Contribution of the apo[a] phenotype to plasma Lp[a] concentrations shows considerable ethnic variation

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Abstract Apolipoprotein[a] polymorphism has been investigated by sodium dodecyl sulfate polyacrylamide (5.37%) gel electrophoresis and immunoblotting using a standardized sample load in four ethnic groups: German, Ghanaian, Chinese, and San (Kalahari Bushmen). A total of 10 different apparent molecular weight (M_r) polymorphs, designated 1 to 10 with increasing M_r, were detected in >99% of all individuals tested (German, 99%; Ghanaian, 99%; Chinese, 100%; San 100%). A null allele is therefore at most an infrequent variant in all populations. Polymorphs 6–10 were common to all four populations, while polymorphs 1–5 appeared to be relatively rare variants not universally detected in each group in the present study. The Chinese had the highest proportion of double-band phenotypes and the observed frequencies were not significantly different from those expected according to simple Mendelian inheritance, whereas the observed apo[a] phenotype distributions of the other three groups did not concur with those expected for Hardy Weinberg equilibrium. The German and Ghanaian groups displayed similar distributions of apo[a] phenotypes while the Chinese and San had significantly higher frequencies of polymorphs 9 and 10. Mean plasma Lp[a] concentrations in Ghanaians (36.2 ± 31.5 mg/dl) were almost 2-fold greater than in Germans (18.7 ± 23.1 mg/dl) and ca 1.65-fold greater than in either Chinese (22.9 ± 18.3 mg/dl) or San (21.1 ± 19.3 mg/dl). A strong inverse correlation was observed between apo[a] M_r and plasma Lp[a] concentration in Germans but this was much less pronounced in Ghanaians. While the mean plasma Lp[a] levels associated with polymorphs 1–6 were similar in both Germans (43.4 ± 30.0 mg/dl) and Ghanaians (49.2 ± 37.6 mg/dl), those Ghanaians with any combination of the polymorphs 9 and 10 had an almost 3-fold greater mean plasma Lp[a] level (20.6 ± 11.3 mg/dl) than their German counterparts (7.8 ± 5.7 mg/dl). It is therefore apparent that: *1*) differences in apo[a] allele frequencies are not primarily responsible for differences in Lp[a] levels between populations; and *2*) the greatest ethnic variation is observed in plasma Lp[a] concentrations associated with the high molecular weight apo[a] polymorphs.—**Helmhold, M., J. Bigge, R. Muche, J. Mainoo, J. Thiery, D. Seidel, and V. W. Armstrong.** Contribution of the apo[a] phenotype to plasma Lp[a] concentrations shows considerable ethnic variation. *J. Lipid Res.* 1991. **32:** 1919–1928.

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The lipoprotein Lp[a] is characterized by a unique glycoprotein, apo[a], that is linked through disulfide bonds to apoB-100 in the lipoprotein particle (for reviews see 1–4). Apo[a] is readily cleaved from the lipoprotein on mild reduction under non-denaturing conditions and can then be separated by ultracentrifugation or affinity chromatography (5–7). Protein sequencing studies (8, 9) and cDNA analysis (10) have shown that this glycoprotein is highly homologous to plasminogen, apparently consisting of a serine protease-like domain, a kringle 5-like domain, and a large number of kringle 4-like repeats.

Apo[a] exhibits remarkable size polymorphism and several different isoforms with apparent molecular weights (M_r) ranging from 280,000 to >800,000 have been reported. Utermann and coworkers (1, 11–14) were able to identify six M_r apo[a] polymorphs in a Caucasian population and on the basis of family studies they proposed that apo[a] polymorphism is controlled by multiple alleles at a single gene locus. Furthermore, their results revealed an inverse correlation between apo[a] size and the plasma Lp[a] concentration. With the methodology of the measured genotype approach, it was calculated that about 40% of the variability in the Lp[a] concentrations was best explained by the size variability at the apo[a] locus in Caucasians (1, 14). Using probes specific for the kringle 4 and kringle 5 domains of apo[a] cDNA, Gavish, Azrolan, and Breslow (15) demonstrated an inverse relationship between the kringle 4/kringle 5 ratio and the plasma Lp[a] concentration.

With their immunoblot procedure Utermann et al. (11, 14) were unable to detect apo[a] polymorphs in about 30% of the individuals tested. More recently, Gaubatz et al. (16) were able to identify 11 different apo[a] M_r polymorphs in a mixed population of over 600 individuals, at least one

Abbreviations: Lp[a], lipoprotein[a]; LDL, low density lipoprotein.

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polymorph being detectable in >99% of the individuals tested. The sensitivity of their procedure was increased through normalizing the sample load of apo Lp[a]. Their results also confirmed the highly significant inverse correlation between the M_r of the apo[a] and the plasma Lp[a] concentration.

In addition to the strong genetic influence determining plasma Lp[a] levels within a given population, significant racial differences in plasma Lp[a] distributions have been observed (1, 17, 18). We therefore investigated apo[a] polymorphism and plasma Lp[a] distribution in four different populations to determine the extent to which ethnic variability in plasma Lp[a] levels is attributable to differences in the type and frequency of apo[a] polymorphs within these populations.

METHODS

Plasma collection and storage

Venous blood was drawn into K-EDTA Vacutainer tubes (1.6 mg EDTA/ml blood) and plasma was promptly separated by low-speed centrifugation. The plasma was frozen within 60 min in 0.5-ml aliquots and transported to Göttingen on dry ice where it was stored at −20°C or −70°C until analysis. All analyses were performed within 3 months of blood sampling.

Study subjects

The German subjects were recruited from laboratory and hospital staff and students of the University of Göttingen. The Ghanaians were also recruited from hospital and laboratory staff and from health individuals with no apparent organic disease at Tema in Ghana. Plasma samples from Chinese males were kindly supplied by Dr. R. Bernhardt (Academy of Sciences, Heidelberg). They represented part of a group of male industrial workers aged 40–60 years, from the city of Wuhan, China, in whom an extensive study of coronary risk factors was undertaken (19). Plasma samples of the San (Bushmen) from Botswana were obtained as part of a WHO study into coronary risk factors and immunological parameters in this population.

ELISA

Lp[a] was quantified using a noncompetitive enzyme-linked immunosorbent assay (20). A polyclonal antiserum was raised in rabbits immunized with Lp[a], and an immunoglobulin fraction monospecific for apo[a] as determined by double diffusion was purified through affinity chromatography over LDL-Sepharose followed by protein-A Sepharose. This fraction exhibited no cross-reactivity to purified plasminogen (IMCO, Sweden) by double diffusion. Polystyrene microtitre plates (96-well immunoplates, Nunc) were coated overnight at 4°C with anti-Lp[a] IgG (0.05 μg protein/well) in coating buffer (0.2 M $Na_2CO_3/NaHCO_3$, pH 10.6). After washing to remove unbound antibodies, the wells were blocked by incubating the plates with 1% gelatin in coating buffer at 20°C for 1 h. After a further wash, 0.1 ml of appropriately diluted Lp[a] standards or samples in PBS, 0.1% Tween, pH 7.2, were added to the wells. Samples were normally diluted 1000-fold; when necessary a higher dilution was used for those samples with high Lp[a] levels that fell outside the linear portion of the standard curve. Each plate contained six dilutions (0.55–70 ng total Lp[a] lipoprotein/well) of a reference Lp[a] standard (Reference Standard Human Lp[a], Immuno, Heidelberg). The same lot number was used in all ethnic groups, and was derived from a single donor with an apo[a] 6/6 phenotype as determined by our procedure. According to Immuno, this standard was a secondary standard (rethrombinized plasma) that has been standardized against an internal primary standard of purified Lp[a] from a single donor prepared by ultracentrifugation, gel filtration, and heparin-Sepharose affinity chromatography. After reconstitution of the lyophilized plasma, the solution was found to be stable for at least 7 days when stored at 4°C. Fresh standards were prepared weekly. The plates were incubated for 2 h at room temperature with the diluted plasma/standards. They were then washed and bound Lp[a] was detected by incubation with biotin-labeled anti-Lp[a] IgG for 1 h at room temperature followed by a 30-min incubation with biotinylated streptavidin peroxidase conjugate (Amersham Buchler, Braunschweig). The substrate 0.4 mM azino-di-(3-ethylbenzthiazolinsulfonic acid)/0.01% H_2O_2 was added for color development and the reaction was stopped after 15 min with 0.5 M H_2SO_4. Absorbance was then read at 405 nm using a Dynatech MR 700 microplate reader. To determine whether plasminogen would interfere with the assay, solutions of purified plasminogen at physiological concentrations of 0.1–0.25 mg/ml were prepared. When diluted 1000-fold these samples did not yield a reading more than 2 standard deviations above background. The present ELISA could detect Lp[a] at a minimum concentration of 4 ng/ml defined as that concentration significantly different from zero at the 95% confidence limits. The within-assay variation determined by assaying three samples with high, intermediate, and low Lp[a] levels 20 times ranged from 3.6 to 4.9%, while the between-assay variability evaluated by assaying the same three samples over 10 consecutive days ranged from 5.0 to 9.1%.

To test the stability of samples frozen and stored at −20°C, 100 plasma samples were measured before and after storage at −20°C for 12 months. A correlation coefficient of 0.94 was observed between the two assays; the slope of the linear regression was 0.95 (y=12 months, x=0 months) with an intercept of 1.62.

Electrophoretic procedures

Plasma samples (20 μl) were diluted with SDS-PAGE sample buffer (15 mM Tris-HCl, 1 mM EDTA, 5% SDS, 2.5% 2-mercaptoethanol, pH 6.8), the volume depending on the original Lp[a] concentration as determined by ELISA. The sample load applied to the gel contained 0.4–0.6 μg of total Lp[a] lipoprotein. SDS-PAGE was carried out according to Laemmli (21) in a Bio-Rad Mini PROTEAN II dual-slab cell using slab gels of 1 mm thickness (length, 8.2 cm; width, 5 cm) with a running gel containing 5.28% acrylamide and 0.09% bisacrylamide, and a stacking gel containing 2.4% acrylamide and 0.16% bisacrylamide. A maximum of 10 samples/gel were applied to avoid edge effects. Electrophoresis was performed at 4°C for 2.5 h at a constant voltage of 150 V. The transfer of protein from the gel to nitrocellulose was basically performed according to the method of Towbin, Staehelin, and Gordon (22) but using a semi-dry apparatus. The gels were equilibrated for 30 min with 25 mM Tris, 0.192 M glycine, and 20% (v/v) methanol, and transfer was performed at 200 mA for 30 min. Apo[a] bands were detected on the nitrocellulose with a purified rabbit IgG fraction to apo[a] followed by a goat anti-rabbit IgG-peroxidase conjugate and color development with 4-chloro-1-naphthol/H_2O_2. The detection limit for this procedure was estimated from serial dilutions of known plasma samples to be around 50 ng applied Lp[a] lipoprotein. Two control plasma containing polymorphs 4, 6, 7, and 8 were run with each gel. If the polymorphs of any particular plasma could not be conclusively characterized from the first experiment, a second SDS-PAGE was carried out using further controls.

Statistical methods

Comparisons of single- to double-band phenotypes were performed by the Wilcoxon-Mann-Whitney test (23). The chi-square goodness-of-fit test (23) was used to compare observed frequencies of phenotypes to expected frequencies. To meet the requirement of expected cell frequencies greater than 5 for the asymptopic chi-square distribution, adjacent cells were combined (24). (The combinations are to be found in Table 3 for each population.) The chi-square test was used to compare phenotype distributions between populations. In the results the chi-square statistic (χ^2), degrees of freedom (DF), and the *P*-values are documented. The statistical significance level was fixed at $\alpha = 5\%$ for all tests. Statistical analyses were carried out using SAS on an IBM 3081 Host at the University Clinic Göttingen.

RESULTS

Distribution of Lp[a] levels in the four populations

Basic lipid and lipoprotein data of the four groups are presented in **Table 1.** Not surprisingly, the highest total cholesterol levels were obtained in the Germans even though this was on average the youngest group. The distribution of Lp[a] levels in the German group was highly skewed (**Fig. 1A**) with a median of 8.5 mg/dl and a mean of 18.7 mg/dl (Table 1). The frequency distributions of the San (Fig. 1D) and Chinese (Fig. 1B) groups were also highly skewed but their medians were shifted to the right compared to the Germans. Differences between these three groups were particularly obvious at low Lp[a] concentrations, 28% of the Germans having a level below 5 mg/dl as opposed to only 8% of the San, and none of the Chinese. The distribution of Lp[a] concentrations in the Ghanaian collective (Fig. 1C) was the least skewed, this group displaying the highest mean and median values. No significant differences were observed between males and females within the German, Ghanaian, or San groups (Table 1).

TABLE 1. Basic lipid and Lp[a] data from the four ethnic groups

Group	Sex	n	Mean (SD)			Lp[a] Concentrations		
			Age	Total Cholesterol	Triglyceride	Mean (SD)	Median	Range
			yr	*mmol/l*			*mg/dl*	
German	M	94	28.9 (9.2)	5.35 (1.17)	1.02 (0.48)	16.1 (20.0)	8.3	1.0- 99.5
	F	72	28.1 (8.8)	5.27 (1.01)	1.25 (0.81)	19.2 (23.9)	8.9	1.5-104.6
	All	166	28.7 (8.8)	5.31 (1.06)	1.16 (0.70)	18.7 (23.1)	8.5	1.0-104.6
Ghanaian	M	69	30.4 (9.8)	4.66 (1.24)	1.27 (0.78)	34.4 (27.5)	27.4	3.5-144.0
	F	121	32.6 (9.2)	4.86 (1.02)	1.20 (0.68)	36.9 (33.6)	25.8	2.6-240.0
	All	190	31.8 (9.4)	4.79 (1.11)	1.23 (0.72)	36.2 (31.5)	26.0	2.6-240.0
San	M	32	39.1[a] (14.4)	3.89 (0.86)	1.00 (0.40)	21.4 (21.2)	16.7	3.7-105.1
	F	35	36.1[a] (14.8)	4.37 (1.42)	0.93 (0.34)	20.9 (17.7)	14.1	4.2- 63.7
	All	67	37.9[a] (15.1)	4.14 (1.18)	0.96 (0.36)	21.1 (19.3)	15.2	3.7-105.1
Chinese	M	88	46.6 (4.9)	3.80 (0.18)	1.36 (0.53)	22.9 (18.3)	15.3	5.7- 92.6

[a]The ages of the San are estimates since in most cases the true age was not known.

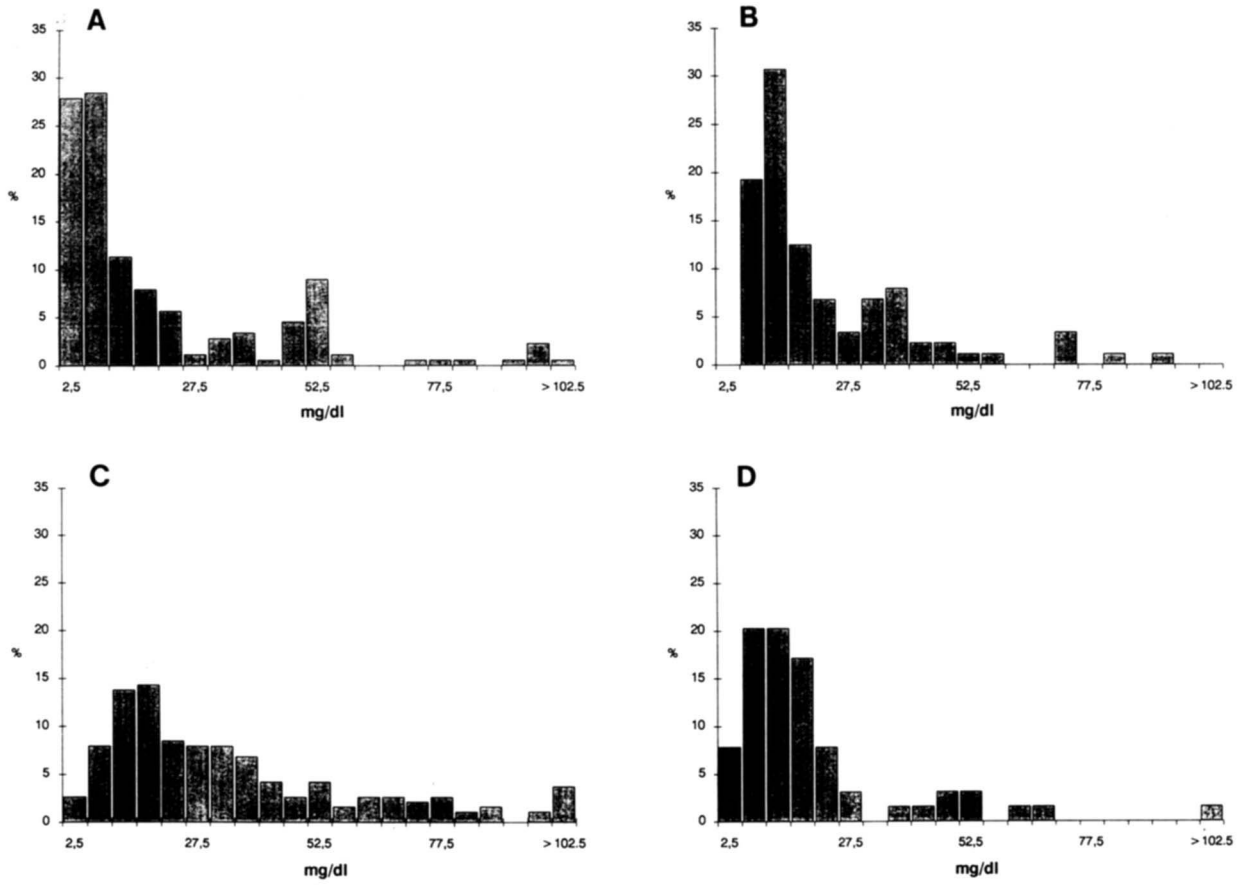

Fig. 1. Distribution of plasma Lp[a] concentrations as determined by ELISA in the four populations; (A) Germans; (B) Chinese; (C) Ghanaians; (D) San.

Apo[a] phenotypes

The sample loads applied to the SDS-PAGE were adjusted to similar Lp[a] values on the basis of the ELISA measurements. Using this system, apo[a] bands could be detected in 507 (99.2%) of the 511 persons investigated and a total of 10 different apo[a] size polymorphs were resolved (**Fig. 2**). In only 4 individuals (2 German, 2 Ghanaian) were there no apo[a] bands observed, although Lp[a] was detectable in plasma with the ELISA. In the majority of the plasmas tested each individual displayed either one or two apo[a] bands. As has been previously noted, fainter lower molecular weight bands were sometimes seen (9, 16). Such bands increased in intensity when samples were subjected to repeated cycles of freezing-thawing or were stored at 4°C for several days; they probably represent proteolytic fragments of apo[a]. We have

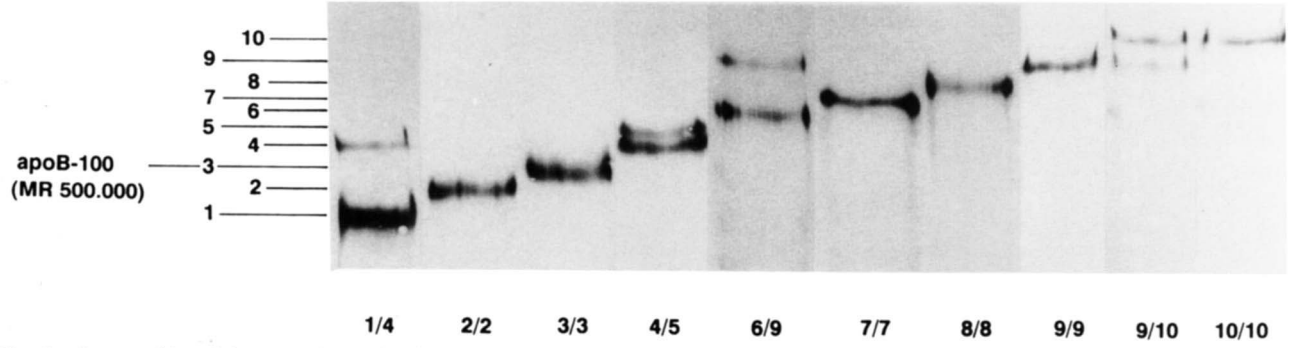

Fig. 2. Immunoblot of the ten polymorphs that were resolved by 5.37% SDS-PAGE.

observed that purified apo[a] is particularly susceptible to proteolytic degradation, a characteristic pattern of lower molecular weight fragments being formed irrespective of apo[a] polymorph type on treatment with elastase (25).

The numbers of single- and double-band phenotypes in the four populations are shown in **Table 2.** The San had the lowest frequency of double-band phenotypes, while the Chinese had the highest. The mean Lp[a] concentrations associated with the single- and double-band phenotypes are also presented in Table 2. Lower Lp[a] levels were associated with the single-band phenotypes in all four groups, although statistical significance was only reached in the Ghanaians ($P < 0.005$), San ($P < 0.05$), and Chinese ($P < 0.05$).

A total of 55 different phenotypes would be possible from 10 different apo[a] polymorphs. In fact only 37 of the 55 possibilities were observed in this study (**Table 3**). The high M_r bands 6–10 were common to all four populations while the lower M_r bands 1–5 were much less frequent and not constantly encountered in every group (Table 3): bands 2, 4, and 5 were observed in Germans, bands 2, 3, 4, and 5 in Ghanaians, bands 1, 3, and 4 in Chinese, but only band 4 in the San.

To test for Hardy-Weinberg equilibrium within the four populations, the observed distributions of phenotypes were compared with those expected from simple Mendelian inheritance by a modified chi-square analysis. Using this procedure the German ($\chi^2 = 84.8$, DF=15, $P < 0.001$), Ghanaian ($\chi^2 = 63.9$, DF=15, $P < 0.001$), and San ($\chi^2 = 25.7$, DF=6, $P < 0.001$) groups were found not to be in Hardy-Weinberg equilibrium. In the case of the Chinese, however, the observed distribution of phenotypes did not differ significantly ($\chi^2 = 2.4$, DF=6, $P = 0.88$) from that predicted on the basis of simple Mendelian inheritance assuming Hardy-Weinberg equilibrium.

Both the Ghanaian and German populations had similar distributions of apo[a] phenotypes (Table 3) which did not differ significantly ($\chi^2 = 5.05$, DF=6, $P = 0.54$) when tested for by chi-square analysis. In the Chinese and San the frequencies of the 9–9, 9–10, and 10–10 phenotypes were notably higher than those seen in the Germans and Ghanaians (0.636 and 0.552 vs. 0.360 and 0.298, respectively). Chi-square analysis revealed that the phenotype distribution of these two populations differed significantly from each other ($\chi^2 = 15.4$, DF=6, $P = 0.018$) and from both the Germans (C vs. Ge; $\chi^2 = 23.8$, DF=6, $P < 0.001$: S vs. Ge; $\chi^2 = 20.6$, DF=6, $P < 0.002$), and the Ghanaians (C vs. Gh; $\chi^2 = 26.06$, DF=6, $P < 0.001$: S vs. Gh; $\chi^2 = 38.8$, DF=6, $P < 0.001$).

The putative allele frequencies, based on the assumption that apo[a] polymorphism is controlled by different alleles at a single gene locus, are presented in **Table 4.**

TABLE 2. Proportion of single-band and double-band phenotypes, and mean plasma Lp[a] concentrations associated with these phenotypes in the four ethnic groups

	Single-Band				Double-Band			
Group	n	%	Mean	SD	n	%	Mean	SD
German	82	50.0	17.1	19.9	82	50.0	20.2	25.9
Ghanaian	89	47.3	28.2	20.3	99	52.7	43.3**	37.6
San	36	54.5	18.0	18.4	30	45.5	25.1*	19.8
Chinese	37	42.0	15.2	7.9	51	58.0	28.4**	21.5

*$P < 0.05$; **$P < 0.005$.

Correlation of apo[a] phenotype to plasma Lp[a] level

Because of the large number of possible phenotypes, we chose to assess the effect of apo[a] phenotype on plasma Lp[a] levels by grouping phenotypes together in the following combinations:

A: Any combination of the polymorphs 1–6;
B: Any one of the polymorphs 1–6 and either of the polymorphs 7 or 8;
C: Any one of the polymorphs 1–6 and either of the polymorphs 9 or 10;
D: Any combination of the polymorphs 7–8;
E: Either of the polymorphs 7 or 8 and either of the polymorphs 9 or 10;
F: Any combination of the polymorphs 9–10.

There is thus a general progression from low to high M_r polymorphs through groups A to F allowing an interpretation of the influence of apo[a] size on plasma Lp[a] levels. The basic statistical data for the Lp[a] concentrations associated with each group are presented in **Table 5.** In the Germans a strong inverse correlation was apparent between plasma Lp[a] concentration and the size of the apo[a] polymorph. The mean and median Lp[a] values increased progressively with decreasing apo[a] molecular weight, there being an almost 6-fold difference between the values observed in group F and those associated with groups A and B. Such a strong correlation was not so apparent in the Ghanaians although even in this group those individuals with the 9–10 combination tended to have the lowest Lp[a] levels of this collective. In comparing the Germans and the Ghanaians, it can be seen that differences between mean Lp[a] values in the two populations are only obvious for the high M_r polymorphs. In groups D to F the Ghanaians had mean and median plasma Lp[a] levels that were 2- to 3-fold higher than their corresponding German counterparts. This holds true even for individual apo[a] phenotypes as illustrated in **Table 6** for the combinations 7/7, 8/8, 8/9, 9,9, and 10,10 in which there were 10 or more individuals associated with each phenotype, thereby allowing a valid comparison. Mean Lp[a] levels in Ghanaians with the 10/10 phenotype were 2.7-fold higher than the corresponding levels in Germans

TABLE 3. Observed and expected frequencies of apo[a] phenotypes in the four ethnic groups

Nr	Phenotype		German		Ghanaian		Chinese		San	
			Obs.	Exp.	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.
1	1	1	0	0	0	0	0	0,003	0	0
2	1	2	0	0	0	0	0	0	0	0
3	1	3	0	0	0	0	0	0,006	0	0
4	1	4	0	0	0	0	0	0,006	0	0
5	1	5	0	0	0	0	0	0	0	0
6	1	6	0	0	0	0	0	0,018	0	0
7	1	7	0	0	0	0	0	0,090	0	0
8	1	8	0	0	0	0	0	0,102	0	0
9	1	9	0	0	0	0	0	0,408	0	0
10	1	10	0	0	0	0	1	0,420	0	0
11	2	2	1	0,023	1	0,023	0	0	0	0
12	2	3	0	0	0	0,054	0	0	0	0
13	2	4	0	0,071	0	0,112	0	0	0	0
14	2	5	1	0,134	0	0,033	0	0	0	0
15	2	6	0	0,335	0	0,252	0	0	0	0
16	2	7	0	0,563	0	0,649	0	0	0	0
17	2	8	1	0,827	1	1,067	0	0	0	0
18	2	9	0	1,078	1	1,212	0	0	0	0
19	2	10	0	0,878	0	0,716	0	0	0	0
20	3	3	0	0	1	0,032	0	0,003	0	0
21	3	4	0	0	0	0,132	0	0,006	0	0
22	3	5	0	0	0	0,039	0	0	0	0
23	3	6	0	0	0	0,298	0	0,018	0	0
24	3	7	0	0	1	0,767	0	0,090	0	0
25	3	8	0	0	0	1,261	0	0,102	0	0
26	3	9	0	0	2	1,432	0	0,408	0	0
27	3	10	0	0	0	0,846	1	0,420	0	0
28	4	4	1	0,053	2	0,137	0	0,003	1	0,032
29	4	5	1	0,201	0	0,081	0	0	0	0
30	4	6	0	0,502	1	0,619	0	0,018	0	0,021
31	4	7	1	0,844	1	1,594	0	0,090	0	0,133
32	4	8	1	1,240	2	2,619	0	0,102	0	0,749
33	4	9	1	1,618	1	2,975	0	0,408	1	1,232
34	4	10	0	1,317	1	1,756	1	0,420	0	0,749
35	5	5	1	0,190	1	0,012	0	0	0	0
36	5	6	0	0,948	0	0,183	0	0	0	0
37	5	7	2	1,595	0	0,472	0	0	0	0
38	5	8	1	2,342	0	0,776	0	0	0	0
39	5	9	2	3,056	1	0,881	0	0	0	0
40	5	10	2	2,487	0	0,520	0	0	0	0
41	6	6	8	1,185	6	0,700	0	0,025	0	0,003
42	6	7	4	3,987	2	3,601	1	0,254	0	0,042
43	6	8	2	5,855	4	5,917	0	0,290	0	0,238
44	6	9	3	7,639	1	6,720	1	1,155	1	0,392
45	6	10	3	6,217	3	3,968	1	1,191	0	0,238
46	7	7	10	3,354	11	4,634	2	0,636	0	0,136
47	7	8	6	9,850	14	15,230	1	1,451	1	1,532
48	7	9	9	12,852	15	17,296	4	5,775	4	2,521
49	7	10	5	10,460	4	10,213	5	5,954	1	1,000
50	8	8	19	7,232	22	12,514	2	0,828	11	11,000
51	8	9	10	18,873	20	28,423	6	6,590	8	14,227
52	8	10	10	15,360	13	16,782	6	6,795	2	8,645
53	9	9	24	12,312	29	16,140	17	13,112	16	28,006
54	9	10	17	20,041	11	19,059	23	27,03853	12	14,227
55	10	10	18	8,156	16	5,627	16	13,940	9	4,323

The following combinations were used to compare observed to expected frequencies in the four ethnic groups: German, numbers 1-34,35-40, 41-42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55; Ghanaian, numbers 1-27, 28-34, 35-42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55; Chinese, numbers 1-45, 46-49, 50-51, 52, 53, 54, 55; and San, numbers 1-47, 48-50, 51, 52, 53, 54, 55.

TABLE 4. Estimated allele frequencies for apo[a] in the four ethnic groups

Isoform (Allele)	Frequency			
	German	Ghanaian	San	Chinese
1	0	0	0	0.006
2	0.012	0.011	0	0
3	0	0.013	0	0.006
4	0.018	0.027	0.023	0.006
5	0.034	0.008	0	0
6	0.085	0.061	0.008	0.017
7	0.143	0.157	0.045	0.085
8	0.210	0.258	0.258	0.097
9	0.274	0.293	0.417	0.386
10	0.223	0.173	0.250	0.398

as opposed to a 1.5-fold difference for the smaller 7/7 phenotype. No major differences were observed, however, between the two groups for those individuals with the polymorph combinations 1-6/1-6 and 1-6/7-8 (Table 5). In the Chinese and San the lowest Lp[a] levels were also associated with the high M_r polymorphs. The mean Lp[a] levels associated with the higher M_r polymorphs in these two populations were intermediate between those of the Germans and the Ghanaians.

DISCUSSION

Using SDS-PAGE and immunoblotting, Utermann and coworkers (1, 11-14) were able to resolve six apo[a] polymorphs with different apparent M_r which they designated F, B, S1, S2, S3, and S4 on the basis of their electrophoretic mobility. The B polymorph migrated with a mobility identical to that of apoB-100, whereas the F polymorph migrated with a faster mobility and the S polymorphs with a slower mobility than apoB-100. Gaubatz et al. (16) subsequently developed an extremely sensitive high resolution method capable of resolving at least 11 different apo[a] size polymorphs in an unselected collective of 692 individuals. The polymorphs were assigned numbers from 1 for the lowest to 11 for the highest apparent M_r, polymorph 3 migrating with a mobility identical to that of apoB-100.

We have now compared apo[a] polymorphism in four different ethnic populations by polyacrylamide gel electrophoresis and immunoblotting using an approach similar to that of Gaubatz et al. (16) in that sample loads were normalized to equivalent amounts of Lp[a] based upon ELISA measurements of the plasma probes. Using this procedure a total of 10 apo[a] bands (numbered 1 to 10 with increasing M_r) could be detected in >99% of all individuals tested from the four ethnic groups. As in the study of Gaubatz et al. (16), our polymorph 3 also had an M_r similar to that of apoB-100.

TABLE 5. Plasma Lp[a] concentrations stratified according to apo[a] phenotype in the four ethnic groups

Apo[a] Phenotype		Serum Lp[a] Concentrations			
		German	Ghanaian	San	Chinese
		mg/dl			
1-6 & 1-6	Mean	43.4	49.2	48.2	
	Median	47.6	37.9		
	Range	2.6-99.1	8.2-13.5		
	n	13	12	1	
1-6 & 7-8	Mean	48.6	44.2	59.4	72.5
	Median	37.2	44.9		
	Range	5.2-104.6	9.3-80.0		
	n	12	10	1	1
1-6 & 9-10	Mean	34.5	56.2	59.4	58.5
	Median	22.9	40.5		73
	Range	2.5-99.9	20.7-108.0		9.7-92.6
	n	11	11	1	5
7-8 & 7-8	Mean	17.5	38.1	22.6	32.1
	Median	12.0	31.2	17.9	31.5
	Range	2.8-71.7	7.1-144	4.2-72.8	16.0-52.0
	n	35	47	12	5
7-8 & 9-10	Mean	13.6	42.4	29.5	32.3
	Median	10.3	28.8	22.1	33.9
	Range	2.5-49.5	2.9-240.0	3.7-67.9	8.6-73.7
	n	34	52	16	21
9-10 & 9-10	Mean	7.8	20.6	15.2	14.5
	Median	6.2	18.5	12.0	12.1
	Range	1.0-24.9	2.6-55.2	4.4-105.1	5.7-37.5
	n	59	56	36	56

During the preparation of our manuscript, Sandholzer et al. (26) published their findings on the effect of apo[a] polymorphism on Lp[a] concentrations in seven ethnic groups. In their study each population was found to contain the five polymorphs B, S1, S2, S3, and S4, and the null type while the F polymorph was only observed in two populations. The higher number of polymorphs reported by ourselves and Gaubatz et al. (16) reflects the greater

TABLE 6. Mean Lp[a] concentrations associated with different apo[a] phenotypes in the German and Ghanaian groups

Phenotype	German			Ghanaian		
	n	Mean	SD	n	Mean	SD
		mg/dl			*mg/dl*	
7/7	10	24.1	17.5	11	37.2	19.7
8/8	19	17.6	16.6	22	33.1	24.4
8/9	10	16.1	16.6	20	39.4	50.2
8/10	10	11.7	9.0	13	30.0	16.0
9/9	24	9.3	6.7	29	23.9	9.3
9/10	17	8.5	5.9	11	21.7	9.9
10/10	18	5.2	2.8	16	13.9	13.1

Only those phenotypes are included in which there were at least 10 individuals with the respective phenotype.

resolution that is afforded by using gel systems with lower acrylamide concentrations (5.37% and 3.85%, respectively) than the 8% gels of Sandholzer et al. (26). An unequivocal determination of the exact number of apo[a] polymorphs using the SDS-PAGE/immunoblot procedure is limited by the resolving power of this technique for proteins of large molecular size. Lackner et al. (27) recently reported the identification of 19 alleles in 102 unrelated Caucasian Americans after digestion of genomic DNA with a restriction endonuclease followed by size fractionation of the DNA fragments using pulse-field electrophoresis. Whether all alleles are expressed remains to be established. In their study of a nine member family, Gaubatz et al. (16) observed several deviations from simple Mendelian inheritance and they suggested that the apparent size of apo[a] polymorphs in plasma is not completely determined by the genes that code for apo[a] but that post-translational processes may also be involved. The determination of apo[a] protein isoforms by immunoblotting after SDS-PAGE should, therefore, still prove to be instructive in our understanding of this glycoprotein, even though it may underestimate the true number of polymorphs present.

In the investigation of Sandholzer et al. (26) and the earlier reports of Utermann and co-workers (1, 11–14), a significant number of individuals were observed in whom no apo[a] band was detectable even though they were positive for Lp[a] by electroimmunoassay. An operational null allele was postulated to account for this finding and was the most frequent allele in three of the populations and the second most frequent allele in a further two populations. However, these findings most probably reflect the insensitivity of their method for detecting apo[a] bands in individuals with low Lp[a] levels. By standardizing the amount of Lp[a] in the sample load for the SDS-PAGE rather than using a constant amount of serum, Gaubatz et al. (16) and ourselves could detect apo[a] bands in more than 99% of samples tested, suggesting that a null allele is, at most, a rare variant. Lackner et al. (27) could also find no evidence for a frequent null allele.

The higher sensitivity obtained through using a standardized sample load leads to an improved detection of double-band phenotypes. In the four populations reported here, the frequency of double-band phenotypes ranged from 45.5 to 58% of all phenotypes, whereas Sandholzer et al. (26) found a much lower proportion ranging between 13.9 and 36.1% in the seven populations studied. Differences are particularly apparent in comparing the frequencies of double-band phenotypes in their Tyrolean (19.9%) and Chinese (21.5%) collectives with our German (50%) and Chinese (58%) groups. It is unlikely that these discrepancies are due primarily to genetic differences. Despite the increased sensitivity of the standardized immunoblot procedure, the numbers of double-band phenotypes are still considerably less than would be expected from the observations of Lackner et al. (27) that 94% of individuals had two different apo[a] alleles by pulsed field electrophoresis. Accordingly, in most immunoblot studies (14, 16, 26) the frequencies of apo[a] alleles were not in Hardy-Weinberg equilibrium, there being an overrepresentation of the single-band compared to the double-band phenotypes. Three of the four collectives in the present study were also not in Hardy-Weinberg equilibrium, but surprisingly the observed frequencies of apo[a] phenotypes in the Chinese, the group with the highest proportion of double-band phenotypes, were not significantly different from those expected from simple Mendelian inheritance. Based on the number of homozygotes and heterozygotes, Lackner et al. (27) concluded that the alleles in their population, as estimated by pulse-field gel electrophoresis, are in Hardy-Weinberg equilibrium.

The possible misclassification of single-band phenotypes by the immunoblot technique due to the presence of a second apo[a] polymorph at a concentration below the detection limit of the immunoblot assay has been discussed by Gaubatz et al. (16). They argued that samples falsely classified as single-band phenotypes should occur most frequently when low M_r polymorphs associated with high Lp[a] concentrations and high apo[a] M_r polymorphs associated with low Lp[a] concentrations are genotypically present. This was, in fact, the opposite of their experimental findings. In agreement with their observations we also found that mean Lp[a] levels of the single-band individuals were lower than those of the double-band phenotypes. This disparity held true irrespective of the ethnic group and it was also observed in the Chinese group even though this was found to be in Hardy-Weinberg equilibrium. A detailed comparison of individuals studied by the two different procedures is required to resolve this phenomenon. One possible explanation for this discrepancy could be that in single-band phenotypes one of the genes is not expressed at the protein level in some individuals.

In this present investigation we found that the high M_r apo[a] polymorphs designated 6–10 were common to all four ethnic groups. The polymorph with the highest frequency in Germans, Ghanaians, and San was band 9, while in the Chinese band 10 (0.398) was slightly more common than band 9 (0.386). Band 9 was also the most common apo[a] polymorph in those individuals screened by Gaubatz et al. (16). It cannot necessarily be concluded, however, that the two polymorphs designated as band 9 in both studies are identical. In all four ethnic groups there was a general decline in frequency with decreasing M_r of the apo[a] polymorph. The low M_r apo[a] polymorphs 1–5 appear to be relatively rare variants and they were not all detected in each of the four populations studied. This may, however, be due to their general low frequency. It remains to be established as to whether there are apo[a] polymorphs unique to any given ethnic group. Although

the polymorphs 1 and 3 were not detected in this group of normal healthy Germans, we have subsequently identified both bands in patients with cardiovascular disease. We have also detected at least one apo[a] polymorph (unpublished observations) with a higher M_r than our polymorph 10 which appears to be analogous to the rare polymorph 11 reported by Gaubatz et al. (16).

Considerable variation was seen between the distribution of Lp[a] levels in the four populations. The German population showed a broad and highly skewed distribution comparable to other Caucasian populations. The mean Lp[a] level of our Chinese population from Wuhan in central China (22.9 mg/dl) was notably higher than that of Singapore Chinese (7 mg/dl) reported by Utermann (1) even though both populations had relatively high frequencies of the high M_r polymorphs. A methodological explanation for this disparity appears unlikely since the mean Lp[a] level of our German collective (17.4 mg/dl) was similar to that of Tyroleans (16.2 mg/dl) observed by Utermann (1). In contrast to the skewed distribution of the German, San, and Chinese groups, the Ghanaians had a much higher mean value and an almost Gaussian distribution. High Lp[a] levels seem, therefore, to be characteristic of all black populations so far studied (17, 18, 26).

In agreement with the results of Utermann et al. (1, 11, 12) and Gaubatz et al. (16), we observed an inverse correlation between the molecular size of the apo[a] polymorph and the respective plasma Lp[a] concentration. In all four populations the highest M_r polymorphs were associated with the lowest Lp[a] concentrations (Tables 5 and 6). However, the mean Lp[a] levels of the Ghanaians in the sub-group F were 1.4-fold greater than those of either the Chinese or San and 2.6-fold greater than those of the Germans. Furthermore, the inverse correlation between the apo[a] polymorph size and the Lp[a] concentration was less pronounced in the Ghanaians than in the Germans and both populations displayed similar Lp[a] concentrations for the low M_r apo[a] phenotypes (subgroups A and B, Table 5). Since the distribution of apo[a] phenotypes and allele frequencies did not differ between the Ghanaians and Germans, these results reveal that the higher levels of Lp[a] observed in the Ghanaian population as compared to the German are not due to differences in apo[a] allele frequencies. Obviously other factors including polygenic influences, variations in Lp[a] synthesis and secretion, and cellular metabolism will also partly determine plasma Lp[a] concentrations. Although the nature of these factors cannot be ascertained from the present study, the extent of their contribution to the plasma Lp[a] level shows greatest ethnic variability in those persons possessing high molecular weight apo[a] polymorphs, a finding that supports the results of Sandholzer et al. (26). One factor that has been shown to influence Lp[a] levels is the LDL-receptor gene (28). Individuals heterozygous for the LDL-receptor defect have significantly higher Lp[a] concentrations than normolipidemic persons with the same apo[a] phenotype. The extent to which Lp[a] is catabolized in vivo by the LDL-receptor is still unclear (29). If the LDL-receptor were to be a major determinant of Lp[a] levels in normolipidemics, then it might be expected that those populations with low cholesterol concentrations and high receptor activities would also have the lowest Lp[a] concentrations. This is clearly not the case. We analyzed total cholesterol concentrations associated with the phenotype combinations presented in Tables 5 and 6, and found no correlation between the mean cholesterol level and phenotype (data not shown) within any given population. For any particular phenotype the German population had higher total cholesterol levels and lower Lp[a] concentrations than the other three populations.

That racial differences can occur in the distribution of apo[a] allele frequencies is suggested by the fact that the San and Chinese groups had higher frequencies of the high M_r polymorphs than either the German or Ghanaian.

The results of these studies have important implications for the proposed association between elevated plasma Lp[a] concentrations and cardiovascular disease. The proportion of individuals in Negroid populations with elevated Lp[a] levels is substantially higher than that of Caucasians (17, 18, 26). In our investigation 45% of the Ghanaians had plasma Lp[a] concentrations of 30 mg/dl or greater as opposed to only 19.5% of Germans. Despite this difference both groups had similar distributions of apo[a] phenotypes. If the absolute Lp[a] level is the predominant factor determining coronary risk due to this lipoprotein then the Negroid population should be at greater risk with respect to Lp[a] than the Caucasian population. The respective LDL-cholesterol concentrations of the two populations will have to be taken into account in such a comparison. If, however, the type of apo[a] polymorph is more important in determining coronary risk, then the risk of the two populations attributable to Lp[a] should be similar.

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