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Research Article

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Lipoproteins Containing Apoprotein B Are a Major Regulator of Neutrophil Responses to Monosodium Urate Crystals

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Abstract. The inflammatory response to intra-articular urate crystals is known to be variable in gouty arthritis. One source of variability may be the modulation of cellular responses by crystal-bound proteins. We have identified three apolipoproteins among the polypeptides bound to urate crystals exposed to plasma. Identification was first based on their coelectrophoresis with polypeptides from isolated lipoproteins and diminution in the protein coat of crystals exposed to lipoprotein-depleted plasma. The apoproteins were immunochemically identified by the Western blotting technique as apoprotein A-I, apoprotein B (apo B), and apoprotein E.

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Lipoprotein-depleted plasma reconstituted with very low, intermediate, and low density lipoproteins (LDL) inhibited crystal-induced CL. High density lipoprotein reconstitution was without effect. Immunodepletion from plasma of all apo B lipoproteins by agarose-bound apo B-specific antibody also removed all inhibitory activity for urate-induced CL. Thus, apo B lipoproteins were shown to be the inhibitory species in plasma. Binding of apo B lipoproteins to urate crystals and inhibition of CL was also seen in the absence of other plasma proteins. In addition, the binding of whole lipoprotein particles to the crystals was verified by detection of crystal-associated cholesterol in addition to the apoprotein.

The effects of LDL on urate crystal-induced CL were stimulus specific. Coincubation of urate crystals and neutrophils in the presence of $10 \mu\text{g/ml}$ LDL resulted in 83% inhibition. In contrast, CL responses to a chemotactic hexapeptide, opsonized zymosan, and *Staphylococcus aureus* were not inhibited by LDL.

The effects of depletion of apo B lipoproteins on plasma suppression of urate crystal-induced CL appeared to be unique. Plasmas or sera depleted of other urate crystal-binding proteins including fibrinogen, fibronectin, C1q, and IgG retained virtually all their CL inhibitory activity. Lipoproteins containing apo B are thus a major regulator of neutrophil responses to urate crystals. These lipoproteins are present in variable concentration in synovial fluid and may exert an important influence on the course of gout.

Introduction

Monosodium urate crystals appear to trigger attacks of gout (1); yet there is striking variability in the inflammatory response to intra-articular crystals (2-5). The ability of urate crystals to induce inflammation is critically dependent upon neutrophils (6). In response to urate crystals, these cells generate superoxide

(7, 8), a chemotactic factor (9, 10), and leukotrienes (11), and release lysosomal enzymes by lytic and nonlytic (12, 13) mechanisms. A number of biologically significant proteins bind to urate crystals (14–17) and may influence crystal-induced neutrophil responses (7, 18). This suggests that variation in the protein coat of these crystals may be one factor in the variable *in vivo* inflammatory response in gout.

Since most synovial fluid proteins are present in plasma (19), and plasma and serum have a more constant composition than gouty synovial fluid (19, 20), investigators have focused on serum and plasma effects on neutrophil-crystal interaction. Serum profoundly inhibits urate crystal-induced neutrophil lysis (18) and a serum coat has been shown by Abramson et al. (7) to reduce urate crystal-induced superoxide generation. In neither study were the inhibitory factor(s) identified. We have recently established detailed biochemical maps of the protein coat of plasma-exposed urate crystals by O'Farrell two-dimensional gel electrophoresis (17) and observed several hitherto unidentified polypeptides, which were increased on the crystal surface relative to plasma. In the present study, we have identified a number of these polypeptides as derived from lipoproteins. We have also found that lipoproteins containing apoprotein B (apo B)¹ account for virtually all of the inhibitory activity of plasma on urate crystal-induced neutrophil chemiluminescence and a major portion of the plasma inhibitory activity in urate-induced neutrophil superoxide generation and cytolysis.

Methods

Crystals and crystal-protein binding studies. Urate crystals were prepared under sterile pyrogen-free conditions by a previously described modification (21) of the method of McCarty and Faires (22) and by the method of Denko and Whitehouse (23) and resuspended to 25 mg/ml in urate-saturated phosphate-buffered saline (PBS) (10 mM Na phosphate buffer, pH 7.4, containing 150 mM NaCl) prepared as previously described (24). Crystals were utilized in unheated form, were negatively birefringent, and ranged predominantly from 15–45 μ m in length. Using polarized light microscopy, it was verified that the smaller crystals in these preparations could be phagocytosed by neutrophils. To coat crystals with plasma proteins, equal volumes of crystals and plasma were coin-cubated at 37°C for 30 min in a total volume of 0.6 ml, unless otherwise indicated. Unbound plasma proteins were removed by washing the crystals through 0.8 ml of a 20% sucrose cushion followed by a wash in 1 ml urate-saturated PBS. These conditions gave reproducible crystal-protein binding patterns on O'Farrell gels as previously described (17).

Other reagents. Opsonized zymosan was prepared by incubation of zymosan (Sigma Chemical Corp., St. Louis, MO) with normal human serum at 37°C for 15 min with agitation followed by repeated washing in PBS and storage at –70°C. A 200-fold excess of opsonized zymosan particles to polymorphonuclear leukocytes (PMN) was used in the chemiluminescence (CL) assay (25). *Staphylococcus aureus* ATCC 25923

was a generous gift from Dr. R. Proctor (University of Wisconsin, Madison, WI) and was grown, washed, and lyophilized as described (26). The chemotactic hexapeptide FNLNLT (*N*-formyl-*N*-leu-leu-phe-*N*-leu-tyr-lys) was the generous gift of Dr. L. Sklar, Scripps Clinic and Research Foundation. All other chemicals were reagent grade.

Lipoprotein depletion, isolation and iodination. Lipoprotein-depleted plasma (LPDP) was prepared by ultracentrifugation (27). Plasma containing 20 mM EDTA was adjusted to a density of 1.25 g/cm³ with solid KBr and centrifuged at 45,000 rpm for 20 h at 4°C in a fixed-angle 50Ti rotor (Beckman Instruments Inc., Spinco Div., Palo Alto, CA). The lipoprotein-containing supernatant was removed and the infranatant taken as LPDP. Reconstituted LPDP was obtained by mixing the supernatant and infranatant. Both LPDP and reconstituted LPDP were dialyzed against 1 liter of 10 mM PBS, pH 7.4, which was changed twice, for a total of 18 h, and their protein concentrations measured and equalized by addition of buffer. Lipoprotein depletion was verified by >95% reduction of cholesterol content of LPDP vs. reconstituted LPDP. Lipoproteins were isolated by sequential ultracentrifugation of pooled plasma from normal fasting subjects using solid KBr for density adjustment as previously described (27). The lipoprotein fractions included very low density lipoprotein (VLDL), $d < 1.006$ g/cm³; intermediate density lipoprotein (IDL), $d = 1.006$ – 1.019 g/cm³; low density lipoprotein (LDL), $d = 1.019$ – 1.063 g/cm³; and high density lipoprotein (HDL), $d = 1.063$ – 1.25 g/cm³. The fractions were dialyzed against 0.15 M NaCl containing 0.3 mM EDTA and 0.0005% alfatocopherol, filter sterilized, and stored sterile for a maximum of 7 d. All lipoprotein concentrations are expressed on the basis of protein as measured by a modification of the Lowry assay using a bovine serum albumin (BSA) standard (28). The lipoproteins used displayed a consistent apoprotein composition as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (27). When labeled lipoproteins were employed, 100 μ g of VLDL, IDL, LDL, and HDL were labeled enzymatically with 1 mCi of ¹²⁵I (Amersham Corp., Arlington Heights, IL) using immobilized lactoperoxidase and glucose oxidase (Enzymobeads, Bio-Rad Laboratories, Richmond, CA). They were dialyzed, characterized, and stored aseptically in the presence of 10% BSA as previously described (29).

Immunodepletion of apo-B lipoproteins. Plasma was depleted of apo B-containing lipoproteins (VLDL, IDL, and LDL) by immunoabsorption with a mouse anti-human apo B monoclonal antibody, V82A6 (27). The monoclonal antibody was immunopurified from ascites fluid as described (29), coupled to CNBr-activated Sepharose 4B (Pharmacia Fine Chemical, Piscataway, NJ), and equilibrated in PBS containing 10 mM EDTA, 10 U/ml trasyolol, and 5 mM benzamidine. 1 ml of plasma was chromatographed on 15 ml of gel containing 26.3 mg of coupled antibody and the nonretained plasma fraction collected. Apo B removal from the plasma was monitored immunochemically by using a solid-phase radioimmunoassay as described (27). The antibody-adsorbed plasma fraction contained <50 ng apo B/mg of plasma protein.

Two-dimensional gel electrophoresis. Urate crystal-bound protein was eluted by boiling for 5 min in 10% glycerol, 5% Nonidet-40, 1.5% SDS, and 2% β -mercaptoethanol, and the protein concentration of the eluates was determined by the Lowry assay using a BSA standard. Samples of purified lipoproteins delipidated by boiling in the same SDS-containing buffer described above, and crystal eluates containing 75 μ g of protein were analyzed by isoelectric focusing followed by SDS-PAGE in an 8–20% exponential gradient slab gel in the second dimension as described previously (17). Protein was detected by the silver-staining protocol of Morrissey (30), and isoelectric points and molecular weights estimated as described previously (17).

1. Abbreviations used in this paper: apo, apolipoprotein; apo A-I, apo B, and apo E, apolipoproteins A-I, B, and E; CL, chemiluminescence; LDH, lactic dehydrogenase; LPDP, lipoprotein-depleted plasma; PAGE, polyacrylamide gel electrophoresis; pl, isoelectric point.

Western blot analysis. For Western blot analysis, the slab gels were washed and electrophoretically transferred to nitrocellulose (0.45 μ m, No. HAWP 304 FO, Millipore Corp., Bedford, MA) as previously detailed (27). The nitrocellulose transfers of O'Farrell gels were soaked overnight in PBS containing 3% BSA, 3% normal goat serum, and 0.01% azide (blocking buffer) to saturate remaining active binding sites, and then incubated with first antibody (hybridoma culture supernatants or ascites fluids) for 18 h at 4°C. Sources of apoprotein-specific antibody were: anti-apoprotein A-I (apo A-I) hybridoma AV63C2 (27); anti-apo B hybridomas V82A6 and V82B1 (27); monospecific rabbit polyclonal anti-LDL serum (29), and anti-apoprotein E (apo E), hybridoma Emab3 (31). The nitrocellulose membranes were washed five times with blocking buffer containing 0.5% TWEEN-20 (Sigma Chemical Co.). Mouse antibody was detected on O'Farrell gel blots by addition of 125 I-goat anti-mouse Ig (0.5 μ Ci/ml) for 4 h at 4°C followed by washing and detection of 125 I by autoradiography as previously described (27). Mouse and rabbit antibodies on blots of single dimensional gels were detected by goat anti-mouse or anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad Laboratories) (32).

PMN luminol-dependent CL. PMNs were isolated from human blood by the method of Boyum (33) utilizing ficoll-hypaque density gradients and dextran sedimentation with modifications as previously described (34). These preparations contained >95% neutrophils. PMN CL assays were performed by using previously described modifications (35) of the method of Wilson et al. (36) and monitored in a Beckman LS-8000 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA) operated in the single photon counting mode. The assay conditions were 2.5×10^5 cells per assay in a final volume of 0.5 ml Hanks' balanced salt solution (HBSS) containing 0.02 M Hepes, 0.25% (wt/vol) BSA, and 2 μ M luminol (Aldrich Chemical Co., Milwaukee, WI). CL was monitored at 22°C at intervals of 0.25 min for 60 min. CL values are expressed as counts per minute per 2.5×10^5 cells after correction for background CL obtained in the absence of stimulation. In selected experiments, results were expressed as the maximum CL response to urate crystals. In each instance, this maximum response was observed within 30 min of PMN exposure to the crystals.

Assay of superoxide O_2^- generation. To measure O_2^- generation, 2 mg/ml urate crystals were added to PMNs at 5×10^6 ml in 1 ml HBSS-0.02 M Hepes-0.25% BSA in the presence of 50 μ M ferricytochrome C (Horse Heart, Sigma Chemical Co.). Experiments were performed in 5 ml polypropylene tubes at 37°C with constant agitation and the reactions were terminated by centrifugation at 500 g for 10 min at 4°C. Cell-free supernatants were decanted and superoxide dismutase-sensitive reduction of cytochrome C was assayed at 551 nm in a Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) (37). Superoxide dismutase (10 μ g/ml) was added to control samples containing crystals. Control samples containing buffer in place of crystals were also included.

PMN cytotoxicity (LDH release) assay. Isolated PMNs in HBSS-0.02 M Hepes-0.25% BSA (6×10^6 cells/ml) and urate crystals (2.5 mg/ml) in a total volume of 0.5 ml were incubated in 5 ml polypropylene tubes at 37°C for 1 h with continuous shaking. Incubations were terminated by addition of 2 ml of iced HBSS and centrifugation at 300 g for 10 min at 4°C. Supernatants were decanted for LDH assay. Certain tubes were terminated at time zero as zero time blanks. Cell suspensions lysed by addition of Triton X-100 in HBSS (final concentration, 0.1% vol/vol) and sonication were used to determine total enzyme activity. LDH was assayed by oxidation of NADH as previously described (18). Results were expressed as percentage of total enzyme activity by the formula (Enzyme release – zero time blanks/total enzyme) $\times$ 100.

Other plasma and protein preparations. Plasma from an asymptomatic patient with acquired agammaglobulinemia was the generous gift of Dr. John Curd, Scripps Clinic and Research Foundation. The IgG concentration of this plasma was <38 μ g/ml by the Mancini method (38). IgM and IgA were undetectable. An aliquot of this plasma was reconstituted with addition of 15 mg/ml IgG (human Cohn fraction II, Calbiochem-Behring Corp., San Diego, CA), which was deaggregated by centrifugation at 28 psi $\times$ 30 min in a Beckman airfuge (39). The chloramine T method was used to label IgG (40).

Fibronectin-depleted plasma and fibronectin were prepared and analyzed by gelatin-Sepharose chromatography and radioimmunoassay, respectively (41). Sham fibronectin-depleted plasma was prepared by chromatography on underivatized Sepharose.

C1q-depleted serum was prepared by Biorex 70 chromatography, analyzed, and reconstituted with C1q as previously described (42).

Determination of crystal-associated cholesterol. To ascertain if native LDL was bound to washed urate crystals, we incubated 12.5 mg/ml urate crystals in PBS buffer containing 0.8 mg/ml LDL (final concentration). Crystals were washed as described above and material was eluted from a 5 mg crystal pellet by incubation with 0.45 ml of 1 M NaCl (14) for 30 min. Urate crystals were removed by sedimentation and total cholesterol was measured in the eluate using the enzymatic spectrofluorometric assay of Heider and Boyett (43) and of Gamble et al. (44). Samples were read in a Perkin-Elmer 650-10S fluorescence spectrophotometer (excitation, 325 nm; emission, 415 nm) (Perkin-Elmer Corp., Norwalk, CT).

Statistical methods. Results were analyzed statistically using the Student's *t* test (two-tailed).

Results

Identification of apolipoproteins bound to urate crystals. We have previously noted several unidentified polypeptides selectively bound to plasma-exposed urate crystals (17). To determine if any of these polypeptides were apolipoproteins, we compared the urate bound polypeptides of plasma and LPDP by two-dimensional O'Farrell gel electrophoresis (17, 45). Three apolipoproteins (apo A-I, apo B, and apo E) were identified among the eluted polypeptides which were consistently absent or reduced when LPDP rather than undepleted plasma was used to coat the urate crystals (Fig. 1, arrows numbered 1–4). The apolipoproteins showed similar mobilities to polypeptides of isolated lipoproteins on O'Farrell gels. In addition, when isolated 125 I-lipoproteins were added to plasma before coating the urate crystals, the 125 I coelectrophoresed with these crystal-bound polypeptides (not shown). Details of lipoprotein class origin and apparent M_r and isoelectric point (pI) for the identified apolipoproteins (apo A-I, apo B, and apo E) appear in Table I. Details of immunochemical identification were as follows. A polypeptide (Fig. 1, arrow number 3) with apparent M_r = 36,000, pI = 4.5–4.8, which was present in isolated VLDL and IDL (gels not shown), contained an epitope reactive with an apo E-specific monoclonal antibody (Fig. 2) and was thus apo E. Similarly, a polypeptide (Fig. 1, arrow number 4) with apparent M_r = 26,000, pI = 4.6–4.8 was identified as apo A-I because it was present in isolated HDL (gels not shown) and possessed an epitope reactive with an apo A-I-specific monoclonal antibody (Fig. 2).

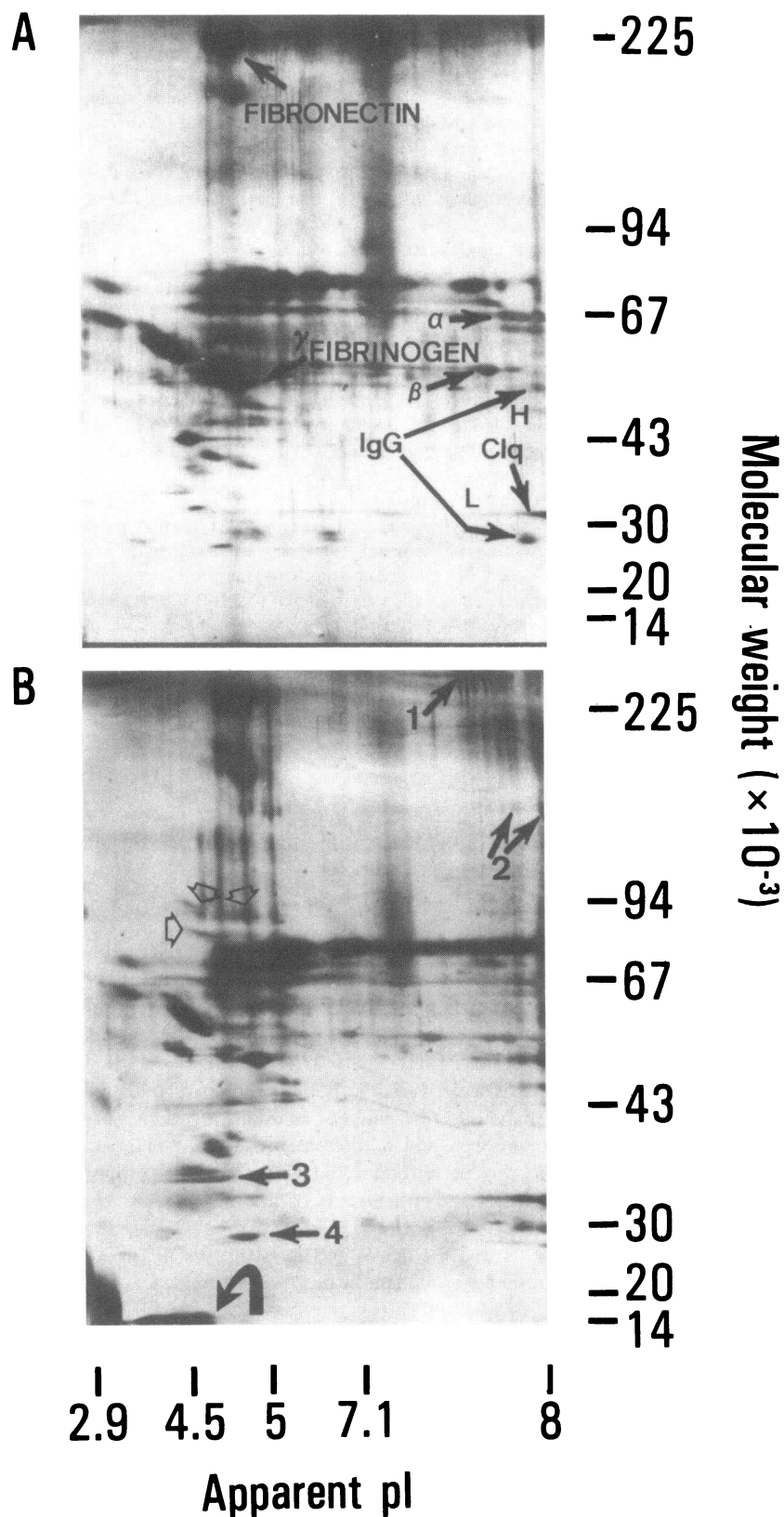


Figure 1. Identification of urate crystal-bound apolipoproteins by two-dimensional O'Farrell gel electrophoresis. After incubation of urate crystals with LPDP (gel A), or reconstituted LPDP (gel B), proteins eluted from the washed urate pellet were run in an isoelectric focusing gel under reducing conditions in the first dimension (right to left) and in an 8–20% exponential gradient SDS-PAGE slab gel in the second dimension (top to bottom). This figure shows the silver-staining patterns of a 75- μ g protein load. Arrows on gel A indicate certain polypeptides represented in abundance on both gels (fibronectin, fibrinogen α , β , and γ chains, IgG heavy (H) and light (L) chains, and Clq). Arrows 1–4 on gel B indicate urate-bound apolipoproteins diminished by plasma lipoprotein depletion (gel A) and identified immunochemically (Table I, Figs. 2 and 3). Open-headed arrows indicate urate-bound polypeptides which comigrated with LDL-derived material but were not identified by Western blot analysis. The curved arrow indicates a group of polypeptides which comigrated with protein from VLDL, IDL, and HDL.

Table 1. Immunochemically Identified Urate Crystal-bound Apolipoproteins on O'Farrell Gels*

Arrow on Fig. 1	Apparent M_r (10^3)	Apparent pI	Diminished by lipoprotein depletion	Co-electrophoresis with apoproteins from:	Identification by Western Blot
1	>250	7.2-8	+	VLDL, IDL, LDL	apo B
2	148	7.2-8	+	LDL	apo B
3	36	4.5-4.8	+	VLDL, IDL	apo E
4	26	4.6-4.8	+	HDL	apo A-I

* Gels shown in Fig. 1.

Urate crystal-bound apo B was identified as follows. Polypeptide number 2 (Fig. 1, apparent M_r = 148,000, pI 7.2-8.0) was present in isolated LDL and was bound by an apo B-specific monoclonal antibody (Fig. 2). In addition, its migration was similar to that described by Kane et al. (46) for the apo B-26 species of LDL and it was thus identified as apo B. Polypeptide number 1 (Fig. 1, apparent M_r > 250,000, pI = 7.2-8.0) was a major crystal-bound species and was observed in gels of isolated VLDL, IDL, and LDL. Its low mobility in SDS gels suggested that it represented apo B-100 (46). However, this polypeptide was only poorly transferred electrophoretically from O'Farrell gels to nitrocellulose paper, and under these conditions, was not detected by overlay with monoclonal anti-apo B antibody. In another approach, isolated LDL and proteins eluted from urate crystals exposed to plasma were applied to 3.3-6% exponential gradient SDS-PAGE gels without prior isoelectric focusing. The gels were electrophoretically transferred to nitrocellulose, as described above, followed by overlay with two different murine monoclonal antibodies and a rabbit polyclonal antiserum specific for human LDL (Fig. 3). Antibody binding was then detected with horseradish peroxidase-conjugated specific IgG. Polypeptides with an apparent M_r of 500,000 and 142,000, respectively, were identified immunochemically as apo B by all three antibodies in urate crystal eluates (Fig. 3; lanes 1 a, 2 a, 3 a). The 500,000 polypeptide was identified also in native LDL (Fig. 3; lanes 1 b, 2 b, 3 b) and presumably represents apo B-100 (46). Another urate-bound apo B polypeptide with apparent M_r = 300,000 was identified by murine monoclonal antibody V82B1 (lane 2 a) and seen as a closely spaced doublet with rabbit polyclonal anti-apo B serum (lane 1 a). Inspection of the polypeptides eluted from urate crystals (Figs. 1 A, B) also revealed that a number of polypeptides, which comigrated with minor species in isolated LDL and possessed apparent M_r from 90,000 to 94,000, were present only in reconstituted LPDP (Fig. 1 B, open-headed arrows). These peptides probably represented apo B fragments (47, 48). In addition, a group of low molecular weight anionic polypeptides (Fig. 1 B, curved arrow), which comigrated with protein in VLDL, IDL, and HDL, was eluted from plasma-exposed urate crystals. These polypeptides were

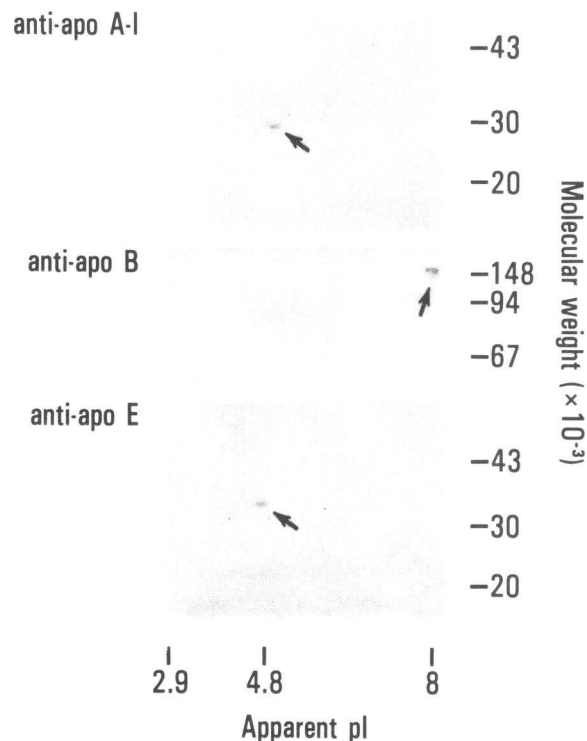


Figure 2. Immunochemical identification of urate crystal-associated apolipoproteins by Western blot. 2-D gels containing 200 μ g of urate crystal-associated plasma proteins were electrophoretically transferred to nitrocellulose membranes and incubated with murine anti-apo A-I, anti-apo B (V82A6), and anti-apo E monoclonal antibodies. Antibody binding was detected by a second incubation with 125 I-labeled anti-mouse Ig followed by autoradiography. The figure shows sections of autoradiographs from three different transfers with arrows pointing to the identified apolipoproteins.

presumed to represent urate-bound apoprotein A-II or one of the apoprotein C subclasses (60), but further identification was not attempted.

Effect of lipoprotein depletion of plasma on the neutrophil-urate crystal interaction. To investigate the effects of crystal-bound lipoproteins on urate crystal-induced neutrophil stimulation, we utilized luminol-dependent CL. CL reflects changes in neutrophil oxidative metabolism (49) and offers the advantages of sensitivity, ease of quantitation, and kinetic measurements on a single sample. Urate crystals induced dose-dependent CL at crystal concentrations from 0.1 to 10 mg/ml (data not shown); a dose of 2 mg/ml urate crystals, which produced a slightly submaximal response, was chosen for CL experiments.

As previously described for urate crystal-induced neutrophil cytolysis (18) and superoxide generation (7), we found that plasma and serum precoating of the crystals suppressed (>50%) the CL response relative to that obtained with uncoated crystals (Fig. 4 A). In contrast, precoating of urate crystals with LPDP led to a CL response quantitatively similar to the response to

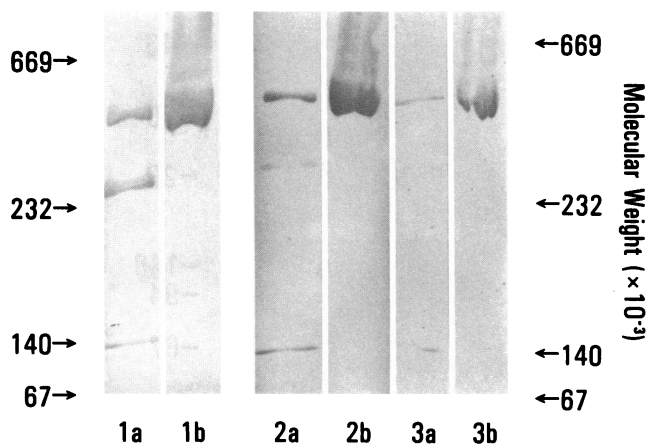


Figure 3. Further identification of urate crystal-bound apo B by Western blot analysis. Single-dimensional 3.3–6% exponential gradient SDS-PAGE slab gels were run under reducing conditions, and then, electrophoretically transferred to nitrocellulose membranes and incubated with anti-apo B antibodies as indicated below. Antibody binding was detected by a second incubation with anti-mouse (or anti-rabbit) IgG conjugated to horseradish peroxidase. The figure is a composite of nitrocellulose transfers from gels containing isolated LDL and plasma proteins eluted from urate crystals. Lanes 1 *b*, 2 *b*, and 3 *b* contained 35 μ g isolated LDL; lanes 1 *a*, 2 *a*, and 3 *a* eluate from 5 mg plasma-exposed urate crystals; lanes 1 *a* and *b* were overlaid with monospecific rabbit polyclonal anti-LDL serum and were from a separate gel; lanes 2 *a* and *b* were overlaid with murine anti-apo B monoclonal antibody V82B1; and lanes 3 *a* and *b* were overlaid with murine anti-apo B monoclonal antibody V82A6.

uncoated crystals (Fig. 4 *B*), despite the fact that numerous plasma proteins were bound to the crystals (Fig. 1 *A*). Reconstitution of LPDP with the $d < 1.25$ g/cm³ fraction of plasma reconstituted the plasma inhibition of CL (Fig. 4). When plasmas were coincubated with cells and crystals, inhibition of CL, which was reversible by lipoprotein depletion, also was observed (data not shown).

To determine if lipoprotein-associated inhibition of urate crystal-induced CL correlated with inhibition of other cellular responses, we assayed superoxide generation and cytolysis. Pre-coating urate crystals with plasma (reconstituted LPDP) inhibited crystal-induced superoxide generation as expected (Table II, 50% inhibition), and this inhibition was significantly reduced by lipoprotein depletion (18% inhibition). Neutrophil lysis was assessed by urate crystal-induced lactic dehydrogenase (LDH) loss (Table III). Lipoprotein depletion from plasma resulted in marked reduction of the suppressive effect of plasma (reconstituted LPDP) on cytolysis.

Identification of apo B-containing lipoproteins as the major inhibitory species. To determine which lipoproteins participated in plasma inhibition of urate crystal-induced neutrophil stimulation, we reconstituted LPDP with physiologic concentrations

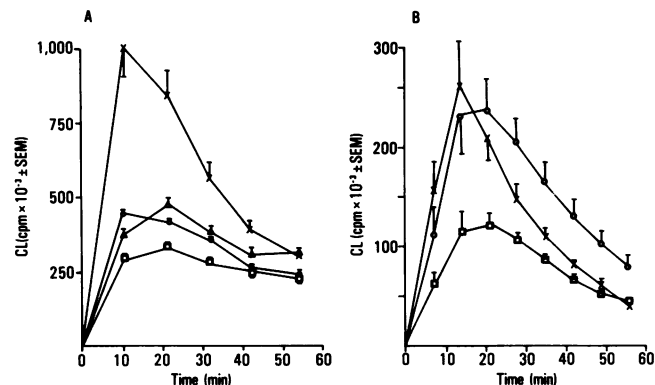


Figure 4. Effect of preincubation of urate crystals with plasma and serum on the crystal-induced CL response. (*A*) Unmodified serum and plasma. 2.5×10^5 PMNs were incubated in HBSS buffer containing 0.02 M Hepes, 0.25% BSA, and 2 μ M luminol at 22°C, and background CL was determined in a Beckman LS-8000 Scintillation counter (Beckman Instruments, Inc., Fullerton, CA) in the out of coincidence mode. At zero time, additions were made in triplicate of 1 mg (2 mg/ml) urate crystals which had been coated with buffer (— $\times$ —), 5 mM EDTA plasma (— $\circ$ —), 0.38% Na citrate plasma (— Δ —), and serum (— $\square$ —) from the same donor. CL was monitored for 60 min at 22°C. CL values are expressed here and throughout the paper as counts per minute corrected for background observed in the absence of stimuli. All CL experiments were performed two or more times and representative experiments are shown. (*B*) Effect of lipoprotein depletion of plasma on inhibition of urate crystal-induced PMN CL. LPDP and reconstituted LPDP were adjusted to equal protein concentrations. Urate crystals at 25 mg/ml (0.3 ml) were incubated with 0.6 ml of these plasmas or buffer and washed. Crystals coated with buffer (— $\times$ —), LPDP (— $\circ$ —), and reconstituted LPDP (— $\square$ —) were added to PMNs and CL was assayed as described.

of isolated VLDL, IDL, LDL, and HDL (50), and used these plasmas to coat urate crystals. The washed crystals were then assayed for their ability to induce CL relative to uncoated crystals. As shown in Table IV *A*, LDL reconstitution had the largest inhibitory effect (78% inhibition of peak crystal-induced CL), VLDL and IDL were active (52% and 46% inhibition, respectively), and HDL was inactive. This suggested that the apo B-containing lipoproteins (VLDL, IDL, and LDL) were the active species. To confirm this, plasma was depleted of apo B-bearing lipoproteins by immunoaffinity chromatography (see Methods). Apo B was undetectable in the antibody adsorbed plasma (<50 ng/mg plasma protein). Apo-B depletion completely abrogated the CL inhibitory effect of plasma (Table IV *B*), and this demonstrated that apo B-containing lipoproteins are a major inhibitor of urate crystal-induced CL.

Binding of individual lipoproteins to urate crystals and their effect on CL. To determine if the binding of plasma lipoproteins to urate crystals and the effects of bound lipoproteins on PMN stimulation were direct or dependent on the presence of other

Table II. Effect of Bound Lipoproteins on Urate Crystal-induced Superoxide (O_2^-) Generation

Stimulus	Cytochrome C reduction (nmol/ 10^6 cells/30 min $\pm$ SEM)	Inhibition of crystal-induced O_2^- generation
		%
No addition (control)	0.63 $\pm$ 0.03	—
Uncoated urate crystals	3.24 $\pm$ 0.15	—
Urate crystals coated with LPDP	2.65 $\pm$ 0.02	18
Urate crystals coated with reconstituted LPDP	1.63 $\pm$ 0.15*	50

PMNs (5×10^6 /ml) in 1 ml HBSS-0.02 M Hepes-0.25% BSA were simulated by the addition of 2 mg/ml uncoated or plasma-coated urate crystals for 30 min at 37°C in the presence of 50 μ M ferricytochrome C. Cell-free supernatants of the reaction mixtures were assayed spectrophotometrically for superoxide dismutase-sensitive reduction of cytochrome C. These are pooled results from two experiments, each performed in triplicate.

* $P < 0.01$ vs. urate crystals coated with LPDP.

bound plasma proteins, we utilized plasma-free systems. We first assessed the binding of isolated, radioiodinated VLDL, IDL, LDL, and HDL to urate crystals (Fig. 5 A). Periphasiologic lipoprotein concentrations were used to reproduce plasma conditions as in the previous experiments. Lipoprotein binding to urate crystals was detected with each of the radioiodinated classes. At physiologic concentrations, the largest quantity of bound lipoprotein was obtained with LDL. The effects of bound purified lipoproteins on urate crystal-induced CL were simultaneously

Table III. Effect of Lipoprotein Depletion on Plasma Inhibition of Urate Crystal-induced PMN Cytolysis

Stimulus: urate crystals coated with:	LDH release	Inhibition relative to uncoated crystals
	% of total $\pm$ SEM	%
PBS (uncoated crystals)	77.2 $\pm$ 0.7	—
LPDP	64.3 $\pm$ 3.7	17
Reconstituted LPDP	29.9 $\pm$ 1.5*	62

Urate crystals (2.5 mg/ml) precoated with LPDP or reconstituted LPDP were incubated in quadruplicate with 6×10^6 PMNs in a total volume of 0.5 ml HBSS-Hepes-BSA at 37°C for 60 min. Percent total LDH released per cell suspension was measured. Results are from an experiment representative of five separate experiments.

* $P < 0.001$ vs. LPDP coated crystals.

Table IV. Effect of Reconstitution of LPDP with Isolated Lipoproteins (A) and Effect of Removal of Apo B-containing Lipoproteins (B) on the Plasma Inhibition of Urate Crystal-induced CL

Stimulus: urate crystals coated with:	Maximum CL (cpm $\pm$ SEM)	Inhibition of CL (%)
		%
A. Buffer (control)	254,040 $\pm$ 56,585	—
LPDP	248,958 $\pm$ 30,032	2
LPDP + VLDL	122,220 $\pm$ 11,162	52
LPDP + IDL	136,223 $\pm$ 2,635	46
LPDP + LDL	56,098 $\pm$ 3,819	78
LPDP + HDL	264,267 $\pm$ 26,246	0
B. Buffer (Control)	523,702 $\pm$ 31,883	—
Apo B-depleted plasma	510,309 $\pm$ 102,669	3
Plasma	275,838 $\pm$ 22,144	47

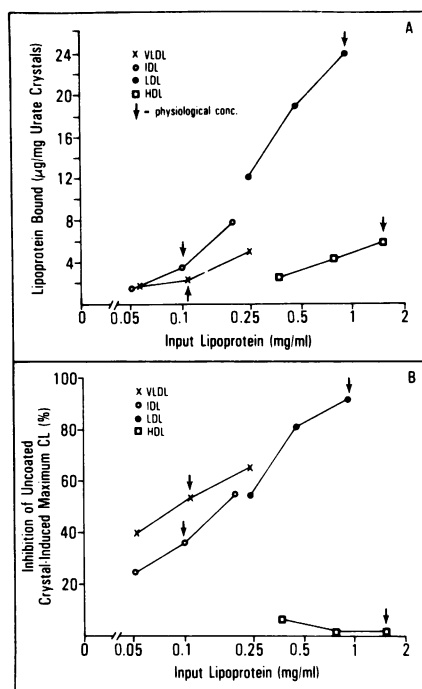
(A.) Lipoproteins were added to LPDP to restore physiologic concentrations (VLDL, 0.12 mg/ml; IDL, 0.1 mg/ml; LDL, 0.9 mg/ml; HDL, 1.5 mg/ml) and the plasmas were incubated with urate crystals. Precoated washed crystals (2 mg/ml) were added in triplicate to PMNs for assay of CL as previously described. Maximum CL refers to the highest CL observed within 30 min of the addition of the crystal stimuli. (B.) In a separate experiment, plasma was depleted of apo B by immunoaffinity chromatography employing a murine monoclonal anti-apo B antibody (V82A6). Apo B-depleted plasma and starting plasma (0.8 ml) diluted to identical protein concentrations were incubated with 0.2 ml of a 25 mg/ml urate crystal suspension. Washed precoated crystals (2 mg/ml) were subsequently added to PMNs in quadruplicate and CL was monitored.

measured (Fig. 5 B). VLDL, IDL, and LDL were inhibitory, and LDL had the greatest quantitative effect at physiologic input concentrations. In contrast, crystal-bound HDL did not inhibit urate crystal-induced CL at the concentrations tested.

To demonstrate association of intact LDL particles with urate crystals, the washed urate crystal pellet was assayed for total cholesterol after incubation of 12.5 mg/ml crystals with a periphasiologic concentration of LDL (0.8 mg/ml). Urate crystal-bound cholesterol (5.3 μ g/mg urate crystal) was detected and confirmed the binding of native LDL.

Stimulus specificity of lipoprotein inhibition of CL. To determine if the inhibitory effect of apo B-containing lipoproteins on CL was stimulus-specific, we added LDL, without washing, to urate crystals, and to two other solid-phase stimuli (*S. aureus* and opsonized zymosan) and one fluid-phase stimulus (chemotactic hexapeptide *N*-formyl-*N*-leu-leu-phe-*N*-leu-tyr-lys). 10 μ g/ml LDL (final sample concentration) inhibited the peak response to urate crystals by >80% but did not inhibit the responses to the other stimuli (Table V).

Effects of other plasma proteins on urate crystal-induced CL. The quantities of protein bound to urate crystals exposed to



compared with uncoated crystal-induced CL(B). Data is plotted semi-logarithmically. Arrows indicate normal fasting plasma concentrations of the respective lipoproteins in fasting individuals. conc, concentration.

LPDP and reconstituted LPDP were measured by the Lowry assay (51) (see Methods) and were not significantly different in nine different determinations (LPDP = 22 ± 4.7 (SEM) μg bound protein/mg crystals, reconstituted LPDP = 28.9 ± 4.5 μg /mg crystals). Values for serum and for citrated and EDTA plasma were similar, lying between 22–26 μg /mg crystals. In addition, other than the lack of lipoprotein polypeptides, the gel patterns of eluates from LPDP-coated crystals were qualitatively similar to those for plasma-coated crystals (Fig. 1). Because bound IgG has been suggested to be important in cellular responses to plasma exposed urate crystals (7, 18, 52), we investigated possible

Figure 5. Quantitation of binding of individual lipoproteins to urate crystals and their ability to inhibit CL in the absence of plasma. Urate crystals at 25 mg/ml (0.3 ml) were incubated with 0.3 ml of ^{125}I -labeled/unlabeled lipoproteins to achieve the lipoprotein concentrations indicated (expressed in terms of protein). Bound ligand was assayed by determination of radioactivity in the washed crystal pellet (A). In parallel, the ability of these coated crystals (2 mg/ml) to induce CL was assayed and

effects of lipoprotein depletion on IgG binding to crystals. Tracer amounts of ^{125}I -labeled IgG were added to urate crystals in the presence of equal concentrations of LPDP and reconstituted LPDP. IgG binding was indistinguishable in the two plasmas (4.7 and 4.6% of the starting IgG counts were bound, respectively). Thus, lipoprotein depletion did not appear to appreciably alter the overall pattern of crystal-bound proteins other than the apolipoproteins.

We also assessed the effects on CL of the removal of other crystal-binding proteins from plasma or serum. Fibrinogen, fibronectin, IgG, and C1q were studied because they have been visualized on O'Farrell gels of plasma proteins eluted from urate crystals (Fig. 1) (17). Fibrinogen did not appear to be important in inhibition of urate-induced CL as serum and plasma had similar effects (Fig. 4 A). Plasma, depleted of fibrinogen, and serum, depleted of C1q, showed no significant changes in the inhibition of CL induced by the precoated crystals (Figs. 6 A and C). In contrast, an IgG-deficient plasma was slightly more inhibitory than the same plasma reconstituted with deaggregated IgG (Fig. 6 B). This was compatible with the previous suggestion of a role for IgG in urate crystal stimulation of neutrophils (7, 18).

Discussion

When the etiologic agent of human gout, the monosodium urate crystal (1, 2) is incubated with plasma, a number of proteins are selectively bound (17). In the present work, apo A-I, apo B, and apo E were identified by electrophoresis and Western blotting techniques. Binding of intact lipoproteins to the urate crystals was substantiated by demonstration of the association of both apo B and cholesterol with the crystals after incubation with LDL. Strikingly, the bound apo B-containing lipoproteins appeared to account for virtually all of the inhibitory activity of plasma on urate-induced PMN CL and a major portion of the plasma inhibition of superoxide generation and cytolysis. This statement is based on the observation that removal of these lipoproteins by density gradient centrifugation or immunodepletion abrogated or markedly reduced the inhibitory effects of plasma on these reactions. Because the major apo B lipoprotein,

Table V. Stimulus Specificity of LDL Inhibition of CL

Inhibitor	Maximum CL response (cpm $\pm$ SEM) to:			
	Urate crystals (2 mg/ml)	Opsonized zymosan (10^6 particles/ml)	<i>S. aureus</i> (100 μg /ml)	FNLPNTL (10 nM)
Buffer (PBS)	2,232,910 $\pm$ 154,515	3,858,677 $\pm$ 722,573	3,558,220 $\pm$ 230,459	776,994 $\pm$ 46,402
LDL (10 μg /ml)	374,740 $\pm$ 2,970	3,820,535 $\pm$ 656,927	3,333,412 $\pm$ 624,046	1,009,914 $\pm$ 23,926

LDL or buffer was preincubated with the stimuli in a volume of 0.1 ml for 30 min at 22°C. Stimuli were then added, without being washed, to triplicate 0.4 ml suspensions of 2.5×10^5 PMNs in HBSS-0.02 M Hepes-0.25% BSA. CL was monitored for 60 min. Indicated stimulus and LDL concentrations are those achieved in the final CL reaction mixture.

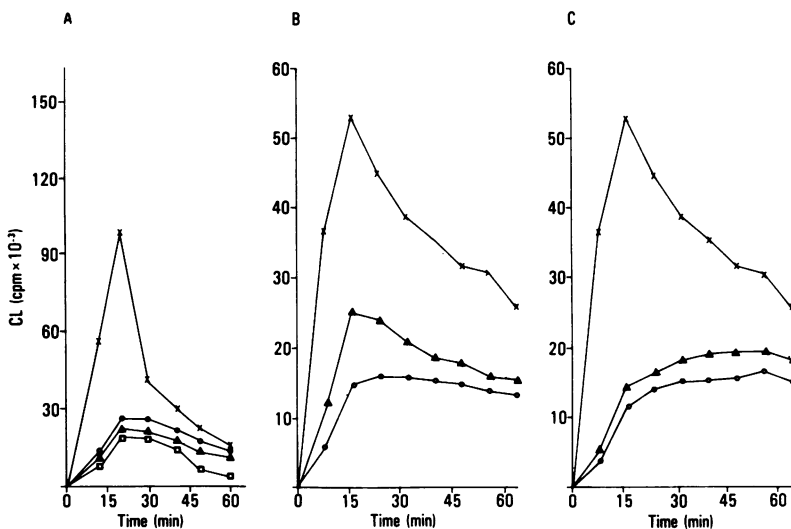


Figure 6. Effects of depletion of other crystal-binding plasma proteins on plasma inhibition of urate crystal-induced CL. (A) Effect of fibronectin: CL responses of PMNs after the addition of urate crystals (2 mg/ml) coated with buffer ($\times$ —), plasma depleted of fibronectin by gelatin-Sepharose affinity chromatography ($\circ$ —), "sham" fibronectin-depleted (chromatographed on underivatized Sepharose) plasma ($\square$ —), and fibronectin-depleted plasma reconstituted with fibronectin to 300 μ g/ml ($\triangle$ —). (B) Effect of IgG: CL responses to urate crystals coated with buffer ($\times$ —), agammaglobulinemic plasma ($\circ$ —), and agammaglobulinemic plasma reconstituted with deaggregated IgG to 15 mg/ml ($\triangle$ —). (C) Effect of C1q: CL responses to urate crystals coated with buffer ($\times$ —), C1q-depleted serum ($\circ$ —), and C1q-depleted serum reconstituted with C1q to 70 μ g/ml ($\triangle$ —).

LDL, has an apparent Stoke's radius ~ 96 Å (53), similar to that calculated from the Stokes-Einstein equation (54) for α -2 macroglobulin (100 Å), the presence of LDL in synovial fluid, like that of α -2 macroglobulin (20), is likely to depend upon the permeability of the synovial membrane. Such predicted wide variations in synovial fluid LDL levels have been observed (19, 55–58) and could greatly affect the inflammatory response to urate crystals or neutrophil peroxidase-dependent catabolism of the crystals (59) in the course of gouty arthritis.

Urate crystal-bound apolipoproteins were visualized on silver-stained two-dimensional O'Farrell gels (Fig. 1, Table I). Identification of each apolipoprotein was suggested by diminution of the spot in question in the O'Farrell gel of the urate pellet of LPDP, and by coelectrophoresis on O'Farrell gels with polypeptides derived from isolated lipoproteins. Western blot analysis provided direct identification of urate crystal-bound apo A-I, apo B, and apo E (Figs. 2 and 3; Table I). A number of smaller LDL-derived polypeptides visualized on silver-stained urate crystal pellet two-dimensional gels (Fig. 1, open arrows), apparent $M_r = 90,000$ – $94,000$, were not detected with monoclonal anti-apo B antibodies in the Western blot system (Figs. 2 and 3). These were probably apo B-related as essentially all the protein of LDL exists as apo B (60). A possible explanation for the failure to detect these as apo B species would be the known differential immunochemical reactivity of various apo B species (27, 47); alternatively, proteolytic cleavage of apo B during preparation (47, 48) and incubation with urate crystals, or the denaturation carried out before electrophoresis may have rendered certain apo B peptides devoid of a suitable epitope for the apo B-specific antibodies.

We investigated the effects of plasma on urate crystal-neutrophil interaction by measurement of luminol-dependent CL, a commonly employed assay system for stimulation of phagocyte oxidative metabolism by particulates (36). Luminol-dependent CL depends on the generation of superoxide and hydrogen per-

oxide and the action of myeloperoxidase (49, 61). Previous studies have shown that preincubation of urate crystals with plasma or serum reduces the ability of crystals to induce neutrophil superoxide generation (7) and cytolysis (18). As expected, crystal preincubation with plasma and serum also inhibited CL (Fig. 4 A). Depletion of lipoproteins from plasma by density gradient ultracentrifugation completely abrogated the inhibition of urate crystal-induced CL produced by precoating the crystals with plasma (Fig. 4 B). Similar effects were observed on coinubation of plasmas with crystals and cells, indicating that the lipoprotein effect is not limited to coated crystals in plasma-free systems. Lipoproteins also showed similar modulatory effects on neutrophil cytolysis (LDH loss, Table III) and superoxide generation (Table II). Thus, lipoproteins modulate a number of neutrophil responses to urate crystals. The inhibitory activity of lipoproteins on urate-induced CL appears to reside exclusively with the apo B lipoproteins. First, in reconstitution experiments, apo B lipoproteins (VLDL, IDL, and LDL), but not HDL, contributed inhibitory activity to LPDP (Table IV A). Second, immunodepletion of apo B lipoproteins by agarose-bound monoclonal antibody also abrogated the inhibitory activity of plasma (Table IV B). Since most of the circulating apo B under normal circumstances is in LDL (60), LDL probably provides the bulk of this activity. This suggestion was borne out by the observation that physiologic reconstitution of LPDP with LDL produced a more potent inhibitory reagent than reconstitution with other lipoproteins (Table IV A).

Because many plasma proteins are bound to these crystals, it is possible that the lipoprotein effect on urate-induced CL is mediated through another plasma protein or is a nonspecific protein effect. However, this seems unlikely for several reasons. First, with the exception of the apolipoproteins, the two-dimensional gel electrophoretic patterns of the crystal eluates were similar for LPDP and reconstituted LPDP (Fig. 1). The amount of urate crystal-bound protein also was comparable for LPDP,

reconstituted LPDP, and unmodified serum and plasma. Second, lipoprotein depletion did not alter the binding of IgG to the crystals. Third, apo B-containing lipoproteins bound directly to the crystals (Fig. 5 A) and inhibited CL (Fig. 5 B) in the absence of other added plasma proteins. Fourth, nonspecific inhibition of urate-induced neutrophil cytolysis by plasma proteins is observed in otherwise protein-free crystal-cell suspensions (18). Thus, all CL, superoxide, and cytolysis assays were performed in the presence of 2–2.5 mg/ml of the “nonspecific protein” BSA, so that additional inhibitory effects, such as those of lipoproteins, could be assessed. Fifth, HDL, bound to crystals in amounts similar to VLDL and IDL, failed to inhibit urate-induced CL (Figs. 5 A and B). Finally, plasmas or sera deficient in, or depleted of, three other major urate-binding plasma proteins still were potent inhibitors of urate-induced CL (Fig. 6).

The ability of apo B-containing lipoproteins to inhibit urate-induced CL was not due to interference with the CL assay or to direct toxic effects on the cells, since response to fluid-phase chemotactic hexapeptide was not reduced by doses of LDL which produced 80% inhibition of urate-induced CL (Table V). In addition, the possibility that inhibition was due to nonspecific effects on PMN-particle interaction or phagocytosis was less likely since responses to two other particulates were unaffected by LDL.

Elucidation of this novel functional property of apo B-containing lipoproteins will require detailed studies of their effects on other events which follow PMN-crystal interaction, effects with other crystals and cell types, and the lipoprotein structural requirements for this activity. It has been suggested that LDL is a major plasma regulator of HeLa cell adhesion to glass (62), which is a negatively charged surface like monosodium urate crystals. In addition, there is evidence that human serum enhances the stimulation by urate crystals of nonmitogenic synovial fibroblast responses in vitro (63, 64). Fibroblasts are known to possess a high affinity receptor for apo B and apo E (65) which might modulate these responses. Thus, the effects observed here may be limited neither to PMNs nor to urate crystals. Furthermore, several transport-unrelated activities of lipoproteins which may modify inflammation-related responses have been recently described (66–71). To these we may add modulation of PMN responses to monosodium urate crystals by apo B-containing lipoproteins. Because synovial fluid levels of LDL may vary from trace amounts in normal states to 40–60% of plasma levels in inflammatory states (19, 55–58), LDL may well represent an important factor in modulation of gouty inflammation.

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