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AMINO ACID SUBSTITUTION FREQUENCY IN HUMAN HEMOGLOBIN[1]

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Hemoglobin A from adult humans does not contain any coded isoleucine[1], thus its presence in hemoglobulin A must originate through errors in transcription or translation, or by somatic mutations arising during DNA replication. Errors in transcription occur infrequently to form altered mRNA, tRNA and rRNA; such errors change the coding in the mRNA and may reduce the fidelity of the tRNA as regards both the kind of amino acid it accepts and the mRNA codons it recognizes[2]. Errors in translation[2] arise through the attachment of wrong amino acids to tRNA (amino acyl synthetase errors) and the imprecise recognition of the mRNA codons by the tRNA anticodons (translational variation). Somatic mutations result from mistakes in replication of DNA; many single base substitution mutations change nonisoleucine into isoleucine codons[3] and these "mutant" cells could have hemoglobin mRNA with isoleucine codons. For this reason, an increase in the quantity of isoleucine in hemoglobin A would be expected in humans who have been exposed to agents that cause base substitution mutations. Radiation may cause base substitution mutations in human somatic cells but this possibility has never been established. This report describes the analysis of the isoleucine content of hemoglobin A from 13 Marshallese who were exposed to fallout from an atomic bomb test in March of 1954 compared with hemoglobin A from 12 Marshallese controls.

Methods

The blood was collected by Dr. Conard in March of 1974 and received in Oak Ridge on April 4, 1974. Hemoglobin was purified initially by molecular seiving on Sephadex G-200 and then by three successive chromatographic separations of the carbonmonoxyhemoglobin, methemoglobin, and metcyanhemoglobin

forms on carboxymethylcellulose (Whatman CM23) using a nonlinear gradient of 1.7 liters of 50 mM sodium phosphate buffer at pH 5.8 and 1.0 liter of 50 mM Na_3PO_4.

Globin was prepared in acidified cold acetone[4] and the α and β chains were separated by chromatography on Whatman CM23[ref.5].

The protein was hydrolyzed in 6N HCl for 21 hrs at 110°C. Two percent of each hydrolysate was used to determine by amino acid analysis[6] the quantity of globin or separated chain in each sample. Tracer amounts of L-leucine-^{14}C and L-isoleucine-4-5^{3}H were added to the remainder of the hydrolysate; amino acids in the hydrolysate were separated on a preparative ion exchange column (1.9 X 60 cm) of 8% sulfonated styrene divinylbenzene copolymer (Beckman, Type 150A). The radiotracers were used to locate fractions containing isoleucine and excluding leucine and also to calculate the percentage of the isoleucine eluting from the preparative column which was actually pooled for the quantitative analysis of isoleucine in the protein hydrolysate. A Beckman Model 120C amino acid analyzer was used for amino acid analyses. The frequency at which isoleucine substitutes for other amino acids in human hemoglobin was calculated by dividing the nanomoles of isoleucine by the nanomoles of all the other amino acids in each sample.

Results

The substitution frequencies of isoleucine for other amino acids in the 25 samples of globin are shown in Table 1. The calculated average exposures to gamma rays and the age at exposure are indicated (Table 1, columns 2 and 3). Eight and 5 of the samples were from persons exposed to 175 and 69 R, respectively; 12 samples were from age- and sex-matched controls. Data in

Table 1 are presented graphically in Figure 1 to show the relationship between the age at exposure and the isoleucine substitution frequency.

For some of the samples, sufficient globin remained to separate the α and β chains and to determine the isoleucine substitution frequency in the separated chains (Table 2). A tracing of the chain separation of sample 24R is shown in Figure 2. The small peaks just ahead of the major β and α chain peaks are commonly observed (see Discussion) and for a few samples these small peaks were analyzed separately (Table 2).

The average isoleucine substitution frequency as a function of radiation exposure is plotted in Figure 3.

Discussion

A slight, but insignificant, increase in the isoleucine substitution frequency was found in controls between ages 20 and 51; the regression line has a slope of 0.0234×10^{-5}. More data on older and younger persons should be collected to determine whether the isoleucine substitution frequency increases linearly with age; it has been suggested that the error rate in protein synthesis should increase exponentially as a function of age[7]. In this regard, the high value obtained for sample 1547 is interesting because she is a 60-year-old woman showing signs of senility. Except in sample 1547, the higher isoleucine substitution frequencies were in samples from exposed individuals; however, some exposed persons had values in the control range (Table 1). T tests show that the 175 R and 69 R groups are not significantly different, $p>.25$; at the 95% confidence limits the former is significantly different from controls, $p<.03$, but the latter is not, $p>.08$. Figure 1 shows that the higher isoleucine substitution frequencies were observed more often

in individuals who were exposed at younger ages, yet the globin from 33R, who was exposed at 1 year of age, had a low content of isoleucine. Radiation-induced lesions may become fixed more frequently in infants than in adults owing to a reduced opportunity for excision-replication repair when a higher percentage of stem cells is dividing. These observations are consistent with findings that the induction of leukemias is higher in prenatal and younger persons exposed to X rays[8] and radiation from an atomic blast[9], respectively; however, more data are needed to establish the extent to which radiation causes a higher isoleucine substitution frequency in the hemoglobin of infants than adults.

The elevated isoleucine substitution frequency could arise by at least two mechanisms; one is by an increase in translational errors and another is by radiation-induced, base substitution mutations in erythropoietic stem cells. If the elevated isoleucine content in hemoglobin of exposed individuals is caused primarily by translational errors, both the α and β chains should have a higher isoleucine content than corresponding controls. On the other hand, base substitution somatic mutations should produce a higher isoleucine content in the polypeptide chain that is the product of the mutated gene because its mRNA would now code for isoleucine. These alternatives may not be readily distinguishable in cases where clones of cells with mutant α and β chain polypeptides appear in the same person. The isoleucine substitution frequencies in the separated α and β chains should equal the values obtained for the globin; this expectation was found (Table 2) but the substitution frequency was consistently higher in the β than in the α chain both in controls and

exposed. In the exposed, the isoleucine content of both chains is elevated but more so in the β than in the α chain. The markedly higher isoleucine content in the β chain of those exposed is consistent with the postulate that the gamma rays induced base substitution mutations in erythropoietic stem cells. The small pre-α and pre-β peaks (Figure 2) contain polypeptides in which oxidation of methionine residues and limited deamidation of asparagine or glutamine residues have occurred, which cause a net increase in the negative charge on these molecules. The pre-α and pre-β regions are more prominent in samples that have been stored for longer periods of time; these samples were stored for 4 to 6 months between preparing the globin and performing the chain separations. The isoleucine content in the pre-α and pre-β regions is higher than would be predicted on the basis of random substitution of isoleucine for other acids owing to single base changes and random oxidation of methionine and deamidation of asparagine and glutamine. The results suggest that molecules with amino acid substitutions are more susceptible to oxidation and deamidation.

Considering the high probability that much of the increased isoleucine in the hemoglobin of the exposed Marshallese resulted from base substitution somatic mutations, an increase in isoleucine incorporation into hemoglobin per rad of exposure can be estimated (Figure 3). The dose required to double the normal substitution frequency of the controls is 100 to 105 R. Based on the data (Table 1 and Figure 3), the isoleucine substitution frequency increased by 0.0320×10^{-5} per amino acid residue per R. However,

the α and β chains contain 141 and 146 amino acid residues, respectively; thus the induced increase in isoleucine is 9.18×10^{-5} in the two polypeptides per R or 4.59×10^{-5} per polypeptide per R. Moreover, there are 20 different amino acids and the average of these would be expected to show a similar increased frequency of substitution for other amino acids as a result of radiation-induced, base substitution mutations; thus the total induced amino acid substitution frequency would be near 9.18×10^{-4} per polypeptide per R. This suggests that for acute exposure the average frequency of induced point mutations at the hemoglobin loci in the erythropoietic stem cells of man may be about three orders of magnitude greater than induced mutations in germinal cells of mice detected by the specific locus method[10].

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Table 1. Substitution frequency of isoleucine for other amino acids in the globin of human hemoglobin from 25 Marshall Island people.

Sample	Exposure	Age at exposure	Age at present	Sex	Substitution frequency ($\times 10^{-5}$)	Average ± SEM ($\times 10^{-5}$)
3R	175 R	15 mo.	21 yr.	M	19.79	
10R	"	30 yr.	50 yr.	M	3.58	
18R	"	24 yr.	44 yr.	F	5.06	
24R	"	13 yr.	33 yr.	F	13.45	8.81 ± 1.96
33R	"	1 yr.	21 yr.	F	4.74	
35R	"	12 yr.	32 yr.	F	5.19	
42R	"	2 yr.	22 yr.	F	10.40	
71R	"	27 yr.	47 yr.	F	8.29	
6A	69 R	1 yr.	21 yr.	M	6.98	
8A	"	17 mo.	21 yr.	F	12.93	
44A	"	3 yr.	23 yr.	M	4.04	5.94 ± 1.92
45A	"	31 yr.	51 yr.	F	3.65	
81A	"	7 yr.	27 yr.	F	2.12	
813U	0 R		20 yr.	M	3.37	
815	"		24 yr.	M	2.17	
929U	"		35 yr.	F	3.47	
836	"		41 yr.	M	2.45	
839	"		46 yr.	F	1.89	
841U	"		41 yr.	F	3.56	3.20 ± 1.52
846	"		51 yr.	F	2.41	
867U	"		46 yr.	F	2.12	
868U	"		51 yr.	F	4.35	
944U	"		49 yr.	M	3.93	
1547	"		60 yr.	F	7.15	
1549	"		21 yr.	M	1.57	

Table 2. Substitution frequency of isoleucine for other amino acids in the separated α and β chains of human hemoglobin.

Sample	Exposure	Substitution frequency ($X\ 10^{-5}$)					
		Globin[a]	Pre-α	α	Pre-β	β	Expected value[b]
8A	69 R	12.93	7.61	2.35	40.36	12.42	15.71
24R	175 R	13.45	4.27	1.25	59.42	7.12	13.93
35R	175 R	5.19	c	4.34	c	8.32	6.33
71R	175 R	8.29	c	5.27	c	13.54	9.41
836	0 R	2.45	c	2.05	c	4.41	3.23
867U	0 R	2.12	1.75	1.99	10.81	3.40	4.53
868U	0 R	4.35	5.57	1.76	8.69	5.04	4.34

[a]Values reported in Table 1.

[b]Values expected when the percentage of each fraction and its substitution frequency are considered in the total.

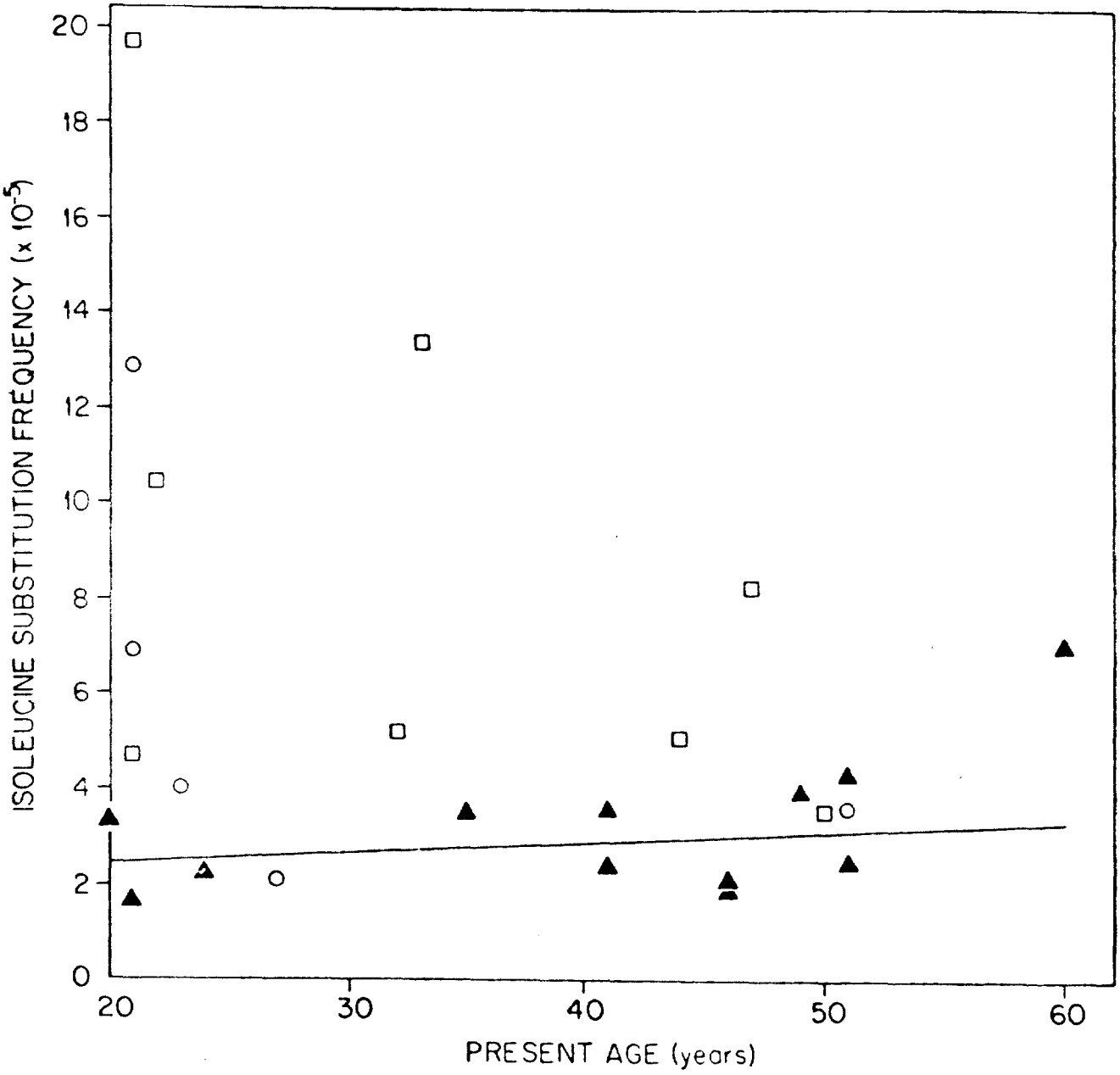
[c]The substitution frequency in this fraction was not determined; the quantity of polypeptide in this fraction represented less than 5% of total.

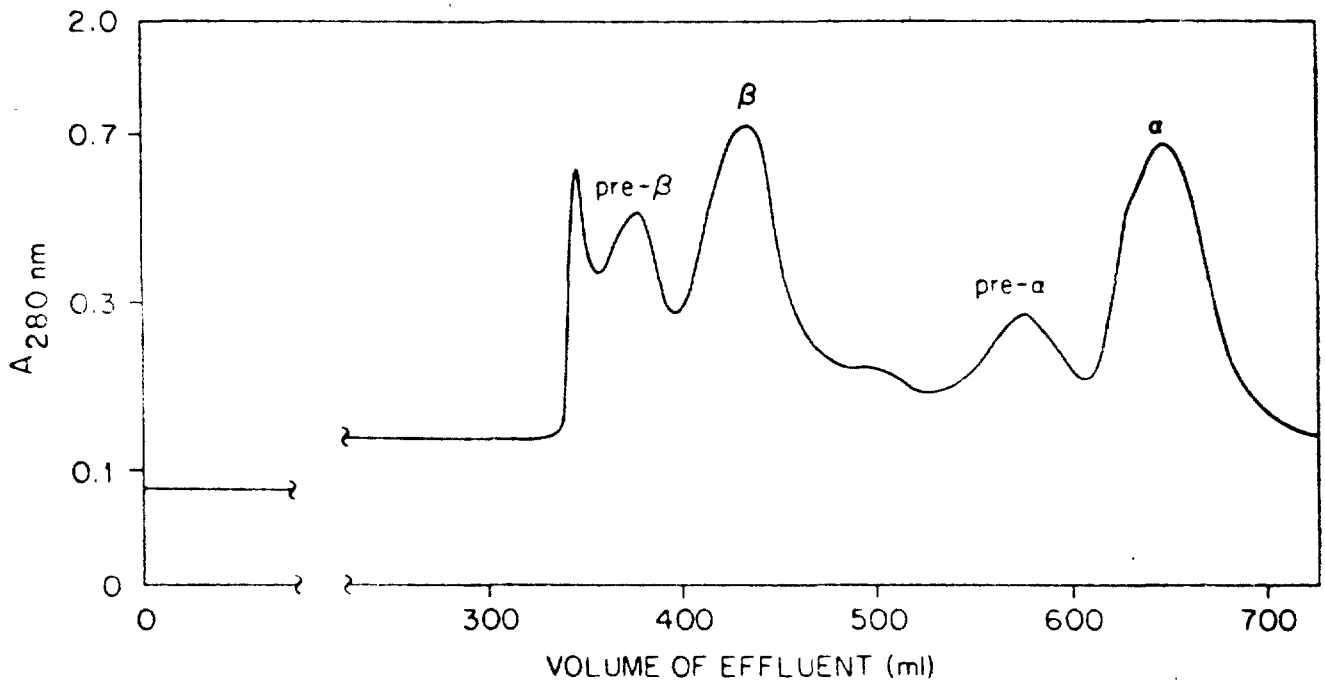
Figure Legends

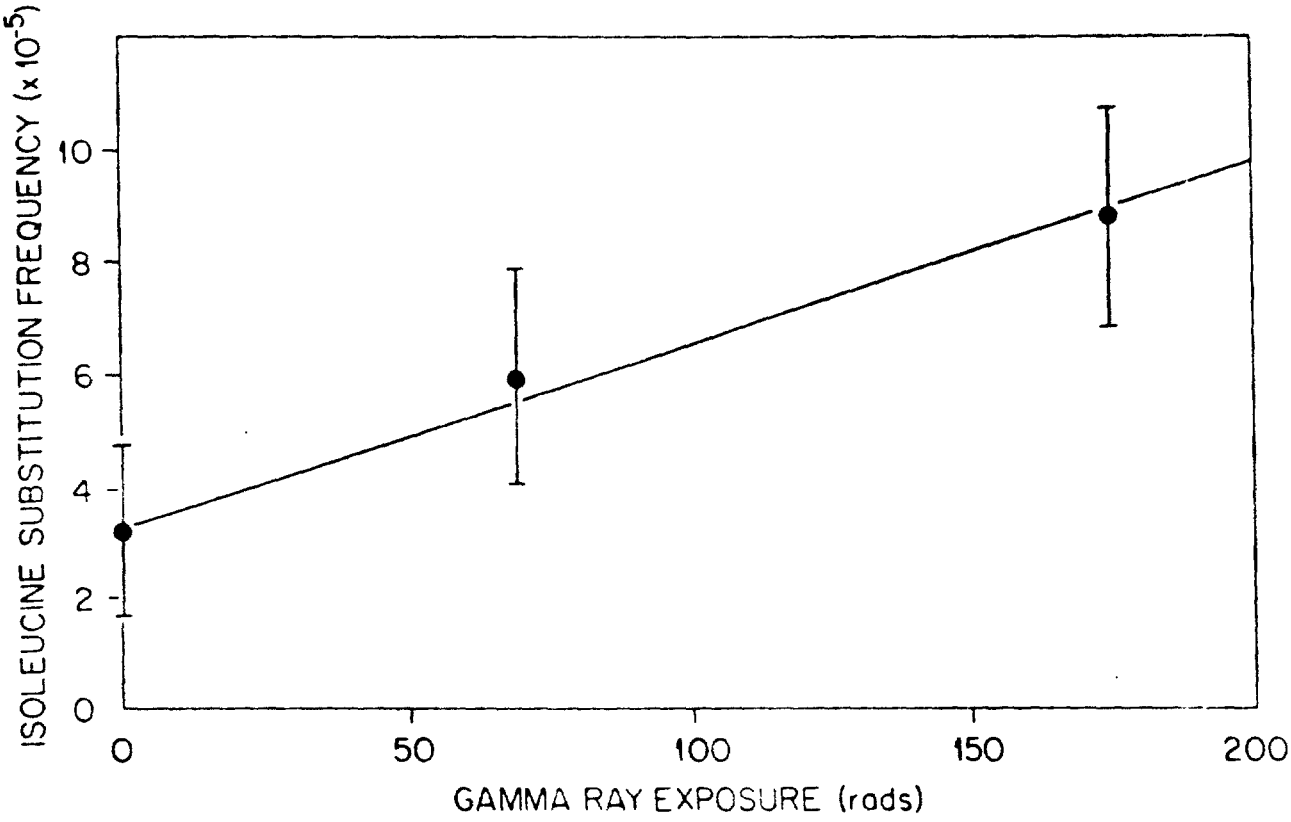
Figure 1. Isoleucine substitution frequencies versus present ages of the 25 Marshallese. The atomic bomb fallout occurred in March of 1954 so those who are now 21 years old were 1 year old when exposed. □ Exposed to 175 R; ○ exposed to 69 R; and ▲ 0 R controls. The computed line for the controls excluding sample 1547 has a slope of 0.0234×10^{-5}; x = 20, y = 2.4038 and x = 50, y = 3.1058.

Figure 2. Separation of the α and β chains of sample 24R on carboxymethylcellulose. Sample dissolved in 5 mM sodium phosphate buffer, pH 6.9; washed into column with 50 ml of the same buffer. Sample eluted with 100 ml of 5 mM sodium phosphate buffer pH 6.9 containing 8 M urea and 50 mM β-mercaptoethanol; then a 600 ml linear gradient of this buffer and 40 mM sodium phosphate buffer pH 6.9 containing 8 M urea and 50 mM β-mercaptoethanol was delivered to the column (1.6 X 40 cm) at a flow rate of 2 mls per minute.

Figure 3. Isoleucine substitution frequency versus gamma ray exposure. The average isoleucine substitution frequency 20 years after exposure is plotted against the exposure these people received in March of 1954. The computed line has a slope of 0.032004×10^{-5}; x = 0, y = 3.3125 and n = 100, y = 6.5129.


ISOLEUCINE SUBSTITUTION FREQUENCY (x 10⁻⁵)
0
2
4
6
8
10
12
14
16
18
20
20
30
40
50
60
PRESENT AGE (years)


2.0
0.7
0.3
0.1
0
$A_{280\ nm}$
pre-β
β
pre-α
α
0
300
400
500
600
700
VOLUME OF EFFLUENT (ml)


ISOLEUCINE SUBSTITUTION FREQUENCY (x 10⁻⁵)
0
2
4
6
8
10
0
50
100
150
200
GAMMA RAY EXPOSURE (rads)