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Tentative Evidence for 3—4 Haematopoetic Stem Cells in Man*

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Summary. Blood specimens from a random sample of 981 South African Negroid females were typed electrophoretically inter alia for their G-6-PD phenotypes. The allele frequency for Gd^B and Gd^{nonB} was found to be 0.8126 and 0.1874 respectively. Calculating the number of individuals expected for each phenotypic class, a highly significant deviation from the Hardy-Weinberg equilibrium became manifest, i.e. there was a deficit of 24.6% of heterozygotes and an excess of 12.3% of each of the two classes of homozygotes.

Several possible reasons for this discrepancy e.g. the effects of pooling sub-samples, selection and misclassifications due to insufficient staining were examined and were found not to be likely explanations for the observed phenomenon. Instead, the result is interpreted as due to only 3—4 stem cells which give rise to the haematopoetic system in man.

Random lyonization of one of each pair of homologous X-chromosomes during early embryogenesis of mammalian females gives rise to two different somatic cell lines which reproduce true to type and thus form a natural mosaic system for developmental studies. The X-linked glucose-6-phosphate dehydrogenase (G-6-PD) polymorphism was utilized in such developmental studies as a suitable marker for calculating the number of primordial stem cells destined to form the

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haematopoetic system (or other tissues) in man. Working with obligatory heterozygotes, two different approaches were adopted for these calculations:

a. Using the electrophoretically determined isoenzyme patterns as marker system, the quantitative variation in the expressed zymograms between heterozygotes provided the data for the calculations (Nance, 1964; Fialkow, 1973).

b. Using enzyme deficiency as the marker, the calculations were based on only that proportion of the phenotypic distribution of heterozygotes showing hemizygous, i.e. deficient, gene expression (Gandini et al., 1968; Gartler et al., 1969; for hair roots).

Calculations based on these approaches have led to an upper estimate of 8—10 primordial precursor cells for the haematopoetic system in man (Nance, 1964; Gandini et al., 1968). In a more recent study (Fialkow, 1973), the corresponding cell pool size was determined to be 13 (95% confidence interval: 8—19). These calculations are based on the assumption that the variation in allele expression is not the consequence of very early X-inactivation. This assumption appears to be justified, since multiple tissue studies revealed that women apparently hemizygous in blood, expressed both alleles in other tissues (Gartler et al., 1969).

The approach adopted in the present study represents a combination of those mentioned above. Instead of scoring enzyme activity (i.e. deficiency vs normal activity) as a marker, individuals were typed qualitatively according to their electrophoretically determined isoenzyme phenotypes. Also, instead of assessing the complete range of phenotypic variation, only that fraction of the heterozygote distribution which shows hemizygotic gene expression is determined. In this way the classification becomes more direct and exact. In addition, instead of working with a limited number of known heterozygotes, a sufficiently large and random sample, consisting of 981 females is tested. Hemizygosity is not determined individually but statistically, according to the observed deviation from the Hardy-Weinberg equilibrium. Under certain reasonable assumptions, to be discussed below, this approach probably leads to a more accurate estimate of the number of primordial precursor cells destined to form the haematopoetic system in man.

Population Samples and Methods

In the course of a survey for genetic polymorphisms among South African Negroid populations, 981 mostly pregnant females and 17 males were tested electrophoretically for their G-6-PD phenotypes. The sample is composed of seven distinct but relatively closely related sub-samples, i.e. Northern Sotho (Pedi), Ndebele, Shangana-Tonga, Western Sotho (Tswana), Zulu, Swazi and Venda, all of which represent Bantu speaking ethnic units. The ethnic differentiation, however, is of little consequence to the present investigation and accordingly the data are treated as a single entity. The origin and composition of the sample is described in greater detail elsewhere (Hitzeroth et al., in press).

The specimens of blood were collected and handled according to standard methods and were stored at —10° to —15° C for several months until they were thawed for analysis in Freiburg (Hitzeroth et al., in press). The G-6-PD phenotypes were demonstrated by means of horizontal starch gel electrophoresis (5 mg of NADP added before degassing), using a discontinous system of phosphate büffer at pH 7.4 and subsequent enzyme specific staining,

using 30 mg glucose-6-phosphate 24 mg NADP, 20 mg nitro-blue tetrazolium chloride (NBT) and a few grains of phenacine methosulphate (PMS) in 150 ml 0.017 M tris-HCl at pH 8.0 as staining solution.

Results

In accordance with results derived from a variety of Negroid populations (Giblett, 1969), our data confirms the occurrence of three G-6-PD alleles, i.e. Gd^A, Gd^B and Gd^{A^-} at polymorphic frequencies also within South African Negroids. Owing to the fact that the enzyme concentration in the hemolysates was not taken into consideration and A and A^- gene products have indistinguishable electrophoretic mobilities, only two G-6-PD phenotypes i.e. "A" and "B" can be determined in males (see Table 1). In females, the alleles Gd^A and Gd^{A^-} can be distinguished only when either is present together with the allele Gd^B, i.e. in A^-B or in AB heterozygotes. Consequently four classes of G-6-PD phenotypes should be observable in females. The "A" and "B" phenotypes are each characterized by a single band, whereas the "AB" phenotype is characterized by two bands with approximately equal staining intensities, and the "A^-B" phenotype by an A-band of distinctly fainter intensity relative to the slower moving B-band (see Fig. 1).

Unexpectedly, an additional electrophoretic phenotype with reciprocal staining property, i.e. "AB^-" was also observed (see Fig. 1 and Table 1). An allele Gd^{B^-}, however, is reported neither to occur in Negroids in general (Giblett, 1969), nor in Southern African Negroids in particular (Reys et al., 1970; Nurse and Jenkins, 1973; Nurse et al., 1974). In accordance with the Lyon hypothesis, we interpret this phenotype as being derived from AB heterozygotes who by chance predominantly have Gd A erythrocytes in their blood. Since this deviation from the mean distribution is assumed to be due to a random process, it follows that the reciprocal phenomenon should also occur with equal frequency, i.e. the intensity of the A-band should be decreased relative to the B-band in an equal proportion of AB heterozygotes, these females therefore should appear phenotypically as A^-B heterozygotes.

Owing to the experimental conditions employed, only the frequency of the allele Gd^B and the combined frequency of the alleles $Gd^A + Gd^{A^-}$, designated Gd^{nonB}, can be calculated directly. The figures for $Gd^B = 0.8126$ (range over all subsamples: 0.7766—0.8433) and for $Gd^{nonB} = 0.1874$, for females, corresponding well with the respective frequencies derived from the male sub-sample in the present

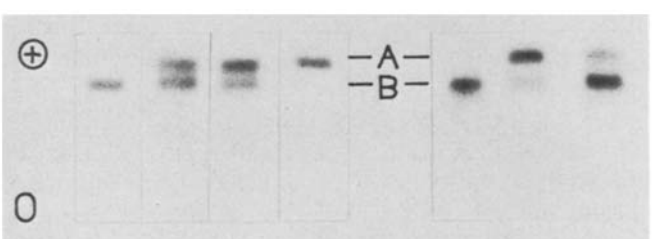

Fig. 1. G-6-PD phenotypes after starch gel electrophoresis and enzyme specific staining; from l.to r. "B", "AB", "AB^-", "A"/"B", "AB^-" and "A^-B"

Table 1. Distribution of electrophoretic G-6-PD phenotypes observed from a sample of South African Negroids, and the calculated number of females expected under Hardy-Weinberg equilibrium conditions for each of the 3 classes of phenotypes

	n	G-6-PD classes						
		B (BB)	nonBB			nonBnonB		Other variants
			(AB	A^-B	AB^-)	(AA	AA^- A^-A^-)	
Males (observed)	17	13		—		4		—
Females (observed)	981	683	78	89	58	71		2
				225				
Females (expected)	979	646.43		298.20		34.36		—
		Excess	Deficit			Excess		
		36.57	73.20			36.64		
		12.26%	24.55%			12.29%		

Allele frequencies: males: $Gd^B = 0.7647$; $Gd^{nonB} = 0.2353$
females: $Gd^B = 0.8126$; $Gd^{nonB} = 0.1874$

The deficit and excess of individuals is expressed as a percentage of the number of heterozygous females expected, i.e. 298.20.

study, as well as with those reported for other samples from South Africa (Nurse and Jenkins, 1973; Nurse et al., 1974). Determining, from the allele frequencies thus calculated, the numbers expected under Hardy-Weinberg equilibrium conditions, and comparing these to the numbers actually observed, a deficit of 24.6% of heterozygotes, as opposed to a surplus of 12.3% for each of the two classes of homozygotes (both figures are in relation to the number of heterozygotes expected) is evident (see Table 1). This difference is highly significant statistically ($P \ll 0.001$).

Discussion and Conclusion

Attempting to explain this result, several possible explanations like sampling and methodical errors, the effects of pooling of distinct sub-samples and of selection, as well as other possible contributory factors must be taken into consideration.

1. Following population genetic theory, a deficit of heterozygotes is to be expected if isolated sub-samples, each in Hardy-Weinberg equilibrium, are pooled and treated as a compound sample (Wahlhund's principle). The magnitude of such an effect is dependent on the variation in size and in allele frequency between the contributing sub-samples (viz. Cavalli-Sforza and Bodmer, 1971). Basing the calculations on the present data, the expected loss of heterozygotes due to the pooling of the given ethnically defined sub-samples is less than 0.5%, relative to the number of heterozygotes expected. This loss is so insignificant that it is extremely unlikely to become manifest as a deviation from a Hardy-Weinberg equilibrium. In fact, for several other, arbitrarily selected, polymorphic systems

for which the testing of this particular sample is completed, i.e. esterase D (Hitzeroth et al., in press), properdin factor B (Mauff et al., 1976) as well as a variety of other serological, serum protein and erythrocyte enzyme markers (in preparation), the number observed for each of the ethnically defined sub-samples separately, as well as for the entire sample, correspond very closely to those expected under equilibrium conditions.

2. However, when considering the G-6-PD results, the highly significant deviation from the Hardy-Weinberg equilibrium not only applies to the pooled sample (see Results), but also to each of the sub-samples separately. Indeed, all sub-samples show a marked deficit of heterozygotes; for the largest single sub-sample alone (Pedi: $n=269$), the deficit of heterozygotes amounts to 29.4%. Again in all cases, the manifested deficit is distributed evenly between both classes of homozygotes.

3. Similarly, when treating the 758 pregnant, as opposed to the 221 non-pregnant women contained in the present sample as two distinct sub-samples, the allele frequencies, $Gd^B=0.8212$ and $=0.7828$, as well as the deficit of heterozygotes, 24.1% and 25.5% respectively, are closely comparable for the two sub-samples. Again, the observed deficit is distributed symmetrically between both classes of homozygotes.

4. The possibility, that in a few instances the intensity of the possible second isoenzyme band (thus identifying the individual as a heterozygote) may have been below the threshold of visibility and that either the unobserved B- or nonB-band would have become identifiable after an extremely prolonged period of staining cannot be ruled out. The possibility of such erroneous classification appears to be limited however, since levels of G-6-PD activity as low as 5% are reported to be discernable in starch gel zymograms (Nance, 1964). Furthermore, following computer simulation analyses (to be discussed in greater detail below), it appears that even an unduly large quota of misclassifications would not affect the interpretration discussed in the present article, to any appreciable extent (see below).

5. Selection is an obvious potential factor capable of distorting the equilibrium distribution of phenotypes. From computer simulation analyses (to be outlined below) it follows, however, that selection against the nonBB heterozygotes (assuming random union of gametes) is not a likely explanation for the observed disequilibric distribution of phenotypes. Furthermore, selection *against* the heterozygotes nonBB, as is implied in the present argument, has not to our knowledge been reported to play any appreciable role at the Gd locus at all.

The possible explanations discussed above, in our opinion, cannot account for the deficit of heterozygotes in the order of magnitude observed from the present data. As was pointed out in the introduction, neither does a very early lyonization provide a likely explanation for the observed phenomenon. Instead, we propose to interpret the results obtained in terms of a very small number of haematopoetic stem cells.

Assuming a mosaic composition of the heterozygous embryo with about an equal proportion of inactivated Gd^B- and Gd^{nonB}-bearing X-chromosomes, and the total number of cells constituting the embryo at that developmental stage of not less than about 32 cells, then the probability that all of three randomly chosen

cells express one particular allele only, is close to 12.5%; with four similarly chosen precursor cells the probability is close to 6.25%. Our data therefore seems most compatible with the hypothesis that 3—4 primordial precursor cells are destined to develop into the haematopoetic system in man.

This hypothesis is based on a number of assumptions, all of which can vary over a range of variation. In order to determine the general effects of these variable assumptions on the arithmetic conclusion arrived at, the data was subjected to a computerized simulation analysis. It is the aim of this procedure to establish the limitations of the underlying assumptions within which the data conforms to the hypothesis postulated above. The specific criteria to be determined is the effect of alternative variables which possibly can lead to a disequilibric distribution of electrophoretic G-6-PD phenotypes closely similar—within 95% confidence limits, as determined by a χ^2-test—to the distribution derived from the present data. The variables that were entered into the simulation analysis are varying frequencies for the alleles Gd^B, Gd^A and Gd^{A^-}; selection of varying intensity *against* the heterozygotes nonBB; a varying rate of misclassification due to insufficient staining and a varying number of primordial precursor cells ($=N$). The quantitative contribution of enzyme activity from each of the three alleles mentioned above, is taken as 100:90:10%, respectively.

It follows from these computations, that the frequency of Gd^B in particular, is a major contributory variable for the estimation of N. A disequilibric distribution of phenotypes closely similar to the one observed from the present data can be simulated on the computer by taking $N \geq 5$ and by varying the frequency of this allele, especially to below 0.75, with frequency $Gd^A < Gd^{A^-}$. However, depending on the rate imputed for misclassification due to insufficient staining and for selection, the frequency of $Gd^B = 0.8126$, as calculated from the present data—with frequency $Gd^A > Gd^{A^-}$—either is a realistic estimate or a slight over-estimate of the true frequency. We therefore are confident that the true frequency for Gd does not exceed the critical interval 0.75—0.85 (see also our frequency estimate for males = 0.7647, the estimates for the sub-samples separately, as well as the estimates determined by other authors, as mentioned above). Within the range of 0.75—0.85 then, and frequency $Gd^A > Gd^{A^-}$, deviations from $N = 3$—4 are dependable mainly on misclassifications due to insufficient staining or to a lesser extent on selection. For the purpose of the subsequent discussion therefore, the frequency of Gd^B is confined to this specific interval, with frequency $Gd^A > Gd^{A^-}$, both of which are realistic assumptions that conform to our data.

Assuming hypothetically, a moderately high selection rate of 20—30% against the nonBB zygotes, this effect can account for a deficit of only 9—14% of heterozygotes (relative to the number expected under equilibrium conditions), which is considerably less than the deficit of 24.6% observed from the present data (see Table 1). In order to account for a loss of heterozygotes of roughly this magnitude due to selection alone, an unrealistically high selection rate of 49—50% would have to be postulated. In all cases, and most obviously with moderate to high selection rates, selection against the heterozygotes leads to a characteristically distorted distribution of phenotypes, i.e. a *decrease* of the least frequent homozygote class and a proportionately *greater increase* of the most frequent homozygote class. Such a characteristic disequilibric distribution of

Table 2. Approximate distribution of misclassified nonBB heterozygotes for different numbers of stem cells (N)

	$N \geq 5$	$N = 4$	$N = 3$
Proportion of misclassifications ascribed to the effect of N	very low	about 50%	close to 100%
Proportion of misclassifications ascribed to insufficient staining	close to 100%	about 50%	very low
Total number of heterozygotes thus misclassified	73.2	73.2	73.2
Estimated number of heterozygotes misclassified as B homozygotes	51.0	47.2	36.6
Estimated number of heterozygotes misclassified as nonB homozygotes	22.2	26.0	36.6

N = number of primordial haematopoetic stem cells. Effect of selection is taken = 0. The dosage effect of the alleles Gd^B: Gd^A: Gd^{A^-} is taken as 100:90:10% respectively. The frequency of Gd^A and Gd^{A^-}, assuming panmixia of gametes, is calculated to be approximately 0.1616 and 0.0258 respectively.

The deficit of heterozygotes observed (i.e. 73.2 individuals or 24.6% of the total number of heterozygotes expected, see Table 1), fits closely with the deficit expected if $N = 3$. In this case, the proportion of misclassifications ascribed to insufficient staining is very low. Taking $N \geq 5$, the effect of N accounts for a small percentage of misclassifications only and the proportion of misclassifications due to insufficient staining, correspondingly, is close to 100%.

Assuming misclassifications due to insufficient staining to contribute substantially to the deficit of heterozygotes, then more heterozygotes are expected to be misclassified as B than as nonB homozygotes. This asymmetry is determined by the frequencies and the dosage effect of the alleles involved (see text)

phenotypes, in fact, is completely incompatible with the strikingly symmetrical *increase in both* homozygote classes observed from the present data. In addition, selection against the heterozygotes at rates as discussed above, is bound to lead to a relatively rapid elimination of the nonB alleles.

Misclassifications of nonBB heterozygotes due to insufficient staining of the two isoenzyme bands is dependent primarily on the dose of enzyme activity contributed by each allele and therefore can be expected to be a non-random process under the given circumstances. If such misclassifications did in fact occur on a substantial scale, nonB alleles (dose of enzyme activity for A = 90%, for A^- = 10%), are expected to have been overlooked more often than B alleles (dose of enzyme activity for B = 100%). Taking this particular quantitative dosage effect as well as the approximate allele frequencies for Gd^A and Gd^{A^-} (assuming panmixia of gametes) into consideration, misclassification due to insufficient staining is expected to introduce a proportionate asymmetry into the observed disequilibric distribution of phenotypes. The magnitude of this effect, given various values of N, is summarized in Table 2.

It will be seen from Table 2 that for $N = 3$, i.e. assuming no misclassifications due to insufficient staining at all, the missing heterozygotes are expected to be distributed completely symmetrically between both classes of homozygotes (see also Table 1). For $N = 4$, i.e. assuming a rate of misclassification due to insufficient staining as high as about 50%, the overall asymmetry in the distribution of the

supernumerary homozygotes is expected to be 1:1.8 (i.e. 26.0:47.2). Assuming almost *all* of the missing heterozygotes to have been misclassified due to insufficient staining, i.e. $N \geq 5$, the asymmetry is expected to be at its maximum —within the limitations of the present observation—i.e. 1:2.3. Such a grossly asymmetric disequilibric distribution, in our opinion, is not compatible with the strikingly symmetric distribution of the supernumerary homozygotes observed from our data.

Within the limits of our postulated conclusion of 3—4 haematopoetic stem cells, indeed provision is made for a maximum of about 50% misclassifications due to insufficient staining (see Table 2). This means that in addition to the effect of $N=4$, as many as 28—29 nonBB heterozygotes could have been misclassified as B homozygotes and 7—8 as nonB homozygotes owing to this source of error alone. In our opinion, this represents the absolute maximum, both in regard to the proportion of erroneous interpretations of electrophoretic zymograms as well as in regard to the compatibility of the asymmetric distribution of the supernumerary homozygotes to be expected under this assumption, as compared to the absolutely symmetric distribution of the supernumerary homozygotes observed from the present data.

It is also evident from our simulation analysis that combinations of several of the variables which were entered into the computations can lead to results compatible with our data, without necessitating $N=3$—4. From an inspection of such cases the underlying combined assumptions (e.g. moderately high selection rates, with a high rate of misclassification due to insufficient staining, at different unrealistic allele frequencies, etc.), appear highly improbable however, and in our opinion, are not relevant. It may also be conjectured that inactivation of the one or other X-chromosome is not always completely random but may occur preferentially, depending on the presence/absence of a particular X-linked gene. Such a hypothetical process, if highly effective, can possibly lead to a disequilibric distribution of G-6-PD phenotypes which may be similar to the one derived from the present data.

In summarising the conclusions from the computer simulation analysis, the hypothesis that 3—4 primordial precursor cells give rise to the haematopoetic system in man, is the arithmetic conclusion most compatible with our data. Alternative explanations to this hypothesis are based on underlying assumptions which proved to be highly improbable.

Assuming this hypothesis to be correct, it predicts the occurrence of boys whose G-6-PD genotype is incompatible with that determined from the blood of their mothers, not to be uncommon at all in these populations. Indeed, cases of this kind of "anomaly" have been reported (Nurse and Jenkins, 1973).

Furthermore the disequilibric distribution of phenotypes as observed, should apply to all X-linked genes provided these loci are subject to random inactivation and the daughter cells are not subject to a subsequent process of selection in the tissue under observation. Applying this conclusion to the Xg blood group system in man, there should be an excess of about 23—46% of $Xg(a^-)$ females when compared to the number expected, calculated from the allele frequency derived from males. Contrary to this expectation, a slight deficit of $Xg(a^-)$ females is observed (Sanger et al., 1971). As was pointed out (Race, 1972) such a

discrepancy is not compatible with a theoretical 4-cell origin of the haematopoetic system, if the Xg locus were subject to inactivation. Since on the basis of our evidence, indeed not more than 3—4 primordial cells develop into the haematopoetic tissue, this lends greater support to the tentative hypothesis forwarded by Race (1972), that the Xg locus probably escapes inactivation (unless an alternative explanation can be invoked e.g. Xg antigens are synthesized by non-erythropoetic cells and subsequently are adsorbed onto erythrocytes).

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