

Localization of the amino acid substitution site in a new variant of human serum albumin, albumin Mexico-2

(albumin Mexico-1/variant screening/peptide mapping/Edman degradation)

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Contributed by Baruch S. Blumberg, February 4, 1980

ABSTRACT Using an electrophoretic screening procedure, we have discovered that two species of human serum albumin Mexico occur that are indistinguishable by conventional electrophoretic methods. We suggest that these species be referred to as albumins Mexico-1 and Mexico-2. Isolation and determination of the partial sequence of the cyanogen bromide fragment of albumin Mexico-2 that differs from the corresponding fragment of the common albumin A revealed this variant to arise from at least a glycine/aspartic acid substitution at position 550. This region of the albumin molecule is involved in the binding of the fatty acid, palmitate.

At least two dozen electrophoretic variants of human serum albumin have been reported (1, 2), but only two of them have been characterized with respect to their primary structures: albumin A (the common albumin) and albumin B, a rare variant found mainly in people of European origin (2, 3).

We have previously reported the existence of an albumin variant (albumin Mexico) that possesses a lower electrophoretic mobility than albumin A at pH 8.6 (4). This variant is relatively common in Indians of Mexico and the southwest United States and reaches polymorphic frequencies in the Pima Indians of Arizona and other Indians (5, 6).

The standard methods for analyzing albumin variants are limited to the detection of surface charges. We have developed a more sensitive screening procedure that involves cleavage of the albumin with cyanogen bromide and analysis of the fragments by polyacrylamide gel electrophoresis in the presence of nonionic detergent (7). Using this method, we have found that two species of albumin Mexico exist that are indistinguishable by conventional techniques. We suggest that these species be termed albumins Mexico-1 and Mexico-2. We have isolated a cyanogen bromide fragment of albumin Mexico-2 that differs from the corresponding albumin A fragment and identified the molecular difference by peptide mapping and sequence determination.

MATERIALS AND METHODS

Variant Screening. Albumin was cleaved at the methionine residues by treating 25 μ l of serum with 50 mg of cyanogen bromide in nitrogen-saturated 70% formic acid overnight followed by lyophilization (repeated once from water). The fragments were reduced in 300 μ l of 0.20 M triethanolamine acetate buffer (pH 8.6), containing 8 M urea, 25 mM methyl amine (cyanate trap), 60 mM dithiothreitol, and 10 mM EDTA for 4 hr at 37°C. The peptides were then alkylated to prevent mixed disulfide formation by adding iodoacetamide to a concentration of 130 mM and reacting for 30 min in the dark at

room temperature. The reaction was stopped by addition of excess dithiothreitol. The samples were diluted 1:5 and precipitated by addition of trichloroacetic acid to 20%. The fragments were collected by centrifugation, washed with acetone/0.2% HCl and acetone, and dried under reduced pressure. All solutions contained 1% thiodiglycol during precipitation. Each sample was then dissolved in 300 μ l of loading solution (8 M urea/1% thiodiglycol/20 mM dithiothreitol) and reduced for about 90 min at 37°C before the gels were loaded.

The fragments (load equivalent to about 2 μ l of serum) were resolved on 12% polyacrylamide gels containing 5 M urea, 6 mM Triton X-100, and 5% acetic acid (7). The same gel conditions were also used for isolation of the variant cyanogen bromide fragments in a preparative gel electrophoresis apparatus.

For comparison, conventional 8% polyacrylamide gels were run with native, uncleaved sera and the Tris/EDTA/borate buffer of Peacock *et al.* (8) or 50 mM Tris-HCl (pH 8.6).

Peptide Analysis. The protocol has been described in detail (9). Briefly, purified cyanogen bromide fragments were digested with trypsin in 0.1 M ammonium bicarbonate (pH 8.1) at a 1:25 enzyme-to-substrate ratio. The incubation was made 0.2 mM in tosylphenylalanylchloromethyl ketone (TPCK) to inhibit residual chymotryptic activity. The insoluble tryptic cores were recovered and digested with thermolysin.

Peptides were resolved by microfingerprinting on Avicell coated plates as described (9) except that electrophoresis was for 90 min. Peptides were visualized with fluorescamine, eluted with and hydrolyzed in 6 M HCl, and subjected to amino acid analysis on a Durrum (Palo Alto, CA) D-500 amino acid analyzer.

Edman Degradation. The manual procedure was performed essentially as described by Edman (10) except that the coupling and cyclization times were doubled and 30% pyridine was used as the aqueous phase (in place of 0.1 M HCl) due to the insolubility of the peptide in the latter. The resulting amino acid thiazolinones were converted to free amino acids by hydrolysis in 6 M HCl containing 0.1% SnCl₂ for 8 hr at 150°C (11).

Materials. Sera. Only samples previously classified by cellulose acetate electrophoresis (Tris citrate buffer, pH 5.4) as being heterozygous or homozygous for the albumin Mexico allele were subjected to the new screening procedure. Eight samples were from the San Carlos Apaches of southeastern Arizona; seven of these were heterozygotes for albumin Mexico-1, as were two samples of unspecified origin. One of the Apache samples was an albumin Mexico-2 heterozygote. Seven samples were from the Pima Indians of the Gila River Community in Arizona; five were found to be heterozygotes for albumin Mexico-2 and two were homozygotes. Two of the heterozygotes were the parents of one of the homozygotes.

Reagents. Electrophoresis-grade acrylamide was from Bio-Rad. Ultrapure urea was obtained from Bethesda Research (Rockville, MD). Cyanogen bromide, fluorescamine, thiodiglycol, trifluoroacetic acid, and iodoacetamide were purchased from Pierce. TPCCK-treated trypsin was from Worthington and thermolysin was obtained from Sigma. Dithiothreitol was from Calbiochem. Phenylisothiocyanate was from Beckman. Pyridine and ethyl acetate used for sequence determination were from Burdick and Jackson (Muskegon, MI).

RESULTS

Fig. 1 compares the electrophoretic mobilities in an 8% polyacrylamide gel of intact, undenatured sera containing albumin phenotypes A/Mexico-1, A/Mexico-2, Mexico-2/Mexico-2, A/B, and A/A. The heterozygotic albumins A/Mexico-1 and A/Mexico-2 possess two bands of equal intensity, one migrating identically to the common albumin (A/A) and one migrating more slowly. The albumin Mexico-2 homozygote has a single band that comigrates with the slow component of both types of albumin Mexico heterozygote. Albumin A/B, shown for comparison, again has one band comigrating with albumin A/A and another band migrating even more slowly than the albumins Mexico.

In order to differentiate between variants that migrate similarly in conventional electrophoretic systems but that may arise from amino acid substitutions in different regions of the albumin molecule, we devised a screening strategy. A small quantity of whole serum was treated with cyanogen bromide to cleave at each of the six methionine residues of albumin. The fragments were then reduced, alkylated, and resolved on polyacrylamide gels containing urea, acetic acid, and Triton X-100. In control experiments (not shown) we found that other serum proteins do not interfere with these analyses. Purified, defatted albumins (3, 12) produce the same pattern. Fig. 2 shows the results obtained by treating the same samples shown in Fig. 1 in this manner. The albumin Mexico-2 heterozygote shows two bands of about equal intensity in the vicinity of fragment CNBr VII (residues 549–585), one comigrating with the normal fragment and one possessing a greater mobility (fragment CNBr VIIM2). The homozygote of albumin Mexico-2 exhibits only the faster migrating variant fragment. These results are indicative of a substitution site in fragment CNBr VII of the variant molecule. Heterozygotic albumin B, shown for comparison, possesses a band in the normal position of fragment CNBr VII and a very fast migrating band, CNBr VIIB, consistent with its postulated lysine/glutamic acid substitution at position 570 (3). The cyanogen bromide fragments of albumin Mexico-1 migrate identically to those of albumin A under these conditions. Thus, the molecular difference between albumins Mexico-1 and A, as indicated by the differences in electrophoretic mobilities of the native proteins (Fig. 1), could not be identified by this system.

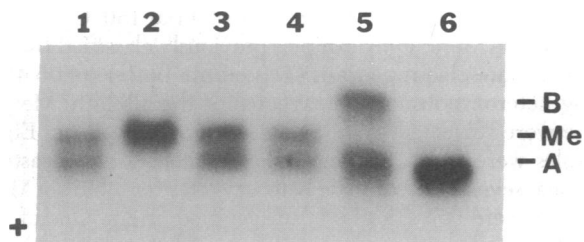


FIG. 1. Electrophoretic mobilities of native variants. Track 1, A/Mexico-1; track 2, Mexico-2/Mexico-2; tracks 3 and 4, A/Mexico-2 (parents of individual in track 2); track 5, A/B; track 6, A/A. Gel was 8% polyacrylamide containing 50 mM Tris-HCl (pH 8.6). Stain was Coomassie brilliant blue R-250.

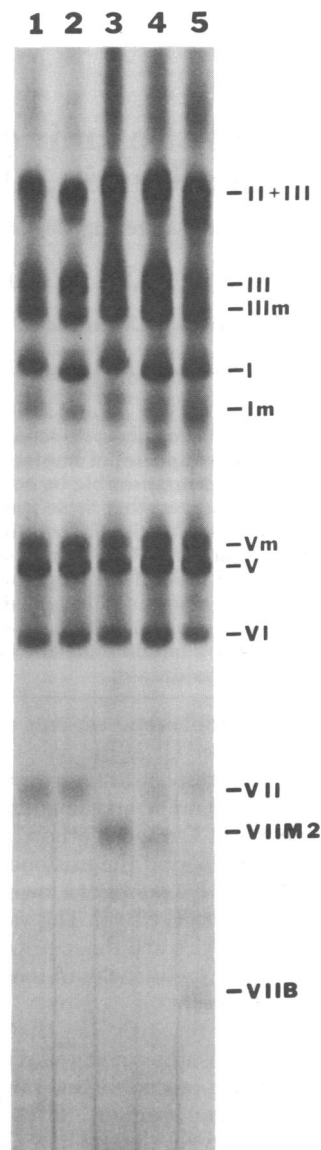


FIG. 2. Resolution of alkylated cyanogen bromide fragments of albumin variants. Gel 1, A/A; gel 2, A/Mexico-1; gel 3, Mexico-2/Mexico-2; gel 4, A/Mexico-2 (parent of individual in gel 2); gel 5, A/B. Gels were 12% polyacrylamide containing 5 M urea, 6 mM Triton X-100, and 5% acetic acid. Stain was Amido black 10 B. Cyanogen bromide fragments were originally identified on the basis of molecular weight and amino acid composition and are numbered by Roman numerals in accordance with their order in the known sequence of albumin A. M2 and B refer to variant peptides found only in albumins Mexico-2 and B, respectively; m refers to modifications probably arising from oxidation of unreacted cysteine or lysine alkylation by iodoacetamide. Fragments CNBr II and IV have not been identified.

In order to localize the amino acid substitution site within fragment CNBr VII of albumin Mexico-2, it was necessary to isolate this fragment in a homogeneous state. This was accomplished by preparative polyacrylamide gel electrophoresis in the presence of urea, acetic acid, and Triton X-100. Fig. 3A shows the elution profile of a preparative gel run obtained from 400 μ l of cleaved and alkylated whole serum from an individual homozygous for albumin Mexico-2. Fragment CNBr VIIM2 eluted first, followed by fragments CNBr VI, V, I, and III and uncleaved CNBr II and III. Fig. 3B shows an analytical gel run of the fragments purified by preparative electrophoresis.

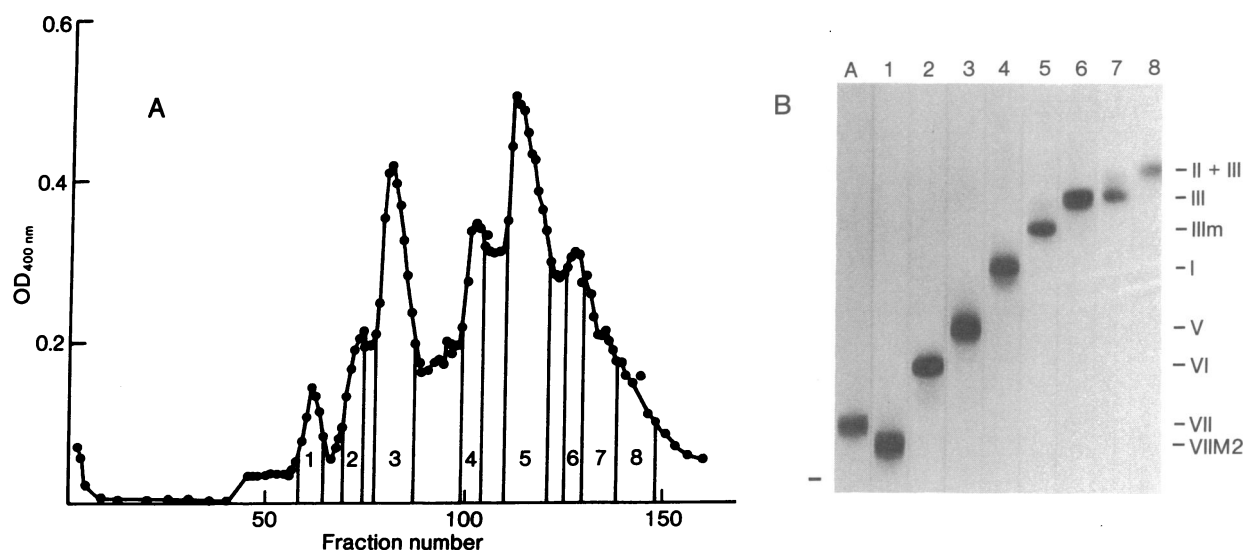


FIG. 3. (A) Purification of CNBr fragments from albumin Mexico-2 by preparative polyacrylamide gel electrophoresis. Gel conditions were as for Fig. 2. Fractions were precipitated by addition of trichloroacetic acid to 20%, pooled as indicated by the vertical lines, and collected by centrifugation followed by washes with acetone/0.2% HCl and acetone. (B) Analytical gels of purified CNBr fragments from albumins Mexico-2/Mexico-2 and A/Naskapi. Gel numbers refer to the fractions indicated in A and gel conditions were the same as for Fig. 2. Gel A is the purified fragment CNBr VII from an albumin A/Naskapi heterozygote.

Fragment CNBr VIIM2 as well as fragments CNBr VI, V, and I appear homogeneous by this method. Also shown is the normally migrating CNBr VII fragment (isolated from an albumin Naskapi heterozygote), which is also homogeneous.

The purified fragments CNBr VII and VIIM2 were digested with trypsin. Peptides that precipitated out during tryptic digestion were recovered and digested with thermolysin. Resolution of the peptides was then accomplished by microfingerprinting. In Fig. 4 the tryptic peptide maps of fragments CNBr VII and VIIM2 are compared. The major difference is the absence of peptide T1 in albumin Mexico-2. Upon elution and amino acid analysis of the peptides, it was found that peptide T1 from CNBr VII was homologous in composition with residues 549-557 in albumin A, which has the sequence Asp-Asp-Phe-Ala-Ala-Phe-Val-Glu-Lys (13). Peptide T1a from CNBr VIIM2 was also homologous in composition with residues

549-557, but lacked one aspartic acid residue and contained one glycine residue (Table 1). The remainder of the tryptic peptides of albumin Mexico-2 were homologous in composition to that expected from the known sequence of albumin A (13). This was in some cases also confirmed by peptide mapping and analysis of the thermolysin peptides (data not shown). The spot migrating just below peptide T5 in CNBr VII appears to be a mixture of derivatives of peptides T1 and T5. Thus, albumin Mexico-2 appeared to arise from the substitution of a glycine for an aspartic acid residue at position 549 or 550.

Due to the presence of two aspartic acid residues in tandem at the NH₂ terminus of fragment CNBr VII, it was necessary to perform two cycles of the Edman degradation on this fragment to determine which aspartic acid residue was substituted. The first cycle released residues in the ratios: aspartic acid, 1.00; glycine, 0.16; and leucine, 0.14. Cycle two produced glycine,

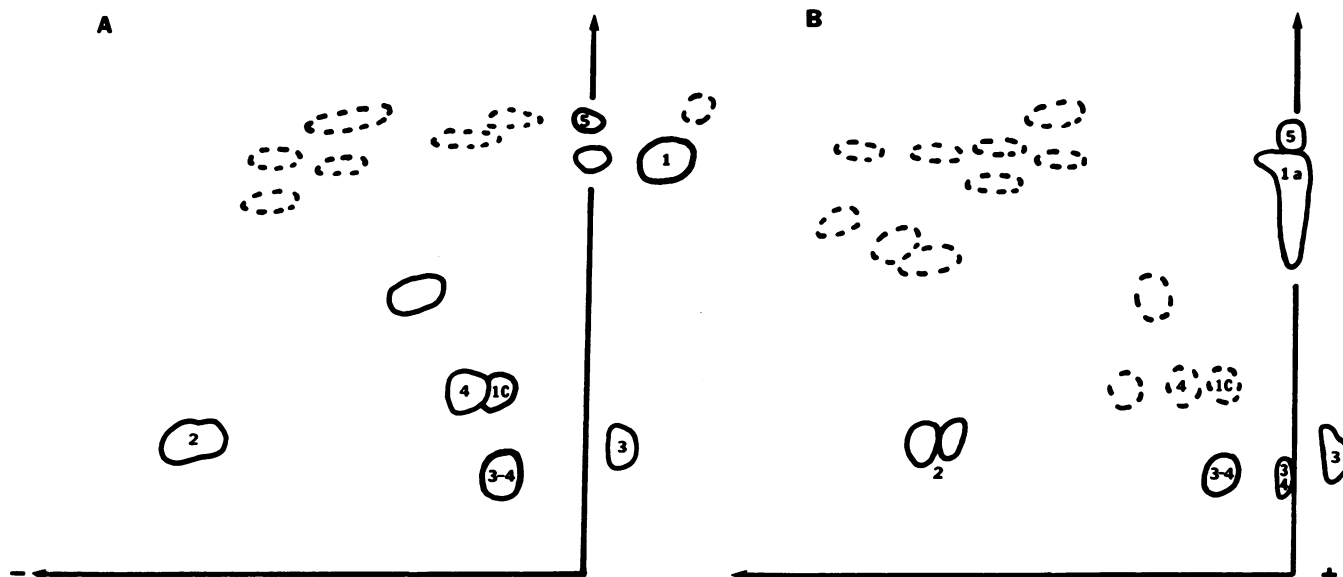


FIG. 4. Tryptic peptide maps of fragments CNBr VII and VIIM2. (A) CNBr VII; (B) CNBr VIIM2. Peptides are numbered in accordance with their order in the known sequence of albumin A. Peptide T1a contains the substitution unique to albumin Mexico-2. The doublet seen in spot 2 may result from cysteine oxidation. Abscissa, electrophoretic dimension, pH 4.7; ordinate, chromatographic dimension.

Table 1. Amino acid composition of peptides T1 and T1a from fragments CNBr VII and VIIM2, respectively

Amino acid	Residues per mole		
	Albumin A*	Peptide T1†	Peptide T1a†
Asp	2.0	1.9	1.0
Thr	0.0	0.0	0.1
Ser	0.0	0.0	0.2
Glu	1.0	1.1	1.3
Pro	0.0	0.0	0.0
Gly	0.0	0.0	1.1
Ala	2.0	2.0	2.2
Val	1.0	1.0	1.0
Met	0.0	0.0	0.0
Ile	0.0	0.0	0.0
Leu	0.0	0.0	0.2
Tyr	0.0	0.0	0.0
Phe	2.0	2.0	1.8
His	0.0	0.0	0.0
Lys	1.0	1.0	1.0
Arg	0.0	0.0	0.1

* From J. R. Brown (13).

† Uncorrected for hydrolysis loss.

1.0; aspartic acid, 0.22; and alanine, 0.18. It is thus aspartic acid 550 that has been replaced by glycine in albumin Mexico-2.

DISCUSSION

The results reported here indicate that albumin Mexico-2 is a new variant, differing from albumin A by the substitution of glycine for aspartic acid at position 550. This loss of one negative charge is consistent with the lower anodic electrophoretic mobility of the native protein at pH 8.6. It is of interest that fragment CNBr VII is also the site of the only other known substitution, the glutamic acid \rightarrow lysine change occurring at position 570 in albumins Oliphant and Ann Arbor [which are electrophoretically indistinguishable from albumin B (3)]. Our finding of a more positively charged fragment CNBr VII in albumin B supports the notion that these three variants are the same protein (3, 14). We have found electrophoretic differences in cyanogen bromide fragments I, V, and VI in other albumin variants (not shown).

The literature on human serum albumin reports over 80 "named variants," but only about two dozen distinct electrophoretic mobilities are detectable at three different pH values on starch gel electrophoresis (1). The use of Triton electrophoresis of cyanogen bromide fragments may alleviate this confusion in two ways. First, it may distinguish between variants that comigrate on conventional gels but have different primary structures by localizing substitution sites to particular regions of the molecule. Second, the acidic, denaturing, and reducing conditions under which the gels are run eliminate many artifacts expected in serum samples suffering from harsh treatment (aggregation, deamidation, oxidation, etc.). Artifacts of this nature may account for some of the published reports of albumin variants.

A further advantage of the Triton method is its ability to resolve neutral substitutions as well as charged ones by virtue of differential Triton binding to regions of proteins possessing high helical probability (15). We have resolved several histone variants differing only in the substitution of one or two neutral amino acids by this method (16, 17). This allows the possible detection of a new class of albumin variants that could not be recognized on conventional systems.

Some named variants (including albumin Mexico-1) exhibit the same pattern as the common albumin A in our system. It

is possible that in some cases this is because the "variant" arises from some of the artifacts mentioned above. In other cases, this could be due to neutral/acidic residue substitutions which would not be detected at pH 3.0 unless the Triton-binding properties were altered. (For albumin Mexico-1, the latter is more likely.) It is conceivable that albumin Mexico-1 also possesses the glycine/aspartic acid substitution found in Mexico-2 but that it is obscured by a second substitution.

Incomplete cleavage by cyanogen bromide is responsible for the appearance of a major band migrating above fragment CNBr III (Fig. 2). This band has a molecular weight of about 23,000 on sodium dodecyl sulfate gels and probably arises from the known partial cleavage of the methionine-cysteine bond at position 123 [fragments CNBr II + III (18)].

Screening for variants of albumin (or other macromolecules) by the method described here could be of great aid to future anthropological studies. The origins and affinities of certain groups of American Indians have been studied through the distributions of albumins Naskapi and Mexico (2). These distributions are consistent with linguistic and archaeological evidence of population movements and contacts (2). However, albumin studies can be misleading in cases where two variants comigrate in conventional electrophoresis systems (such as cellulose acetate or starch gel) but are not molecularly identical. The two Mexico variants illustrate this point. Albumin Mexico was first reported in 1967 (4) and since that time has been found in American Indian groups from the southwestern United States of Guatemala (2). Because all of these surveys have used conventional electrophoresis techniques, it is impossible to determine what proportion of samples classified as Mexico is Mexico-1 or Mexico-2. Triton electrophoresis of cyanogen bromide fragments of eight serum samples from the San Carlos Apaches and two samples of unspecified origin that had been classified as heterozygous for albumin Mexico showed only one of them to have the fast migrating CNBr VII fragment characteristic of albumin Mexico-2. Thus far, the variant has been observed in seven Pima Indians—the homozygous female and her heterozygous parents reported here and in an unrelated male homozygote and three unrelated heterozygotes. Additional surveys will be necessary to establish the distributions and gene frequencies of both albumins Mexico-1 and Mexico-2. Such studies may shed additional light on the population relationships of Indian groups in the Southwest and Mexico.

Because albumin is the major carrier of many physiologically active substances and drugs in the blood, it is possible that some variants have different functional properties. This notion is supported by our finding that heterozygous albumins Mexico and Naskapi exhibit a small but significant decrease in binding affinity for the drug warfarin (19). The aspartic acid/glycine substitution at position 550 in albumin Mexico-2 occurs in a part of an α -helical region that is sensitive to small changes in the helix nucleation parameter. According to the predictive rules of Chou and Fasman (20), the substitution of helix-indifferent aspartic acid by the strong helix breaker, glycine, is sufficient to cause a gap in the helix of about four residues. Because this region of fragment CNBr VII is part of a fragment (loops 7–9) that appears to be involved in palmitate binding (21), a change in helical content may influence the binding capacity of this variant for fatty acids. This hypothesis can be tested.

Single amino acid substitutions in hemoglobin variants produce several disease states due to altered functional properties of the molecule (22), and it is possible that albumin variants play a role in the development of disease in American Indian populations where variants are common. Both albumin Mexico and diabetes occur with high frequencies in several American Indian tribes of the Southwest (5, 6, 23). A study on the co-oc-

currence of albumin variants and diabetes in individual persons may provide interesting results.

We thank Drs. Peter Bennett, David Pettite, and Lowell Weitkamp for providing some of the serum samples used in this study and Ms. Judy Mamas for technical assistance. This work was supported in part by Grant RR-05539 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health, by Grants CA-06551, CA-06927, and CA-15135 from the National Institutes of Health, and by an appropriation from the Commonwealth of Pennsylvania.

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