High-Field ^{13}C NMR Studies of Certain Normal and Abnormal Human Plasma Lipoproteins

Abstract. *High-field (63.4 kilogauss) Fourier transform nuclear magnetic resonance spectroscopy of ^{13}C in natural abundance has been used to study the structural organization and molecular dynamics of constituent lipids of normal human very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). The same method was used to study the abnormal β-VLDL of two type III hyperlipoproteinemia patients having markedly differing ratios of VLDL cholesterol to triglyceride (0.3 and 0.6, respectively). Resolution obtained at 63.4 kilogauss has made possible the assignment of several additional resonances of cholesterol ring carbon atoms, not resolved in earlier studies at lower fields, in the VLDL spectra. The rotational reorientation of the ring portion of cholesteryl esters in VLDL (normal) and β-VLDL (abnormal) is not highly anisotropic and is similar to that for cholesteryl esters dissolved in excess triolein. The rotations of cholesteryl esters in LDL are more highly anisotropic and significantly more restricted. The results suggest that the structural organization of the lipid components in β-VLDL resembles that found in normal VLDL but differs significantly from that for normal LDL.*

Natural abundance Fourier transform ^{13}C nuclear magnetic resonance (NMR) spectroscopy at low magnetic field (14.2 kilogauss) has been used in our laboratory to study the structural organization of the intact lipoproteins of human plasma. The major features of the carbon NMR spectra of high-density lipoproteins (HDL), low-density lipoproteins (LDL), and very-low-density lipoproteins (VLDL) have been assigned to the lipid moiety of these complexes (*1*). Semiquantitative information regarding segmental motion of several fatty acid carbon atoms was gained by measurement of spin-lattice relaxation times (T_1), and the reorientation of the cholesterol ring was investigated by T_1 and linewidth measurements of the cholesterol C6 resonance (*2*).

Although there are significant theoretical and instrumental difficulties (*3*), high-field ^{13}C NMR relative to low-field ^{13}C NMR (< 30 kilogauss) should have advantages of increased sensitivity and resolution, at least for certain carbon types (*3, 4*). Experimentally for the case of human plasma lipoproteins, we have found a significant improvement in both sensitivity and resolution at high field (63.4 kilogauss) relative to low fields (14.2 and 23.5 kilogauss) where similar small sample volumes (~ 1.5 to 2.0 ml) are employed. We report here studies on beta very-low-density lipoproteins (β-VLDL) from two patients with familial type III hyperlipoproteinemia (*5, 6*), a disease which is characterized by an elevation of both plasma cholesterol and triglyceride and by cholesterol-rich beta-migrating VLDL (β-VLDL), which float in the ultracentrifugal fraction density < 1.006. The ^{13}C NMR spectra of these abnormal lipoproteins are compared with spectra of normal VLDL and LDL and with results obtained for anhydrous lipid systems.

Spectra were obtained with a 67.88-Mhz (63.41 kilogauss) NMR spectrometer that accepts 10-mm sample tubes. The instrument consists of the Bruker model HX 270 superconducting magnet equipped with a Bruker ^{13}C probe and home-built radio-frequency electronics. The data accumulator consists of the Nicolet model 1085 computer equipped with a pulse sequencer. The ^1H irradiation for proton-decoupling was centered at 3.6 parts per million (ppm) downfield from the ^1H resonance of tetramethylsilane (TMS).

VLDL and LDL were isolated from pooled fresh plasma collected from three young, healthy donors after they had fasted overnight. In the normals VLDL and LDL were separated and isolated in KBr of varying concentrations: VLDL, density = 0.95 to 1.019; and LDL, density = 1.019 to 1.063 (*7*). Densities were checked by pycnometry. Electrophoresis on 2 percent agarose gels in sodium barbital buffer, *p*H 8.6, revealed only a single band for each lipoprotein class.

Blood samples were drawn from type III hyperlipoproteinemia patients who had fasted for 12 hours (overnight); these patients had been consuming their usual average diet without recent weight loss. Lipids and lipoproteins were evaluated by lipoprotein electrophoresis, measurements of total HDL cholesterol (C-HDL), LDL cholesterol (C-LDL), and VLDL cholesterol (C-VLDL) and triglycerides (*8*). Diagnosis of type III hyperlipoproteinemia was based on the chemical index reported by Morganroth *et al.* (*5*) and Fredrickson *et al.* (*6*), in which patients with a ratio of C-VLDL to plasma triglyceride of 0.3 or greater were considered to have type III; and 0.25 to 0.29, possible type III, within the plasma triglyceride range of 150 to 1000 mg per 100 ml (*6*).

In patient S.E., floating β-lipoprotein, β-VLDL, was present; the ratio of C-VLDL to plasma triglyceride ranged from 0.29 to 0.34. The second patient (C.H.) showed substantial elevation of cholesterol and triglyceride, a ratio of C-VLDL to triglyceride of 0.52 to 0.61, and β-VLDL. Additional first-degree relatives of both patients had type III hyperlipoproteinemia.

In subjects with type III hyperlipoproteinemia, the VLDL fraction (including β-VLDL) was isolated by centrifugation of the plasma without density adjustment for 16 hours at 100,000*g* (*9*). A single, turbid layer which floated was separated and used without further purification.

Samples were prepared for NMR study by concentration of the lipoprotein in dialysis tubing placed against dry Sephadex G-50. Previous experience indicates that the nature of the ^{13}C NMR spectra of plasma lipoproteins is the same whether samples are concentrated by ultrafiltration or dialysis. The technique used here is the milder of the two. All lipoprotein preparations were concentrated by the same procedure. Moreover, it has been established that the ^{13}C NMR spectra of lipoproteins are concentration independent over the range studied (*2*). The lipoprotein samples were dialyzed for 48 hours against 0.2*M* KBr, 0.01*M* phosphate, $10^{-4}M$ EDTA at *p*H 7.4.

The cholesteryl oleate–triglyceride (COT) sample was prepared by addition of cholesteryl oleate to triolein to make a 1 : 2 molar ratio (0.37 weight ratio) of the ester to triglyceride for comparison with the VLDL ^{13}C NMR spectra. Lipids were obtained from NuChek and used without further purification. The COT sample was maintained at about 60°C for 30 minutes prior to NMR analysis and vortexed at maximum setting on a Lab-Line Super Mixer to ensure complete mixing of the lipids, and appeared as a colorless isotropic solution at 35°C. The NMR spectrometer probe temperature was near 35°C for all samples.

In Fig. 1 are shown the complete ^{13}C NMR high-field spectra for VLDL, the two β-VLDL samples, and LDL. Selected resonances are noted; the chemical shifts for these resonances are ± 0.1 ppm of values previously obtained (*2*). All narrow resonances from cholesterol ring carbons were assigned to cholesteryl esters (*10*). No resonances attributable

to carbon atoms of proteins were observed. The ^{13}C NMR spectra reflect differences both in the types of lipid and quantity of the particular lipid in the lipoprotein complex. The relative content of phospholipid, as indicated by the intensity of the choline $\overset{+}{N}(CH_3)_3$ peak was much higher, for example, in LDL than in VLDL or in the β-VLDL samples. Quantitative differences in the cholesteryl ester/triglyceride ratio between the two β-VLDL samples are reflected in the β-VLDL spectra. The ratio of the peak height of the triglyceride CH peak to that for the cholesteryl ester C3 peak was slightly greater in the VLDL spectrum than in the β-VLDL-1 spectrum (insets), in agreement with chemical analysis showing a C-VLDL/triglyceride weight ratio for β-VLDL (~ 0.3) that was moderately greater than that for normal C-VLDL triglyceride (≤ 0.25) (*5*, *6*). The much higher C-VLDL/triglyceride ratio of β-VLDL-2 (0.6) is reflected in the diminished ratio of the triglyceride CH to cholesteryl ester C3 peak heights. In LDL, where the cholesteryl ester/triglyceride weight ratio is high (~ 3.5), no resonances for the glycerol backbone of triglycerides were identifiable.

The linewidths of most resonances in the relatively cholesterol-rich β-VLDL (β-VLDL-2) are somewhat greater (~ 1.5 times) than linewidths of the corresponding resonances in β-VLDL-1. An even larger increment of broadening was observed in general on going from the β-VLDL-2 spectrum to the LDL spectrum. The small differences in linewidths of corresponding resonances in the VLDL and β-VLDL-1 were within error limits imposed by variations in instrumental broadening and variation of sample temperatures, while the differences observed for β-VLDL-2 relative to VLDL and β-VLDL-1 were considered to be just outside the experimental limits of error.

The aliphatic regions (~ 10 to 80 ppm downfield from TMS) of β-VLDL-1, VLDL, and COT are shown in Fig. 2. Assignments for cholesterol carbons of the cholesteryl ester moieties are indicated in Fig. 2 by numbers corresponding to the standard numbering system for cholesterol. All cholesterol resonances were assigned by comparison with reported chemical shifts for cholesteryl acetate in dioxane and chloroform; the chemical shifts observed in the lipid and lipoprotein spectra are within 0.2 ppm of these reported values (*11*). In the COT spectrum, all cholesterol carbon atoms were detected, except for C7 and C8, which are probably buried in the intense

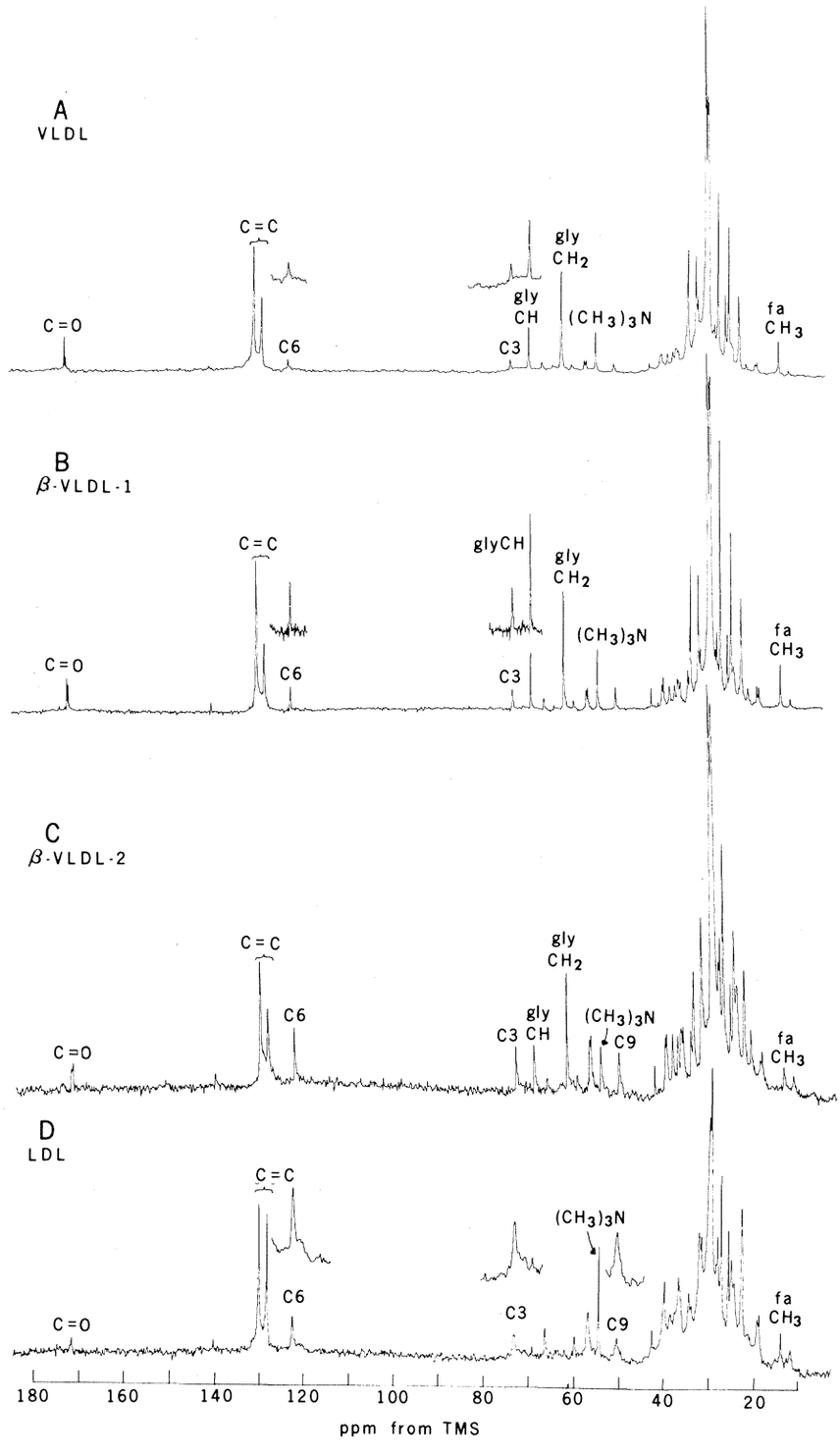

Fig. 1. Proton decoupled natural abundance ^{13}C Fourier transform NMR spectra of human serum lipoproteins at about 35°C, recorded at 67.9 Mhz with a recycle time of 0.180 second. Spectral width is 13,888 hertz (~ 200 ppm); 16K transforms were used to improve digital resolution of 4K (4096) accumulation points. The inserts are printed with a twofold vertical expansion. The signal-to-noise ratio was enhanced by the use of digital broadening of 1.1 hertz on the main spectra and 2.2 hertz on the inserts, except for the LDL insert which has 4.8-hertz broadening. (A) VLDL, 55 mg of lipoprotein per milliliter, after 380,000 scans; (B) β-VLDL-1, 110 mg of lipoprotein per milliliter, after 262,144 scans; (C) β-VLDL-2, 120 mg of lipoprotein per milliliter, after 131,072 scans; and (D) LDL, 50 mg of lipoprotein per milliliter, after 131,072 scans. Peaks are identified by the following symbols: C=O, (lipid) carbonyl; C=C, olefinic of fatty acyl esters; C6, cholesterol (ester) C6: C3, cholesterol (ester) C3; gly CH and gly CH_2, glycerol CH and CH_2 of triglycerides; $(CH_3)_3N$, choline methyl of phospholipids; C9, cholesterol (ester) C9; fa CH_3, fatty acyl CH_3. The chemical shift scale is in parts per million (ppm) downfield from TMS.

resonance at 32.3 ppm, and C26 and C27, which are buried in the intense resonance at 22.8 ppm.

In the spectrum of β-VLDL-1, C15 and C23 of the cholesterol ring system were not clearly resolved, and C12 was not unambiguously detectable. Other than these differences the resemblance of the β-VLDL-1 spectrum and the COT spectrum is remarkable. The linewidths of all resonances were slightly broader in the β-VLDL-1 spectrum, while the small additional increase in linewidths for resonances in the VLDL spectrum led to significantly poorer resolution for several of the cholesterol resonances. However, increased resolution as a result of the higher magnetic field strength makes possible the observation of several additional cholesterol carbons; in previous lipoprotein spectra obtained at low field (14.2 kilogauss), no methylene carbons of cholesterol were clearly detectable, and several methine carbons (C20, C14, and C17) were not clearly resolved (*1*, *2*).

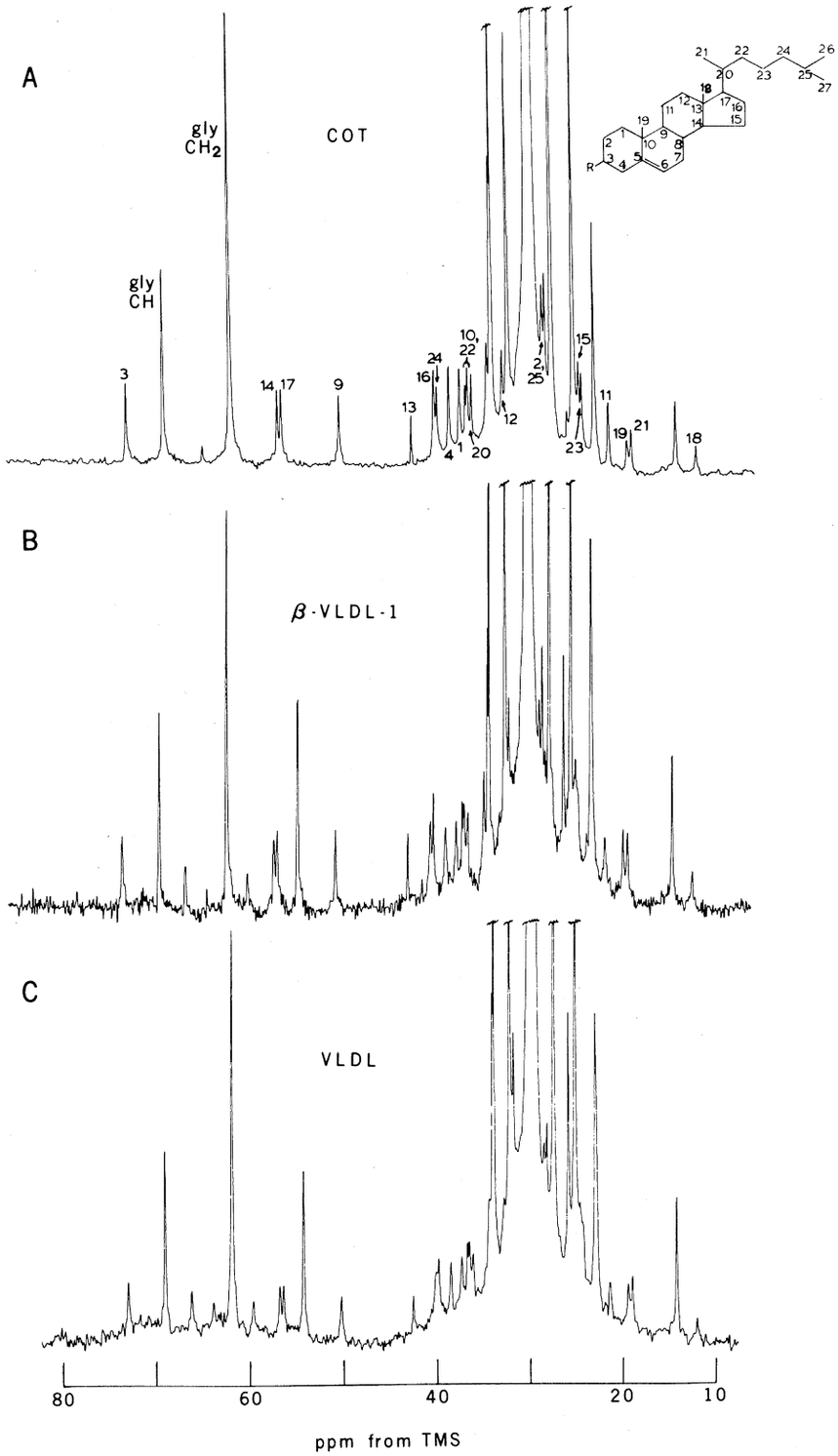

Fig. 2. Aliphatic region (~ 10 to 80 ppm from TMS) of proton decoupled natural abundance ^{13}C Fourier transform NMR spectra at 67.9 Mhz for (A) cholesteryl oleate in triolein (COT), (B) β-VLDL-1 (C-VLDL/triglyceride ratio = 0.3), and (C) normal VLDL. Conditions for VLDL and β-VLDL-1 are the same as those given in Fig. 1, with 1.1-hertz digital broadening. The COT spectrum was recorded after 4096 scans with a recycle time of 0.180 second and 1.1-hertz digital broadening introduced on a 16K transform. Peaks are identified as follows: peak numbers correspond to cholesterol carbon numbers as shown in the inset; gly CH and gly CH_2 identify glycerol CH and CH_2 carbons of triglycerides.

The narrow linewidths of cholesterol resonances of the cholesteryl esters in the Fig. 2 spectra indicate that all carbon atoms of the cholesterol moiety, both in the steroid ring and the aliphatic side chain, have a great deal of rotational or segmental mobility (or both). The narrow linewidths of the fatty acyl carbon resonances similarly reflect rapid segmental motions of the carbon atoms, although corresponding carbon atoms of phospholipids, triglycerides, and cholesteryl esters cannot be distinguished (*2*). In the high-field spectrum of LDL (Fig. 1) all cholesterol resonances were significantly broader than for β-VLDL and VLDL. In contrast, cholesteryl esters dissolved in a small amount of triolein and pure cholesteryl esters in isotropic melts yielded high-field ^{13}C NMR spectra with significantly broader resonances than those observed for the above systems, except LDL.

In the LDL spectrum, the cholesterol C6 and C3 resonances exhibited a broad component in addition to a narrow component, as shown in the inset, which may reflect unesterified cholesterol (*10*). No resonances attributable to unesterified cholesterol were present in the VLDL or β-VLDL spectra.

The similarity of the cholesteryl ester environments in the normal and abnormal VLDL and the cholesteryl ester–triglyceride mixture is also suggested by a comparison of the cholesteryl ester C6 and C3 linewidths. The ^{13}C NMR studies at low field (23.5 kilogauss) with pure cholesteryl esters and cholesteryl esters dissolved in triolein have demonstrated that anisotropic motions of the cholesteryl ring can be monitored by differences in the C6 and C3 (or C9) linewidths, which reflect motions of the ring system in approximately perpendicular directions (*12*). For the COT mixture, the cholesteryl ester C6 and C3 linewidths were equivalent, suggesting isotropic rotation

of the cholesteryl ring. In the VLDL and both β-VLDL spectra the linewidth ratio of C3 (or C9) to C6 linewidth is 1.2 ± 0.1, while in LDL this ratio is ~ 2.0. Thus the motions of the cholesterol ring system in the cholesteryl esters of β-VLDL and VLDL were nearly isotropic, while in LDL these motions were significantly more anisotropic.

Early studies of the chemical composition of β-VLDL suggested that β-VLDL differs from LDL only in its higher triglyceride content: the ratios of cholesterol to protein and phospholipid to protein in β-VLDL are similar to those found for LDL when the relative proportions of lipids in β-VLDL are calculated, excluding triglyceride (*13*). In addition the apoprotein was found to be essentially all apoLDL (apoB) by a variety of criteria (*13, 14*). In subsequent studies on the apoproteins of β-VLDL, increased amounts of apoB, decreased amounts of apoC proteins, and an increased amount of an "arginine-rich apoprotein," relative to VLDL, were found (*15*). Moreover, the "arginine-rich apoprotein" (apoE) has been shown to consist of three components, one of which is missing in β-VLDL but not in VLDL (*16*).

Our ^{13}C NMR results suggest that the structural organization of the observed lipid components (particularly the cholesteryl esters) in β-VLDL resembles that found in normal VLDL, and differs substantially from that found in LDL.

The close resemblance of the ^{13}C NMR spectra of normal VLDL and the β-VLDL-1 indicate that the features of the high-resolution lipid spectra obtained for these lipoproteins, namely, the linewidths and relative intensities, are determined principally by the lipid composition and not by the nature of the apoprotein components. The ^{13}C NMR spectrum of the β-VLDL sample having a higher C-VLDL/triglyceride ratio (β-VLDL-2) shows an increased intensity in cholesteryl ester resonances but otherwise yields a spectrum similar to that for normal VLDL, while the ^{13}C NMR spectrum of normal LDL differs significantly from those for the normal VLDL and β-VLDL samples. Thus, the nature of the ^{13}C NMR spectra of β-VLDL lipids is not appreciably affected by the increased content of apoB in β-VLDL relative to normal VLDL. Furthermore, the rotational reorientation of the cholesterol ring of the cholesteryl esters observed in the ^{13}C NMR spectra of normal VLDL and β-VLDL is probably not highly anisotropic and is similar to that for cholesteryl ester dissolved in excess triolein. These results are compatible with the results of Sata *et al.* (*17*) which suggest that cholesteryl esters in both normal VLDL and β-VLDL occupy a triolein liquid core.

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HLA Antigens and Corticosteroid Response

Abstract. *Compared with normal individuals, patients with primary open-angle glaucoma have increased prevalences of HLA-B12 and B7 antigens and are more responsive to glucocorticoids. Lymphocytes from both ocular normotensive and glaucomatous individuals with the HLA-B12 antigen require significantly ($P < .02$) lower concentrations of prednisolone to inhibit phytohemagglutinin-induced transformation.*

Significantly increased prevalences of HLA-B12 (55 percent) and HLA-B7 (48 percent) antigens are found in both black and white patients with primary open-angle glaucoma (POAG) when compared with similar subjects with normal intraocular pressure (20 percent B12 and 18 percent B7) (*1–3*). Of clinical importance, the presence of HLA-B12 or B7 in glaucoma suspects with increased intraocular pressure increases the risk of development of glaucomatous damage to the optic nerve (*3*). Furthermore, a family history of glaucoma is more prevalent among those glaucoma suspects as well as POAG patients with B7 or B12 antigens, as compared to comparable patients who lack these antigens (*4*). From the epidemiologic point of view, populations such as the Australian aborigines with no HLA-B12 or B7 antigens (*5*) are found to have no POAG (*6*).

POAG patients are more responsive to topical ocular application of corticosteroids than are other normotensive individuals (*7*). POAG patients demonstrate significantly greater reduction of plasma (in vivo) cortisol when given 0.25 mg of dexamethasone than do normal subjects (*8*). Similarly, in vitro, phytohemaggluti-

Table 1. HLA-B 12 and B 7 antigens and prednisolone inhibition of lymphocyte transformation. Probabilities for compared values are indicated by superscript letters: [a], $P < .001$; [b], $P < .02$; and [c], $P < .02$

	Primary open-angle glaucoma		Ocular normotensive	
	Patients (No.)	Pred I_{50}*	Patients (No.)	Pred I_{50}*
Total	25†	37 ± 12[a]	30‡	76 ± 20[a]
With B12	13 (52 percent)	32 ± 10[b]	7 (23 percent)	60 ± 7[c]
Without B12	12	42 ± 9[b]	23	81 ± 20[c]
With B7	14 (56 percent)	37 ± 10	6 (20 percent)	71 ± 16
Without B7	11	38 ± 13	24	77 ± 21

*The concentration prednisolone (nanograms per milliliter) for 50 percent inhibition of phytohemagglutinin-induced lymphocyte transformation (mean ± standard deviation). †18 white, 15 female, 64 ± 8 years old. ‡21 white, 18 female, 55 ± 11 years old.