The EMBO Journal vol.5 no.3 pp.615–622, 1986

Overproduction of human Cu/Zn-superoxide dismutase in transfected cells: extenuation of paraquat-mediated cytotoxicity and enhancement of lipid peroxidation

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Communicated by Y.Groner

The 'housekeeping' enzyme Cu/Zn-superoxide dismutase (SOD-1) is encoded by a gene residing on human chromosome 21, at the region 21q22 known to be involved in Down's syndrome. The SOD-1 gene and the SOD-1 cDNA were introduced into mouse L-cells and human HeLa cells, respectively as part of recombinant plasmids containing the *neo*^{R} selectable marker. Human and mouse transformants were obtained that expressed elevated levels (up to 6-fold) of authentic, enzymatically active human SOD-1. This enabled us to examine the consequences of hSOD-1 gene dosage, apart from gene dosage effects contributed by other genes residing on chromosome 21. Human and mouse cell clones that overproduce the hSOD-1 had altered properties; they were more resistant to paraquat than the parental cells and showed an increase in lipid peroxidation. The data are consistent with the possibility that gene dosage of hSOD-1 contributes to some of the clinical symptoms associated with Down's syndrome.

Key words: chromosome 21/Down's syndrome/gene dosage/hSOD-neo^R transformants/oxidative stress

Introduction

Down's syndrome (DS) is the most common genetic abnormality occurring once per 1000 live births (Hook, 1981). It is characterized by severe mental retardation as well as a wide variety of physiological defects such as reduced viability, morphogenetic abnormalities, increased incidence of leukemia, high susceptibility to infections and some signs of premature aging (reviewed in Smith and Berg, 1976; Burgio *et al.*, 1981; de la Cruz and Gerald, 1981). Lejeune *et al.* (1959) demonstrated that patients with DS have in their cells an extra copy of chromosome 21, a condition called trisomy 21. The causative factor for most of the trisomy is an ovarian non-disjunction at meiosis, while non-disjunction of paternal origin occurring at the first or second meiotic division accounts for ~20% of DS (Hassold and Jacob, 1984; Bond and Chandley, 1983). Patients with DS usually have a karyotype with 47 chromosomes (46 plus one additional 21). However, cases of DS have been identified in which only a portion of chromosome 21 is present in triplicate, usually translocated to another chromosome. This finding has permitted the localization of the region 'responsible' for the syndrome on segment 21q22 — the distal portion of the long-arm of chromosome 21 (reviewed by Summit, 1981). Although the relationship between trisomy 21 and DS has been known for >25 years, there is no effective treatment and very little is known about the way in which the additional chromosomal segment (21q22) causes the disease (Smith and Warren, 1985). It is generally assumed that the extra chromosome or segment codes for normal products and that the abnormalities found in the syndrome are produced by an excess of some of those proteins (see de la Cruz and Gerald, 1981; Epstein *et al.*, 1981, 1982; Scoggin and Patterson, 1982; Patterson *et al.*, 1982). The gene encoding the 'housekeeping' enzyme Cu/Zn-superoxide dismutase (SOD; EC 1.15.1.1) resides on chromosome 21 at the 21q22 segment known to be involved in DS (reviewed by Francke, 1981; Epstein and Epstein, 1981; Sinet, 1982). It may therefore be considered as a suspect for involvement in the etiology of the syndrome.

During oxidative metabolism, unstable, highly reactive oxygen radicals are formed both spontaneously and as a result of enzymatic activity of oxidative enzymes (Malmstrom, 1982). Cu/Zn-superoxide dismutase (SOD-1) is a protective enzyme responsible for maintaining lower levels of superoxide radicals within the cell. It acts by catalyzing the dismutation reaction, i.e. the interaction of two superoxide radicals, in such a way that one is reduced and the other oxidized, to yield hydrogen peroxide and oxygen: $O_2^- + O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$ (reviewed by Fridovich, 1978, 1979). A number of studies have led to the consensus that the superoxide radical (O_2^-) itself is not particularly harmful. The danger stems from its ability to interact with H_2O_2 to generate singlet oxygen ($^1O_2$), and hydroxy radicals ($OH\cdot$); both are extremely active and highly cytotoxic forms of oxygen (Badway and Karnovsky, 1980). A gene dosage response of SOD-1 in trisomy 21 has been shown in blood cells and cultured fibroblasts (Francke, 1981; Epstein and Epstein, 1981; Sinet, 1982), and it was suggested that the increased SOD-1 activity in trisomy 21 cells could lead to an accumulation of noxious concentrations of hydrogen peroxides (Sinet, 1982). More recently, Brooksbank and Balazs (1984) have reported that, in addition to increased activity of SOD-1, *in vitro* lipoperoxidation is also enhanced in cerebral cortex homogenates of DS fetuses.

To understand at the molecular level how an extra set of what seem to be normal genes produces DS, the genes involved in the pathology associated with the syndrome should be identified by demonstrating a connection between overproduction of their gene products and clinical symptoms of DS. It is for this reason that we isolated the human SOD-1 (hSOD-1) gene and studied its molecular structure and expression (Lieman-Hurwitz *et al.*, 1982; Sherman *et al.*, 1983, 1984; Levanon *et al.*, 1985; Danciger *et al.*, 1985). Here we describe the expression of hSOD-1 cDNA and the hSOD-1 gene introduced into human and mouse cells as recombinant plasmids containing the *neo*^{R}-selectable marker. The selected G418-resistant transformants produce authentic, enzymatically active hSOD-1. Clones that overproduce hSOD-1 have altered properties; they are more resistant to paraquat and show an increased lipid peroxidation.

Results

Generation of human and mouse cell lines containing integrated copies of hSOD-1 recombinant plasmids

The aim was to introduce extra copies of hSOD-1 cDNA into

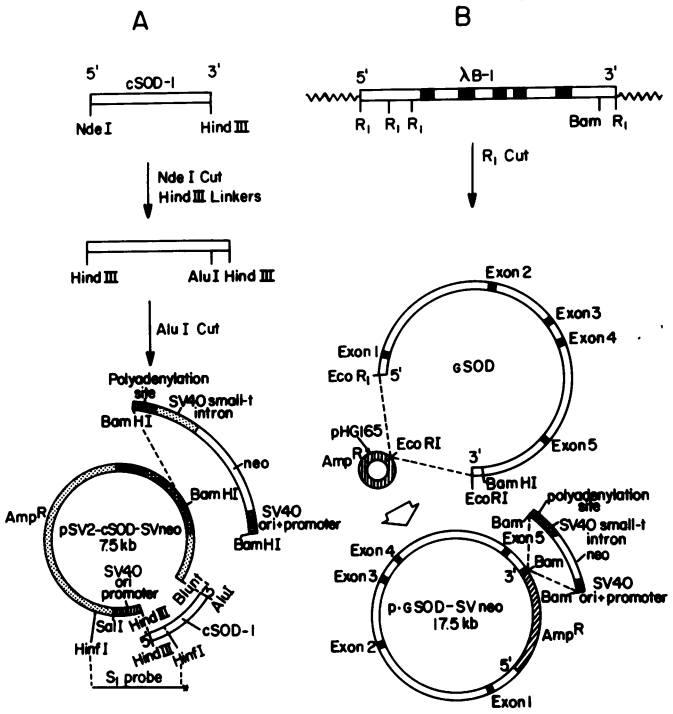

Fig. 1. Structure of hSOD-1 recombinant plasmids. The details of the construction are given in Materials and methods. **(A)** Shuttle vector expressing the hSOD-1 cDNA (cSOD). pBR322 DNA is represented by the stippled segment and contains the pBR322 origin of DNA replication and the β-lactamase gene. The open arcs represent either the *neo* gene or the hSOD-1 cDNA. The hatched segment contains the SV40 origin of DNA replication, the early promoter and enhancer. The stippled and cross-hatched segments contain the SV40 small-t antigen intron and the site at which the SV40 early transcript is polyadenylated. **(B)** Shuttle vector expressing the hSOD-1 gene (gSOD). pHG165 DNA is represented by the hatched segments and contains the pBR322 origin of DNA replication, the β-lactamase gene and the polylinker. The open arc represents the *neo* gene, and the cross-hatched the SV40 regulatory elements indicated above. The solid black regions are the exons of the hSOD-1 gene.

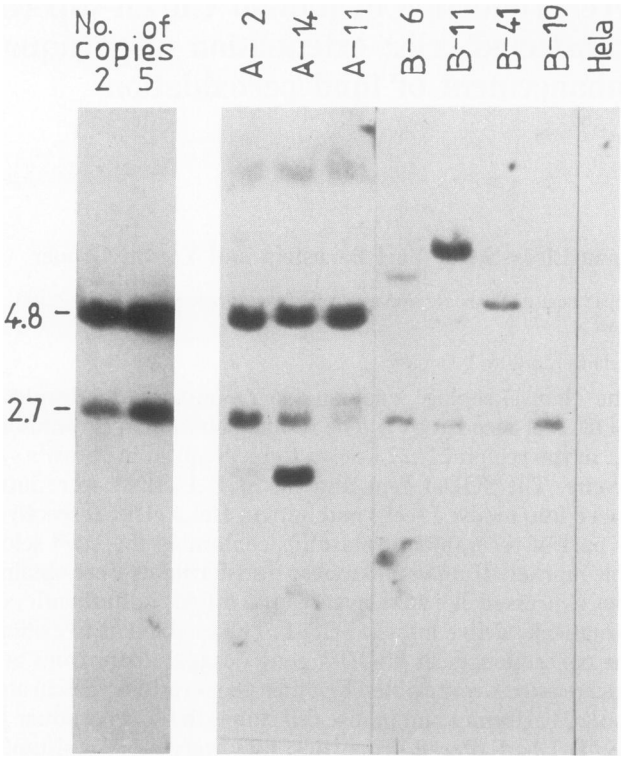

Fig. 2. DNA sequences derived from the transfected plasmid pSV2-cSOD-SVneo in high mol. wt. DNA extracted from G418-resistant HeLa cells. DNA (20 μg) from selected clones was digested with *Bam*HI, electrophoresed in 1% agarose gel and transferred to nitrocellulose filters. The blots were hybridized with ^{32}P-labeled pSV2-neo DNA. The 4.8-kb fragment contains the hSOD-1 cDNA and the pBR322 sequences (see Figure 1A), while the 2.7 kb fragment encompasses the *neo* transcription unit. To estimate the number of integrated plasmids, DNA equal to two and five copies of pSV2-cSOD-SVneo was added to the HeLa cells DNA prior to digestion with *Bam*HI, shown at the left side.

homologous recipient HeLa cells, extra copies of the gene into heterologous recipient mouse L cells and to obtain clones of cells overproducing hSOD-1. A cDNA clone which contains the entire coding region (Sherman *et al.*, 1983) was trimmed at the 3′-untranslated region and inserted into a pSV2 vector (see Mulligan and Berg, 1980) between the SV40 promoter and small-t intron (Figure 1A). A 2.7-kb *Bam*HI fragment containing the *neo* transcriptional unit (Southern and Berg, 1982) was then inserted into the unique *Bam*HI site 3′-proximal to the hSOD-1 cDNA. In this construct (pSV2-cSOD-neo) both the hSOD-1 cDNA and the *neo* gene are under the control of SV40 regulatory elements. Expression of the *neo* gene confers upon cells resistance to the toxic aminoglycoside G418 (Southern and Berg, 1982). A second plasmid, p-gSOD-SVneo (Figure 1B), contained in addition to the *neo* transcriptional unit a 12-kb *Eco*RI-*Bam*HI fragment encompassing the cellular hSOD-1 gene previously isolated and characterized (Levanon *et al.*, 1985). Cells were transfected by the calcium phosphate precipitation method (Graham and Van der Eb, 1973; Wigler *et al.*, 1978) and selected for G418 resistance. Colonies that grew in the presence of the antibiotic were collected, and a few dozen of them were expanded into cell clones and examined for hSOD-1 activity and for the presence of integrated SOD-1 and *neo* sequences in their chromosomal DNA. Several hSOD-1 producers, as well as non-producers, were selected for further study. High mol. wt. DNA from HeLa-derived G418- resistant clones, originally transfected with pSV-cSOD-neo, was digested with *Bam*HI and analysed by blot hybridization (Figure 2). This enzyme cuts the plasmid twice, separating the *neo* and SOD-1 transcription units (Figure 1A) and is therefore diagnostic of an intact SOD-1 fragment. The seven clones analysed contained 1−5 copies of plasmid in their genome (Figure 2). In clones A-2, A-11, and A-14, the SOD-1-containing sequences occurred mostly as a 4.8-kb band corresponding to the larger *Bam*HI fragment of the transfecting plasmid, indicating a 'head-to-tail' arrangement of the integrated copies. As indicated below, these clones expressed the integrated plasmids as enzymatically active hSOD-1, whereas B-6, B-11 and B-19, which apparently lack the intact 4.8 kb, did not. Comparable genomic blot analysis of the L-cell-derived transformants produced with the gene-containing plasmid p-gSOD-SVneo also revealed a pattern indicating integration of 2−10 tandemly arranged copies (data not shown).

Expression of hSOD-1 by the integrated plasmids

The synthesis of hSOD-1 mRNA and protein by the human and mouse transformed clones was analysed in a variety of ways. In human cells the unique functional gene — located on chromosome 21 — expresses two mRNAs of 0.7 and 0.9 kb. They differ in length at their 3′-untranslated region and the 0.7-kb is four times more abundant than the 0.9-kb mRNA (Sherman *et al.*, 1983, 1984; Levanon *et al.*, 1985). To characterize and quantitate the hSOD-1 RNA made in the mouse L-cell-derived clones,

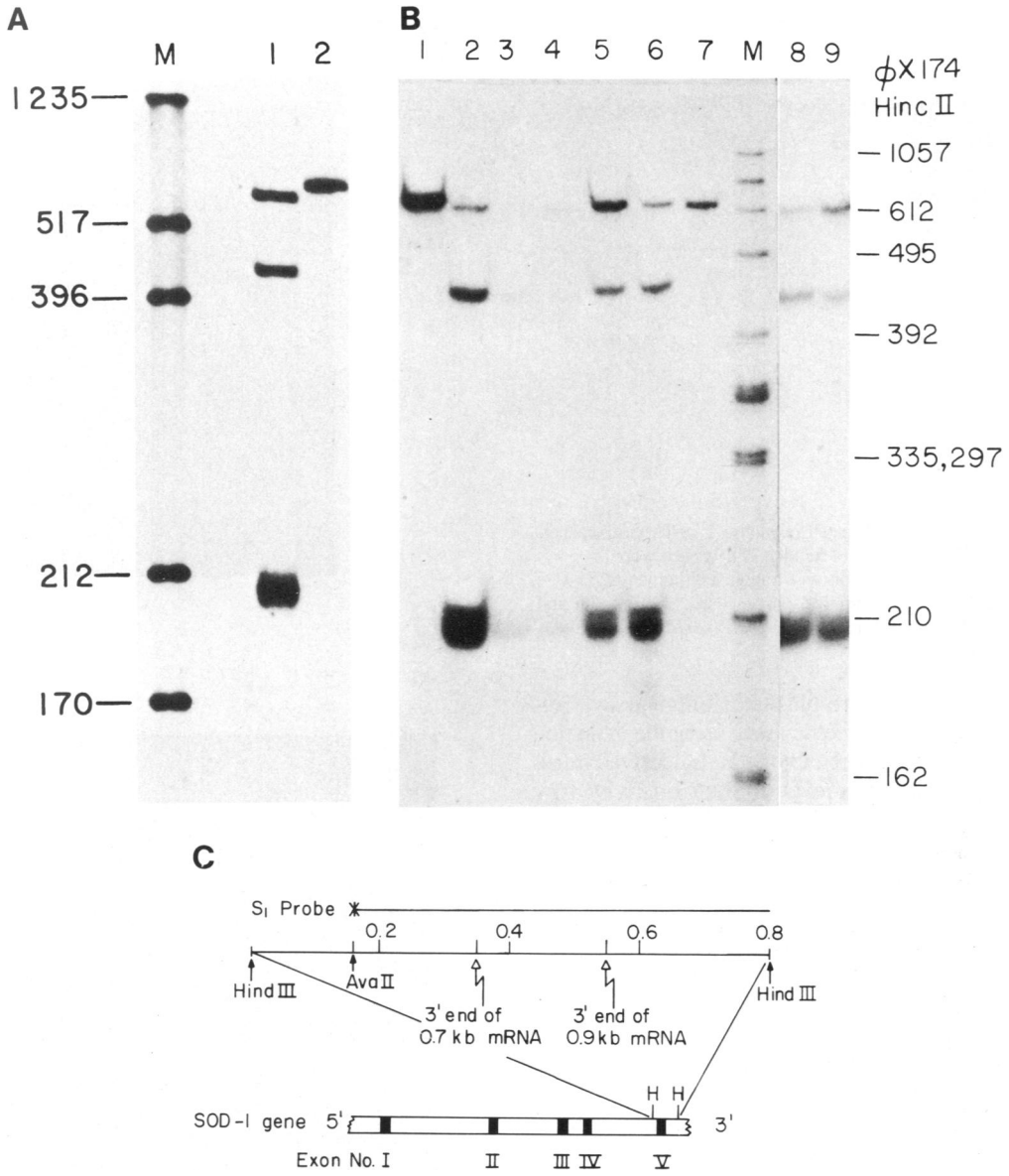

Fig. 3. S1 nuclease analysis of hSOD-1-specific mRNAs. The *Hin*dIII−*Ava*II fragment shown at (C) was labeled at the 3′ of the *Ava*II site, strand separated and hybridized to: **(A) lane 1:** 10 μg of poly(A)$^+$ RNA from human placenta, **lane 2:** control, minus RNA. **(B)** 20 μg of total cytoplasmic RNA from the various L-cell-derived hSOD-1 clones. **Lanes 1−6** correspond to clones 51, 99-C, 73, 81, 88 and 60. **Lane 7** contains RNA from the parental L-cells. **Lanes 8** and **9** represent a longer exposure (4 days rather than one) of lanes **3** and **4**.

a 636-bp *Ava*II−*Hin*dIII genomic fragment which spans the 3′ exon sequences and extends 250 bp further downstream, was labeled at the 3′ end of the *Ava*II site and used for S1 nuclease analysis (Berk and Sharp, 1977; Weaver and Weissmann, 1979) (Figure 3C). Analysis of RNA extracted from the parental line and from one of the G418-resistant hSOD-1-negative clones (G-51) is shown (Figure 3) along with several clones that produced different amounts of hSOD-1 transcripts. All the mouse clones expressing the hSOD-1 gene produced the two mRNA species at a ratio similar to that found in human cells (Figure 3A and B). RNA extracted from the HeLa-derived clones was similarly analysed by S1 nuclease using as a probe the 660-bp *Hin*fI−*Hin*fI fragment encompassing the SV40 promoter and the 5′ region of the SOD-1 cDNA (see Figure 1). Of the seven G418-resistant clones examined (as listed in Figure 2) A-2, A-11 and A-14 had substantial amounts of vector-derived hSOD-1 RNA, while B-6, B-11, B-14 and B-19 had not (data not shown).

The ability of the L-clones to translate the hSOD-1 RNA was assayed by immunoprecipitation. Transformants were labeled with [^{35}S]cysteine and extracts were examined by *Staphylococcus*-protein A immunoprecipitation with rabbit anti-hSOD-1. A protein of mol. wt ~16 000 co-migrating with authentic hSOD-1 was precipitated from all the clones except #51 (Figure 4). In addition to the hSOD-1, the mouse protein of mol. wt 14 000 was also detected, that is because the anti-hSOD-1 serum cross-reacts with the mouse SOD-1 (Figure 4). The relative intensity of the two bands can serve as an indicator for the amount of hSOD-1 produced by the various clones; and it is clear that clones 60 and 99-C expressed between four and five times more hSOD-1 than did clones 73 or 81.

The enzymatic activity of hSOD-1 in the transfected cells was determined after gel electrophoresis which separated the human

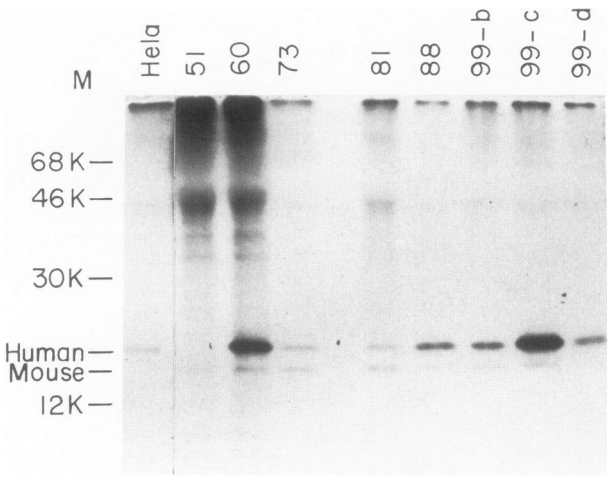

Fig. 4. Synthesis of hSOD-1 protein detected in mouse L-cell transformants. Cells were labeled with [^{35}S]cysteine for 4 h, total cell lysates were prepared, subjected to immunoprecipitation with rabbit anti-human SOD-1 serum and analysed by SDS-gel electrophoresis. The positions of human and mouse SOD-1 are indicated. 51 to 99 are the various L-cell-derived clones containing the hSOD-1 gene.

and mouse enzymes, using the nitro-blue tetrazolium assay (Figure 5), as well as by spectrophotometric assay using the inhibition of nitrite formation method (Table I). Of the HeLa-derived transformants, B-6 had, as expected, a level of SOD-1 activity very similar to that of the parental line, whereas A-11 and A-14 contained more activity (Figure 5A). Among the L-cell derivatives, clone 51 was similar to the parental line and the other clones produced between two and five times more enzymatically active hSOD-1 (Figure 5B and Table I). In summary, the collection of G418-resistant transformants contained clones expressing various levels of hSOD-1 RNA and protein. The recombinant plasmids encoding the hSOD-1 were integrated into the cellular DNA and the hSOD-1 protein made was enzymatically active.

Cellular effects resulting from overproduction of hSOD-1

The herbicide paraquat (1.1′dimethyl-4,4′-bipyridinum dichloride) is a non-selective weed killer, highly cytotoxic and lethal to animals (reviewed in Autor, 1977). It is known to increase the production of superoxide radicals ($O_2^{\cdot-}$) by a mechanism that involves reduction of paraquat by NADPH-diaphorase to the relatively stable paraquat radical ($PQ^{\cdot+}$) which reacts rapidly with oxygen to produce the superoxide radical (Farrington *et al.*, 1973; Hassan and Fridovich, 1979; Moody and Hassan, 1982). In animals, paraquat toxicity mainly affects the lungs; it was proposed that the pulmonary lesions are related to peroxidation of membrane lipids (Bus *et al.*, 1977). It was reported that paraquat stimulates SOD activity in *Escherichia coli* (Hassan and Fridovich, 1977) and in *Salmonella typhimurium* (Moody and Hassan, 1982) and that due to the increase in SOD activity the cells become more resistant to paraquat toxicity (Moody and Hassan, 1982). It was therefore interesting to analyse the effects of paraquat on the human and mouse cells overproducing hSOD-1. The cells were treated for 48 h with different concentrations of paraquat and the extent of survival was determined (Figure 6). The HeLa-derived overproducer clone A-11 was totally resistant to paraquat concentrations which killed all the cells of B-6 — a G418-resistant clone that expresses only the native SOD-1. Clone A-2 which overexpresses hSOD-1 to a lesser extent than A-11 was less resistant but not as sensitive as B-6. Surprisingly, A-14 which produced higher levels of hSOD-1 than A-11 was

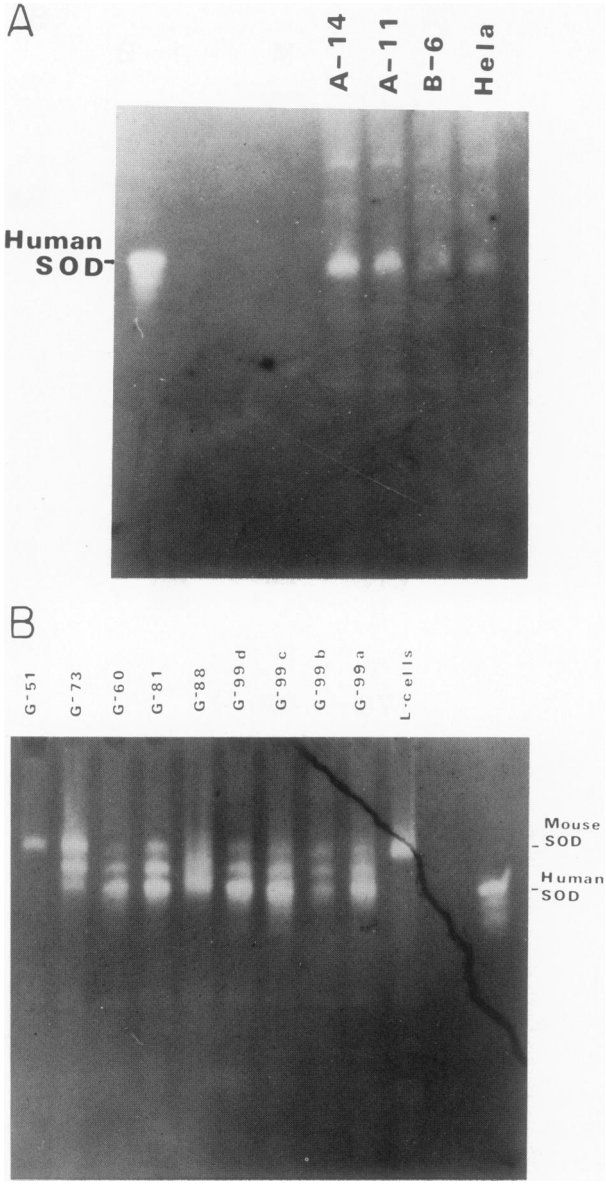

Fig. 5. Enzymatic activity of hSOD-1 in HeLa cell- and L-cell-derived transformants. In each case an NP-40 extract of 1.0×10^6 cells was made, loaded on 10% acrylamide-urea gel and electrophoresis was performed for 2 h at 150 V. For measurements of SOD-1 activity the gels were treated as described in Materials and methods. **(A)** HeLa cell-derived clones. **(B)** L-cell-derived clones. Position of mouse and human SOD-1 are indicated. The middle band present in the L-cell-derived clones expressing the hSOD-1 is probably a hybrid dimer of human subunit plus mouse subunit.

less resistant (Figure 6A). Similar results were obtained with the L-cell-derived clones. G-51, a hSOD-1-negative clone, was sensitive to even 0.2 mM of paraquat, a concentration not affecting clone 60, a high hSOD-1 producer. Again, clone 99-C which possessed higher hSOD-1 activity than clone 60 was less resistant to the drug (Figure 6B). Based on the knowledge that H_2O_2 is the product of $O_2^{\cdot-}$ dismutation, our tentative conclusion is that the combination of paraquat and higher hSOD-1 activity in A-14 and 99-C resulted in an increased production of H_2O_2 to the point where it became more toxic than the paraquat-generated $O_2^{\cdot-}$. Moreover, even in the hSOD-1 overproducers, the protection against paraquat cytoxicity is limited and A-11, G-60 and G-99-C will die upon longer (>48 h) exposure to paraquat concentrations as low as 0.05 mM. We assume that this cell death is due to

Table I. Specific activity of SOD-1 in L-cell transformants

Clone	Specific activity U/mg protein	Specific activity relative to L-cells
L-cells	4.9	1.0
51	4.9	1.0
73	5.0	1.0
99-A	16.6	3.4
60	17.7	3.6
99-C	28.5	5.8

Cell extracts (0.5% NP-40) were prepared and the specific activity of SOD-1 (units per mg protein) was determined as described in Materials and methods. Units (U) of SOD-1 represent the KCN-sensitive activity in the extracts and was deduced from a standard curve obtained with purified hSOD-1. Each number represents the average of specific activity measurements on three different extracts.

Table II. Lipid peroxidation in L-cell transformants

Cells	pmol MDA/μg protein			
	in vivo	*in vivo* (+PQ)	*in vitro*	*in vitro* (+PQ)
Parental L-cells	0.074	0.483	45.45	108.00
Clone 60	0.109 (147)	0.371	82.25 (180)	126.45
Clone 99-C	0.139 (188)	0.632	87.20 (191)	131.80

Malondialdehyde formation *in vivo* and *in vitro* was determined as described in Materials and methods. Paraquat at 0.4 mM was added for 24 h prior to harvesting of cells. Values are representative of 11 separate determinations, with different batches of clones 60 and 99-C, all of which showed significant increase (compared with the parental L-cells) of MDA formation both *in vivo* and *in vitro*. Those extracts were also assayed for hSOD-1 activity as described in Table I and Figure 5 to verify the consistency of the increase in enzymatic activity. Numbers in parenthesis represent percent of the values in L-cells.

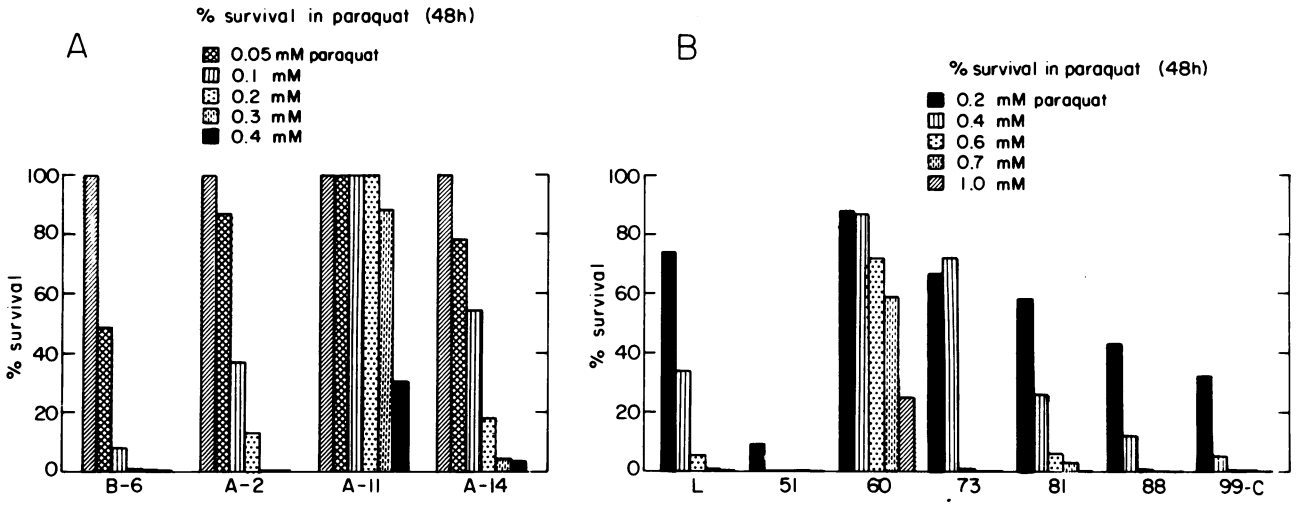

Fig. 6. Increased resistance to paraquat of the transfected clones overexpressing hSOD-1. Paraquat cytotoxicity was measured as described in Materials and methods. **(A)** HeLa cell-derived clones. **(B)** L-cell-derived clones.

the accumulation of lesions caused by elevated levels of hydrogen peroxide and the generation of hydroxy radicals in the clones possessing high hSOD-1 activity. The knowledge that perturbation in the delicate balance between the rates of hydrogen peroxide formation and its removal can cause lipid peroxidation (see Yagi, 1982; Kanner and Harel, 1985), as well as the more directly related observations of enhanced lipid peroxidation, possibly linked to increased SOD-1 activity, in homogenates of DS brains (Brooksbank and Balazs, 1984) prompted us to examine the degree of lipid peroxidation in clones overproducing hSOD-1. The extent of lipid peroxidation in two of the L-derived hSOD-1 overproducers (clones 60 and 99-C) was determined and compared with the level found in the parental L-cells (Table II). The widely used thiobarbituric acid (TBA) reaction (Niehaus and Samuelsson, 1968; Brooksbank and Balazs, 1984; Kanner and Harel, 1985) was employed. Although the exact mechanism of the reaction has not been elucidated it was shown that lipid hydroperoxides undergo decomposition through several possible intermediates to produce malondialdehyde (MDA) which reacts with TBA to yield a pink coloured TBA pigment with characteristic absorption and fluorescence spectra (Dahle *et al.*, 1962; Pryor *et al.*, 1976; Yagi, 1976). Both *in vivo* and *in vitro* lipid peroxidation were significantly enhanced in clones 60 and 99-C (Table II). The process was augmented by paraquat and as before (Figure 6B) its effect was more pronounced in clone 99-C than in clone 60 (Table II). These results support the contention that the increased activity of hSOD-1 created within the cells an oxidative stress leading to enhanced lipid peroxidation.

Discussion

The physiological and mental abnormalities associated with DS are complex and probably involve more than an extra dose of a single gene (Epstein *et al.*, 1981; Smith, 1985). A gene implicated in the syndrome would most likely have the following features: (i) reside at the 21q22 segment of chromosome 21, (ii) be overexpressed in all, or certain tissues of DS fetuses, and (iii) be connected by virtue of its overproduction or mistiming of expression to clinical symptoms of DS. The hSOD-1 gene fulfills requirements (i) and (ii), but do higher levels of hSOD-1 produce phenotypic changes? In an attempt to answer this question and to analyse how an excess of a normal gene product affects cell physiology, a series of cultured cells overproducing hSOD-1 was generated and examined for consequent metabolic changes. Plasmids containing the selectable marker *neo* and either the hSOD-1 cDNA or the hSOD-1 gene were transfected into human and mouse cells, respectively and G148-resistant clones overproducing various amounts (up to 6-fold) of enzymatically active hSOD-1 protein were isolated (Figures 4 and 5 and Table I). These hSOD-1 overproducers enabled us to study the cellular response to elevated levels of the enzyme in a more defined background, distinct from cellular effects caused by gene dosage of

other chromosome 21-encoded genes. When the transfected cells were exposed for 48 h to paraquat, a herbicide known to act as an *in vivo* generator of superoxide radicals, the clones overproducing hSOD-1 were substantially more resistant than control cells (Figure 6). In previous reports it was shown that *E. coli* (Hassan and Fridovich, 1977) and *S. typhimurium* (Moody and Hassan, 1982) cells become more resistant to paraquat toxicity by inducing SOD activity (~4-fold). Preventing this induction greatly augmented paraquat toxicity. The results presented here are therefore in good correlation with the data obtained for *E. coli* and *S. typhimurium* although the human Cu/Zn-SOD and the bacterial Mn-SOD are different proteins (see Oberley, 1982). As for the correlation between hSOD-1 overexpression and resistance to paraquat, it was interesting to note that although all the transformants were totally protected against the toxic G418 due to the expression of the *neo*^{R} gene, the clones that possess the highest hSOD-1 activity (A-14 and 99-C) were less resistant to paraquat, suggesting the existence of additional processes in which elevated activity of hSOD-1 is not advantageous. Moreover, even the most resistant clones (A-14 and -60) were gradually deteriorating in the presence of relatively low concentrations (0.05 mM) of paraquat, indicating that the elevated levels of hSOD-1 did not protect these cells from the cumulative damage caused by paraquat by-products. Based on the knowledge that hydrogen peroxide is produced by the dismutation of superoxide, our conclusion is that a combination of paraquat and the higher hSOD-1 of A-14 and 99-C resulted in increased production of H_2O_2 and other active forms of oxygen ($OH\cdot$, $^1O_2$) to the point where they became more toxic than just the (O_2^-) generated by paraquat. However, even in the absence of paraquat, higher levels of hSOD-1 may interfere with the normal metabolism of reduced oxygen species and create an oxidative stress. In this context, it should be mentioned that so far we have failed to isolate a transfected clone possessing >6-fold hSOD-1 activity, suggesting that higher hSOD-1 levels may be noxious to the cell. Gene transfer experiments in which the hSOD-1 expression is controlled by an inducible promoter should clarify this point.

Under normal conditions, a delicate balance exists between the rate of hydrogen peroxide formation via dismutation of superoxides and its removal by glutathione peroxidase (GSHPx). Increased activity of hSOD-1, as in the transfected clones, could lead to an increase in the steady-state levels of hydrogen peroxides, resulting in formation of hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$), which may enhance lipid peroxidation (see Yagi, 1982). Lipid peroxidation is defined as a non-enzymatic breakdown of unsaturated fatty acids, giving rise to peroxy radicals and ultimately to malondialdehyde. The physiological consequences of increased lipid peroxidation result from the damage caused to cellular membranes and organelles and their associated enzymes. The overall mechanism of the process is not yet completely understood but the involvement of hydrogen peroxides, hydroxyl radicals and singlet oxygen is firmly established (Yagi, 1982). For example, Kanner and Harel (1985) have recently shown that even small amounts of H_2O_2 could interact with metmyoglobin and generate active radicals which initiate membranal lipid peroxidation. The data presented in Table II clearly show a connection between the higher level of hSOD-1 activity and lipid peroxidation (both *in vivo* and *in vitro*). Furthermore, the lipid peroxidation was enhanced by paraquat (0.4 mM) even in those clones having high hSOD-1, indicating that H_2O_2 or other oxygen derivatives are involved in the process. The transformants in Table II overexpressed 3.6 and 5.8 times more SOD-1 than the parental L-cells, whereas in DS the documented SOD-1 activity amounted to only 1.5 times the normal level. Nevertheless, the clear correlation in Table II between elevated hSOD-1 activity and lipid peroxidation support the idea of Brooksbank and Balazs (1984) (see below) that in brains of DS fetuses there is a higher potential for lipid peroxidation due to the higher activity of SOD-1. In addition, we should consider the possibility that during embryo development SOD-1 activity in certain regions of DS brain increases above the regular level of 1.5 times.

In DS erythrocytes, glutathione peroxidase activity is significantly increased along with that of SOD-1 (Sinet *et al.*, 1975; Frischer *et al.*, 1981; Kedziora *et al.*, 1982), while catalase levels are normal (Pantelakis *et al.*, 1972). This is believed to be an adaptive response to the elevated levels of hydrogen peroxides produced by the augmented SOD-1 activity, rather than a gene dosage effect, since the gene coding for GSHPx is located on human chromosome No. 3 (Wijnen *et al.*, 1978). However, as was reported by Brooksbank and Balazs (1984) this adaptive mechanism does not function in the brain of DS fetuses in which SOD-1 activity was raised but GSHPx activity was not. Furthermore, *in vitro* lipid peroxidation was substantially elevated in those brain specimens (Brooksbank and Balazs, 1984). More recently, these authors reported that during brain development of DS fetuses, a perturbation in the metabolism of essentially fatty acids occurs, distorting their incorporation into phosphoglycerides (Brooksband *et al.*, 1985). Thus, also in brains of DS fetuses there seems to be a correlation between potential increase of lipid peroxidation and elevated SOD-1 activity. Since the molecular structure of the lipid component has a profound effect on the properties of the membranes, enhanced lipid peroxidation may be related to the reported functional abnormalities in DS membranes, e.g. decrease in Na^+/K^+ ATPase and serotonin uptake in DS platelets (McCoy and Enns, 1978), increased adhesiveness of DS fetal fibroblasts *in vitro* (Wright *et al.*, 1984), as well as abnormal electric membrane properties of DS neurons in cell culture (Scott *et al.*, 1982). It will be interesting to determine whether neuronal cells programmed to express elevated levels of hSOD-1 show any of the above-mentioned defects.

While various other genes residing in the 21q22 segment are undoubtedly involved in the etiology of the Down's phenotype, the data presented here are consistent with the possibility that gene dosage of hSOD-1 leads to increased lipid peroxidation and thus contributes to some of the clinical symptoms associated with Down's syndrome.

Materials and methods

Construction of recombinant plasmids expressing hSOD-1

pSV2-cSOD-SVneo. A cDNA clone containing the entire coding region of hSOD-1 was utilized (Lieman-Hurwitz *et al.*, 1982; Sherman *et al.*, 1983). An *Nde*I site (CATATG) containing the authentic ATG was created at the 5′ terminus of the cDNA clone (Hartman *et al.*, unpublished). This clone was digested with *Nde*I, the 5′-protruding terminus was filled-in and ligated to *Hin*dIII linkers. The plasmid was then digested with *Alu*I (which removes 45 nucleotides of the 3′-untranslated region) plus *Hin*dIII and the resulting 520-bp fragment was inserted at the proper orientation into a pSV2 derivative (Subramani *et al.*, 1981) described below, to create the pSV2-cSOD (Figure 1A). A 2.7-kb *Bam*HI fragment encompassing the *neo* transcriptional unit was removed from pSV2-neo (Southern and Berg, 1982) and inserted into the unique *Bam*HI site of pSV2-cSOD 3′-proximal to the hSOD-1 cDNA (Figure 1A). Expression of both the hSOD-1 cDNA and the *neo* gene was controlled by SV40 regulatory elements (early promoter, enhancer, termination and polyadenylation site). The plasmid used in the construction of pSV2-cSOD was derived from a modified pSV2-DHFR (Canaani and Berg, 1982), which contains the *Eco*RI–*Sal*I fragment of pML (Lusky and Botchan, 1981), by removing the DHFR cDNA in three consecutive steps; (i) digestion with *Bgl*II, (ii) repair to blunt ends, and (iii) digestion with *Hin*dIII, followed by isolation of a 4.3-kb pSV2 vector.

p-GSOD-SVneo. The gene coding for hSOD-1 was previously isolated from human genomic libraries (Levanon *et al.*, 1985). A 13-kb *Eco*RI fragment from λB-1 containing the entire hSOD-1 gene as well as 0.55 kb and 5.0 kb from the 5′- and 3′-flanking sequences, respectively was inserted into the *Eco*RI site of pHG165 (a pBR322 derivative containing a poly linker) (Figure 1B). This plasmid was digested with *Bam*HI which cuts twice; in the poly linker and in the 3′ region of the gene, removing a 1.5 kb 3′-flanking fragment. A 2.7-kb *Bam*HI fragment containing the *neo* transcriptional unit described above was then ligated to the p-GSOD construct to create p-GSOD-SVneo (Figure 1B).

Growth and transfection of cells

Human HeLa and mouse L cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing penicillin, streptomycin and 10% fetal calf serum. The antibiotic G418 (GIBCO, USA) was stored in DMEM (8 mg/ml) and diluted into culture medium as needed. Supercoiled plasmid DNA was introduced into tissue culture cells (10 μg for $\sim 5 \times 10^6$ cells) as a calcium phosphate precipitate (Graham and Van der Eb, 1973; Wigler *et al.*, 1978) followed by a glycerol shock after 4 h. About 48 h later, the cells were trypsinized and replated at a 1:10 dilution. Selection for G418 at 800 μg/ml was instituted 16 h later. G418-resistant sublines were maintained in medium containing 400 μg/ml antibiotic and screened for the expression of hSOD-1. Selected clones were grown to mass culture for biochemical analysis.

Enzymatic assays of hSOD-1

Gel electrophoresis (adapted from Beauchamp and Fridovich, 1971) was performed in 10% polyacrylamide slab gels containing 8 M urea. Gels and running buffer were without SDS and samples were not heated prior to electrophoresis. Superoxide dismutase was localized by soaking the gels in 2.45×10^{-3} M nitroblue tetrazolium for 20 min, followed by immersion for 15 min in a solution containing 0.028 M tetramethylethylenediamine, 2.8×10^{-5} M riboflavin and 0.036 M potassium phosphate at pH 7.8. The gels were then placed in glass trays and illuminated for 5–15 min. During illumination the gels became uniformly blue except at positions containing superoxide dismutase. Illumination was discontinued when maximum contrast between the achromatic zone and the general blue colour had been achieved. The gels were then photographed.

Inhibition of nitrite formation was determined by a spectrophotometric method (adapted from Elstner and Heupel, 1976), nitrite formation from hydroxylammonium chloride was determined under the following conditions: The reaction mixture (0.5 ml) contained 250 μl SOD standard or cell extract in 65 mM phosphate buffer, 25 μl xanthine (1.142 mg/ml), 25 μl hydroxylammonium chloride (0.69 mg/ml), 125 μl KCN (4 mM) or H_2O, 75 μl xanthine oxidase (0.1 U/ml). The mixture was incubated at 25°C for 20 min followed by addition of 0.5 ml α-naphthylamine (1 ng/ml) and 0.5 ml sulfonilic acid (3.3 mg/ml). Incubation was continued at room temperature for 20 min and the O.D. at 530 nm was determined.

Assay of paraquat cytotoxicity

G418-resistant clones and parental cell lines were grown in DMEM supplemented with 10% fetal calf serum. Twenty-four hours before application of paraquat, cells were seeded in 9 mm microwells at 4×10^4 cells/100 μl/well. Paraquat, at various concentrations, was applied in triplicate and the cells were further incubated for 48 h. Viability of the cells was determined by a modification of the procedure described by Finter (1969). Cells were incubated with neutral red for 2 h, excess dye was washed away and the neutral red that was taken up by the cells was extracted with Sorensen's citrate buffer–ethanol mixture and quantitated colorimetrically at 570 nm with a MicroELISA Autoreader (Dynatech, Alexander, VA).

Assay of lipid peroxidation (adapted from Yagi, 1976; Boehme *et al.*, 1977)

Cells, 5×10^5 per 9 cm plate, were maintained for 48 h in DMEM containing penicillin, streptomycin, 4% fetal calf serum and 50 μM linoleic plus arachidonic acids. DMEM containing 10% fetal calf serum was then substituted and the cells were maintained for a further 24 h. Cell monolayers were washed once with phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA) and three times with plain PBS. Cells were then scraped off, quantitatively transferred to an Eppendorf tube, washed with 50 mM Tris (pH 7.0), resuspended in 400 μl of the same buffer and lysed using a Dounce homogenizer. To determine the extent of *in vivo* lipid peroxidation, aliquots (75–150 μl) of cell extracts were immediately removed into an equal volume of 10% trichloroacetic acid (TCA). Following centrifugation at 12 000 *g* for 15 min, two volumes of 0.67% TBA were added to the supernatants and the reaction mixtures were heated for 15 min in boiling water. The TBA-positive material was determined by measuring the relative intensity at 553 nm using a fluorometer (Perkin-Elmer MPF-44A) with excitation at 515 nm. Blanks without cell extracts were similarly processed. For determination of *in vitro* lipid peroxidation, aliquots (75–150 μl) were removed into glass tubes containing 10 μM $FeSO_4$ and 250 μM ascorbic acid in a final volume of 300 μl and tubes were incubated uncovered by shaking at 37°C for 2 h. Reactions were stopped by TCA and TBA-positive material was determined as before, except the absorbance at 532 nm was measured by a spectrophotometer (Beckman DU-5). A standard curve of 0–10 nmol MDA was prepared from 1,1,3, 3-tetraethoxypropane (Sigma) and read either by the fluorometer or by the spectrophotometer; the results are expressed as pmol MDA per μg protein.

Other procedures used in this work have been described previously; labeling, extraction and immunoprecipitation of SOD-1 from human and mouse cells in Lieman-Hurwitz *et al.* (1982), DNA-blot hybridization and S1 nuclease analysis in Sherman *et al.* (1983, 1984) and Levanon *et al.* (1985).

Acknowledgements

We thank Dan Canaani and Paul Berg for providing the pSV2 vectors, Jacob Hartman and Marian Gorecki for the SOD-1 plasmid containing the *NdeI* site and Jonathan Kuhn for the plasmid pHG165. We also wish to thank Ephraim Yavin for his help and comments concerning the analysis of fatty acids and lipid peroxidation and Meir Shinizky for helpful discussions about lipid peroxidation and membrane functions. This work was supported by a Basic Research Grant No. 1-906 from the March of Dimes Birth Defects Foundation; by BioTechnology General Corp., (Israel), by Minerva Foundation, Munich, FRG, and by the Weizmann Institute's Leo and Julia Forchheimer Center of Molecular Genetics.

References

Autor,A.P., ed. (1977) *Biochemical Mechanisms of Paraquat Toxicity*, Academic Press, NY.

Badwey,J.A. and Karnovsky,M.L. (1980) *Annu. Rev. Biochem.*, **49**, 695–726.

Beauchamp,C. and Fridovich,I. (1971) *Ann. Biochem.*, **44**, 276–278.

Berk,A.J. and Sharp,P.A. (1977) *Cell*, **12**, 721–732.

Boehme,D.H., Kosecki,R., Carson,S., Stern,F. and Marks,N. (1977) *Brain Res.*, **136**, 11–21.

Bond,D.J. and Chandley,A.C. eds. (1983) *Aneuploidy*, Oxford University Press, London.

Brooksbank,B.W.L. and Balazs,R. (1984) *Dev. Brain Res.*, **16**, 37–44.

Brooksbank,B.W.L., Martinez,M. and Balazs,R. (1985) *J. Neurochem.*, **44**, 869–874.

Burgio,G.R., Fraccaro,M., Tiepolo,L. and Wolf,U. eds. (1981) *Trisomy 21*, Springer, Berlin.

Bus,J.S., Aust,S.D. and Gibson,J.E. (1977) in Autor,A.P. (ed.), *Biochemical Mechanisms of Paraquat Toxicity*, Academic Press, NY, pp. 157–174.

Canaani,D. and Berg,P. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5166–5170.

Dahle,L.K., Hill,E.G. and Holman,R.T. (1962) *Arch. Biochem. Biophys.*, **98**, 253–261.

Danciger,E., Dafni,N., Bernstein,Y., Laver-Rudich,Z., Neer,A. and Groner,Y. (1985) in press.

de la Cruz,F.F. and Gerald,P.S. eds. (1981) *Trisomy 21 (Down's Syndrome) Research Perspective*, University Park Press, Baltimore, MD.

Elstner,E.F. and Heupel,A. (1976) *Ann. Biochem.*, **70**, 616–620.

Epstein,C.J. and Epstein,L.B. (1981) in de la Cruz,F.F. and Gerald,P.S. (eds.), *Trisomy 21 (Down's Syndrome) Research Perspective*, University Park Press, Baltimore, MD, pp. 253–260.

Epstein,C.J., Epstein,L.B., Cox,D.R. and Weil,J. (1981) in Burgio,A.R., Fraccaro,M., Tiepolo,L. and Wolf,U. (eds.), *Trisomy 21*, Springer-Verlag, NY, pp. 155–172.

Epstein,C.J., Epstein,L.B., Weil,J. and Cox,D.R. (1982) *Ann. N.Y. Acad. Sci.*, **396**, 107–118.

Farrington,J.A., Ebert,M., Land,E.J. and Fletcher,K. (1973) *Biochim. Biophys. Acta*, **314**, 372–381.

Finter,N.B. (1969) *J. Gen. Virol.*, **5**, 419–427.

Francke,U. (1981) in de la Cruz,F.F. and Gerald,P.S. (eds.), *Trisomy 21 (Down's Syndrome) Research Perspective*, University Park Press, Baltimore, MD, pp. 237–251.

Fridovich,I. (1978) *Science*, **201**, 875–880.

Fridovich,.I. (1979) in Eichhorn,G.L. and Marzilli,L.G. (eds.), *Advances in Inorganic Biochemistry*, Elsevier/North-Holland, NY, pp. 67–90.

Frischer,H., Cher,L.K., Ahmad,T., Justice,P. and Smith,G.F. (1981) *Prog. Clin. Biol. Res.*, **55**, 269–283.

Graham,F.L. and Van der Eb,A.J. (1973) *Virology*, **52**, 456–467.

Hassold,T.J. and Jacobs,P.A. (1984) *Annu. Rev. Genet.*, **18**, 69–97.

Hassan,H.M. and Fridovich,I. (1977) *J. Biol. Chem.*, **252**, 7667–7672.

Hassan,H.M. and Fridovich,I. (1979) *Arch. Biochem. Biophys.*, **196**, 385–395.

Hook,E.B. (1981) in de la Cruz,F.F. and Gerald,P.S. (eds.), *Trisomy 21 (Down's Syndrome) Research Perspective*, University Park Press, Baltimore, MD, pp. 3–68.

Kanner,J. and Harel,S. (1985) *Arch. Biochem. Biophys.*, **237**, 314–321.

Kedziora,J., Lukaszewicz,R., Koter,M., Bartosz,G., Pawlowska,B. and Aitkin,D. (1982) *Experientia*, **38**, 543–544.

Lejeune,J.M., Gautier,M. and Turpin,R. (1959) *C.R. Acad. Sci.*, **248**, 1721–1722.

Levanon,D., Lieman-Hurwitz,J., Dafni,N., Wigderson,M., Sherman,L., Bern-

stein,Y., Laver-Rudich,Z., Danciger,E., Stein,O. and Groner,Y. (1985) *EMBO J.*, **4**, 77–84.
Lieman-Hurwitz,J., Dafni,N., Lavie,V. and Groner,Y. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2808–2811.
Lusky,M. and Botcham,M. (1981) *Nature*, **293**, 79–81.
Malstrom,B.G. (1982) *Annu. Rev. Biochem.*, **51**, 21–59.
McCoy,E.E. and Enns,L. (1978) *Pediatr. Res.*, **12**, 685–689.
Moody,C.S. and Hassan,H.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2855–2859.
Mulligan,R.C. and Berg,P. (1980) *Science*, **209**, 1422–1427.
Niehaus,W.G., Jr. and Samuelsson,B. (1968) *Eur. J. Biochem.*, **6**, 126–130.
Oberley,L.W., ed. (1982) *Superoxide Dismutase*, CRC Press, Boca Raton, FL.
Pantelokis,S.N., Karaklis,A.G., Alexion,D., Verdas,E. and Valaes,T. (1972) *Am. J. Hum. Genet.*, **22**, 184–193.
Patterson,D., Jones,C., Scoggin,C.H., Miller,Y.E. and Graw,S. (1982) *Ann. N.Y. Acad. Sci.*, **396**, 69–81.
Pryor,W.A., Stanley,J.P. and Blair,E. (1976) *Lipids*, **11**, 370–379.
Scoggin,C.H. and Patterson,D. (1982) *Arch. Intern. Med.*, **142**, 462–464.
Scott,B.S., Pettit,T.L., Becker,L.E. and Edwards,B.A.V. (1982) *Dev. Brain Res.*, **2**, 257–270.
Sherman,L., Dafni,N., Lieman-Hurwitz,J. and Groner,Y. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5465–5469.
Sherman,L., Levanon,D., Lieman-Hurwitz,J., Dafni,N. and Groner,Y. (1984) *Nucleic Acids Res.*, **12**, 9349–9365.
Sinet,P.M. (1982) *Ann. N.Y. Acad. Sci.*, **396**, 83–94.
Sinet,P.M., Michelson,A.M., Bazin,A., Lejeune,J. and Jerome,H. (1975) *Biochem. Biophys. Res. Commun.*, **67**, 910–915.
Smith,G.F. (1985) *Ann. N.Y. Acad. Sci.*, **450**.
Smith,G.F. and Berg,J.M. (1976) *Down's Anomaly* 2nd ed., Churchill Ltd., London.
Smith,G.F. and Warren,S.T. (1985) *Ann. N.Y. Acad. Sci.*, **450**, 1–9.
Southern,P.J. and Berg,P. (1982) *J. Mol. Genet.*, **1**, 327–341.
Subramani,S., Mulligan,R. and Berg,P. (1981) *Mol. Cell. Biol.*, **1**, 854–864.
Summitt,R.L. (1981) in de la Cruz,F.F. and Gerald,P.S. (eds.), *Trisomy 21 (Down's Syndrome) Research Perspective*, University Park Press, Baltimore, MD, pp. 225–235.
Weaver,R.F. and Weissman,C. (1979) *Nucleic Acids Res.*, **7**, 1175–1193.
Wigler,M., Pellicer,A., Silverstein,S. and Axel,R. (1978) *Cell*, **14**, 725–731.
Wijnen,L.M.M., Monteba-Ven Heusel,M., Pearson,P.L. and Khan,P.M. (1978) *Cytogenet. Cell Genet.*, **22**, 232–235.
Wright,T.C., Destrempes,M., Orkin,R. and Kurnit,D.M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 2426–2430.
Yagi,K., ed. (1982) *Lipid Peroxidation in Biology and Medicine*, Academic Press, NY.
Yagi,K. (1976) *Biochem. Med.*, **15**, 212–216.

Received on 4 November 1985; revised on 24 December 1985