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Leucocyte Alkaline Phosphatase and Erythrocyte Glucose-6-phosphate Dehydrogenase in Down's Syndrome*

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Following the discovery that Down's syndrome was due to trisomy, many investigators have reported increased activities of some cellular enzymes in mongoloid subjects (see review by Rosner, Ong, Paine, and Mahanand, 1965). The rationale underlying these studies is the possibility that the increased enzyme activities observed may reflect dosage effects of genes located on the trisomic chromosome. However, the data published so far have not been consistent with any simple hypothesis (Nadler, Inouye, Justice, and Hsia, 1967).

The present study was designed to assay two enzymes, leucocyte alkaline phosphatase (LAP) and erythrocyte glucose-6-phosphate dehydrogenase (G6PD) in mongoloid and non-mongoloid mentally retarded subjects. Though these enzymes have previously been measured in comparable subjects, no data have appeared which indicate that the observed variability between subjects may be genetic, i.e. evidence demonstrating that inter-individual variability significantly exceeds intra-individual variability. In the present study two blood specimens drawn from the same subjects one to three weeks apart were assayed to provide data for partitioning variability. Further, though LAP activities have been measured using both biochemical and histochemical methods, little information on the comparability of these alternative methods has appeared. In this study both histochemical and biochemical assays of LAP activities were performed on blood specimens from the same person.

The choice of the two enzymes for this study was based in part on the report by Rosner *et al.* (1965) who studied five enzymes including LAP and G6PD. They found significantly higher activities for all five enzymes among standard trisomy-21 mongoloids than among translocation mongoloids or controls, the latter two groups being comparable. Moreover, the distributions of activities among the standard type of mongoloids did not overlap with corresponding distributions of either translocation mongoloids or controls. The most direct genetic interpretation of their findings, the presence of a locus regulating the activity of each enzyme on the trisomic chromosome, fixation of a single allele at each locus, each locus on the short non-translocated portion of the trisomic chromosome, and multiplicative dosage effects in each case seemed collectively improbable. On the other hand, the possibility of distinguishing translocation mongoloids from the standard trisomy-21 mongoloids by biochemical assays implied by their results led us to duplicate their methods as closely as possible with a larger number of subjects.

Material and Methods

Subjects for the present study included 28 institutionalized mongoloids found by chromosome analysis to be trisomic for chromosome 21 (22 according to Yunis, Hook, and Mayer, 1965). Translocation mongoloids were to be studied following the comparison of standard mongoloids and controls. Subjects for the control group were 28 non-mongoloid mentally retarded subjects matched to the mongoloid subjects for sex and within one year for age. Patients with detectable signs of infection or other illness were excluded. All subjects were Caucasians ranging in age from 7 to 28 years ($\bar{x}=17{\cdot}7$).

Procedure

Blood specimens were obtained by venepuncture using 10 ml. heparinized syringes or 'vacutainers', and were

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mixed and chilled in an ice-bath for transportation to the laboratory. Specimens were coded and randomized before assay. Each group of assays included both mongoloid and corresponding control subjects.

Preparation and Assays of White Cells. Whole blood was mixed with an equal volume of 6% fibrinogen* and cells were allowed to sediment for 8–15 minutes. The supernatant, containing white and red cells in about a 1:1 ratio, was spun down and washed once with cold isotonic saline. Because efforts to obtain a white cell suspension essentially free of red cells using phytohaemagglutinin were only moderately successful, a hypotonic treatment was applied. The saline suspension of white cells was diluted 1:3 with distilled water and allowed to stand for 10 minutes. The suspension was then centrifuged and washed three times with cold isotonic saline. Preliminary tests of aliquots of the same white cell suspensions given hypotonic treatment and white cells isolated without hypotonic treatment indicated that the treatment had no detectable effect on enzyme activity. After the third washing, the pellet of white cells was resuspended in Krebs-Ringer bicarbonate buffer at *p*H 7·5. Aliquots of this suspension were colorimetrically assayed in duplicate for alkaline phosphatase activity using Sigma reagents*; protein was assayed by the method of Lowry, Rosebrough, Farr, and Randall (1951). Assays differing significantly from pre-established test mean differences were repeated. LAP activity was expressed as Sigma units per mg. protein. Kaplow's (1955) method was used for histochemical assays of LAP activity of thin blood films. Prepared slides were coded and randomized by one of us and scored blindly by another.

Preparation and Assays of Red Cells. The red cells remaining after the fibrinogen sedimentation were spun down and washed three times with isotonic saline. The upper portion of the red cell sediment was drawn off with the supernatant following each washing. The final red cell suspension was stored at 4° C. until assayed. Preliminary results confirmed a report by Mellbye and Scott (1964) that G6PD activity was unstable in haemolysates. Hence, haemolysates were prepared just before assay. Aliquots (0·2 ml.) of packed cells were diluted to 4 ml. with distilled water, mixed, incubated at room temperature for 10 minutes, and centrifuged at 1700 × g to remove stroma. The G6PD activity of 0·1 ml. aliquots of the clear haemolysate was assayed according to the method of Biezenherz, Boltze, Bucher, Czok, Garbade, Meyer-Arendt, and Pfleiderer (1953), using Boehringer Mannheim reagents. Duplicate assays were run and repeated if differences significantly exceeded the pre-established mean differences.

Cyanmethhaemoglobin reagent† and standard† were used for haemoglobin determination and results were expressed as units of G6PD activity per g. haemoglobin. All assays were completed within 5 hours after blood had been drawn. As mentioned above, both LAP and G6PD activities were measured twice for some subjects in specimens collected 1 to 3 weeks apart.

* Sigma Chemical Company.

† Hycel, Inc., Houston, Texas.

Results

Analysis of Sample Variances. The two analyses of variance in Table I were based upon duplicate assays of G6PD and colorimetric LAP activities of two blood specimens from the same individuals (11 mongoloids, 10 controls for G6PD, and 14 mongoloids, 10 controls for LAP assays). Analyses for each enzyme were run separately for mongoloids and controls to detect possible differences in contributions to variability in the two groups. Corresponding variances of G6PD activities for mongoloid and controls did not differ significantly (e.g. inter-individual variances were 10,213 and 11,034 for mongoloids and controls, respectively). In the separate LAP analyses, the intra-individual variance for controls was significantly higher than the corresponding variance for mongoloids ($p < 0·05$), but this resulted from only 2 subjects who differed considerably in the two determinations. All other variances were comparable. Obviously, experimental error makes a very small contribution to total variation in both assays. The number of subjects was too small to demonstrate a significant difference between groups for either enzyme ($F = 3·54$, $p > 0·05$, and $F = 0·83$, $p > 0·2$ for G6PD and LAP, respectively). The fact that the

TABLE I

ANALYSIS OF VARIANCE OF DUPLICATE G6PD AND COLORIMETRIC LAP ASSAYS IN TWO BLOOD SPECIMENS COLLECTED FROM THE SAME MONGOLOID AND CONTROL SUBJECTS

Source	SS	d/f	Variance	F	p
		G6PD Activity			
Between groups	35,955	1	35,955	3·54	> 0·05
Between individuals	193,197	19	10,168	2·95	< 0·05
Within individuals	72,470	21	3,451		
Error	10,890	42	259		
Total	312,512	83			
		Colorimetric LAP Activity			
Between groups	0·2404	1	0·2404	0·83	> 0·20
Between individuals	6·4025	22	0·2910	2·51	< 0·05
Within individuals	2·7845	24	0·1160		
Error	0·1019	48	0·0021		
Total	9·5293	95			

TABLE II

DESCRIPTIVE STATISTICS FOR G6PD AND COLORIMETRIC AND HISTOCHEMICAL LAP ACTIVITIES IN MONGOLOID AND AGE- AND SEX-MATCHED NON-MONGOLOID MENTALLY RETARDED SUBJECTS

	G6PD*		Colorimetric LAP†		Histochemical LAP‡	
	No.	$\bar{x} \pm SE_{\bar{x}}$	No.	$\bar{x} \pm SE_{\bar{x}}$	No.	$x \pm SE_{\bar{x}}$
Male mongoloids	14	393 ± 12	14	0·56 ± 0·08	14	119 ± 8·4
Female mongoloids	14	418 ± 15	14	0·46 ± 0·07	25	114 ± 7·9
Total	28	— —	28	0·51 ± 0·03	39	115 ± 5·8
Male controls	14	335 ± 15	14	0·34 ± 0·05	14	81 ± 10·0
Female controls	14	367 ± 14	14	0·45 ± 0·06	25	92 ± 6·5
Total	28	— —	28	0·39 ± 0·04	39	88 ± 5·4

* Activity expressed as G6PD units/g. haemoglobin.
† Activity expressed as Sigma units LAP/g. protein.
‡ Activity expressed as LAP scores.

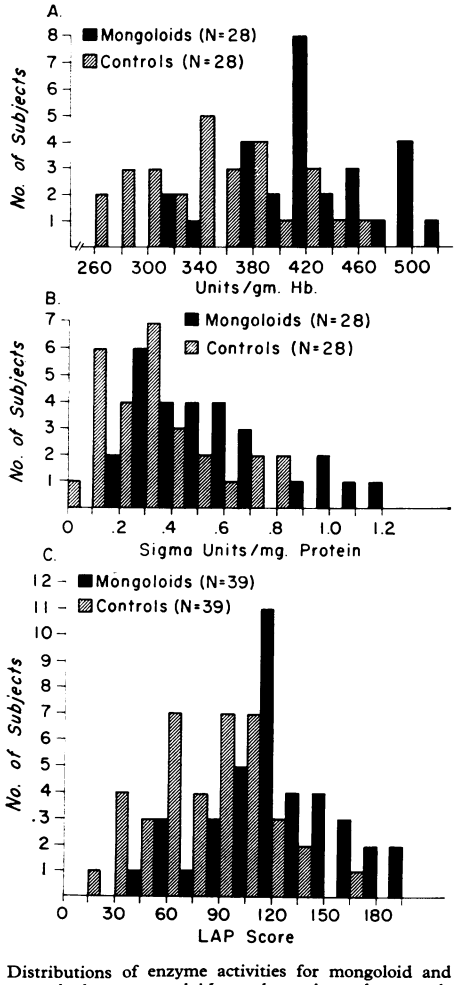

FIG. Distributions of enzyme activities for mongoloid and age- and sex-matched non-mongoloid retardates: A, erythrocyte glucose-6-phosphate dehydrogenase; B, colorimetric leucocyte alkaline phosphatase; C, histochemical leucocyte alkaline phosphatase.

inter-individual variance significantly exceeds the intra-individual variance in both cases suggests that the data satisfy minimal requirements for meaningful genetic study. However, these comparisons indicate some individual constancy without providing much information on inconsistency. Correlation coefficients calculated from means of the duplicates between times were of only moderate size ($r_{xy} = 0{\cdot}55$, $p < 0{\cdot}01$; $r_{xy} = 0{\cdot}54$, $p < 0{\cdot}01$ for LAP and G6PD, respectively). The coefficients of alienation ($1 - r^2$) indicate that most of the variability in time (about 70%) remains unaccounted for by these correlations. In other words, most of the components influencing intra-individual variability were not common to both time periods.

G6PD Activity. Descriptive statistics for all assays are given in Table II. Mean G6PD activities were higher for females than for males in both mongoloid and control subjects but did not reach conventional significance levels. Davidson, Migeon, Borden, and Childs (1963) demonstrated significantly higher G6PD activities for females in a large series. Mean mongoloid G6PD activities were significantly higher than those of controls in both sexes ($t = 2{\cdot}93$, $p < 0{\cdot}01$ and $t = 2{\cdot}53$, $p < 0{\cdot}02$ for males and females, respectively). Intergroup t tests and intragroup regression analyses indicate that these differences do not arise from differences in haemoglobin values. Though mean activities for males (335) and females (367) were comparable to that reported by Rosner *et al.* (1965) for controls (336), mean activity for mongoloids in the present study (408) was about half the mean value reported by Rosner *et al.* (820).

The distributions of G6PD activities given in Fig. A illustrate the almost complete overlap between values for mongoloid and control subjects in contrast to the absence of overlap in the values of

Rosner *et al.* Though the distributions for both mongoloid and control subjects exhibit suggestive antimodes, present data are too few to detect significant departure from the normal unimodal distribution. Further, the distributions of large samples of normal adults did not reveal antimodes (Davidson *et al.*, 1963). The means and variations in erythrocyte G6PD activities in the present study are comparable to those reported by Shih, Wong, Inouye, Makler, and Hsia (1965) for mongoloids and controls except that means in the latter study were about 20% higher for both groups.

Colorimetric LAP Assays. Though sex differences in the colorimetric assays of LAP activities could not be detected in either mongoloid or control samples, the mean for male mongoloids (Table II) differed significantly from the control mean ($t = 2{\cdot}42$, $p < 0{\cdot}05$), whereas the corresponding data for females did not ($t = 1{\cdot}22$, $p > 0{\cdot}20$). Trubowitz, Kirman, and Masek (1962) were able to detect a significant difference between mongoloids and controls in samples of comparable size and age. Alter, Lee, Pourfar, and Dobkin (1963) found a highly significant difference between mongoloid and control subjects under 10 years of age but could detect no significant difference between older age-groups. Since only one mongoloid-control pair in the present study was under 10 years of age, the absence of a significant difference between female mongoloids and controls is compatible with the findings of Alter *et al.* (1963). The latter investigators also found that neutrophil LAP activities exhibited negative regression with subjects' ages, particularly striking under 10 years of age. Though LAP activities for mongoloids showed a negative regression on age of the same magnitude ($-0{\cdot}00094$) as that found by Alter *et al.* ($-0{\cdot}00074$), the value was not significant. In contrast, LAP activities for control subjects exhibited a significant positive regression on age ($b_{yx} = 0{\cdot}19 < 0{\cdot}05$). The use of different bases for the expression of enzyme activity may account for the disparity. The LAP activities of the present study for both mongoloids and controls are significantly lower than those reported by Rosner *et al.* (1965) using the same method, but possibly their subjects were younger than ours. Again, though this study and others demonstrated significant differences in leucocyte LAP activities between mongoloid and control subjects, the distributions have generally been overlapping (see Fig. B) whereas those of Rosner *et al.* exhibited no overlap.

The colorimetric LAP activities were correlated with erythrocyte G6PD assays ($r_{xy} = 0{\cdot}29$, $p < 0{\cdot}05$). Similarly, Mellman, Oski, Tedesco, Maciera-Coelho, and Harris (1964) found significant positive correlations between leucocyte galactose-1-phosphate uridyl transferase and both G6PD and acid phosphatase. The biological basis for these associations remains obscure.

Histochemical LAP Assays. Histochemical assays of LAP activities yielded significant differences between means for mongoloid and control in both sexes ($t = 2{\cdot}85$, $p < 0{\cdot}01$ and $t = 2{\cdot}10$, $p < 0{\cdot}05$ for males and females, respectively), though it was necessary to study additional females before the difference between mongoloid and control females became significant. The mean LAP scores in Table II agree remarkably well with those reported by King, Gillis, and Baikie (1962), and are in substantial agreement with several other studies (Trubowitz *et al.*, 1962; O'Sullivan and Pryles, 1963; Lennox, White, and Campbell, 1962), employing histochemical methods. In all cases, means for mongoloids were significantly higher than control means, and some mongoloids had normal values (Fig. C). An intra-individual correlation of moderate size was found between the colorimetric and histochemical LAP assays of the present study ($r_{xy} = 0{\cdot}49$, $p < 0{\cdot}001$). However, because histochemical and colorimetric assays were performed on specimens from the same subjects obtained at different times, the effects of time were confounded with effects due to differences in technique. Hence, no conclusions about comparability of the two techniques can be drawn. The fact that the association between techniques is of the same order as the intra-individual correlation using the same technique (colorimetric) suggests that the two methods may be measuring the same entity, but further study is needed.

When studies on mongoloids and controls were completed, it became apparent from the considerable overlap between mongoloid and control distributions and the large intra-individual variability in G6PD and LAP activities that study of the small number of translocation mongoloids available to us would almost certainly lead to equivocal results. Hence, the study of translocation mongoloids was not pursued.

Discussion

The data of the present study agree with previous studies in that mongoloids appear to have significantly higher levels of erythrocyte G6PD activity and LAP activities. However, though analyses indicate that methods employed have small experimental errors, they also demonstrate that intra-individual variability from time to time would lead

to large misclassification errors if one were to assign genotypes on the basis of observed phenotypic assays. Hence, it is not surprising that more rigorous genetic analyses have not been performed. Further, the present data and those of previous studies do not provide information on the validity of the enzyme assays for detecting the overdose effects expected in trisomic diseases. G6PD, for instance, is known to be an adaptive enzyme from studies of rat liver (Johnson, Moser, and Sassoon, 1966) and alkaline phosphatase is shown to be adaptive from study of human cells in tissue culture (Cox and Pontecorvo, 1961) and variations in activity associated with several disease states (Rosner and Lee, 1965). One cannot be assured that total enzyme or enzyme-producing capacity has been assessed in any studies. In addition, the homeostatic mechanism regulating these enzyme systems may prevent phenotypic expression of overdose effects as is true for G6PD activity in normal females.

From the observed increase in LAP activities among patients with trisomy-18, Down's syndrome, and Klinefelter's syndrome, Nadler, Inouye, and Hsia (1966) suggested that the increases were nonspecific. That assertion can, of course, be made for virtually all signs and symptoms associated with chromosomal anomalies, since they occur, albeit with differing frequencies, in some normal subjects and in different abnormal karyotypes (e.g. the single flexion crease on the 5th digit in Down's syndrome, trisomy-18, and normal persons). This fact of life is a prime difficulty in studying diseases due to chromosomal additions in contrast to the more specific biochemical defects associated with single gene substitutions.

Three papers (Rosner *et al.*, 1965; Nadler *et al.*, 1967; King *et al.*, 1962) include most of the hypotheses and conjectures put forth to account for the observations on enzyme activity in Down's syndrome and these will not be reiterated. Fitting a simple hypothesis to the enzyme differences observed between mongoloid and non-mongoloid subjects is precluded by three principal enigmas, the correlations between independent enzymes found in the present study and in the work of Mellman *et al.* (1964), the normal levels in mongoloid platelets (Shih and Hsia, 1966) and fibroblasts (Nadler *et al.*, 1967) of enzymes known to be raised in mongoloid leucocytes, and the fact that mongoloid to non-mongoloid mean activity ratios have as often as not disagreed with the theoretical 1·5:1 ratio expected on the basis of simplifying assumptions (in the present study the ratios were 1·2, 1·3, and 1·3 for G6PD, and the two assays of LAP activity, respectively). These puzzles may not be resolved until our meagre knowledge of enzyme induction and repression in man is significantly augmented. Further, though theoretical models for gene frequency analyses in trisomy have been developed by Bateman (1960), Shaw and Gershowitz (1962), Penrose (1963), Goodman (1965), and Goodman and Thomas (1966), the necessary assignment of phenotypes or genotypes to the relatively continuous distributions of enzyme activities reported to date would be purely arbitrary. The continuous distribution in both mongoloid and control populations in LAP and G6PD activities and the comparable variances of mongoloid and control subjects suggest that polygenic regulation, multiple environmental influences, or both, are operative in many of the enzyme systems studied. Results obtained by Brandt, Frøland, Mikkelson, Nielsen, and Tolstrup (1963) with erythrocyte galactose-1-phosphate uridyl transferase approached theoretical expectancies most closely but it has been shown subsequently by Hsia, Inouye, Wong, and South (1964) that the rise in enzyme activity observed in mongoloid leucocytes is not found in erythrocytes or in fibroblasts (Nadler *et al.*, 1967).

Distinguishing translocation mongoloids from the standard type using biochemical means is of both clinical and theoretical interest. The fact that findings in the present study did not corroborate those of Rosner *et al.* (1965) indicates that further studies will be needed to realize this goal.

Summary

Leucocyte alkaline phosphatase (LAP) and erythrocyte glucose-6-phosphate dehydrogenase (G6PD) activities were measured colorimetrically in 28 mongoloid and 28 non-mongoloid mentally retarded subjects. Histochemical tests of LAP activities were also determined in 39 mongoloid and 39 non-mongoloid mentally retarded subjects. Analyses of variance showed that intra-individual variability was significantly smaller than inter-individual variability for both G6PD and colorimetric LAP assays, suggesting that genetic variability was being detected between individuals. However, the small though significant correlation coefficients calculated from repeat determinations suggest that endogenous and exogenous factors make significant contributions to variability in time within individuals.

Mean G6PD activities and mean histochemical LAP scores were significantly higher for mongoloids than for controls in both sexes. The mean for colorimetric LAP assays was significantly higher for male mongoloids than for male controls, but the corresponding difference for females was not sig-

nificant. The distributions of mongoloid and control values for all three assays showed almost complete overlap, in contrast to the findings of Rosner *et al.* (1965) who found no overlap in smaller samples. It was concluded that translocation mongoloids could not be distinguished from standard type mongoloids using the methods employed in this study.

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