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The same substitution, glutamic acid → lysine at position 501, occurs in three alloalbumins of Asiatic origin: Albumins Vancouver, Birmingham, and Adana

(serum albumin/bisalbuminemia/genetic polymorphism/plasma protein variants/point mutation)

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ABSTRACT **A strategy is described for identifying structural changes in genetic variants of human serum albumin (alloalbumins). By use of this strategy we have determined an amino acid substitution in three alloalbumins of Asiatic origin. The same amino acid exchange, glutamic acid → lysine at position 501, occurs in albumins Vancouver and Birmingham, both from families that migrated from northern India, and also in albumin Adana from Turkey. This exchange corresponds to a single base mutation in the codon GAG to AAG and accords with the slow mobility of the three albumins at pH 8.6. Each of the three alloalbumins had been reported to be a new variant, yet they have the same substitution. These results emphasize the need for structural study of genetic variants that have been differentiated only by nonspecific physical criteria such as dye binding and electrophoretic mobility. We know of no other description of the substitution involved in an alloalbumin originating from the Indian subcontinent. However, the same change of glutamic acid → lysine at position 501 may be present in several other named variants reported for populations in north India and the surrounding regions.**

More than a decade ago about 100 genetic variants (allotypes) of human serum albumin bearing differing geographical or ethnic names had been reported on the basis of identification by clinical electrophoresis or by genetic screening with a series of electrophoretic methods (1, 2), but few had been subjected to structural study until recently. Already, more variants are known for albumin than for any other protein except hemoglobin, and many more may be discovered because albumin has a rate of molecular evolution three times that of hemoglobin (3). Yet, up to 1978 some 85 different point mutations (single amino acid exchanges) had been established for abnormal human hemoglobins (4) compared to only 1 for a variant albumin (5). This disparity reflects two factors: (*i*) Unlike abnormal hemoglobins, alloalbumins are not associated with disease or a significant effect on physiological function, and most are very rare (6). (*ii*) The albumin molecule consists of a single polypeptide chain with 585 amino acids and 17 disulfide bridges, a circumstance that magnifies the difficulty of structural determination of a single substitution (7, 8). However, investigation of human albumin variants has recently been stimulated by the advent of improved methods for structural study of proteins, by interest in protein genetics and evolution, and by knowledge of the complete protein (8) and genomic (9) sequence of albumin. As summarized by Takahashi *et al.* (10) and Minchiotti *et al.* (11), some 15 different amino acid substitutions and nucleotide mutations have so far been identified for genetic variants of human serum albumin, chiefly in individuals of European, Amerindian, and Japanese ancestry. Here we report another substitution (glutamic acid replaced by lysine at position 501, denoted 501 Glu → Lys). This occurs in both albumin Vancouver (12) and albumin Birmingham (13) and establishes the structural change in an albumin allotype carried by families originating from the Indian subcontinent. The same substitution is present in albumin Adana, found in a single individual in Turkey (14, 15).

The strategy we used for structural study of these alloalbumins consists of CNBr cleavage of the purified alkylated albumin followed by isoelectric focusing to identify the CNBr fragment in which the substitution occurs. The variant CNBr fragment is purified by HPLC and digested with trypsin or V8 protease. A peptide map of the digest is made by HPLC, and the variant peptide is purified and its amino acid composition and sequence are determined. An automated tandem HPLC system for tryptic peptide mapping (16) was also used for albumin Adana.

By use of this strategy the same amino acid exchange, 501 Glu → Lys, was identified in albumin Vancouver from a Fiji Indian family living in Canada (12) and in albumin Birmingham from an Indian family that had migrated to England from the Punjab (13). Each of the three alloalbumins had been described as "a new variant," and albumins Vancouver and Birmingham had been reported to differ from each other and also from albumin Kashmir on the basis of dye-binding studies and their electrophoretic mobility in certain systems (12, 13). Our finding that albumins Vancouver and Birmingham are identical confirms the necessity for structural study of genetic variants of any protein that may have been identified and differentiated only by nonspecific physical criteria. The same substitution (501 Glu → Lys) may be present in other alloalbumins designated Kashmir (6) and Afghanistan (2) and may be as common among certain ethnic groups in north India as albumin B (570 Glu → Lys) is among people of European ancestry (1, 2, 6, 11).

MATERIALS AND METHODS

Sera. A fresh specimen of lyophilized serum (5 ml) from an individual with the albumin Vancouver trait was provided by J. Frohlich (Vancouver, Canada). Lyophilized serum (2 ml) from the index case of albumin Birmingham was provided by R. Jefferis (Birmingham, U.K.). In both instances inheritance had been proved, the donor was heterozygous, and the variant had previously been described (12, 13). By use of

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cellulose acetate Microzone electrophoresis at pH 8.6 (Beckman) we compared these sera to reference sera containing alloalbumins for which we had determined the substitution. The same scheme was used for purification and characterization of the albumin in both cases.

Structural study of albumin Adana had been undertaken earlier by Franklin *et al.* (14) and again by Takahashi *et al.* (15). In both studies the substitution had been localized to CNBr fragment CB6 (residues 447–548), but the site and nature of the amino acid exchange had not been determined because of the size of CB6 and the small sample available.

Purification and Characterization of Albumins. No attempt was made to separate the normal and variant albumins, and the total albumin was purified in two steps. In the first step for albumins Vancouver and Birmingham 1 ml of serum was subjected to ion-exchange HPLC on a Bio-Gel TSK DEAE-5PW column (7.5 × 75 mm) (Bio-Rad). The HPLC system has been described (16). A gradient from 0% to 60% over 60 min with a flow rate of 1 ml/min was used from buffer A (0.01 M NH_4HCO_3) to buffer B (1.00 M NH_4HCO_3). The second step was gel filtration chromatography with a Toyo Soda TSK G4000SW column (7.5 × 600 mm) (Altex, Berkeley, CA) and isocratic elution with 0.1 M NH_4HCO_3 at a flow rate of 0.5 ml/min. Albumin Adana was purified by a similar procedure (16). The purified albumin was characterized by cellulose acetate Microzone electrophoresis at pH 8.6 (Beckman model R101) and then was reduced and carboxymethylated and cleaved with CNBr as described (17). As a reference standard for normal (common) albumin (albumin A) we used a commercial albumin from pooled human plasma (lot 102578, Calbiochem–Behring). This was carried through all the procedures applied to the albumins prepared from the heterozygous sera.

Screening and Purification of CNBr Fragments. Analytical isoelectric focusing of the CNBr fragments (10, 17) localized the substitution in all three variant albumins to CB6, a polypeptide containing 102 amino acid residues (positions 447–548). To purify CB6, the CNBr fragments were separated preparatively by reversed-phase HPLC on a Vydac C_{18} column (4.6 × 250 mm) (The Nest Group, Southboro, MA).

HPLC Peptide Profiles and Identification of Variant Peptide. In the first experiments the purified CB6 was digested with trypsin (16). A tryptic peptide profile map was made by reversed-phase HPLC on a Vydac C_{18} column. Tryptic peptides of human albumin are given the prefix T and are numbered consecutively in their order in the sequence (16).

After the analyses of the tryptic peptides indicated that the substitution was probably 501 Glu → Lys and thus at the N terminus of T67, we undertook to confirm this by preparing peptides of CB6 from albumins A and Vancouver with *Staphylococcus aureus* V8 protease (Miles). This digestion was at 37°C for 4 hr under conditions in which this enzyme is rather specific for glutamic residues and was predicted to yield a single confirmatory overlapping peptide (enzyme-to-substrate, 1:20, wt/wt) (pH 7.8, 0.1 M NH_4HCO_3/5 mM EDTA). The peptide profile of the V8 protease digest was mapped by reversed-phase HPLC on a Vydac C_{18} column. The V8 peptides are given the prefix S and are numbered consecutively in their order in the sequence of human albumin. Under the conditions used the V8 protease produces a single peptide from albumin A (S50-51, residues 496–505); a single peptide (S50-51*, residues 496–505) is also obtained from albumin Vancouver, but it has the substitution 501 Glu → Lys.

Structural Studies. The CNBr fragments of the purified alloalbumins were initially identified by their retention time in the HPLC elution profile and by isoelectric focusing with use of known CNBr fragments of albumin A as reference standards. The identification was confirmed by amino acid analysis (Beckman amino acid analyzer, model 121M) and in some cases by automated sequence analysis of the CNBr fragments, using the Beckman model 890C sequencer (10, 15–18). The tryptic and V8 protease peptides were identified by amino acid analysis with reference to the published sequence of human albumin (8, 9). The substitution in the variant peptides in the HPLC profile was established by automated sequence analysis.

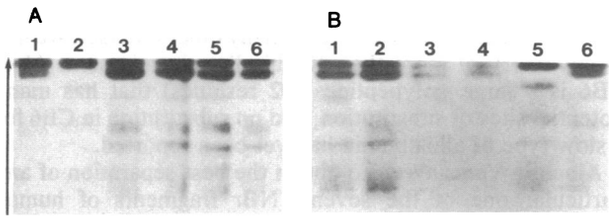

FIG. 1. Electrophoretograms at pH 8.6 of alloalbumin-containing sera with and without trypsin treatment as a test for proalbumins. Heterozygous sera (20 μl) were digested with 13 μg of trypsin at room temperature for 10 min, and digestion was stopped with soybean trypsin inhibitor (18). (*A*) Lane 1, proalbumin Gainesville serum; lane 2, proalbumin Gainesville serum with trypsin treatment; lane 3, albumin Vancouver serum; lane 4, albumin Vancouver serum with trypsin treatment; lane 5, albumin B (Oliphant) serum; lane 6, albumin B (Oliphant) serum with trypsin treatment. Arrow indicates the direction of electrophoretic migration. (*B*) Lane 1, albumin Birmingham serum with trypsin treatment; lane 2, albumin Birmingham serum; lane 3, albumin Bantu serum with trypsin treatment; lane 4, albumin Bantu serum; lane 5, proalbumin Gainesville serum with trypsin treatment; lane 6, proalbumin Gainesville serum. The fastest-migrating band is normal albumin (albumin A).

RESULTS AND DISCUSSION

The Vancouver and Birmingham alloalbumins had a slow mobility in Microzone electrophoresis at pH 8.6, as if each reflected an amino acid substitution corresponding to an increase in charge of 2+ similar to that of albumin B (570 Glu → Lys) (5, 10, 11) and also to proalbumins of the Christchurch type (−1 Arg → Gln) (18) (Fig. 1). However, the mobility of the Vancouver and Birmingham albumins was not affected by limited incubation with trypsin, and thus they did not appear to be proalbumins (Fig. 1). Furthermore, isoelectric focusing of the CNBr fragments of all three alloalbumins indicated that the amino acid substitution was localized to CB6 (see figure 1 of ref. 15 for Adana; see Fig. 2 for Vancouver and Birmingham). However, we had no *a priori* reason to suspect that the three alloalbumins had an identical substitution. In the first place, each had been declared to be "a new variant," and albumins Vancouver and Birmingham had been distinguished by dye-binding ability and other

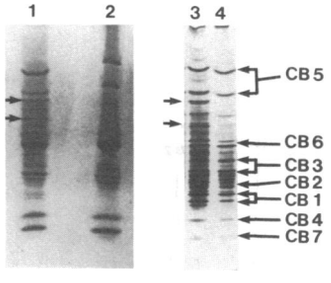

FIG. 2. Isoelectric focusing of CNBr fragments of normal human albumin A and genetic variants Vancouver and Birmingham. Lane 1, albumin Vancouver; lanes 2 and 4, albumin A; lane 3, albumin Birmingham. Arrows indicate bands for CNBr fragments with an amino acid substitution resulting in a charge change. CNBr fragments may have two charge forms because of the homoserine–homoserine lactone equilibrium.

criteria (12). Furthermore, we studied the three variants at different times while working on many others. In fact, we had an inventory of more than 100 bisalbuminemic sera. Also, CB6 is a large polypeptide (102 residues) that has many potential sites of substitution, and no substitution in CB6 for a slow type of alloalbumin had yet been reported.

Albumin Vancouver. To obtain the best separation of any particular one of the seven CNBr fragments of human albumin, the conditions for purification on a preparative scale must sometimes be modified. For some alloalbumins the variant CNBr fragment (e.g., CB4-C in albumin Cooperstown) separates well from the normal (CB4-A) (17); in other instances the variant CNBr fragment may overlap with a different normal CNBr fragment. In the case of albumin Vancouver the normal and variant CB6 fragments overlapped, but they separated adequately from the broad overlapping peaks of CB3, CB1, and CB5 (Fig. 3).

The tryptic digest of this CB6 peak gave an HPLC profile in which the normal T67 peptide was reduced in relative amount and was adjacent to a new peptide peak (Fig. 4). The latter was designated T67* after amino acid analysis showed an apparent substitution of one glutamic residue by a lysine. From the stoichiometry it appeared that the substitution could have occurred at either Glu-501 or Glu-518 in the 19-residue T67 peptide. If the exchange was at Glu-518, it might be difficult to complete the sequence analysis to the end of the peptide. Yet, if the exchange was at Glu-501, proof by sequence analysis would be easy but an overlap into the preceding sequence would not be established. Thus, we elected to purify a second preparation of CB6 and make a V8 protease digest to guide the strategy for sequence analysis of the tryptic peptide T67*.

The HPLC profile of the V8 protease digest of CB6 from albumin Vancouver (Fig. 5) showed the presence of a new peptide, designated S50-S51*, which was absent from the profile of CB6 from albumin A. There was a concurrent decrease in the peak labeled S50-51. Amino acid analysis was done for all the V8 protease peptides from albumin A and one of the alloalbumins. This showed that the two small potential V8 peptides, S50 and S51, which are adjacent in the sequence of albumin A, were linked as a single peptide despite the presence of a potential V8 protease-susceptible site at Glu-501. The amino acid composition and sequence analysis of S50-51* indicated it was a peptide composed of S50 and S51

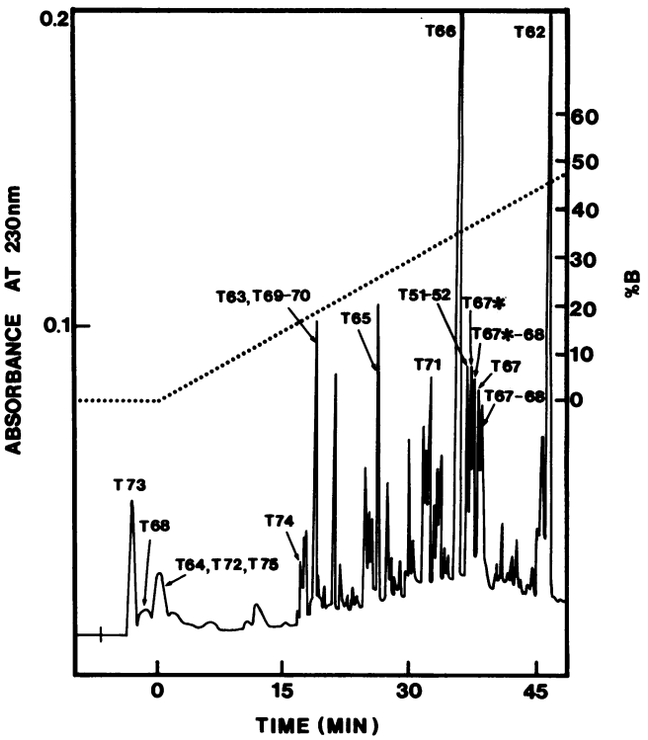

FIG. 4. HPLC elution profile on a Vydac C_{18} column of tryptic peptides isolated from CB6 of albumin Vancouver. The lyophilized tryptic digest was dissolved in 0.1% trifluoroacetic acid (buffer A) and eluted at a flow rate of 1 ml/min over 60 min with a linear gradient from 0% to 60% buffer B (acetonitrile/0.1% trifluoroacetic acid). T67* and T67*-68 denote the variant peptides containing the amino acid substitution.

with a Glu → Lys substitution at Glu-501 (Fig. 6). Automated sequence analysis of the T67* tryptic peptide described above confirmed the 501 Glu → Lys exchange.

Albumin Birmingham. Preceding work (12) as well as our own studies described above had shown that this alloalbumin had many characteristics similar to those of albumin Vancouver. In particular, both had a slow 2+ mobility, and we had localized the substitution to CB6 in both cases. Thus, a CNBr digest was made of the albumin purified from the

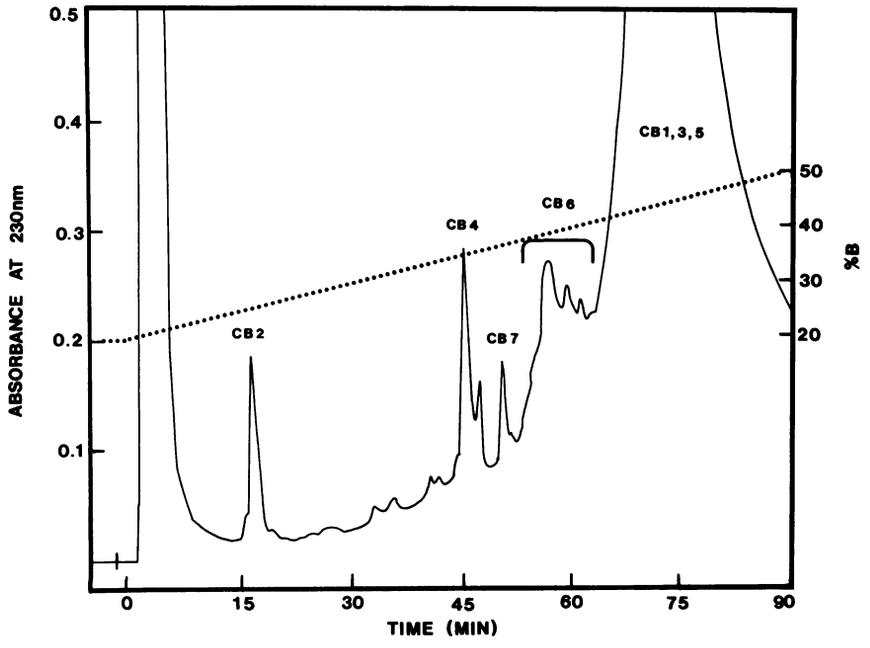

FIG. 3. HPLC elution profile in a Vydac C_{18} column of CNBr fragments of reduced and carboxymethylated albumin Vancouver. The CNBr fragments in buffer A (0.1% trifluoroacetic acid) were eluted at a flow rate of 1 ml/min over 90 min with a linear gradient from 20% to 50% buffer B (acetonitrile/2-propanol; 2:1, vol/vol) containing 0.1% trifluoroacetic acid. The CB6 fractions containing both normal and variant CB6 (bracket) were pooled for further structural characterization.

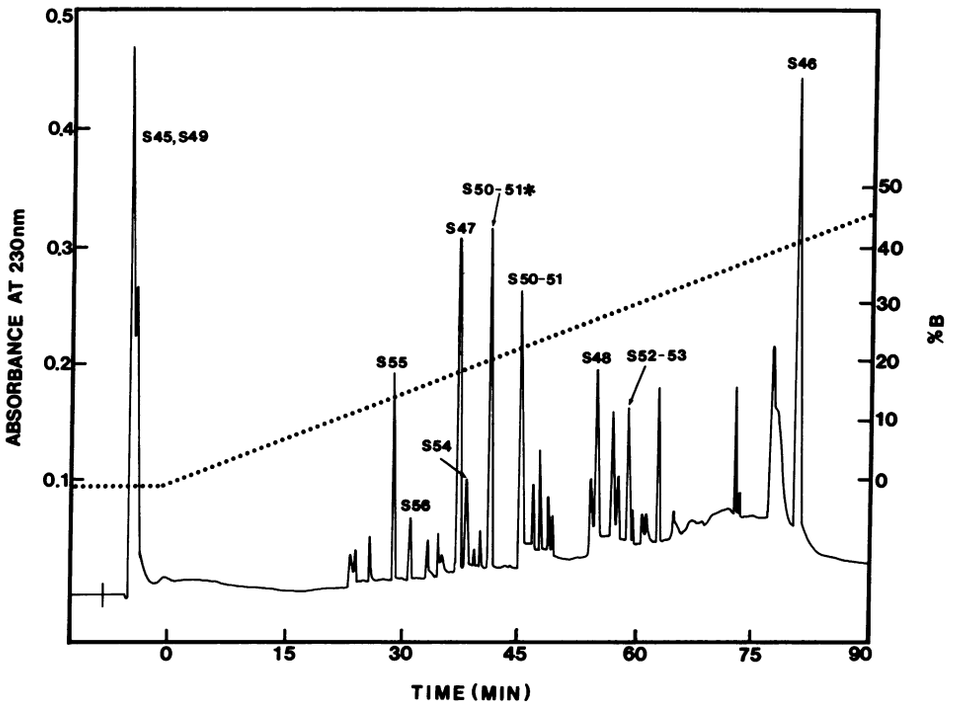

FIG. 5. HPLC elution profile on a Vydac C_{18} column of V8 peptides isolated from CB6 of albumin Vancouver. The conditions are the same as for the tryptic digest of CB6 (Fig. 4) except that the gradient was from 0% to 50% buffer B for 100 min. S50-51* denotes the variant peptide containing the amino acid substitution, and S50-51 is the normal peptide.

Birmingham serum. The chromatogram in reversed-phase HPLC of this digest was essentially identical to that shown in Fig. 3 for the CNBr digest of albumin Vancouver. A tryptic digest of CB6 Birmingham was prepared and submitted to HPLC exactly as for CB6 Vancouver. The HPLC profiles of the tryptic peptides of CB6 from the two alloalbumins were nearly identical. Both showed the appearance of the variant peptide T67*. Automated sequence analysis of T67* from CB6 of albumin Birmingham established the substitution 501 Glu → Lys (Fig. 6). We had insufficient albumin Birmingham to confirm the substitution by making an independent V8 protease digest of this alloalbumin.

Albumin Adana. The serum specimen (C180458) had been collected in 1979 from a single patient in a hospital in Adana, Turkey. We had received the last remaining sample of the Adana serum (<1 ml) from B. Blumberg (Philadelphia) prior to receipt of the Vancouver and Birmingham specimens. In previous work (15, 16) we had confirmed the report of Franklin *et al.* (14) that CB6 was the site of substitution in this alloalbumin, but we had not identified the amino acid exchange. In our earlier study the total albumin from the Adana serum was purified and alkylated and was divided into two aliquots. One aliquot of the purified albumin from the Adana serum was digested with trypsin and was chromatographed by the automated tandem HPLC method (16). Many peptide peaks had been identified by amino acid analysis, especially those from CB6, but no clue to the site of substitution in the Adana albumin was obtained. However, all the peptides were stored for future study.

In the present work a review was made of our previous amino acid analyses of the stored peptides from the tandem HPLC map, especially those that had a retention time about that predicted for T67 and T67*. A small peptide peak was identified that had a stoichiometry close to that expected for T67*. The sample was submitted to automated sequence analysis. An unambiguous sequence for 19 residues was obtained, which was identical to that of T67* given in Fig. 6. This established the substitution 501 Glu → Lys in albumin Adana. The second aliquot of the Adana albumin was digested with CNBr. The digest was analyzed by isoelectric focusing, which again confirmed that the substitution was in CB6. The CB6 fragment was purified by HPLC and digested

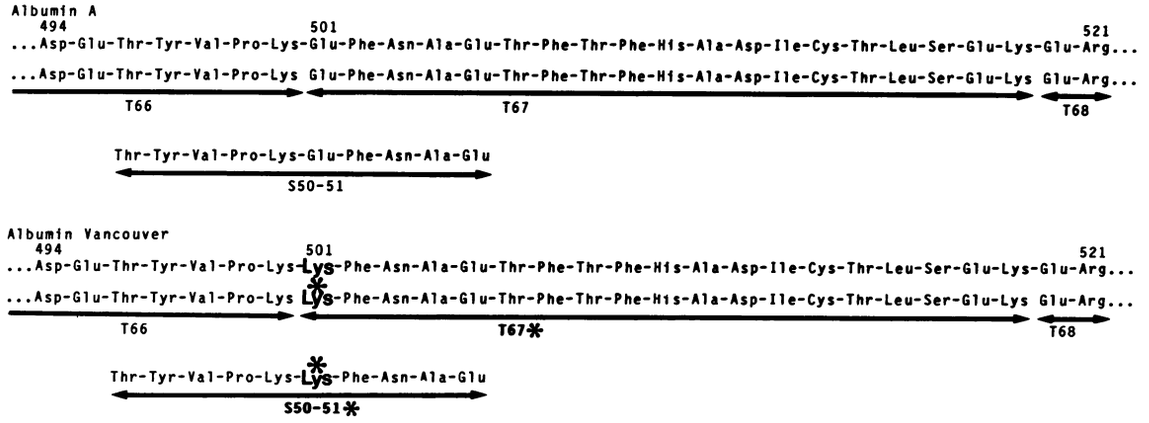

FIG. 6. Amino acid sequence analysis of albumin Vancouver variant peptides. The sequence for the intact protein is given in the upper line of each set. The asterisk marks the substitution 501 Glu → Lys. The text indicates the peptides for which sequence analysis was done for albumins Birmingham and Adana.

with trypsin. In the HPLC profile of the tryptic digest two peaks occurred at the position expected for T67*. Sequence analysis showed that one of these peptides was T67*. This confirmed that the substitution in albumin Adana was 501 Glu → Lys, the same as in albumins Vancouver and Birmingham.

Significance of the Identical Substitution in the Three Alloalbumins. The three alloalbumins have the same amino acid substitution, 501 Glu → Lys. This change gives a net increase in charge of 2+. This accords with their slow mobility at pH 8.6 and other pH values, and it accounts for the fact that they migrate similarly to albumin B and proalbumins. This amino acid exchange accords with the mutation of a single nucleotide base in the codon GAG for position 501 (9) to AAG. In addition to establishing another inherited substitution in the albumin molecule, namely 501 Glu → Lys, these results have significance with respect to criteria for typing albumin variants and for the ethnic and geographic distribution of this and other variants. Each of the three alloalbumins had been described as "a new variant." Although the criteria for distinguishing albumin Adana as a unique variant had not been recorded (14), the report came from a laboratory experienced in electrophoretic typing of genetic variants of albumin and other blood proteins. An experienced laboratory had compared albumins Vancouver and Birmingham to each other by five electrophoretic methods and also for their ability to bind four different dyes (12). Sufficient differences in electrophoretic mobility in several systems and in dye binding were found that the authors concluded albumin Vancouver was a new variant (12). They also concluded it differed from the Asiatic variant called albumin Kashmir.

With respect to the possible differences in dye binding by certain alloalbumins, an interesting question is to what extent such differences may affect the measurement of their concentration relative to albumin A, as estimated by the scanning of electrophoretograms. Albumin Vancouver had earlier been reported to have a unique ratio of the two bands (A, 35%; variant, 65%) (12). However, the fresh specimen we studied had a ratio close to 1:1, which is similar to that reported for most bisalbuminemic sera (6). Deviation from a 1:1 ratio could reflect an inherent difference in the rate of expression of the allelic genes or different rates of degradation of the normal and variant albumins. However, it is obvious that quantitative measures other than dye binding would have to be used to validate such studies.

Ethnic and Geographic Distribution of the 501 Glu → Lys Substitution. Despite the names Vancouver, Birmingham, and Adana—each representing a different continent—the gene for all three albumins originated in Asia. The index individual for albumin Vancouver was born in Fiji, of East Indian ancestry (12). The maternal parents had migrated to Fiji from northern India (Lucknow). The paternal great-grandparents had migrated to Fiji from an unknown part of India but spoke Urdu, which is the official language of Pakistan and is widely used in India. Albumin Birmingham was present in an Indian family from the Jullander district of the Punjab (13). No family data are available for the single individual with albumin Adana. However, this city in southeastern Turkey is on the historic trade routes from the Indian subcontinent. Moreover, Kaur *et al.* (19) concluded that albumins Kashmir and Adana were identical within the limits of resolution of the methods they used, including similarity in the electrophoretic mobilities of CB6 from both albumins.

Despite the difference in dye binding of albumins Birmingham and Kashmir, it is likely that they are identical variants because they have the same electrophoretic mobility in six different media. Also, Kashmir and the Punjab are neighboring regions of northern India. Schell and Blumberg (1) have summarized a number of examples of albumin variants similar to Kashmir that have been found in Indian populations. They concluded that "albumin Kashmir is restricted to families of Indo-Dravidian descent, but it occurs more often than any of the other restricted variants." Indeed, albumins Kashmir and Afghanistan also appear to be identical on the basis of mobility in a series of systems (2). Unfortunately, the civil unrest in this whole region has prevented willing colleagues from providing us with samples to test our hypothesis. Furthermore, this makes it difficult to assign a precedent name for the variant, as is the custom when several are shown to be identical (1). The precedent name is probably albumin Kashmir. However, we propose that for the present this alloalbumin type be designated "albumin 501 Glu → Lys (Birmingham, Vancouver, Adana)." It should be recognized, however, that our results do not exclude the future finding of the same substitution in individuals who do not have north Indian ancestry.

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