The Biochemical and Genetic Basis for the Microheterogeneity of Human R-Type Vitamin B_{12} Binding Proteins

By Soo Young Yang, Peter S. Coleman, and Bo Dupont

R-type vitamin B_{12} binding proteins (R proteins) from human granulocytes, erythrocytes, plasma, and other body fluids were characterized by isoprotein banding patterns on autoradiograms after resolution via thin-layer polyacrylamide isoelectric focusing (IEF) gel electrophoresis. R proteins obtained from various tissue sources in a given individual show tissue-specific electrophoretic patterns. The desialated R proteins obtained following in vitro treatment with neuraminidase are, however, the same for any given individual and do not show tissue specificity. The differences seen in native R proteins (i.e., transcobalamin I, III, and others) obtained from different tissues are due to variations only in the sialic acid content. Granulocytes from patients with chronic myelogenous leukemia (CML) contain both TC I and TC III, and these R proteins can be released in vitro by lithium stimulation. Normal granulocytes contain only TC III. Differences in desialated R proteins from individual to individual are due to a genetic polymorphism controlled by a single genetic locus (designated TCR) with two alleles, 1 and 2, which are found to be codominantly expressed in heterozygous individuals. The allelic variants of the desialated R proteins found in different blood cells and body fluids are controlled by only one genetic locus.

VITAMIN B_{12} (COBALAMIN) is rarely found in the free state in the body, but is usually attached to specific proteins. In the review by Allen, vitamin B_{12} binding proteins were classified according to their structural and functional properties: intrinsic factor (IF) in gastric juice mediates the absorption of the vitamin B_{12} from the gastrointestinal tract; transcobalamin II (TC II) occurs only in the plasma and facilitates transport of the vitamin from blood to various tissues; and finally, the R proteins occur in most body fluids, including the plasma, and can also be found in some cells. The plasma contains two R proteins known as transcobalamin I (TC I) and transcobalamin III (TC III). The term “R protein” was originally devised by Simons and Gräsbeck to distinguish vitamin B_{12} binding proteins from IF in human gastric juice. It was termed protein “R” because of its rapid electrophoretic mobility. This term is now used to denote cobalamin-binding proteins from various sources such as saliva, leukocytes, milk, plasma, and amniotic fluids. These proteins are immunologically identical even though they may differ in molecular weight, electrophoretic mobility, and carbohydrate content. Because many features are common among the R proteins from various tissues, Stenman introduced the term “cobalophilin” for the R proteins.

Two fractions of R proteins in plasma can be distinguished by DEAE cellulose chromatography. One binds strongly to DEAE cellulose and has been termed TC I. The other, which binds only weakly to DEAE, is called TC III. TC I is mostly saturated with endogenous B_{12} in normal plasma. In vivo, TC III normally is not found to any significant extent in plasma (the half-life in vivo is less than 5 min). But in vitro, unsaturated TC III is released from granulocytes, and this release can be inhibited by fluoride ion. The biochemical and genetic characteristics of TC II have recently been resolved. Based on immunologic and biochemical similarities between R proteins from different tissues, it has been speculated that these proteins have a common phylogenetic origin and could be controlled by one genetic locus. Recently, Azen and Denniston have described a genetic polymorphism of R proteins of saliva. These authors demonstrated that the banding patterns of neuraminidase-treated samples from saliva, tears, milk, and leukocytes in isoelectric focusing electrophoresis were similar, but not identical, and could be explained by an autosomal inheritance of two codominant alleles of one genetic locus. These previous studies did not make it possible to explain the relationship between TC I, TC III, and other R proteins and to explain the biochemical nature of the microheterogeneity of these proteins.

The present study demonstrates that TC I, TC III, and other native R proteins found in different normal tissues have different banding patterns on autoradiograms of isoelectric focusing (IEF) gels. These differences are tissue specific and caused by different degrees of sialation. Our study also demonstrated that leukemic granulocytes contain both TC I and TC III and that both these proteins can be released in vitro from the leukemic cells by lithium stimulation. Normal granulocytes contain only TC III. Desialated R

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proteins, however, all have identical IEF banding patterns for a given individual, and this uniform IEF pattern is independent of the tissue of origin. Each individual's R protein banding pattern is determined by a single autosomal genetic locus with two codominantly expressed alleles.

MATERIALS AND METHODS

Plasma and Serum Collection

Plasma and serum from venous blood were drawn into glass tubes (Vacutainer tubes, Becton-Dickinson, Rutherford, N.J.). Serum samples were obtained by allowing clot formation at room temperature for 30 min, followed by retraction of the coagel after 1 hr at 4°C. The serum was separated by centrifugation at 4°C, and divided into aliquots that were stored at -70°C until used. Plasma was obtained by collecting blood samples in Vacutainer tubes (Becton-Dickinson) containing 10.5 mg of Na2EDTA. Plasma was separated immediately after collection by centrifugation at 4°C, divided into aliquots, and stored at -70°C.

Erythrocyte Lysates

One milliliter of packed red blood cells was obtained from EDTA or anticoagulant-treated blood. The red blood cells were washed twice with 0.9% NaCl and lysed by addition of 1 ml of distilled water. The red cell membrane lysate was extracted with 1 ml CCl4, and the suspension was vortexed vigorously and then centrifuged for 10 min at 600 g. The aqueous supernatant was stored at -70°C.

Saliva

Human saliva was collected from healthy individuals. The samples were placed on ice within 30 min of collection. The saliva was then centrifuged at 600 g for 20 min at 4°C and the supernatant was stored at -70°C.

Amniotic Fluid (AF)

Human amniotic fluid samples were obtained from diagnostic amniocenteses.

Isolation of Granulocytes

Granulocytes were isolated as described by Willoughby et al.23 Greater than 95% of the leukocytes were classified as granulocytes. Their viability was consistently greater than 98% as judged by the dye exclusion of trypan blue. Less than 0.2% residual erythrocytes were found in the granulocyte suspension. The isolated granulocytes were suspended in Hanks’ solution containing 0.1% CaCl2. The release of R proteins from isolated granulocytes was induced by lithium treatment as described by Scott et al.24 Briefly, LiCI (0.2 M) was added to the isolated granulocyte suspension (20 x 10⁶/ml) to yield a final concentration of 0.01 M of LiCl. The mixture was left at room temperature overnight, and the supernatant was collected after centrifugation and stored at -70°C.

Release of R Proteins From Isolated Granulocytes

The release of R proteins from isolated granulocytes was induced by lithium treatment as described by Scott et al.24 Briefly, LiCl (0.2 M) was added to the isolated granulocyte suspension (20 x 10⁶/ml) to yield a final concentration of 0.01 M of LiCl. The mixture was left at room temperature overnight, and the supernatant was collected after centrifugation and stored at -70°C.

Removal of Sialic Acids From R Proteins

Samples of R proteins were mixed with an equal volume of acetate buffer (0.05 M sodium acetate, pH 5.5, containing 0.15 M NaCl, 0.1% CaCl₂) and neuraminidase (500 U/ml, Behring Diagnostics, Somerville, N.J.). The mixture was incubated at 37°C for 16–24 hr for all experiments unless otherwise stated. Dialysis of the reaction mixture against acetate buffer did not increase significantly the removal of sialic acid and therefore such dialysis was not used as the standard method for neuraminidase treatment of R protein.

Consistent and reproducible results of isoelectric banding patterns following neuraminidase treatment were obtained when R proteins were labeled with 125I vitamin B₁₂ before the enzyme treatment. The banding patterns of R proteins labeled after desialylation by neuraminidase were not as apparent as R proteins labeled before treatment with neuraminidase. The loss of affinity for the radiolabeled ligands following the treatment of neuraminidase was much greater in samples of saliva and granulocytes as compared with erythrocytes, amniotic fluid, and tears. Both acidic and basic components of desialated R proteins, however, appear to have almost the same degree of affinity for the vitamin B₁₂ ligands as the native forms of R proteins. The evidence for B₁₂ affinity of desialated R isoproteins can be obtained from the observation that fresh saliva contains a significant amount of native desialated isoproteins (Fig. 1).
The banding patterns of the isoproteins with pI > 4.5 occurring in fresh saliva can be identified as clearly as the sialated isoproteins (pI < 4.5). Similar observations were made for the affinity of R proteins towards radioisotopes. The R proteins, once labeled with ¹⁹⁷⁷Co vitamin B₁₂, did not lose the ligands for long periods of time at 4°C. The radiolabeled R proteins isolated via DEAE or Sephadex G-150 column chromatography could be identified on the IEF gel after storage for 3 mo at 4°C, although basic shifts of bands were observed due to the spontaneous release of sialic acid from the more acidic R proteins (data not shown). R proteins from saliva and granulocytes, however, do lose their affinity for radioligands relatively quickly. The IEF bands could not be detected when R proteins from granulocytes and saliva were labeled with ¹⁹⁷⁷Co-B₁₂ after incubation at 37°C for 16 hr or after storage at 4°C for 2-3 wk. R proteins from erythrocytes were the most stable of all R proteins tested with respect to their affinity for ¹⁹⁷⁷Co-vitamin B₁₂.

**Labeling Vitamin B₁₂ Binding Proteins**

¹⁹⁷⁷Co-cyanocobalamin (specific activity 100-300 μCi/μg, 0.454 μg/ml, Amersham, Arlington, Ill.) was diluted in phosphate-buffered saline (pH 7.2) to final concentration of 4.54 × 10⁴ pg/ml. Samples of R proteins were mixed with an equal volume of ¹⁹⁷⁷Co-B₁₂ solution and incubated at 37°C for 15 min. This radiolabeled mixture was used for IEF gel electrophoresis.

**Analytical Thin-Layer Isoelectric Focusing Gel (IEF) Electrophoresis**

Analytical thin-layer isoelectric focusing in polyacrylamide gels was accomplished by modification of the method described by Karlsson et al. Polyacrylamide gels were made from an acrylamide stock solution containing 29.1% (w/v) acrylamide and 9.9% (w/v) N,N'-methylenebisacrylamide (Bio Rad Laboratories, Rockville Center, N.Y.). The gel contained a final concentration of 5% acrylamide, 5% glycerol, 1.5% taurin (w/v), Eastman Co., Rochester, N.Y.) and 2% (w/v) SERVALYTE (Accurate Chemical Scientific Corp., Hicksville, N.Y.) yielding a pH range of 2-8 over the length of the gel.

Samples (10 μl) (body fluids, cell lysates, etc.) were applied to filter paper (Whatman no. 1, England) strips 0.5 × 1 cm, aligned with the electric field, and placed close to the cathode. Electrofocusing was performed on an LKB 2117 Multiphor apparatus for 3 hr at 5°C. The voltage was slowly increased from 200 V to 1000 V during the 3-hr electrofocusing. At the beginning of the electrofocusing, the current was approximately 25mA and decreased during the run to 10 mA. Following electrophoresis, the gel was vacuum dried and exposed to x-ray film (Kodak, Eastman, Rochester, N.Y. or Cronex, Dupont Co., Minneapolis, Minn.) at −70°C for at least 7 days.

The pH gradient across the gel was determined by slicing the dried gel (0.5 × 10 cm), and eluting each slice with double distilled water (1 ml) for 24 hr, and the pH values of the aqueous eluants were measured directly.

**DEAE Cellulose Chromatography for Separating TC I and TC III**

Chromatography was performed as described by Burger et al. at 4°C on a column (1 × 20 cm) packed with DEAE cellulose (DE-52), Whatman, England). The column was equilibrated with 0.02 M potassium phosphate, pH 7.5. The column was eluted with a linear phosphate gradient in which the mixing solution contained 50 ml of equilibrating buffer and the reservoir contained 50 ml of 0.3 M KH₂PO₄, pH 4.5.

**Preparation of Antiserum**

Antiserum to human saliva was prepared by inoculating a rabbit with a mixture containing equal volumes of fresh single donor saliva (which had been previously centrifuged and filtered on 0.2 μm pore filters; Gelman, Ann Arbor, Mich.) together with complete Freund’s adjuvant. Rabbits received a total of 4 intracutaneous injections of adjuvant mixture (1 ml) at 10-day intervals, and the antibody was tested using immunodiffusion plates against human saliva.

**Immunodiffusion**

Immunodiffusion tests were performed for ¹⁷⁷⁷Co-B₁₂-saturated vitamin B₁₂ binding proteins against the antiserum to human saliva on 2% agarose gels in 0.05 M Veronal buffer, pH 8.6. After 24 hr, excess free ¹⁷⁷⁷Co-B₁₂ within the diffusion gel was removed by washing with normal saline for 2 days, and the gel was then dried. The radioactive precipitin lines were demonstrated by autoradiography.

**Population and Family Studies**

Samples (granulocytes, erythrocyte lysates, or saliva) from unrelated individuals in different populations were used in this study. Paternity in the families studied was corroborated in all cases by HLA typing and/or by red cell antigen typing.

**Statistical Analysis**

The genetic basis for the polymorphic variants of R protein was tested using the Hardy-Weinberg equation. The validity of the Hardy-Weinberg equation for the proposed hypothesis was tested using the chi-square (χ²) test. The allelic segregation of R protein variants was studied in randomly selected families, and the analysis was applied to the distribution of genotypes among the siblings.

**Results**

**IEF Gel Electrophoresis of Native Vitamin B₁₂ Binding Proteins**

Figure 1 shows the autoradiogram of isoelectric focusing (IEF) banding patterns of R proteins from different tissue sources. The R proteins of granulocytes and erythrocytes demonstrate very similar banding positions within the pH range of 2.5-4. Plasma sample of a patient with chronic myelogenous leukemia (CML) gave a very strong band at pH 2.3. There were no neuraminidase-resistant B₁₂ binding proteins observed on IEF gels in the plasma samples obtained from CML patients and normal individuals. The B₁₂⁻unsaturated TC II, which is present in a considerable amount in the plasma, could be demonstrated in the same samples by autoradiogam following 7%-9% acrylamide gel electrophoresis as described by Yang et al. (data not shown). The R proteins from saliva revealed considerably more isoprotein bands across a much wider pH range than R proteins from other tissues and body fluids, and the isoelectric focusing patterns of saliva R proteins varied considerably depending on when sample collection occurred and the quantity collected. For example, saliva samples obtained after meals or late in the day or samples taken in large quantities at one time (more than 1 ml) gave
no isoprotein at pH 2.3. There were, however, bands of increased intensity towards the higher pH range of the gel with samples obtained under the above conditions. Vitamin B$_{12}$ binding proteins in amniotic fluid (AF) obtained at early pregnancy (prior to 19 wk of gestation) were quite different from the R proteins described above and displayed several isoprotein bands in the pH range 5.16–5.75. In contrast, the vitamin B$_{12}$ binding proteins of AF obtained from pregnancy later than 19 wk of gestation yielded an unresolved pattern that was very similar to the pattern obtained with tears. The banding patterns of the native R proteins were tissue specific (data not shown).

**TC I and TC III**

Figure 2 shows the elution profiles of TC I and TC III released in vitro from granulocytes from a patient with CML using a linear gradient system on a DEAE column. Two peaks of R proteins were observed (solid line Fig. 2). One peak corresponded to TC III and the other peak corresponded to TC I. The height of the TC I peak is much greater in this sample than the peak of TC III. The binding of endogenous vitamin B$_{12}$ to the transcobalamin occurs extracellularly.$^{1,14}$ Since the vitamin B$_{12}$-unsaturated TC I and TC III was obtained in vitro from isolated granulocytes, the height of the peaks may reflect the total amounts of TC I and TC III. The TC I peak is not observed in the R proteins released in vitro from normal granulocytes. The TC III standard shown in Fig. 2 was obtained by normal granulocyte stimulation in vitro with lithium chloride. The TC I standard was isolated from the plasma of a patient with CML by DEAE cellulose chromatography using a discontinuous gradient system.$^{7,25}$

The TC I and TC III protein obtained from granulocytes of patients with CML can also be identified by autoradiography of IEF gels. Figure 3 shows the isoprotein patterns of R proteins from normal granulocytes and the R proteins obtained from both plasma and isolated granulocytes of patients with CML. The R proteins from granulocytes of these patients contained both TC I (focused at pH 2.3) and TC III (focused at pH 2.5–4), while the normal granulocytes contain only TC III. Plasma from patients with CML always contain vitamin B$_{12}$-unsaturated TC I and sometimes TC III. Normal plasma contains a small fraction of B$_{12}$-unsaturated TC I and trace amounts of TC III.$^{26,27}$ These studies indicate that the B$_{12}$-unsaturated TC I occurring in plasma of CML patients originates from the CML granulocytes, which have increased sialation and secretion of R proteins.

**Reaction of R Proteins With Anti-human Saliva Antibody**

Antiserum against R proteins was prepared in rabbits (see Materials and Methods). This antiserum was found to give only one precipitin line on immunodiffusion plates analyzed by autoradiography after addition of $^{57}$Co-vitamin B$_{12}$-labeled samples from saliva, lysates from erythrocytes, granulocytes, tears, plasma from patients with CML, or samples of AF collected in late pregnancy. No precipitin line was observed with EDTA-treated normal plasma. It appears that autoradiography of the immunodiffusion reactions may not be sensitive enough to reveal the small amounts of unsaturated TC I and TC III in EDTA-treated normal plasma. The precipitin line of each of these different samples showed reactions of identity with each other,
indicating that the R proteins in all samples were immunologically indistinguishable (Fig. 4).

**IEF Banding Patterns of Neuraminidase-Treated R Proteins**

Neuraminidase treatment of R proteins obtained from blood cells and body fluids revealed dramatic changes in the patterns of bands observed by isoelectric focusing and autoradiography. The banding patterns of vitamin B₁₂ binding proteins following neuraminidase treatment were found to be identical in all samples from the same individual regardless of the tissue of origin of the R proteins. Figure 5 demonstrates the banding patterns of the R proteins following neuraminidase treatment of samples of blood cells, saliva, and tears obtained from the same individual (the plasma sample is, however, from an unrelated patient with CML). Two major components of the R protein with isoelectric point (pI) at 4.91 and 5.09 were observed when the sialic acids had been removed. It was noted that the component at pI 4.91 could be further desialated to give rise to the form at pI 5.09. This result could be obtained either by treatment of the sample with higher concentrations of neuraminidase and/or by means of longer incubation periods. These more drastic incubation conditions, however, resulted in the progressive disassociation of the radiolabeled ligands from the R proteins, and the isoproteins of R proteins therefore could not be detected. While the R protein band with pI 5.09 probably is completely desialated, the band with pI 4.91 may contain at least one neuraminidase-resistant sialic acid residue per molecule. The relative resistance of the isoprotein (pI 4.91) to neuraminidase may be due to an R protein conformation that limits the accessibility of neuraminidase to this remaining sialic acid residue.

**Phenotypic Variations of R Protein Banding Patterns in the Random Population**

Isoelectric focusing of R proteins with samples from different individuals after neuraminidase treatment resulted in three different banding patterns. These three R protein variants could be identified in samples from granulocytes, erythrocytes, and saliva, and the different R protein phenotypes are shown in Fig. 6. Two distinct bands at pI 5.09 (the main desialated protein) and 4.91 (the neuraminidase-resistant R protein) are seen in samples from certain individuals. These two bands were designated according to the nomenclature used for saliva R protein by Azen and Denniston. The genetic locus coding for the R protein was tentatively designated as TCR (transcobalamin R protein). The most commonly observed R protein phenotype in all populations tested was designated as TCR 1. Another set of bands slightly more basic than...
Table 1. R Protein Phenotypes in Different Tissues of Single Individuals

<table>
<thead>
<tr>
<th>No. of Individuals Tested</th>
<th>Phenotypes</th>
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<tr>
<td></td>
<td>Granulocytes</td>
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<tr>
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</tr>
<tr>
<td>9</td>
<td>1-2</td>
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<tr>
<td>8</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>NT</td>
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NT, not tested.

Phenotypes 1 and 2 are the two allelic forms of the desialated R proteins described in the text.

Fig. 6. Phenotypes of R proteins from random donors of granulocytes (Gr), saliva (Sal), and erythrocytes (RBC). The three different phenotypes are designated by the symbols 1, 2, and 1-2. 57Co-vitamin B12-labeled samples were treated with neuraminidase for 24 hr at 37°C before isoelectric focusing on 5% polyacrylamide gels containing 6% glycerol, 1.5% taurin (w/v), and 2% "Servalytes," pH range 2-8. All phenotypes of the R proteins in saliva samples show complete acidic conversion (see text).

Fig. 7. Autoradiogram of saliva R protein on IEF gel. Samples 2, 3, and 4 were treated with neuraminidase for 16 hr at 37°C while sample 1 was untreated. These samples were obtained from 4 different individuals of the same TCR phenotype. The samples had been stored at −80°C for 6 mo before neuraminidase treatment.

The two TCR 1 bands was designated as TCR 2. Of the two isoprotein bands in TCR 2, the acidic band is not as apparent as the alkaline band (see phenotypes labeled 2 in Fig. 6). Similarly, the intensity of the alkaline isoprotein band of the TCR 2 is not as strong as that of the TCR 1 on IEF gels. Individuals possessing a combination of the 1 and 2 were designated as TCR 1-2. The isoprotein bands from saliva also show an acidic shift that is probably attributable to glycosidase enzymes present in saliva, but is not due to neuraminidase activity. The acidic conversion of the saliva R protein is shown in Fig. 7. All saliva samples in Fig. 7 had been stored at −80°C over 6 mo. Saliva samples (nos. 2, 3, 4) treated with neuraminidase show three different stages of R protein desialation, resulting in a situation in which sample no. 2 does not show any acidic conversion, sample no. 3 is semiconverted, and sample no. 4 shows complete conversion of the desialated isoprotein, which is independent of neuraminidase activity. The acidic conversion of the isoprotein at pH 5.09 can also be observed in saliva sample (no. 1) without neuraminidase treatment. The three different patterns of R protein bands that are observed in saliva samples 2, 3, and 4 in Fig. 7 were obtained from saliva samples with the same phenotype (TCR 1), and the extent of the conversion cannot be predicted. The same type of isoprotein conversion was also observed in R proteins from erythrocytes and granulocytes, but only when the 57Co-B12-labeled and neuraminidase-treated samples were left at 4°C for a prolonged period of time (>1–2 wk). As a result of these banding conversions in saliva R proteins due to varying degrees of deglycosylation, care should be taken when stored saliva samples are used for phenotyping of R proteins. The banding shift of R isoproteins is not always consistent in saliva samples, even when these are treated under standardized conditions.

In order to examine whether the expression of R protein phenotypes from different tissues within the same individual is controlled by the same or by separate genetic loci, the typing of R proteins from granulocytes, erythrocytes, and saliva from the same individuals was carried out. The results are summarized in Table 1. Of 71 individuals tested, 51 were of the TCR 1, 18 of the TCR 1-2, and 2 of the TCR 2 type. Only one R protein phenotype was observed for any individual regardless of which tissues or body fluids were
sampled, and all tissues for each individual displayed one phenotype only, supporting the hypothesis that all R proteins are controlled by only one genetic locus.

**Population and Family Studies of R Protein Phenotypes**

The simplest genetic model to explain the distribution of three R protein phenotypes in the population is an autosomal mode of inheritance, where a single genetic locus (called TCR) controls the expression of two codominant alleles (TCR*1 and TCR*2). Individuals with only one pair of isoproteins (1 or 2) are assumed to be homozygous for the alleles at that locus, and individuals with all four isoprotein bands (1-2) are assumed to represent the heterozygous individuals. Table 2 shows the observed and expected phenotype frequencies and the gene frequencies obtained from a random white population, an American black population, and a Chinese population. Although there appeared to be no significant differences in the phenotype frequencies found between males and females, the gene frequencies differed significantly between different racial groups. The TCR*1 was the most common allele in all populations tested. The TCR*2 was observed at a frequency of 12% in whites, but only at 1% among American blacks. Not a single individual in the random Chinese population of 136 individuals carried the TCR*2. Observed phenotype frequencies in the white population did not differ significantly from those expected by assuming Hardy-Weinberg equilibrium ($\chi^2 = 1.04; 0.5 < p < 0.75, d.f. = 1$).

One-hundred-one informative families with a total of 329 children were studied for the segregation of the electrophoretic variants of the TCR alleles 1 and 2. The paternity of the children in each of these families was corroborated by HLA typing. The family data are summarized in Table 3. Unexpected variants different from the 1 or 2 alleles were not observed in any of the children in these families. The segregation ratio for the different genotypes obtained in this family material was consistent with a simple mendelian mode of inheritance of two codominantly expressed alleles at a single autosomal genetic locus ($\chi^2 = 0.476; 0.50 < p < 0.75, d.f. = 1$).

**DISCUSSION**

The banding patterns obtained for native $^{57}$Co-B$_{12}$-labeled R proteins were studied by autoradiography of IEF gel electrophoresis. Extensive microheterogeneity of R proteins from each tissue was found to be due to differences in the amounts of sialic acid contained in the native R proteins. The banding patterns were tissue specific and did not differ significantly from individual to individual. Varying numbers of narrowly spaced bands were found in the pI range from 2.3 to 5.0. It is now generally believed that R proteins from different sources are a mixture of isoproteins with varying degrees of carbohydrate content, particularly sialic acid. However, the biochemical and genetic relationship between TC I, TC III, and other R proteins have not been documented. Stenman et al. postulated that serum TC I seen in chronic myeloid leukemia came from granulocytes in which sialic acid was added to TC III to make TC I. Stenman further suggested that at least two different groups of R proteins existed: one with acidic isoproteins (pI 2.3-4.0) containing TC I and TC III occurring in all the cells and fluids, and one more basic protein (pI 4.0-5.0) only occurring in saliva and milk. Testing of this hypothesis has brought conflicting results. Rachmilewitz et al. have suggested that as cells progress through the myeloid maturation series, they shift from the production of TC I to production of TC III, and that TC I production is increased in the pathologic states (e.g., CML) associated with proliferation of

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**Table 2. R Protein Phenotype Numbers and Gene Frequencies in Random Populations**

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<thead>
<tr>
<th>Population</th>
<th>Phenytypes</th>
<th>Gene Frequencies</th>
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<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>White</td>
<td>350 (350.0)</td>
<td>98 (95.5)</td>
</tr>
<tr>
<td>American black</td>
<td>47 (47.04)</td>
<td>1 (0.95)</td>
</tr>
<tr>
<td>Chinese</td>
<td>136</td>
<td>—</td>
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*White: $\chi^2 = 1.04, 0.5 < p < 0.75, d.f. = 1$.

**Expected numbers (shown in parentheses) were calculated from the observed gene frequencies based on the Hardy-Weinberg equilibrium ($p + q$)^2 where $p$ and $q$ represent observed gene frequencies.*
immature myeloid cells. Hall,²⁹ however, could not find any evidence that granulocytes produce TC I.

Our study presents direct evidence that both TC I and TC III originate from normal granulocytes and granulocytes from CML patients. The present study has also allowed the comparison of normal granulocyte R proteins (TC III) released in vitro by lithium stimulation with plasma TC I obtained from patients with CML. These studies have also demonstrated that all desialated R proteins from one individual have identical IEF banding patterns, while the native R proteins only differ with regard to sialic acid content. The banding patterns of desialated R proteins, regardless of their tissue origins, were the same. The patterns were very similar to those of desialated saliva R proteins observed by Azen and Denniston.²² They observed, however, an extra acidic band in samples from leukocytes. We have not observed the extra acidic ⁵⁷Co-B₁₂-labeled protein in samples obtained from lithium-stimulated granulocytes.

The existence of the desialated R proteins in milk and saliva may depend on two factors: the level of sialyltransferase activity and the level of sialic acids in a given tissue within which R protein is synthesized. Another factor could be the number of galactose residues that serve as acid acceptors for the terminal sialic acid residues. Depending on the rate of salivation versus the rate of glycosylation of R proteins, one might consider that rapidly released glycoproteins may be incomplete (lack of terminal sugar moieties) and thus be undersialated. Such a hypothesis acquires support from the observation by us (data not shown) and other investigators³⁰³¹ that R proteins in milk and saliva are much smaller in size (estimated by gel filtration chromatography) compared with R proteins from blood cells and other body fluids. Stenman⁵ has also observed that the isoprotein population displaying a high pI distribution (4.0–5.0) also possessed a smaller average molecular size determined by gel filtration. A marked difference in the carbohydrate composition of R proteins from different tissue sources has been reported, particularly with regard to the sialic acid content.¹⁹²⁰ Plasma TC I, amniotic fluid, and hepatoma cells containing R proteins had a relatively high sialic acid content and a low content of fucose residues, whereas saliva, milk, and TC III had a lower sialic acid and higher fucose content. The carbohydrate composition of TC III and the normal granulocyte vitamin B₁₂ binding protein have been reported to be very similar, and these two proteins were found to contain approximately half as much sialic acid and twice as much fucose as TC I. This finding may account for the fact that fucose and sialic acid are interchangeable as terminal sugar residues³² and that the sialic acid residues sometimes are replaced by fucose. Notwithstanding these facts, only sialic acid moieties contribute to the net acidic charge of the glycoprotein, and as a result, are entirely and uniquely responsible for differential IEF gel patterns obtained in this study.

The genetic study of polymorphism for the desialated saliva R proteins by Azen and Denniston²² demonstrated that the banding patterns obtained by IEF gel electrophoresis could be explained by a simple genetic model with one locus and two codominantly expressed alleles. Our study also demonstrates that the same genetic model applies for the desialated R proteins from erythrocytes, granulocytes, and saliva. The expression of the desialated TCR variants was always the same when samples were taken from different tissue sources of the same individual (Table 1). Furthermore, the present study provides the biochemical and immunochemical evidence that the same gene controls the R protein production in different cells and body fluids.

The polymorphic variants of R protein described in this study have been observed in whites and American blacks, but not in the Chinese population. The rare TCR*₂ occurs with a gene frequency of 0.12 in whites, but only with a frequency of 0.01 in American blacks. These findings are in agreement with the findings of Azen and Denniston.²² Congenital deficiency of R proteins in two brothers described by Carmel and Herbert³³ can best be explained by assuming that this disease is a simple autosomal recessive or is X-linked. This particular R-protein-deficient family was further studied by Hall and Begley,³⁴ who confirmed the total absence of R proteins from any tissues (i.e., serum, saliva, gastric juice, cerebrospinal fluid, and granulocytes) sampled from these brothers. The present study provides the biochemical and genetic documentation for the theory that all desialated R proteins are genetically controlled by a single locus.

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