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# Amino acid substitutions in albumin variants found in Brazil

**(alloalbumins/genetic polymorphism/population markers/point mutation)**

KUNIO ARAI[†‡], KAREN HUSS[†§], JEANNE MADISON[†], FRANK W. PUTNAM[†¶], FRANCISCO M. SALZANO[‖], MARIA H. L. P. FRANCO[‖], S. E. B. SANTOS[††], AND MARIA J. M. FREITAS[‡‡]

[†]Department of Biology, Indiana University, Bloomington, IN 47405; [‖]Departamento de Genética, Instituto de Biociências, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil; [††]Departamento de Genética, Centro de Ciências Biológicas, Universidade Federal do Pará, Belém, PA, Brazil; and [‡‡]Laboratorió de Genética, Instituto de Ciências Biológicas, Universidade Federal do Amazonas, Manaus, AM, Brazil

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**ABSTRACT** **Conventional horizontal starch-gel electrophoresis in four buffer systems and structural studies were performed on four albumin variants, and the findings were compared with similar previous data. Albumins Coari I and Porto Alegre I have a previously unreported amino acid substitution (glutamic acid replaced by lysine at position 358, denoted 358 Glu → Lys). The alteration in albumin Porto Alegre II (501 Glu → Lys) is the same as that found for three alloalbumins of Asiatic origin, designated Vancouver, Birmingham, and Adana. Albumin Oriximiná I has the same exchange as albumin Maku (541 Lys → Glu). Some of these findings can be explained only by the occurrence of independent mutations at the same site in the albumin gene. They also point to a third cluster of mutations in that gene. indicating hypermutability in some of its segments.**

Systematic electrophoretic screening of plasma for a series of genetic variants has been extensively conducted in Brazil (1–5). In studies including the albumin locus the Porto Alegre group conducted population surveys of a total of 14,650 persons. Of these, 9615 individuals surveyed at 13 locations were classified phenotypically as of mixed ancestry (White/Black in Porto Alegre and in the interior of Rio Grande do Sul, and White/Black/Indian elsewhere), and 2110 individuals at 4 locations as White. In addition, 2925 Indians of 17 different tribes were included in the surveys. Most of these studies have been published by the Porto Alegre group, including a series of articles pertinent to the present investigation (1–5). As a result of these studies 12 instances of albumin variants have been detected. Franco and Salzano (5) gave details about the probands and used horizontal starch gel electrophoresis in four buffer systems to compare 10 of these alloalbumins with each other and also with 6 reference albumin variants. Some of the variants could not be distinguished from each other; for example, Coari I, Porto Alegre I, and several others were indistinguishable. Likewise, Oriximiná I from a triracial individual appeared to be the same as the Amerindian variant Maku (3).

Because of mutual interests arising out of previous investigation of alloalbumins occurring in North and South American Indians, Japanese, and Caucasians (6–11), we undertook a collaborative structural study of four albumin variants from Brazil that were available for analysis. The results obtained are presented herein.

## MATERIALS AND METHODS

The four variants studied structurally were Coari I, Porto Alegre I, Porto Alegre II, and Oriximiná I, named after the towns where they have been discovered. Porto Alegre is the capital of Brazil's southernmost state (30°10′ S, 51°5′ W), while Coari (4°5′ S, 63°8′ W) and Oriximiná (1°45′ S, 55°8′ W) are located, respectively, in the northern states of Amazonas and Pará, in the Amazonian region. All of the variants were from heterozygous individuals. Familial occurrence was established for Porto Alegre I and Oriximiná I but not for Coari I or Porto Alegre II. Plasma samples from the probands were first restudied in Porto Alegre by using starch gel electrophoresis in four different buffer systems as described by Franco and Salzano (5): (*i*) sodium acetate/EDTA, pH 5.3; (*ii*) Tris/EDTA/borate, pH 6.9; (*iii*) Tris/EDTA/borate, pH 6.1; and (*iv*) Tris/lithium succinate/citrate, pH 6.0. In these tests the four variants were compared with Manaus I, Mura I, and 10 other reference albumin types, including some specimens for which the structural change had earlier been determined in Bloomington.

The structural studies were done in Bloomington and involved the same general strategy, with some modification as required in individual cases. The strategy consists of six steps, the principles and details of which have been described (6–11). The steps are: (*i*) cellulose acetate (Microzone) electrophoresis at pH 8.6; (*ii*) purification of the total albumin (normal albumin A plus variant) by HPLC, reduction and carboxymethylation, and cleavage with CNBr (7, 10, 11); (*iii*) analytical isoelectric focusing of the CNBr digest to identify the CNBr fragment in which the substitution was localized (7, 10); (*iv*) HPLC peptide mapping on a preparative scale to purify the variant CNBr fragment (10, 11); (*v*) HPLC peptide mapping of a tryptic or *Staphylococcus aureus* V8 protease digest of the purified CNBr fragment (10, 11); and (*vi*) amino acid analysis of the variant peptide(s) with the Beckman model 121M amino acid analyzer and automated sequence determination with the Beckman model 890C sequencer (6). Tryptic peptides are designated T and V8 protease peptides are designated S, and both types are numbered consecutively in their predicted order in the amino acid sequence (6, 11).

## RESULTS AND DISCUSSION

**Starch Gel Electrophoresis.** Examples of starch gel electrophoresis at pH 6.1 and pH 6.9 are given in Fig. 1. At pH 6.1 and 6.9 Maku, Oriximiná I, and Mura I had a fast mobility (−2 net charge relative to normal albumin), and they appeared identical in all four buffer systems. Coari I and Porto Alegre I had a slow mobility (about +2) and could not be differentiated from each other under these conditions, or at pH 5.3 or pH 6.0. In all four buffer systems Porto Alegre II, which had a slow mobility (≥ +2), could readily be differentiated from Coari I and Porto Alegre I (see Fig. 1 for pH 6.1 and pH 6.9). However, despite some slight differences in

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[‡]Present address: Josai University, Department of Chemistry, Faculty of Science, Keyakidai, Sakado, Saitama 350-02, Japan.
[§]Present address: Pitman-Moore, Terre Haute, IN 47802.
[¶]To whom reprint requests should be addressed.

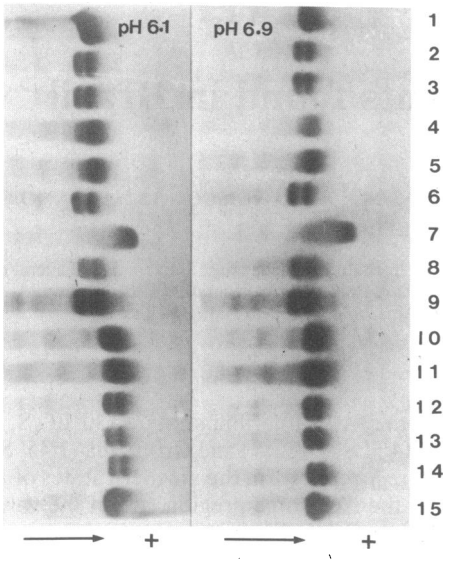


FIG. 1. Comparative mobility of human albumin variants in two Tris/EDTA/borate systems, pH 6.1 and pH 6.9. The anode is at the right. From top to bottom: A (normal serum) (lane 1), Coari I (lane 2), Porto Alegre I (lane 3), Gainesville (Christchurch type of proalbumin, serum 3433) (9) (lane 4), Pollibauer (Lille type of proalbumin, serum 6535) (9) (lane 5), Porto Alegre II (lane 6), artefact (denatured B serum 3006) (lane 7), Vancouver (11) (lane 8), Manaus I (lane 9), Cooperstown (Reading type) (10) (lane 10), Mexico (serum 276682) (8) (lane 11), Maku (lane 12), Oriximiná I (lane 13), Mura I (lane 14), and A (normal serum) (lane 15). All the sera with variants are from individuals heterozygous at the albumin locus. References cited for non-Brazilian sera give the source of the serum and the results of structural study of the variant.

apparent mobility, it was difficult to differentiate Porto Alegre II unambiguously from Vancouver and Manaus I. Fig. 1 shows that all of the variants mentioned above could readily be distinguished at pH 6.1 and 6.9 from albumin Mexico (slow, +1, aspartic acid replaced by glycine at position 550, denoted 550 Asp → Gly) (8), albumin Cooperstown (fast, −1, 313 Lys → Asn) (10), and the two types of proalbumins, Gainesville (Christchurch type, −1 Arg → Gln) (9) and Pollibauer (Lille type, −2 Arg → His) (9). Although not shown in Fig. 1, the latter four variants could be distinguished from each other by a combination of the pH 5.3 and pH 6.0 systems and also at pH 8.6.

**Structural Studies.** These will first be described by albumin type and then integrated with previous observations presented elsewhere.

*Albumin Coari I.* Coari I was detected in a single individual in a study of 762 individuals of a triracial (White/Black/Indian) population in Coari (5). In contrast to most alloalbumins, Coari I could be partially separated from albumin A by use of our standard method: a combination of anion-exchange HPLC on a TSK-DEAE 5 PW column (Anspec, Ann Arbor, MI) and gel filtration with a TSK 4000W column (Anspec). Isoelectric focusing of the CNBr digest indicated the substitution was in CNBr fragment CB5 (residues 330–446). During the initial purification of the CNBr fragments there was difficulty in the separation of CB3 and CB5. However, these peptides were separated successfully by use of a combination of gel filtration (Sephadex G-75 superfine) and reversed-phase HPLC on a Vydac $C_{18}$ column (Anspec), and a pure CB5 fragment was obtained. This consisted of about two-thirds variant CB5 and one-third normal CB5. The tryptic digest was made, and the tryptic peptides were separated by HPLC on a Vydac $C_{18}$ column (11) (Fig. 2). The HPLC peptide profile of the Coari I CB5 was compared to a similar profile of the tryptic peptides of albumin A, which had been identified by amino acid analysis. The variant Coari I peptide

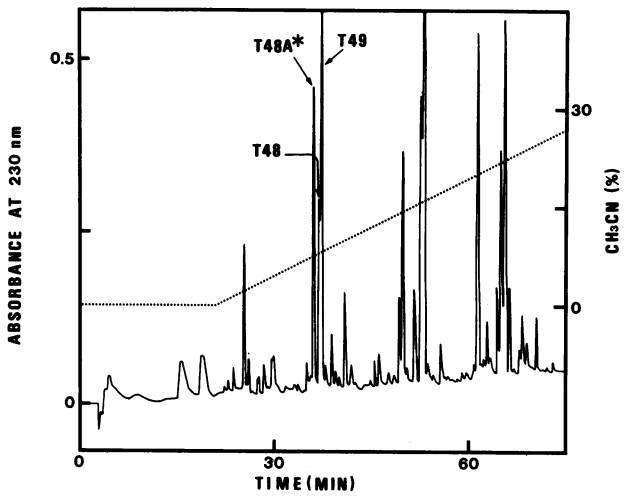


FIG. 2. HPLC elution profile on a Vydac $C_{18}$ column of the tryptic peptides of CB5 from albumin Coari I. The lyophilized tryptic digest was dissolved in 0.1% trifluoroacetic acid (buffer A) and eluted at a flow rate of 1 ml/min over 100 min with a linear gradient from 0 to 50% buffer B (acetonitrile/0.1% trifluoroacetic acid). T48 and T49 are peptides of albumin A. T48A* denotes a variant peptide with an amino acid substitution.

T48A* had the same amino-terminal hexapeptide sequence as the normal T48, but it differed because the substitution 358 Glu → Lys provided a new site for tryptic cleavage, causing the loss of the carboxyl-terminal Lys-359 (Fig. 3). This substitution had not previously been reported for an alloalbumin, and it required confirmation because of the lack of an overlap into the following sequence.

To confirm the structural change in Coari I a V8 protease digest was made of a second preparation of the CB5 variant fragment. Only the Vydac $C_{18}$ HPLC step was used for preparation. The variant CB5 was separated from normal CB5, but it was contaminated with CB3. The V8 protease peptides were separated by HPLC on an Ultrasphere ODS column (Anspec). Fraction 31 (F31 in Fig. 4) contained the variant peptide (designated S37*-38); this was purified by rechromatography on a Vydac $C_{18}$ column (see Fig. 4 *Inset*). Reference to Fig. 3 shows that peptide S37*-38 has a sequence identical to that of S37 plus S38 in albumin A except for the substitution 358 Glu → Lys, which removes the V8 protease-specific site responsible for cleavage between S37 and S38.

The amino acid substitution 358 Glu → Lys in Coari I has not previously been reported. The codon for this position is GAG (12). The substitution is attributable to a point mutation in the codon GAG to AAG. The amino acid exchange corresponds to an increase in charge of +2. This explains the slow mobility of Coari I. However, the mobility is not quite as slow as that of albumin B, which has a 570 Glu → Lys exchange. This probably reflects differences in the secondary and tertiary structure of albumin at the two positions.

*Albumin Porto Alegre I.* Porto Alegre I was found by clinical electrophoresis in a White person of Uruguayan origin and in her mother. This was the only instance detected in a survey of 1178 phenotypically White persons in Porto Alegre, which has a mainly Caucasoid population. The proband lived in Porto Alegre in the south of Brazil, several thousand miles from Coari. This variant could not be distinguished electrophoretically from Coari I by starch gel electrophoresis in the four buffer systems (Fig. 1), and our structural study established that the two alloalbumins had the same amino acid substitution, 358 Glu → Lys.

The strategy for structural study of Porto Alegre I was similar to that for Coari I, except that only the tryptic peptides of CB5 were studied. Analytical isoelectric focusing

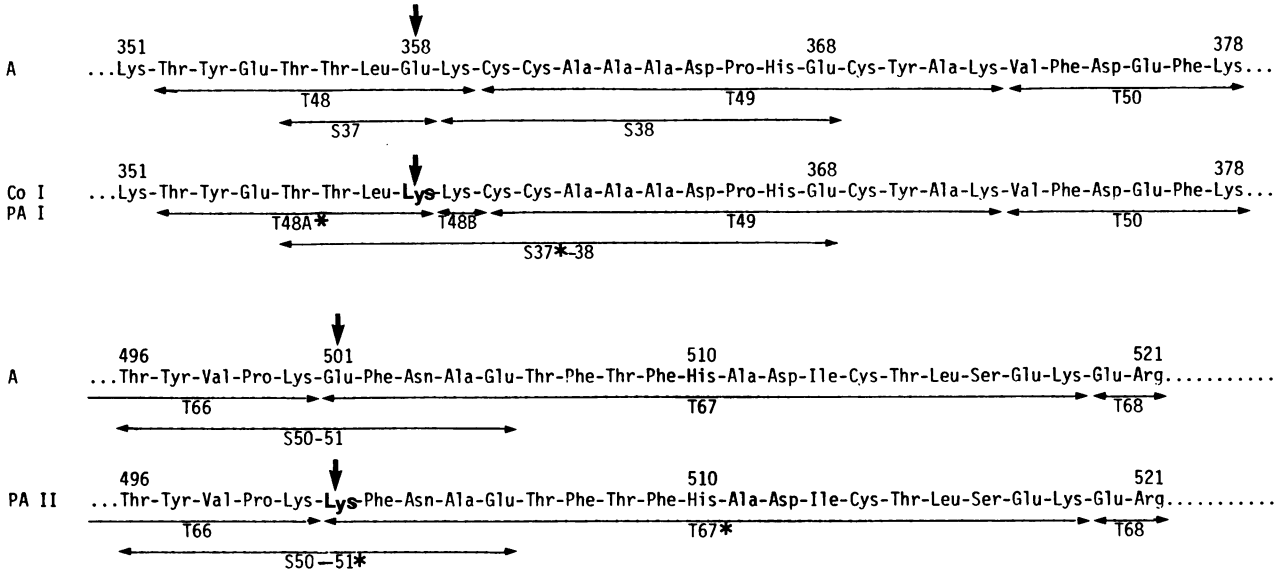


FIG. 3. Amino acid sequence analysis of variant peptides from albumins Coari I (Co I), Porto Alegre I (PA I), and Porto Alegre II (PA II) compared to the sequence of peptides from normal albumin (A). The peptide designations are defined in the text. The amino acid substitutions in the variant peptides are shown in boldface letters and are indicated by vertical arrows.

of a CNBr digest of the purified carboxymethylated albumin indicated that there was a substitution in CB5. Preparative separation of the CNBr digest was done in two steps: gel filtration on a TSK G3000 SW column (Anspec), then HPLC on a Vydac $C_{18}$ column. This procedure separated CB3, but the normal and variant CB5 fragments were not completely separated, so they were pooled. A tryptic digest of the CB5 fragments was chromatographed by HPLC on Vydac $C_{18}$ column. This yielded the pure normal peptide T48. Rechromatography of another peptide peak on Vydac $C_{18}$ yielded T48A* and T49. Determination of the amino acid composition and sequence of T48A* established the amino acid substitution 358 Glu → Lys, the same as in Coari I. This supported the electrophoretic study, which showed that Porto Alegre I and Coari I could not be distinguished by starch gel electrophoresis in four buffer systems, or at pH 8.6 in cellulose acetate electrophoresis.

A third variant, Belém IV, which was not available for structural study, could not be distinguished electrophoretically from Porto Alegre I and Coari I (5). Belém IV had been found in a survey of 1581 subjects from Belém, a city on the northern coast of Brazil (1°25′ S, 48°30′ W). The population of Belém is largely triracial (White/Black/Indian), and the variant was present in a father and son of mixed racial ancestry. Although Belém IV was not studied, it probably has the 358 Glu → Lys substitution. Santa Ana is another named alloalbumin that cannot be distinguished electrophoretically from the three described above (5). This albumin was discovered by clinical electrophoresis (13). It was present in 19 members of a Mexican family that moved to Santa Ana,

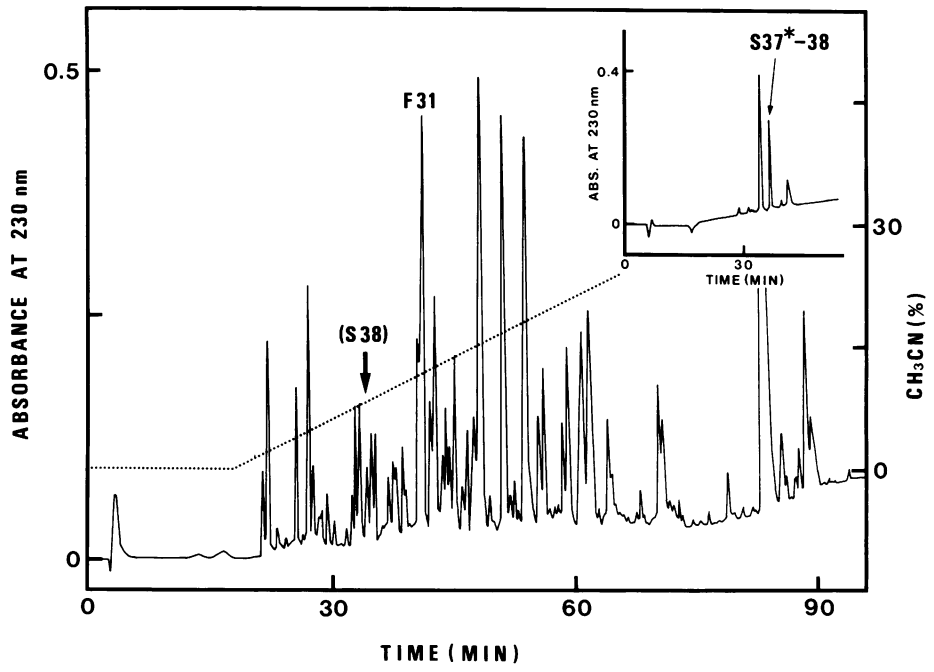


FIG. 4. HPLC elution profile on an Ultrasphere ODS column of V8 protease peptides of CB5 from albumin Coari I. The conditions are the same as for the tryptic digest (Fig. 2). (S38) marks the peak eluted at the retention time for the normal S38 peptide of albumin A. F31 is the fraction rechromatographed with the same column and conditions (see *Inset*). S37*-38 is the variant peptide from albumin Coari I.

California. Because Caucasian ancestry is the only racial characteristic shared by the carriers of the four alloalbumins, it is likely that the 358 Glu → Lys mutation originated in the Caucasian gene pool.

*Albumin Porto Alegre II.* This alloalbumin was detected by clinical electrophoresis (5). The carrier was of mixed African/Caucasian (Portuguese) ancestry and was the only carrier identified in a survey of 2743 biracial (Black/White) persons in Porto Alegre. The four grandparents had been born in Brazil, but relatives were not available for study. In previous studies (5) Porto Alegre II had exhibited the slowest mobility of all the variants compared by starch gel electrophoresis at neutral or acidic pH in the four buffer systems, even slower than albumin B or the two types of proalbumins. In the present work it was difficult to differentiate Porto Alegre II, Vancouver, and Manaus I (Fig. 1).

The general strategy for structural study of Porto Alegre II was similar to that described in *Materials and Methods.* However, because of an apparent similarity of Porto Alegre II to albumins Vancouver and Birmingham of Asiatic origin, the procedure described by Huss *et al.* (11) for those variants was followed in detail for Porto Alegre II. As in those cases, isoelectric focusing identified CB6 (residues 447–548) as the variant CNBr fragment. Purification of the pooled normal and variant CB6 fragments was accomplished by HPLC (see figure 3 of ref. 11). HPLC separation of a tryptic digest of the pooled CB6 fragments yielded tryptic peptide T67 from albumin A and T67* from Porto Alegre II (Fig. 3). Peptides T67 and T67* were identified by their retention time and by amino acid composition, but there was insufficient material for sequence analysis. A V8 protease digest was also made of CB6 under conditions favoring cleavage after glutamic acid. HPLC purification yielded the normal peptide S50-51 and the variant peptide S50-51* (for diagram of HPLC profile see figure 5 of ref. 11). Sequence analysis of S50-51* established the substitution 501 Glu → Lys. In the V8 digest of albumin A the two potential peptides S50 and S51 are generally linked to form the peptide S50-51. In the V8 digest of an alloalbumin with the 501 Glu → Lys substitution, they are necessarily linked as S50-51* because the mutation removes the V8-susceptible site.

The substitution 501 Glu → Lys produces a net increase in charge of +2. This accounts for the slow mobility of Porto Alegre II in all five pH systems used for electrophoretic study. The amino acid exchange accords with the mutation of a single nucleotide base in the codon GAG for position 501 (12) to AAG.

**Identity of Albumins Porto Alegre II, Vancouver, Birmingham, and Adana.** It was a surprise to find that the amino acid substitution 501 Glu → Lys present in Porto Alegre II is the same as that reported by us for three alloalbumins of Asiatic origin designated Vancouver, Birmingham, and Adana (11). The first two of these were present in unrelated families that had migrated from northern India; actually, the family with the Vancouver trait had lived in Fiji for several generations. Albumin Adana was identified in a single individual in Adana, Turkey. The family of the proband of Porto Alegre II had lived in Brazil for three generations and was of African/Caucasian (Portuguese) descent. It seems most likely that the Porto Alegre II variant resulted from an independent mutation of the albumin gene.

**Independent Mutations at the Same Site in the Albumin Gene.** Several other examples are known of alloalbumins with identical substitutions that are present in families of diverse ethnic background living in widely separated geographical regions. By structural study we have proven that the substitution 570 Glu → Lys is present in albumins of the B type that are found in individuals descended from five different European nationalities, and also in several Japanese and in one Cambodian (14). An albumin of the Reading type (313 Lys → Asn) is expressed by families of three different European descents, one living in New York (albumin Cooperstown), a second in New Zealand (albumin Canterbury), and the third in Italy (albumin Tagliacozzo) (10). We and others have confirmed by structural study that proalbumins of the Christchurch type (−1 Arg → Gln) and the Lille type (−2 Arg → His) are present in Caucasians of several European ancestries and in Japanese, and the Lille type is also present in a Chinese (9, 14).

*Albumins Oriximiná I and Maku.* Albumin Oriximiná I was discovered in a mother and daughter of mixed racial descent in a study of 191 individuals who lived in the triracial (White/Black/Indian) Amazonian community of Oriximiná. On the basis of its electrophoretic mobility, albumin Oriximiná I was classified as the same as albumin Maku, which is named after an almost extinct Indian tribe (15). Maku is a fast albumin that was originally identified in one of the last surviving persons who could speak Maku (15). A variant indistinguishable electrophoretically from Maku was encountered with an allele frequency of 0.019 in the Wapishana tribe (16). Takahashi *et al.* (7) determined the amino acid substitution in a specimen of Wapishana origin and found it to be 541 Lys → Glu. Our structural study described below established that Oriximiná I and Maku (Wapishana) have the same amino acid substitution.

Essentially the same procedure was used for analysis of Oriximiná I as described above and as earlier reported for Maku (7). Isoelectric focusing of a CNBr digest of the carboxymethylated albumin (A/Oriximiná I) showed that the substitution was in CNBr fragment CB6 (residues 447–548). CB6 was purified by HPLC on a Vydac $C_{18}$ column. A tryptic digest of CB6 was fractionated by HPLC in the standard procedure. T73 (Ala-Thr-Lys) and T74 (Glu-Gln-Leu-Lys), the expected tryptic peptides from albumin A for positions 539–541 and 542–545, respectively, were identified by their retention time and by amino acid analysis. A variant peptide T73*-74 that was derived from Oriximiná I was isolated. This had the amino acid sequence Ala-Thr-*Glu*-Glu-Gln-Leu-Lys, which established the substitution 541 Lys → Glu. This mutation removes a site for tryptic cleavage, thereby generating the variant peptide. The 541 Lys → Glu exchange introduces a change in net charge of −2, which accords with the fast mobility of this variant. This point substitution is explicable by a single-base alteration in the codon AAA to GAA.

Because albumins Oriximiná I and Maku have the same amino acid substitution, the Oriximiná variant undoubtedly originated from the Indian gene pool in this neo-Brazilian community. Studies with 13 polymorphic systems resulted in the following estimates of accumulated admixture in this triracial population: 57% White, 15% Black, and 28% Indian (3). It is likely that albumin Mura I also has the substitution 541 Glu → Lys because this alloalbumin cannot be distinguished electrophoretically from Maku and Oriximiná I, and it was detected in an Indian during a genetic study of 206 members of the Mura tribe.

**Structural Study as a Means for Validation of Genetic Polymorphism Based on Electrophoretic Screening.** Systematic electrophoretic screening with a standardized system has commonly been used in large population surveys to identify genetic polymorphism expressed in plasma or blood polypeptides (1–5, 17, 18). Generally only electromorphs are identified. It is always essential to undertake a systematic comparison with use of a series of buffer systems and a number of reference standards. The advantage of this screening procedure is that large-scale surveys of many polypeptides and enzymes can be conducted. However, structural study of the variants identified is difficult and time-consuming, and it is rarely attempted. Undoubtedly the most extensive survey of this type is the Biochemical Genetics

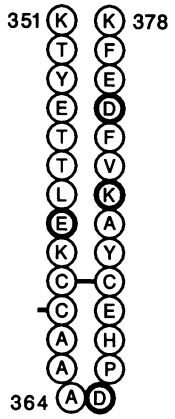


FIG. 5. Amino acid sequence in one-letter code for a region of CB5 covering residues 351–378. Bold circles mark single-point amino acid exchanges in albumin genetic variants: 358 Glu → Lys, albumins Coari I and Porto Alegre I; 365 Asp → His, albumin Parklands (19); 372 Lys → Glu, albumins Naskapi and Mersin (8); and 375 Asp → Asn, albumin Nagasaki 2 (7).

Study of the Radiation Effects Research Foundation at Hiroshima and Nagasaki (17, 18). In a search for mutations affecting protein structure in children of atomic bomb survivors more than $10^6$ locus products were examined for alterations in electrophoretic mobility. Among the many genetic variants identified electrophoretically were five types of alloalbumins. We have either reported (7) or have since determined (unpublished data) the amino acid changes in all five types of alloalbumins. Each type has a different amino acid substitution, and none is identical to those reported here. This result adds much credence to the validity of systematic electrophoretic screening, albeit sequence analysis of the variant polypeptide or gene must still be the final criterion.

Likewise, the present study has validated the systematic electrophoretic comparison of albumin variants in Brazil (1–5). Albumins Coari I and Porto Alegre I could not be distinguished electrophoretically, and we found they had the same substitution, 358 Glu → Lys, one which had not previously been reported. Albumin Porto Alegre II was difficult to distinguish electrophoretically from Vancouver and Manaus I, and it had the same substitution as Vancouver (501 Glu → Lys). We have found this change in three alloalbumins of Asiatic origin, Vancouver, Birmingham, and Adana (11); thus, we assume Porto Alegre II arose by an independent mutation. In addition, albumins Oriximiná I and Maku were indistinguishable electrophoretically, and we found they had the same structural change, 541 Lys → Glu.

These studies also indicate the importance of studying populations from widely separated geographical areas and distinct ethnic groups. Only in this way can we have a truly comprehensive picture of the extent and kind of genetic variation present in our species.

**Clustering of Mutations.** Two clusters of mutation in the albumin gene have previously been noted—one at the amino terminus (6), the other at the carboxyl terminus (14). A third cluster has been identified as the result of this and other recent work of our laboratory. Amino acid exchanges have been identified at four sites in a sequence of 18 amino acids from residues 358–375: Coari I and Porto Alegre I at Glu-358, Parklands at Asp-365 (19), Naskapi and Mersin at Lys-372 (6, 8), and Nagasaki-2 at Asp-375 (7) (Fig. 5). This exceeds the number reported to date in the first 357 amino acids of the albumin molecule. Such clusters may indicate hypermutability of certain segments of the albumin gene.

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