.2–2,000 pg/0.1 ml) and the concentration corresponding to 50% B/Bo was determined. The cross-reactant 5-HETE standard was subsequently employed at concentrations 50–500,000/0.1 ml and the concentration corresponding to 50% B/Bo was similarly determined. Percent cross-reactivity was calculated from the formula: 12-HETE concentration at 50% B/Bo over 5-HETE concentration at 50% B/Bo  $\times 100$ . The antiserum to 5-HETE cross-reacted with 12-HETE by 0.21%. The antiserum to 12-HETE cross-reacted with 5-HETE by 0.25%. To assess RIA noise levels, tissue-free glomerular incubation media ( $n = 3$ ) were subjected to extractions and chromatographic analyses as described above, and HPLC eluates corresponding to LTC<sub>4</sub>, LTB<sub>4</sub>, and 5-HETE retention times were subsequently assayed simultaneously with eluates obtained from analyses of glomerular extracts. To perform the assays, in 0.1 ml of LT or HETE eluates reconstituted in RIA buffer, 7000–8,000 cpm of <sup>3</sup>H-ligand dissolved in 100  $\mu$ l of buffer was added followed by 100  $\mu$ l of antibody used at proper dilution. RIA reactants were subsequently incubated at 4°C for 18 h and the antibody bound was separated from free by the addition of dextran-coated charcoal. To assess the ability of the antibodies employed to react with LT or HETE comigrating with the appropriate standard, immunoreactivity was assayed in all fractions after HPLC of a mixture of LTC<sub>4</sub>, LTB<sub>4</sub>, 5-HETE, and 12-HETE standards (100 ng each). The immunoreactivity profiles are demonstrated in Fig. 1, which also shows the RIA “noise” levels obtained after extractions and chromatographic separations of 2 ml of glomeruli-free incubation medium. Since no exogenous LTC<sub>4</sub> is contained in manufactured RPMI medium 1640, these levels are most likely due to compounds in this medium that cross-reacted with the LTC<sub>4</sub> antiserum employed. The biochemical studies described in glomeruli isolated from rats with NSGN were based on the assumption that glomeruli purified by differential sieving at each time point after induction of the disease are representative of glomeruli in histologic sections at that point. It is possible, however, that inflamed glomeruli are of a different size compared to normal glomeruli. Therefore, the isolation technique, designed to maximize purification of normal glomeruli, may not be entirely appropriate for immunologically injured glomeruli. To address this issue, eicosanoid synthesis was also determined in renal cortical slices obtained in a group of rats ( $n = 6$ ) with NSGN at 3 h after induction of the disease using high doses of anti-GBM Ig (150  $\mu$ g/g of body weight) and values were compared to those obtained in a control group receiving nonimmune rabbit serum ( $n = 6$ ). In these experiments, cortical slices of 1 mm thickness were obtained using a Stadie-Riggs tissue slicer (Arthur Thomas Co.,

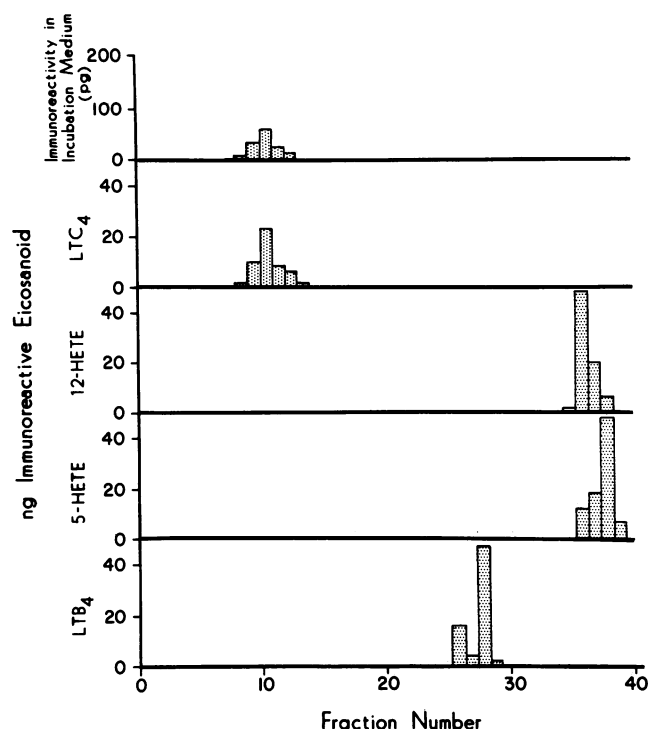


Figure 1. Immunoreactivity profiles in fractions of an HPLC-purified mixture of 5-HETE, 12-HETE, LTB<sub>4</sub>, and LTC<sub>4</sub> (100 ng each) as well as of tissue-free incubation medium (uppermost panel). Fractions were individually assayed for all four eicosanoids. Note that in tissue-free medium immunoreactivity in fractions corresponding to LTC<sub>4</sub> retention time was detectable.

Philadelphia, PA) and incubated in 2 ml of RPMI-1640 in polypropylene tubes at 37°C for 45 min. At the end of incubation, slices were homogenized using a tissue homogenizer (Polytron, Brinkmann Instruments Co., Westbury, NY) and homogenates were extracted and processed for biochemical studies in a manner identical to that described for glomeruli. Results of eicosanoid measurements in all groups were expressed as mean  $\pm$  SE of picograms or nanograms of LT or HETE per milligram of tissue protein. For statistical comparisons of immunoassayable glomerular HETE and leukotriene values obtained in the various groups of rats with NSGN and respective controls, Student's *t* test for unpaired observations was employed.

**Identification of LTB<sub>4</sub> and 5-HETE.** For further identification of the compound comigrating with authentic LTB<sub>4</sub>, nanogram amounts generated from pooled glomerular extracts of six rats with NSGN killed at 1 h after induction of the disease were purified by HPLC as described above. Fractions corresponding to retention times identical to those of LTB<sub>4</sub> were subsequently dried under vacuum, reconstituted in 1 ml of HPLC grade methanol, and placed in the cuvette of a diode array spectrophotometer (model 8450, Hewlett-Packard Co., Palo Alto, CA). An absolute absorbance UV spectrum was then obtained by scanning the region from 220 to 320 nm. The spectrum was compared to that obtained from HPLC-purified LTB<sub>4</sub> standard with respect to configuration and absorbance maxima. The compound was further characterized by Fourier-transform infrared spectroscopy (FTIR). HPLC-purified samples were reconstituted in 100  $\mu$ l of methanol and transferred dropwise on the sodium chloride plate of an FTIR spectrometer (Beckman FT-2000 or Nicolet MX1, Nicolet Instrument Corp., Madison, WI) capable of providing a continuous throughput interferogram. The NaCl plate was transparent throughout the 4,000 to 625  $\text{cm}^{-1}$  wavenumber region and scanning was performed between 4,000 and 1,000 wavenumbers under a continuous flow of nitrogen.

The spectrum obtained was compared to that obtained from the HPLC-purified LTB<sub>4</sub> standard. For identification of 5-HETE, nanogram amounts generated from pooled glomerular extracts were subjected to HPLC using a reverse-phase column system as described above and a system using a normal phase 10- $\mu$ m silica cartridge. The latter system employed elutions in hexane/isopropanol/acetic acid (100:8:0.5) for 10 min followed by a gradient to 70% hexane/acetic acid (125:1) over 100 min. Retention times were compared with those of authentic 5-HETE and eluates were subjected to UV and FTIR spectroscopy as described for LTB<sub>4</sub>.

## Results

Fig. 2 demonstrates a high-pressure liquid chromatogram obtained from pooled glomerular extracts of rats with NSGN at 3 h after infusions of low (A) and high (B) doses of nephrotoxic serum. 12-HETE was the main product obtained in extracts from the low-dose group whereas both 5-HETE and LTB<sub>4</sub> were present in extracts from the high-dose group. An uniden-

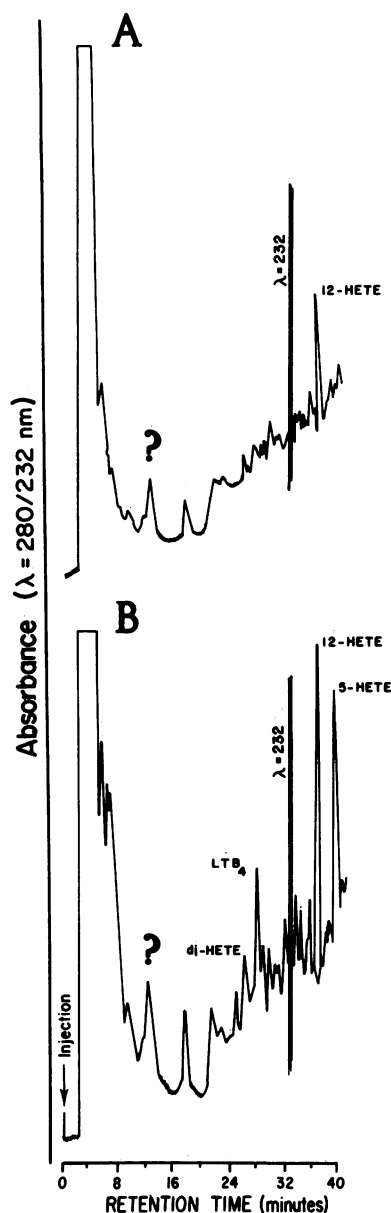


Figure 2. High pressure liquid chromatograms obtained from pooled glomerular extracts of rats with NSGN using (A) low and (B) high doses of anti-GBM Ig. 12-HETE was the major eicosanoid detected in extracts from the low-dose group, whereas both 5-HETE and LTB<sub>4</sub> were present in extracts from the high-dose group. An unidentified peak with retention time similar to that of LTC<sub>4</sub> was also present.

tified peak at 14.4 min was present in extracts from both groups and had a retention time similar to that of LTC<sub>4</sub> standard. However, the amounts of immunoassayable LTC<sub>4</sub> found in the corresponding eluates were below the detection limits of the LTC<sub>4</sub> RIA (Table I). In Fig. 3, the absolute absorbance spectrum of HPLC-purified LTB<sub>4</sub> obtained from pooled glomerular extracts is shown (lower diagram) to demonstrate the configuration of a conjugated triene with absorbance maxima of 261, 270, and 281 which were identical to those of HPLC-purified LTB<sub>4</sub> standard. Fig. 3 also demonstrates the FTIR spectrum of glomerular (A) and standard (B) LTB<sub>4</sub> to indicate areas of bending and stretching vibrations due to the presence of the various groups and bonds in the LTB<sub>4</sub> molecule as indicated. In Fig. 4, the absolute absorbance and FTIR spectra of glomerular 5-HETE are shown.

Table I summarizes the HETE and LT values in isolated glomeruli and renal cortical slices obtained from rats studied at 3 h after induction of NSGN using low and high doses of nephrotoxic serum. 12-HETE was the only eicosanoid detectable under basal conditions in control (no injury) glomeruli and in the low-dose group and was also the most abundant in all groups. Basal synthesis of immunoassayable 5-HETE and LTB<sub>4</sub> was detectable in the high-dose group only (924±219 and 104±24 pg/mg of glomerular protein, respectively). A23187 (2  $\mu$ M) induced significant production of these two eicosanoids in the low-dose group. However, their levels (all in ng/mg glomerular protein) were not statistically different from respective controls (5-HETE, 12.5±2.9 compared to 12.9±1.8; LTB<sub>4</sub>, 2.4±0.5 compared to 2.1±0.4). In the high-dose group, A23187 markedly enhanced glomerular 5-HETE and LTB<sub>4</sub> levels (5-HETE, 63.1±7.8 compared to 12.9±1.8,  $P < 0.05$ ; LTB<sub>4</sub>, 7.1±1.1 compared to 2.1±0.4,  $P < 0.05$ ). Immune injury enhanced both basal and stimulated 12-HETE production regardless of the dose of anti-GBM Ig employed (Table I). 12-HETE was the only detectable product in cortical slices and

Table I. Immunoassayable HETE and LTB<sub>4</sub> Values Derived from Isolated Glomeruli and Cortical Slices under Basal and A23187-stimulated Conditions

Source	Basal synthesis			
	12-HETE	5-HETE	LTB <sub>4</sub>	LTC <sub>4</sub>
pg/mg protein				
No injury (glomeruli)	190±70	<30	<17	<120
No injury (cortical slices)	42±12	<30	<17	<120
NTS: low dose (glomeruli)	450±170*	<30	<17	<120
NTS: high dose (glomeruli)	1970±230†	924±219	104±24	<120
NTS: high dose (cortical slices)	107±12*	136±32	59±14	<120
Source	Stimulated glomerular LTB <sub>4</sub> synthesis			
	12-HETE	5-HETE	LTB <sub>4</sub>	LTC <sub>4</sub>
ng/mg protein				
No injury	17.0±2.5	12.9±1.8	2.1±0.4	<120
NTS: low dose	70.7±9.0	12.5±2.9	2.4±0.5	<120
NTS: high dose	175±30†	63.1±7.8*	7.1±1.1*	<120

NTS, nephrotoxic serum.

\*  $P < 0.05$ ; †  $P < 0.01$ , compared to no injury.

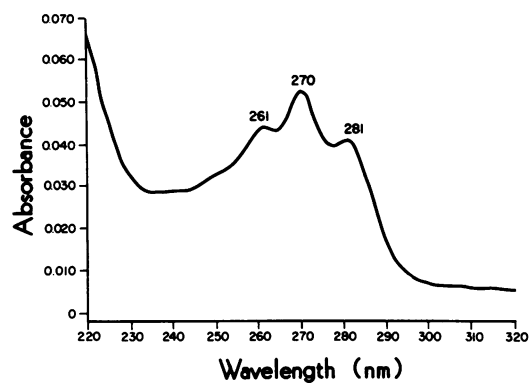
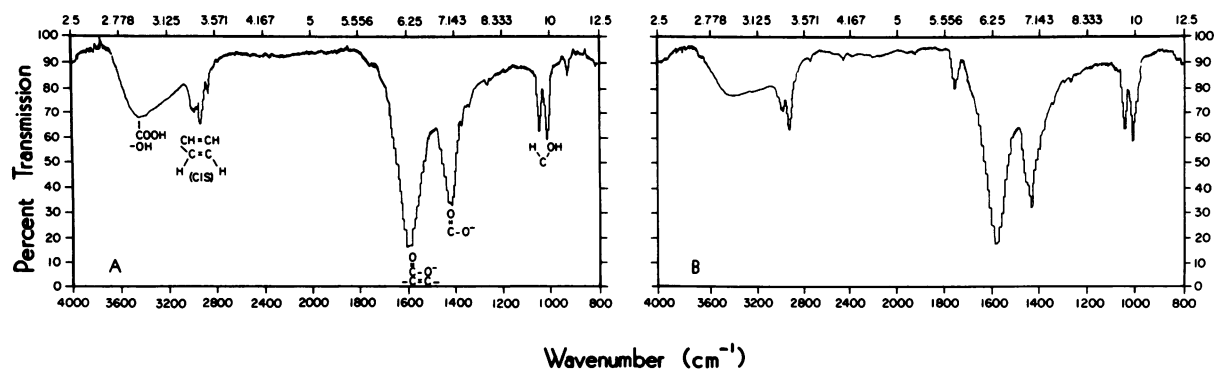


Figure 3. Absolute absorbance (lower panel) and FTIR spectrum (upper panel) of glomerular HPLC-purified LTB<sub>4</sub>. Note configuration of a conjugated triene with absorbance maxima at 261, 270, and 281 nm. Bending and stretching vibrations in the FTIR spectrum of

(A) glomeruli-derived and (B) standard LTB<sub>4</sub> correspond to the various groups and double bonds of the LTB<sub>4</sub> molecule as indicated. Instrument: Beckman FT-2000.

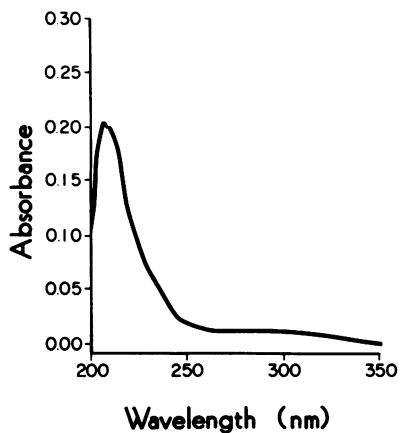
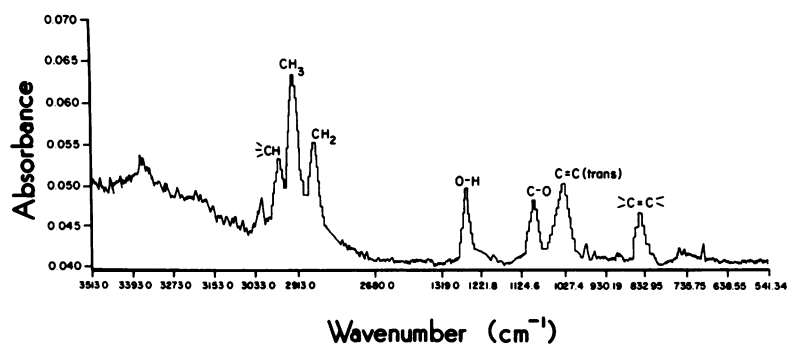


Figure 4. Absolute absorbance (lower panel) and FTIR spectra of glomerular 5-HETE. Instrument: Nicolet MX-1 FT 48.

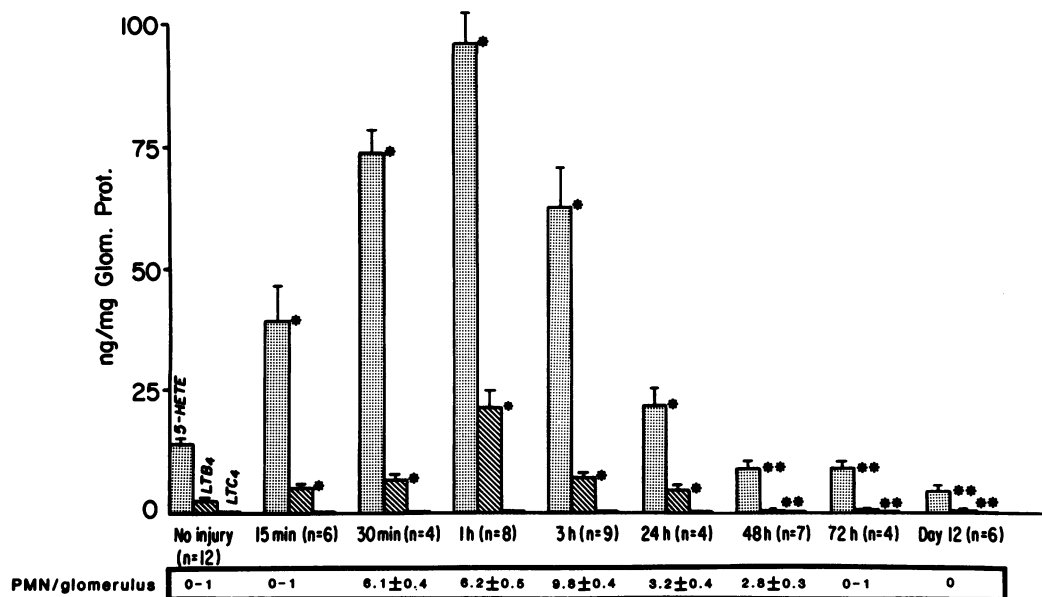


Figure 5. Glomerular 5-HETE and LT (mean  $\pm$  SEM) in response to A23187 at various time points after a single intravenous administration of anti-GBM Ig (105  $\mu$ g/g of body weight) shown in parallel with glomerular PMN infiltration. Enhanced 5-HETE and LTB<sub>4</sub> synthesis commenced at 15 min, peaked at 1 h, and returned toward control levels by 24 h. At 48 and 72 h and on day 12 their levels were significantly lower than control. (\*) Values significantly higher than in the no injury group; (\*\*) values significantly lower than in the no injury group (see text).

its basal synthesis was enhanced in slices obtained from the high dose nephrotoxic serum group ( $42 \pm 12$  compared to  $107 \pm 12$  pg/mg protein). Basal 5-HETE and LTB<sub>4</sub> synthesis was measurable in the latter group only ( $136 \pm 32$  and  $59 \pm 14$  pg/mg protein, respectively). In Fig. 5, glomerular 5-HETE and LT synthetic profiles in the presence of A23187 are demonstrated at the various points of study after a single intravenous infusion of high doses of anti-GBM Ig. In parallel, the extent of glomerular PMN infiltration assessed in cortical tissue (PMN per glomerulus,  $n = 80-240$ ) is shown. In glomeruli isolated from control rats (no glomerular injury,  $n = 12$ ) and incubated with A23187 (2  $\mu$ M) the immunoassayable 5-HETE and LTB<sub>4</sub> levels were  $12.9 \pm 1.8$  and  $2.1 \pm 0.5$  ng/mg glomerular protein, respectively (Fig. 5). Enhanced production of these two eicosanoids was observed at 15 min after infusion of anti-GBM Ig when no change in PMN infiltration had occurred, peaked at 1 h when neutrophil infiltration was  $6.2 \pm 0.5$  PMN per glomerulus, and returned toward control, yet still signifi-

cantly higher levels by 24 h when significant PMN infiltration was still present ( $3.2 \pm 0.4$ ). At 48 and 72 h and on day 12, basal 5-HETE and LTB<sub>4</sub> levels were undetectable (data not shown). At these time points (Fig. 5), A23187 induced significantly lower production of 5-HETE and LTB<sub>4</sub> compared to control levels. Thus, the values obtained at 48 h ( $n = 7$ ) for 5-HETE were  $8.1 \pm 1.4$  compared to  $12.9 \pm 1.8$  in controls,  $P < 0.05$ . The values for LTB<sub>4</sub> were  $0.38 \pm 0.08$  compared to  $2.1 \pm 0.5$  in controls,  $P < 0.05$ . In Table II the MPO activity assayed in glomerular preparations after induction of NSGN is shown. Note the temporal correlation between glomerular MPO activity and glomerular PMN infiltration (Fig. 5) which are in apparent agreement.

In Fig. 6 the effects of complement and PMN depletion on glomerular 12- and 5-HETE and LT in response to A23187 stimulation are shown in parallel with the extent of PMN infiltration. Anti-PMN serum administered before induction of NSGN-induced peripheral neutropenia (PMN counts  $< 200/\text{mm}^3$ ), abolished glomerular PMN infiltrates (Fig. 6), and reduced glomerular MPO activity (Table II), compared to PMN-replete animals with NSGN studied in parallel (3 h). At 3 h after induction of NSGN, 5-HETE levels in the PMN-depleted rats ( $n = 12$ ) were reduced compared to the control (PMN replete) group ( $n = 9$ ) ( $48 \pm 4$  compared to  $63.1 \pm 7.8$  ng/mg glomerular protein,  $P < 0.05$ ) but remained markedly higher compared to levels obtained in glomeruli from rats without glomerular injury ( $12.9 \pm 1.8$  ng/mg glomerular protein,  $n = 12$ , Fig. 5) or from a PMN-depleted group of normal rats ( $13.4 \pm 0.7$  ng/mg glomerular protein,  $n = 5$ , data not shown). There were no significant differences in LTB<sub>4</sub> or 12-HETE levels (Fig. 6). Complement depletion using purified CVF was associated with peripheral neutropenia (PMN counts  $350-550/\text{mm}^3$ ) and significantly reduced but did not abolish glomerular PMN infiltrates or MPO activity at 3 h after induction of NSGN (Fig. 6, Table II). Compared to the complement-replete group ( $n = 9$ ), in the complement-depleted group ( $n = 10$ ) there was a marked decrement in glomerular 5-HETE and LTB<sub>4</sub> levels (5-HETE,  $10.3 \pm 1.8$  compared to  $63.1 \pm 7.8$ ,  $P$

Table II. MPO Activity in Glomerular Preparations after Induction of NSGN

Preparation	MPO
	$U \times 10^{-3}$
No injury	1.4
NSGN: 15 min	1.9
NSGN: 1 h	37.9
NSGN: 3 h	47.4
NSGN: 24 h	17.4
NSGN: 48 h	3.8
NSGN (3 h): PMN depleted	2.1
NSGN (3 h): complement-depleted	6.5
CVF-treated	4.4

Values reflect the mean of duplicate observations.

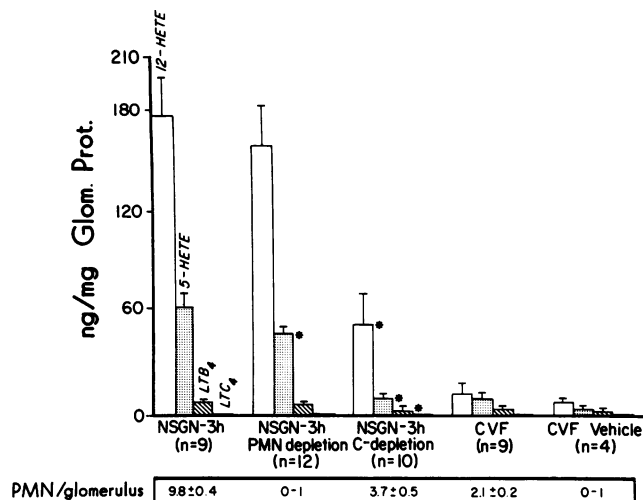


Figure 6. Effects of PMN and complement depletion on glomerular HETE and  $LTB_4$  levels shown in parallel with glomerular PMN infiltration. Eicosanoid values are mean $\pm$ SEM and were obtained from A23187-stimulated glomerular preparations. PMN depletion partially but significantly reduced 5-HETE levels and had no effect on those of 12-HETE or  $LTB_4$ . Complement depletion markedly reduced glomerular 5-HETE and  $LTB_4$  to levels indistinguishable from CVF-treated controls and significantly reduced 12-HETE production. CVF-treated controls tended to have higher glomerular 12- and 5-HETE values compared to rats receiving CVF vehicle. (\*) Values significantly lower than in the NSGN (3-h) group (see text).

$< 0.01$ ;  $LTB_4$ ,  $3.9 \pm 0.8$  compared to  $7.1 \pm 1.1$ ,  $P < 0.05$ , Fig. 6). The eicosanoid levels in the complement-depleted group were similar to those obtained in glomeruli isolated from CVF-treated control rats ( $n = 9$ ) (5-HETE,  $10.3 \pm 0.8$ ;  $LTB_4$ ,  $3.9 \pm 0.9$  ng/mg glomerular protein, Fig. 6). In the complement-depleted group, there was also a significant reduction in 12-HETE production compared to the complement-replete group ( $52 \pm 17$  compared to  $175 \pm 30$  ng/mg glomerular protein,  $P < 0.01$ , Fig. 6). The levels of this eicosanoid, however, remained significantly higher than in the CVF-treated controls ( $52 \pm 17$  compared to  $11.9 \pm 1.9$  ng/mg glomerular protein,  $P < 0.01$ ). In the latter group, appreciable glomerular PMN infiltration was noted ( $2.1 \pm 0.2$ ) and the levels of 12- and 5-HETE tended to be higher compared to the CVF vehicle-treated group (12-HETE,  $11.9 \pm 1.0$  compared to  $8.0 \pm 2.5$ ; 5-HETE,  $10.3 \pm 0.8$  compared to  $3.9 \pm 0.6$ , Fig. 6). These differences did not reach statistical significance.

Table III summarizes the  $PGE_2$  and  $LTB_2$  values obtained in glomerular preparations incubated with CVF and in appropriate controls. Note that CVF did not induce significant increments in  $PGE_2$  synthesis. This was observed whether serum free medium or normal rat serum with RPMI was employed.  $LTB_4$  levels were below the detection limits of the RIA.

## Discussion

The results of this study demonstrate that in anti-GBM antibody-induced glomerular injury, the spontaneous (basal) production of glomerular 12-, 5-HETE, and  $LTB_4$  is enhanced and the type of eicosanoid synthesized is related to the amount of anti-GBM antibody employed. Although both doses of

Table III. Immunoassayable  $PGE_2$  and  $LTB_4$  Values in Glomerular Incubation Media after Introduction of CVF

Glomerular incubations	$PGE_2$	$LTB_4$
	ng/mg protein	pg/mg protein
NRS/RPMI (1:10) ( $n = 6$ )	$37.7 \pm 3.5$	$< 17$
NRS/RPMI + CVF 2 U ( $n = 4$ )	$27.6 \pm 1.5$	$< 17$
NRS/RPMI + CVF 10 U ( $n = 6$ )	$47.9 \pm 6.0$	$< 17$
RPMI + CVF 10 U ( $n = 5$ )	$18.3 \pm 5.0$	$< 17$
RPMI	$15.2 \pm 3.7$	$< 17$

NRS, normal rat serum.

anti-GBM Ig (30 and 105  $\mu$ g/100 g of body weight) induced glomerular injury (proteinuria) and glomerular PMN infiltration at the early heterologous phase (3 h) of injury, detectable basal production of 5-HETE and  $LTB_4$  was noted only in glomeruli and cortical slices obtained from rats with NSGN induced using high doses of anti-GBM Ig (Table I). In contrast, enhanced 12-HETE production occurred regardless of the dose of anti-GBM serum employed. The similarities in eicosanoid synthetic profiles obtained under basal incubation conditions in preparations of isolated glomeruli and in cortical slices from rats with NSGN and controls (Table I) support the argument that biochemical events occurring in isolated glomeruli are most likely representative of events occurring in histologic sections. The phospholipase activator A23187 was used in order to provide a stimulus of eicosanoid production in addition to stimuli originating from intraglomerular events occurring after induction of NSGN. As such, it provided maximum in vitro unmasking of arachidonate lipoxygenase activities. Under these conditions, nanogram quantities of 5-HETE and  $LTB_4$  were present in glomeruli isolated from rats with NSGN using low anti-GBM Ig doses (30  $\mu$ g/g of body weight). However, these amounts were not significantly different than those obtained in control glomeruli (Table I). 12-HETE levels were detectable under both basal and stimulated conditions and, in contrast to 5-HETE and  $LTB_4$ , they increased regardless the dose of anti-GBM antibody employed (Table I). These observations indicate that the 12-lipoxygenase activity is the most abundant in both normal and in immunologically injured glomeruli.

In contrast to the synthetic profiles of 12-HETE and of arachidonate cyclooxygenation products (prostaglandins and thromboxanes) described previously, the enhanced synthesis of 5-HETE and  $LTB_4$  in NSGN commenced and returned toward control levels earlier (at 15 min and 24 h, respectively) (Fig. 5). Augmented synthesis of 12-HETE, prostaglandins and thromboxane  $A_2$  begins later (3 h) and is maintained (Tx $B_2$ , 12-HETE) throughout the period of study of the disease (4, 6). Also, in contrast to other eicosanoids, the ability of immunologically injured glomeruli to produce 5-HETE and  $LTB_4$  in response to A23187 stimulation was impaired after the early heterologous phase of NSGN (48 h, 72 h, day 12). At these points of study the levels of 5-HETE and particularly those of  $LTB_4$  were significantly lower than in the "no injury" group (Fig. 5). This could be indicative of either an injury-induced impairment of the enzymic synthesis of these eicosanoids occurring at later stages of NSGN or a depletion of preformed



pools during earlier stages of the disease. The previously described inability to enhance conversion of [ $^3\text{H}$ ]arachidonate to [ $^3\text{H}$ ]5-HETE in glomeruli isolated from rats with NSGN at 48 h (4) supports the possibility of injury-induced impairment of enzymic synthesis.

The role of PMN infiltration in contributing to glomerular 12-, 5-HETE, and  $\text{LTB}_4$  production was assessed by (a) correlating glomerular eicosanoid levels with glomerular PMN infiltrates in cortical tissue sections and with MPO activity in preparations of isolated glomeruli and (b) by performing PMN depletion experiments. As shown in Fig. 5 and Table II, enhanced 5-HETE and  $\text{LTB}_4$  production occurred at 15 min after induction of NSGN when no apparent glomerular PMN infiltration was evident by light microscopy and no marked increase in glomerular MPO activity occurred. Furthermore, 5-HETE and  $\text{LTB}_4$  levels began to decline at 3 h when both glomerular PMN infiltration (Fig. 5) and MPO activity (Table II) peaked. Moreover, at 48 h, the stimulated production of these eicosanoids fell to below control levels despite continued PMN infiltration ( $2.8 \pm 0.3$ ) and increased MPO activity. PMN depletion abolished glomerular PMN infiltration at 3 h after induction of NSGN (Fig. 6), reduced MPO activity (Table II), and decreased but did not eliminate 5-HETE production. Moreover, there was no significant effect on 12-HETE and  $\text{LTB}_4$  levels (Fig. 6). The lack of temporal correlation between the extent of glomerular PMN infiltration and eicosanoid levels, the discrepancy between the extent of PMN infiltration and levels of 5-lipoxygenation products, and the inability to markedly reduce these levels despite significant reduction of glomerular PMN infiltrates in PMN depleted animals indicate that infiltrating PMN only partially contributed to glomerular eicosanoid production.

In contrast to PMN depletion, the complement depletion experiments indicate that glomerular 5-HETE and  $\text{LTB}_4$  production is markedly dependent on complement deposition whereas production of 12-HETE is dependent to a lesser extent (Fig. 6). In the de complemented group of rats, 5-HETE and  $\text{LTB}_4$  production was reduced to levels no different than those of CVF-treated controls (Fig. 6), despite the significant presence of glomerular PMN ( $3.7 \pm 0.5$ ) and MPO activity (Table II). The mechanism of complement-mediated increase in glomerular arachidonate lipoxygenation products is unclear. A similar phenomenon was observed in the early heterologous phase of NSGN (5 h) with respect to thromboxane synthesis (13). In NSGN, glomerular injury is complement deposition dependent (14) and intraglomerular activation of complement could be associated with generation of anaphylatoxins. Of these, C5a has been shown to activate 5-lipoxygenase in various tissues such as lung as well as in purified and mixed cell populations resulting in release of monoHETE, diHETE, and  $\text{LTB}_4$  (15).

The cellular source(s) of glomerular HETE and leukotrienes in NSGN is unclear. Recruited platelets do not appear to be the major source of 12-HETE (4) and PMN only partially contribute to glomerular 5-HETE levels (Fig. 6). Moreover, enhanced  $\text{LTB}_4$  production was described in a model of glomerular injury induced by cationized bovine  $\gamma$ -globulin in which glomerular inflammatory cell infiltrates are minimal or absent (16). In normal rat glomeruli, synthetic activities have been described for  $\text{LTB}_4$  and  $\text{LTC}_4$  (17, 18), indicative for the presence of arachidonate 5-lipoxygenase pathway, and detect-

able immunoassayable levels of  $\text{LTB}_4$  were obtained (17). The significance of the enhanced production of 5-HETE and  $\text{LTB}_4$  remains to be explored. In contrast to other eicosanoids, their augmented production occurs very early (15 min) after induction of glomerular injury and is short-lived (24 h). It is possible that the early events triggered by the anti-GBM antibody binding, such as complement activation, are crucial in stimulating the arachidonate 5-lipoxygenase pathway, the products of which could, in turn, exert chemotactic effects, enhance expression of C3b receptors (20), and release of lysosomal enzymes (20), thereby contributing to glomerular capillary wall injury.

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