Am J Hum Genet 29:563–570, 1977

Dosage Effects for Superoxide Dismutase-1 in Nucleated Cells Aneuploid for Chromosome 21

WILLIAM W. FEASTER,[1,2] LILLIAN W. KWOK,[1] AND CHARLES J. EPSTEIN[1]

INTRODUCTION

Despite the frequency of aneuploidy in man, little is known about the mechanisms by which chromosome imbalance results in reduced viability and abnormalities of morphogenesis and mental function [1]. Although several explanations have been suggested, there is still little firm evidence relating to the direct effects of the imbalance. It is generally assumed that the presence of an extra chromosome will result in the production of commensurately increased amounts of the gene products controlled by that chromosome (to 150% of the normal amount) and that absence of a chromosome will result in commensurately less (50%). However, this gene dosage supposition has yet to be rigorously established in man and other mammals.

There are many reports concerning increased activities of various enzymes in the cells of patients with trisomy 21, often to the expected 150% of normal (see [2] for review). However, the relevance of many of these findings to any type of gene dosage effect has been negated by the realization that such activity changes do not occur in all cell types and that they may occur even when the enzyme in question is not controlled by chromosome 21. In general, the offending cell has been the erythrocyte, and evidence for a gene dosage effect obtained with red cells must be viewed with caution.

In 1973 Tan et al. [3], using mouse-human somatic cell hybrids, presented evidence assigning the locus for indophenol oxidase-B (IPO-B) to human chromosome 21. IPO-B was later found to be equivalent to the cytoplasmic superoxide dismutase (SOD-1, E.C.1.15.1.1). Subsequent studies [4–8] demonstrated a 50% increase in SOD-1 activity in erythrocytes and platelets from Down syndrome patients, and the amount of SOD-1 protein present in erythrocytes was similarly increased [9]. In associated investigations, Sinet et al. [10] also detected a 55% increase in the activity of glutathione peroxidase in erythrocytes from subjects with trisomy 21.

In view of the earlier difficulties with the interpretation of enzyme activities in trisomy 21 erythrocytes, we felt that further dosage studies in *nucleated* cells were important. This report presents the results of our work with SOD-1 in fibroblasts,

Received December 8, 1976; revised March 21, 1977.

This work was supported by grants from the National Institutes of Health (GM-19527), the Maternal and Child Health Service (Project No. 445), and the National Foundation–March of Dimes. C. J. Epstein is an investigator of the Howard Hughes Medical Institute.

[1] All authors: Department of Pediatrics, University of California, San Francisco, California 94143. Address reprint requests to C. J. Epstein.

[2] Present address: Department of Pediatrics, University of California, San Diego, California 92103.

lymphocytes, and polymorphonuclear leukocytes and with glutathione peroxidase in fibroblasts.

MATERIALS AND METHODS

Isolation of Leukocytes

Fresh heparinized blood (5–10 ml) from noninstitutionalized individuals with trisomy 21 and from normal controls was mixed 1:1 with phosphate buffered saline (PBS), layered on Hypaque-Ficoll gradients, and centrifuged following the procedure of Böyum [11]. The lymphocyte rich mononuclear layer and the polymorphonuclear containing buffy coat layer were obtained, washed twice in PBS, and twice in Hank's balanced salt solution (BSS) with 15% fetal calf serum (FCS) and 5 U heparin/ml. The lymphocytes were washed one additional time in PBS and the pellet collected. The red cells in the granulocyte-containing buffy coat layer were lysed twice with 0.83% ammonium chloride in 0.05 M Tris, pH 7.67, for 10 min at 37°C. The remaining polymorphonuclear cells were then washed twice with Hank's BSS-FCS and once with PBS and the pellet collected. Total yield varied from 2 to 16 $\times$ 10^6 cells per sample. The lymphocytes were judged to be over 90% pure, and the polymorphonuclear granulocytes, 99% pure by examination of stained smears.

Fibroblast Cultures

Normal and trisomic cell lines were initiated from gluteal skin punch biopsies. Other normal and trisomic lines were obtained from Drs. E. Schneider and R. Summitt, and two monosomic lines (GM-137 and GM-230) from the Human Genetic Mutant Cell Repository, Camden, N.J. The cells were grown in plastic T-flasks in Eagle's minimal essential medium containing 20% FCS, penicillin, streptomycin, and L-glutamine. All cultures were screened and found to be negative for mycoplasma contamination prior to assay. The cells used for analysis were harvested with 0.2% Pronase at the fourth to seventh passage. The cells were washed three times in PBS and the final pellets were resuspended in 200–600 μl PBS and lysed by three cycles of freeze-thawing. After centrifugation at 15,000 g for 30 min, the supernatants were collected and stored at −60°C until assayed.

Superoxide Dismutase-1 Assay

SOD-1 was assayed for its ability to inhibit the superoxide mediated reduction of nitroblue tetrazolium (NBT). The assay, similar to those previously described [12, 13], was adapted to a total volume of 300 μl to accommodate small amounts of cell supernatant. Assays were run at 22°C in matched microcuvettes in a Gilford 2,000 recording spectrophotometer. The reaction mixture, in a final volume of 300 μl, consisted of 0.02 M sodium bicarbonate, pH 10; 0.1 mM EDTA; 0.013 mM xanthine; 3 μM phenazine methosulfate; 27 μM nitroblue tetrazolium; and from 2 to 30 μl of cell supernatant. The assay was started by adding 10 μl of xanthine oxidase at an appropriate dilution to yield a change in absorbance at 418 nm of 0.024 to 0.036 OD U/min. In each set of assays, normal and aneuploid samples were intermixed, and their identity was not known until after completion of the calculations.

One unit of enzyme activity is defined as that amount which causes a 50% inhibition of the rate of NBT reduction. Protein determinations were done by a micromodification of the Lowry technique. Glutathione peroxidase in fibroblast extracts was assayed as described by Beutler [14].

RESULTS

Inhibition of SOD Activity by Cyanide

Superoxide dismutase exists in two forms, the cyanide-sensitive soluble cupro-zinc SOD-1 and the cyanide resistant mitochondrial manganese-containing SOD-2. Studies were performed to determine whether any SOD-2 was represented in the SOD activity

being measured. Sodium cyanide at a concentration of 0.01 M was added to assays of leukocyte and fibroblast supernatants, and SOD activity was completely inhibited in all instances. Therefore, it was concluded that the mitochondrial SOD-2 is inactive under the assay conditions being used and that the measured activity represents SOD-1. This was not unexpected since the high pH of 10 was chosen for the assay because of its known inhibitory effect on SOD-2 activity. As a result of this outcome, it was not considered necessary to add cyanide to subsequent assay mixtures.

SOD-1 Activity in Leukocytes

The SOD-1 activities of polymorphonuclear granulocytes and lymphocytes are shown in table 1; the activity of SOD-1 in lymphocytes is about three times that of the granulocytes. In each case, the differences between the mean activities of trisomic and normal cells are statistically significant ($P < .005$ with one-tailed Student's t test, or $< .01$ with the Wilcoxon rank sum test). The ratios of mean trisomic to normal activities are 1.38 and 1.40 for the granulocytes and lymphocytes, respectively.

SOD-1 Activity in Fibroblasts

The data from the fibroblast studies are shown in table 2. There is no overlap between the trisomic and normal and the normal and monosomic groups, and the mean SOD-1 activities are statistically different at the $P < .005$ and $< .01$ confidence levels, respectively. The ratios of mean trisomic to normal and of mean monosomic to normal activities are 1.8 and 0.6, respectively.

The trisomic and monosomic cell lines were assayed several months apart, and as can be seen from the data, the normal control values were different at the two times. However, since each set of control cells was assayed at the same time as the respective aneuploid set and the identities of the individual samples were not known during the assay, the observed activity and ratio differences between aneuploid and normal are considered valid.

TABLE 1

ACTIVITY OF SUPEROXIDE DISMUTASE-1 IN TRISOMIC AND NORMAL LEUKOCYTES

POLYMORPHONUCLEAR LEUKOCYTES		LYMPHOCYTES	
Trisomy 21	Control	Trisomy 21	Control
0.077	0.048	0.172	0.118
0.060	0.043	0.244	0.169
0.051	0.058	0.149	0.147
0.078	0.043	0.172	0.112
0.070	0.062	0.175	0.127
0.061	0.049	...	0.137
...	0.033	...	0.099
Mean ±SD	0.066 ± 0.011	0.048 ± 0.010	0.182 ± 0.036	0.130 ± 0.023
P	< .005,*	.01†	< .005,*	.005†
Ratio	1.38		1.40	

NOTE. — Enzyme activity = U/mg protein.
* One-tailed Student's t test.
† Wilcoxon rank sum test.

TABLE 2

ACTIVITY OF SUPEROXIDE DISMUTASE-1 IN NORMAL, TRISOMIC, AND MONOSOMIC FIBROBLASTS

	Trisomy 21	Control	Monosomy 21	Control
	0.121	0.062	0.089	0.122
	0.144	0.069	0.037	0.097
	0.095	0.057	0.067	0.093
	0.090	0.059	0.063	0.128
	0.112	0.058	...	0.094
	...	0.067		
Mean ± SD	0.112 ± 0.022	0.062 ± 0.005	0.054 ± 0.021	0.107 ± 0.017
P	< .005,*	< .005†	< .01,*	.01†
Ratio	1.81		0.60	

NOTE.—Enzyme activity = U/mg protein.
* One-tailed Student's *t* test.
† Wilcoxon rank sum test.

Glutathione Peroxidase Activity in Fibroblasts

The same samples used for the comparison of SOD-1 activities in normal and trisomic fibroblasts were also used for the assay of glutathione peroxidase activity. The activity of this enzyme in trisomic fibroblasts was 9.49 ± 2.32 (SD) μmol NADPH oxidized per hour/mg protein and in control fibroblasts, 8.46 ± 1.55 μmol per hour/mg protein. The difference between these means is not significant. Furthermore, when glutathione peroxidase and SOD-1 activities in individual cell lines are compared with one another (fig. 1), no correlation between the two can be detected ($r = .07$).

DISCUSSION

If gene dosage is indeed the primary determinant of the amount of a gene product contained within a cell, it would be expected that the ratios of the amounts of the product will be 0.5:1.0:1.5 when cells monosomic, disomic (normal), and trisomic for the chromosome carrying the locus in question are compared. The results obtained in the present studies with SOD-1 in cells aneuploid for chromosome 21 are in reasonable agreement with this expectation. As previously found by others with non-nucleated red cells and platelets [4–8], trisomy 21 results in a significant increase in SOD-1 activity, to between 1.4 and 1.8 times that found in control cells, in lymphocytes, granulocytes, and fibroblasts. Conversely, monosomy 21 results in a decrease to 0.6 of that in control fibroblasts. The fact that the observed ratios are not precisely 1.5 and 0.5, respectively, is not unexpected in view of the impossibility of controlling all possible relevant factors. With regard to the fibroblasts, these factors may include the age of the donor, the site of the biopsy, the actual number of replications in vitro, and the donor's overall genotype. For the leukocytes, the genotype, as well as exposure to infections and to other agents which may affect leukocyte proliferation and activity, must be considered.

As with red cells, caution is necessary in using the results obtained with the lymphocytes and granulocytes as proof of a true dosage effect. Although we did not measure the activities of enzymes controlled by other chromosomes, the studies of Nadler et al. [15] demonstrated that nonspecific increases in the activities of several

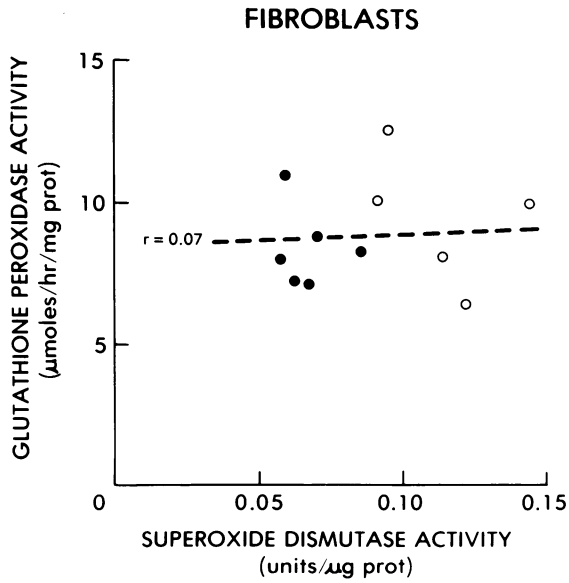

FIG. 1.—Relationship between glutathione peroxidase and superoxide dismutase-1 activities in trisomy 21 *(open circles)* and control *(closed circles)* fibroblasts. No significant correlation ($r = .07$) is present.

enzymes are found in trisomic lymphocytes and polymorphonuclear leukocytes purified on glass fiber columns. On the other hand, available evidence indicates that cultured fibroblasts do not share the difficulties of erythrocytes and leukocytes [2], and nonspecific elevations in enzyme activity have not been reported. Furthermore, utilization of fibroblasts permits the analysis of monosomic as well as trisomic cells. We feel, therefore, that our fibroblast data provide strong evidence for a true dosage effect for SOD-1 in chromosome 21 aneuploidy. Since platelets may also be free of such nonspecific alterations [2], the earlier data on their SOD-1 activities [8] can also be considered as valid evidence for gene dosage. With these results in hand, it is now possible to consider seriously the tentative localization of SOD-1 to band 21q22.1 as proposed by Sinet et al.[16].

In contrast to the results with SOD-1, the measurement of glutathione peroxidase activity in fibroblasts does not support a dosage effect related to chromosome 21, one of two possible interpretations to explain the elevated activity in erythrocytes [10]. As mentioned previously, there is considerable precedent for not accepting apparent enzyme dosage effects in trisomy 21 erythrocytes [2, 17] as indicating localization of the gene to this chromosome, and in general, it does not appear wise to attempt to map genes by dosage studies in erythrocytes alone. If such an attempt is made, as it has been successfully done with erythrocyte acid phosphatase in partial trisomy for part of the short arm of chromosome 2 [18], then it is essential to show that nonspecific changes do not occur in the activities of enzymes known to be governed by other chromosomes.

There is a considerable body of literature relating to the existence of gene dosage effects in cells from *diploid* individuals heterozygous for various enzyme deficiency

states (see Hsia et al. [19] for review) or for surface antigens such as $Rh_0(D)$ [20], HLA [21], and H-2 (in the mouse) [22]. Similarly, proportional changes in enzyme activities have been found in various *heteroploid* [23] and *polyploid* [24] animal cell lines. However, our results with SOD-1 in human cells allow this locus to be added to a relatively small list of others for which exact and specific gene dosage effects have been demonstrated in *aneuploid* situations in man and other mammals. In cultured cells derived from human fetuses with trisomy 16, the activity of adenine phosphoribosyltransferase was found to be 1.69 times that of control cells [25]. Mouse embryos trisomic for chromosome 1, which carries the *Id-1* locus, have 1.53 times the amount of isocitrate dehydrogenase found in control embryos [26]. Eggs and very early embryos derived from normal 40,XX female mice have twice the activity of the X-linked enzymes, glucose-6-phosphate dehydrogenase, hypoxanthine-guanine phosphoribosyltransferase, and phosphoglycerate kinase, as do eggs derived from 39,XO females [27, 28]. Leukocytes from humans with an XXYY or XYY chromosome constitution may have about twice the amount of Y antigen on their surfaces than do cells of XY individuals [29].

Another locus known to be present on human chromosome 21 is *AVG*, (antiviral gene), which controls the ability of a cell to respond to interferon [3]. When aneuploid and normal cells were studied, it was found that the trisomy 21 fibroblasts are three to seven times more sensitive to the protective effects of interferon against viral infection [30, 31], while monosomic fibroblasts are only 0.2 to 0.5 times as sensitive [31]. The most likely explanation of the departure of these results from the expected ratios is the fact that the response being measured is several steps removed from the primary action of the *AVG* product. Recent evidence suggests that this product is a surface receptor to which interferon must bind and the concentration of which is related to the number of chromosome 21s in the cell [32].

SUMMARY

Assays of the activity of chromosome 21 determined superoxide dismutase-1 (SOD-1) in lymphocytes and polymorphonuclear granulocytes have demonstrated 38% and 40% increases, respectively, in cells from individuals with trisomy 21. Similarly, SOD-1 activity in trisomic fibroblasts is increased by 81%, while cells monosomic for chromosome 21 have only 60% of normal activity. Taken together with the data on SOD-1 activities in trisomic erythrocytes and platelets, the present results firmly confirm the existence of a true dosage effect for this enzyme in cells aneuploid for chromosome 21. However, the results of assays of the activity of glutathione peroxidase in trisomic fibroblasts did not confirm the possibility previously reported of a chromosome 21 related dosage effect for this enzyme.

ACKNOWLEDGMENTS

We wish to thank Mrs. Gisella Abbo-Halbasch and Mrs. Georgianne Tucker for expert technical assistance and Dr. Lois B. Epstein for valuable help in developing the lymphocyte and granulocyte isolation techniques in this work.

REFERENCES

1. SANDLER L, HECHT F: Genetic effects of aneuploidy. *Am J Hum Genet* 25:332–339, 1973
2. HSIA DY-Y, NADLER HL, SHIH L: Biochemical changes in chromosomal abnormalities. *Ann NY Acad Sci* 171:526–536, 1968
3. TAN YH, TISCHFIELD J, RUDDLE FH: The linkage of genes for the human interferon induced antiviral protein and indophenol oxidase-B traits to chromosome G-21. *J Exp Med* 137:317–330, 1973
4. SICHITIU S, SINET PM, LEJEUNE J, FRÉZAL J: Surdosage de la forme dimérique de l'indophenoloxydase dans la trisomie 21, secondaire au surdosage génique. *Humangenetik* 23:65–72, 1974
5. SINET PM, ALLARD D, LEJEUNE J, JEROME H: Augmentation d'activite de la superoxyde dismutase dans la trisomie pour le chromosome 21. *C R Acad Sci [D] (Paris)* 278:3267–3270, 1974
6. GILLES L, FERRADINI C, FOOS J, PUCHEAULT J, ALLARD D, SINET PM, JEROME H: The estimation of red cell superoxyde dismutase activity by pulse radiolysis in normal and trisomic 21 subjects. *FEBS Lett* 69:55–58, 1976
7. CROSTI N, SERRA A, RIGO A, VIGLINO P: Dosage effect of SOD-A gene in 21 trisomic cells. *Hum Genet* 31:197–202, 1976
8. SINET PM, LAVALLE F, MICHELSON AM, JEROME H: Superoxide dismutase activities of blood platelets in trisomy 21. *Biochem Biophys Res Commun* 67:904–909, 1975
9. FRANTS RR, ERIKSSON AW, JONGBLOET PH, HAMERS AJ: Superoxide dismutase in Down syndrome. *Lancet* 2:42–43, 1975
10. SINET PM, MICHELSON AM, BAZIN A, LEJEUNE J, JEROME H: Increase in glutathione peroxidase activity in erythrocytes from trisomy 21 subjects. *Biochem Biophys Res Commun* 67:910–915, 1975
11. BÖYUM A: Separation of leukocytes from blood and bone marrow. *Scand J Clin Lab Invest* 21, suppl. 97:77–89, 1968
12. FRIED R: Enzymatic and non-enzymatic assay of superoxide dismutase. *Biochimie* 57:657–660, 1975
13. SALIN ML, MCCORD JM: Superoxide dismutase in polymorphonuclear leukocytes. *J Clin Invest* 54:1005–1009, 1974
14. BEUTLER E: *A Manual of Biochemical Methods,* 2d ed. New York, Grune and Stratton, 1975
15. NADLER HL, MONTELEONE PL, INOUYE T, HSIA DY-Y: Lymphocyte and granulocyte enzyme activity in patients with Down's syndrome. *Blood* 30:669–673, 1967
16. SINET PM, COUTURIER J, DUTRILLAUX B, POISSONNIER M, RAOUL O, RETHORE MO, ALLARD D, LEJEUNE J, JEROME H: Trisomie 21 et superoxyde dismutase-1 (IPO-A). Tentative de localization sur las sous bande 21q22.1. *Exp Cell Res* 97:47–55, 1976
17. LAYZER RB, EPSTEIN CJ: Phosphofructokinase and chromosome 21. *Am J Hum Genet* 24:533–543, 1972
18. MAGENIS RE, KOLER RD, LOVRIEN E, BIGLEY RH, DUVAL MC, OVERTON KM: Gene dosage: evidence for assignment of erythrocyte acid phosphatase locus to chromosome 2. *Proc Natl Acad Sci USA* 72:4526–4530, 1975
19. HSIA DY-Y: The detection of heterozygous carriers. *Med Clin North Am* 53:857–874, 1969
20. MASOUREDIS SP, DUPUY ME, ELLIOT M: Relationship between Rh_o(D) zygosity and red cell Rh_o(D) antigen content in family members. *J Clin Invest* 46:681–694, 1967
21. DUMBLE L, MORRIS PJ: Improved HL-A typing of cadaveric renal donors. I. Quantitation of HL-A gene dosage. *Tissue Antigens* 5:103–107, 1975
22. BOYSE EA, STOCKERT E, OLD LJ: Isoantigens of the H-2 and Tla loci of the mouse. Interactions affecting their representation on thymocytes. *J Exp Med* 128:85–95, 1968
23. FARBER R: Gene dosage and the expression of electrophoretic patterns in heteroploid mouse cell lines. *Genetics* 74:521–531, 1973
24. WESTERVELD A, VISSER RPLS, FREEKE MA, BOOTSMA D: Evidence for linkage of

3-phosphoglycerate kinase, hypoxanthine-guanine phosphoribosyltransferase, and glucose-6-phosphate dehydrogenase loci in Chinese hamster cells studied by using a relationship between gene multiplicity and enzyme activity. *Biochem Genet* 7:33–40, 1972
25. MARIMO B, GIANELLI F: Gene dosage effect in human trisomy 16. *Nature* 256:204–206, 1975
26. EPSTEIN CJ, TUCKER G, TRAVIS B, GROPP A: Gene dosage for isocitrate dehydrogenase in mouse embryos trisomic for chromosome 1. *Nature* 267:615–616, 1977
27. EPSTEIN CJ: Expression of the mammalian X-chromosome before and after fertilization. *Science* 175:1467–1468, 1972
28. KOZAK LP, MCLEAN GK, EICHER EM: X-linkage of phosphoglycerate kinase in the mouse. *Biochem Genet* 11:41–47, 1974
29. WACHTEL SS, KOO GC, BREG WR, ELIAS S, BOYSE E, MILLER OJ: Expression of H-Y antigen in human males with two Y chromosomes. *N Engl J Med* 293:1070–1072, 1975
30. TAN YH, SCHNEIDER EL, TISCHFIELD J, EPSTEIN CJ, RUDDLE FH: Chromosome 21 dosage: effect on the expression of the interferon induced antiviral state. *Science* 186:61–63, 1974
31. EPSTEIN LB, EPSTEIN CJ: Localization of the gene *(AVG)* for the antiviral expression of immune and classical interferon to the distal portion of the long arm of chromosome 21. *J Infect Dis* 133 (suppl.):A56–A62, 1976
32. REVEL M, BASH D, RUDDLE FH: Antibodies to a cell-surface component coded by human chromosome 21 inhibit action of interferon. *Nature* 260:139–141, 1976

INTERNATIONAL SYMPOSIUM ON GENETIC DISEASES AMONG ASHKENAZI JEWS

The First International Symposium on Genetic Diseases among Ashkenazi Jews, sponsored by the National Foundation for Jewish Genetic Diseases, will be held at the Barbizon Plaza Hotel, New York City, on December 12–14, 1977. Cochairmen of the symposium are: Drs. R. M. Goodman, Chaim Sheba Medical Center, Tel Hashomer, Israel, and A. G. Motulsky, University of Washington, Seattle, Washington. Attendance is limited to 300. For further information, contact Ms. Joan Samsen, Executive Director, NFJGD, Room 702, 608 Fifth Avenue, New York, N. Y. 10020. Phone: 212–247–7130.