Induction of Superoxide Dismutase in Leukocytes by Paraquat: Correlation With Age and Possible Predictor of Longevity

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Reactive oxygen species (ROS) are thought to play a role in the aging process as well as in a number of human diseases states. Superoxide dismutase (SOD), an enzyme that scavenges the superoxide anion (O_2^-) is constitutively expressed in leukocytes and other tissues. When assayed in peripheral blood leukocytes (PBL), constitutive SOD activity shows little variation among individuals of different ages. We have found that significant induction of SOD activity occurs in PBL incubated in vitro with paraquat, an agent known to cause intracellular O_2^- production. This induction was found to be highly age dependent; lymphocytes from 36 healthy subjects aged 20 to 40 years showed an increase of 85% ± 10%, versus an increase of only 8% ± 1% for lymphocytes from 30 healthy subjects aged 65 to 79 years ($P < 10^{-4}$). Forty subjects, aged 67 to 73 years, who were healthy at the time of assay of leukocyte SOD induction were followed up 5 years later. Nineteen of these subjects had died; all 19 had shown SOD induction of less than 10% (range, 0% to 7%; mean, 2.4%). In contrast, of the 21 survivors (range, 2.5% to 50%; mean, 21%), 12 had shown SOD induction greater than 10%, and 7 had shown SOD induction ≥35% ($P < 10^{-3}$). Thirteen of the 19 deaths were attributable to malignancy or cerebrocardiovascular disease. Preservation of leukocyte SOD inducibility appears to correlate with longevity in elderly individuals and may be of value in predicting resistance to malignancy or fatal cardiovascular events.

IN RECENT YEARS reactive oxygen species (ROS) and lipid peroxides have been implicated in the pathogenesis of a large number of diseases[1-24] and environmental toxicities.[25-27a] These include many intractable diseases such as Behçet's disease,[1] mucocutaneous lymph-node syndrome (MCLS),[2] rheumatoid arthritis (RA),[3] dermatitis herpetiformis,[4] Crohn's disease, diabetes mellitus, infectious diseases such as hepatitis, and dermatologic diseases such as treatment-resistant dermatitis,[16,17] ulcers of mucosa and skin,[14,16] burns[18] and wounds,[14] and keloid formation.[19] In addition, oxygen toxicity due to ROS and/or lipid peroxides, to some extent generated by modern environmental pollutants, has been implicated in carcinogenesis[23,24] and aging.[7,20]

Superoxide dismutase (SOD) is a class of enzyme that effectively scavenges ROS and inhibits lipid peroxidation.[28-30] Genetic polymorphism in man has been observed for both SOD isomers, Cu, Zn-SOD (locus on chromosome 21) and Mn-SOD (locus on chromosome 6). We have shown that individuals with a strong SOD induction capacity tend to respond more promptly to treatment of diseases that involve ROS than do those whose SOD induction capacity is low.[16,31] Furthermore, there have been reports that constitutive SOD activity decreases with age,[32-34] although we and others have not been able to confirm this finding.[20,31,35]

From the foregoing it may be hypothesized that genetic or acquired variation in SOD activity among individuals may correlate with resistance or susceptibility to disease. When assayed in healthy individuals, constitutive SOD activity in leukocytes or tissues shows little variation.[20]

In bacteria[36] and plants,[37,38] it has been observed that exposure to oxidative stress cause a marked increase in SOD activity. We have recently reported that although SOD activity is not different between young and elderly individuals, the SOD induction capacity is diminished in the elderly compared with that in young adults when assessed under conditions of oxygen stress.[31,39,40] In addition, significant individual differences that are independent of age are seen in the levels of SOD activity induced by oxygen stress. We have used an assay system in which leukocytes are exposed to the herbicide paraquat, a compound known to stimulate production of O_2^- [41,42] with subsequent formation of OH · to assess the induction of SOD activity by oxygen stress. In the presence of optimal concentrations of paraquat, peripheral blood leukocytes (PBL) from healthy young adults show up to twofold increases in SOD activity. This assay method revealed significant differences in SOD inducibility between aged and non-aged healthy individuals. Further studies indicated a possible correlation of the assay results with disease susceptibility and longevity in elderly subjects.

MATERIALS AND METHODS

Subjects consisted of 99 nonaged healthy volunteers (51 males aged 20 to 54 years, 48 females aged 20 to 54 years) and 43 aged asymptomatic individuals (20 males aged 65 to 88 years, 23 females aged 65 to 96 years).

In addition to this cross-sectional comparison between the SOD induction capacities of aged individuals and that of young adults, 40 subjects, aged 67 to 73 years who were asymptomatic upon entry into the study, were assessed for SOD induction capacity by the paraquat method, and 5 years later morbidity and mortality were assessed in this group.

In preliminary experiments optimal concentrations of paraquat were found that had minimal effect on cell viability. Viability was determined by trypan blue dye exclusion for both lymphocytes and neutrophils, by the phagocytizing capacity using oil Red O for neutrophils, and by responsiveness to phytohemagglutinin (PHA) for lymphocytes at varying concentrations of paraquat ($2.45 \times 10^{-4} - 2.45$ mmol/L). Preliminary time-course experiments were also carried out to determine the optimal incubation time with paraquat for maximal leukocyte SOD induction.

Peripheral blood neutrophils and lymphocytes were isolated by

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Ficoll-Hypaque (FH) gradient centrifugation under conditions previously shown to maintain maximum cell viability.[4] Neutrophils or lymphocytes (3×10^6/mL) suspended in RPMI medium containing 10% fetal calf serum (FCS) were incubated with 2.45×10^{-3} or 2.45×10^{-2} mmol/L paraquat (Wakojunyaku Kogyo Co, Osaka, Japan) at 37°C for 18 hours in a humidified 5% CO_2 atmosphere. Thereafter cells were harvested by centrifugation and sonicated, and the supernatants were obtained after centrifugation at 14,800*g* for 20 minutes.

For the assay of SOD activity, 0.2 mL of supernatant was added to the xanthine-xanthine oxidase O_2^- generating system. This system consisted of 16.5 μmol/L ferricytochrome *c*, 0.1 mmol/L hypoxanthine, and 1.25 mmol/L EDTA in total volume of 2 mL of 125 mmol/L phosphate buffer. After the addition of cell supernatant, 0.006 U/mL of dialyzed xanthine oxidase was added to generate O_2^-. Under these assay conditions the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50% (ie, to a rate of 0.0125 absorbance at 550 nmol/L U/min) was defined as 1 U of activity. Since percent inhibition in leukocytes is not linear above approximately 1 U, it was adjusted according to Asada's formula.[43]

The SOD induction capacity of each leukocyte supernatant was calculating from the following formula:

$$\% \text{ SOD Induction} = \left(\frac{b - a}{a}\right) \times 100,$$

where a is SOD activity in the absence of paraquat and b is SOD activity in the presence of paraquat.

For the neutrophil phagocytosis assay, emulsions of paraffin oil containing oil red O were prepared as previously described,[44] except that a lipopolysaccharide solution was replaced with normal human serum. The emulsion was incubated with an equal volume of normal human serum at 37°C for 30 minutes for opsonization. Neutrophils (2×10^7 cells/0.9 mL Krebs-Ringer phosphate buffer (KRP)) were preincubated with paraquat for 6 or 18 hours and washed. Thereafter 0.1 mL of the opsonized emulsion was added to the neutrophil suspension, and the mixture was incubated for 5 minutes at 37°C. Then 9 mL ice-cold KRP was added to the solution to stop the reaction. The cells were washed three times with ice-cold KRP to remove the paraffin oil droplets that had not been ingested. Paraffin oil containing oil red O was extracted from the cells by the method of Bligh and Dyer[45] using chloroform and methanol (vol/vol, 1:2), and the optical density of the chloroform layer was determined at a wavelengths of 525 nm.

The lymphocytes that had been preincubated for 6 or 18 hours with paraquat were examined for their responses to PHA. Briefly, 3×10^6 lymphocytes suspended in RPMI 1640 medium containing 20% heat-inactivated pooled human AB serum and 2×10^5 mitomycin-C–treated monocytes were incubated 3 days at 37°C in a humidified 5% CO_2 atmosphere in the presence of 10 μg/mL PHA. Lymphocyte blastogenesis was assessed by the DNA uptake of tritiated thymidine (^{3}H) Tdr (2 Ci/mmol/L, New England Nuclear, Boston, MA) during the last 24 hours of culture.

The results were expressed as the mean ± SD of replicate assay. Statistical significance was determined by χ^2 and by Student's *t* test.

RESULTS

In preliminary experiments at paraquat concentrations of 2.45×10^{-3} and 2.45×10^{-2} mmol/L and an incubation time of 18 hours, substantial induction of SOD activity was seen in both lymphocytes and neutrophils while cell viability remained greater than 90% (Figs 1 through 3). Lymphocytes showed a greater capacity for SOD induction than neutrophils, particularly with an 18-hour incubation period (Fig 1). For each individual, determinations of SOD induction were carried out with both neutrophils and lymphocytes, each at paraquat concentrations of 2.45×10^{-3} and 2.45×10^{-2} mmol/L. For each individual subject, the percent induction reported in this study was the highest of the four separate assays.

In preliminary experiments, the addition of low concentrations of PHA to the lymphocyte culture during the incubation with paraquat increased the % SOD induction, but it did not significantly improve lymphocyte viability (Fig 2). Furthermore, the presence of PHA for longer periods (48 to 96 hours) reduced cell viability, as shown in Figs 2 and 3. Therefore in this study lymphocytes were incubated for 18 hours in the presence of 2.45×10^{-3} mmol/L or 2.45×10^{-2} mmol/L paraquat without addition of PHA.

Lymphocytes from 36 subjects aged 20 to 40 years

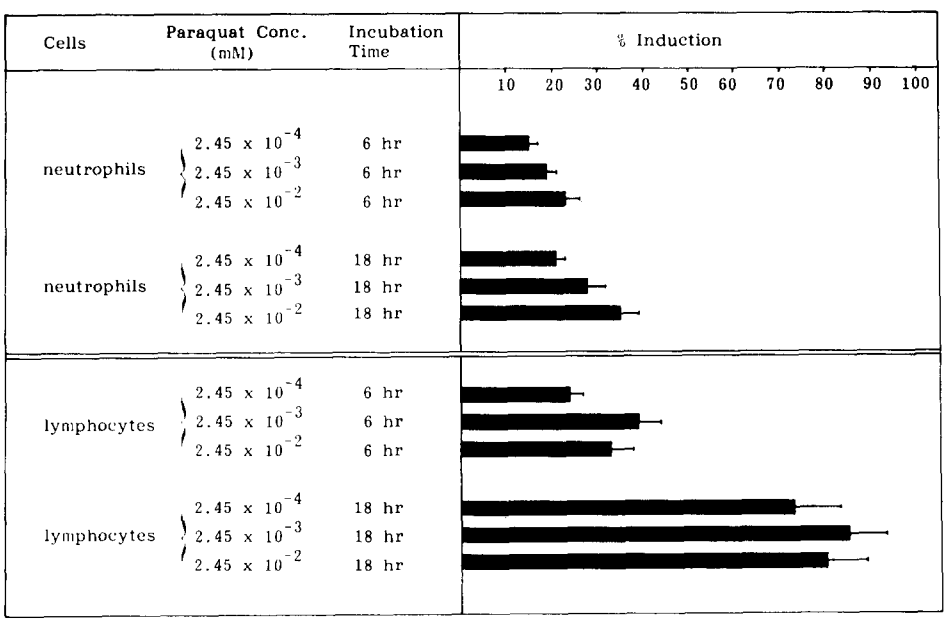

Fig 1. Effect of paraquat concentration and time of incubation on percent SOD induction in neutrophils and lymphocytes from young adults. Leukocytes from 10 healthy donors, aged 20 to 40 years, were assessed in triplicate. Results are expressed as mean ± SEM.

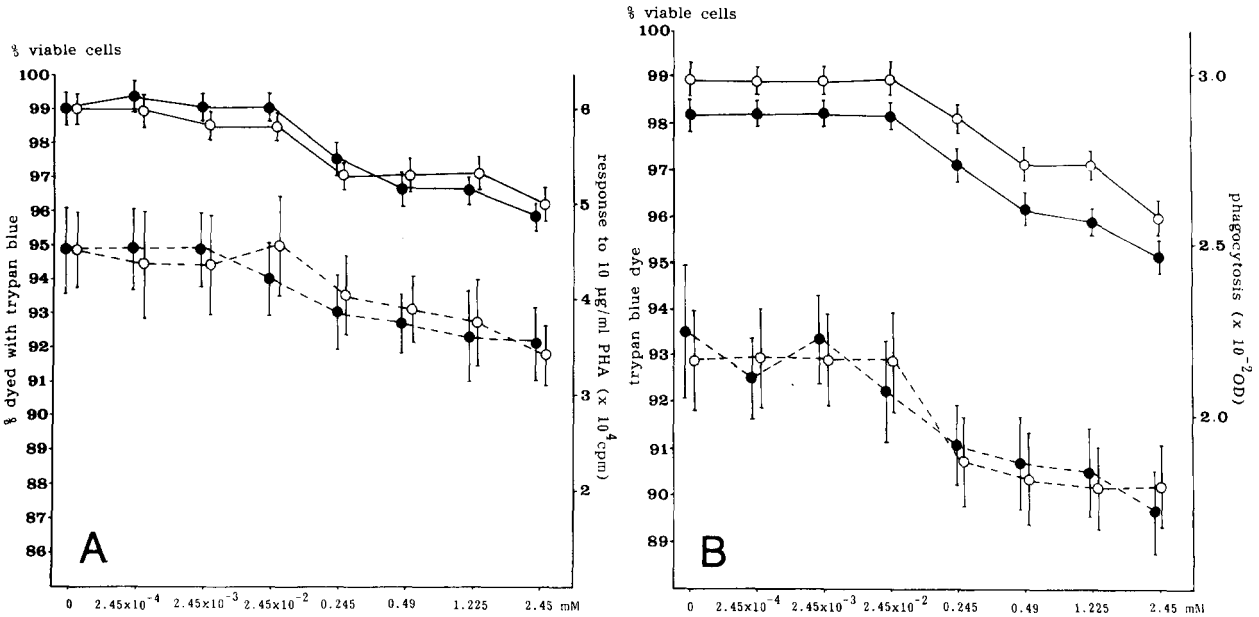

Fig 2. Correlation of cell viability with paraquat concentration. (A) Lymphocytes. Cell viability as assessed by trypan blue exclusion (——) and proliferative response to 10 μg/mL PHA (----). ○, 6-hour incubation; ●, 18-hour incubation at 37°C with the indicated concentrations of paraquat. (B) Neutrophils. Cell viability by trypan blue (——) and by phagocytosis using paraffin oil (----). ○ and ● are as in A. Donors were as in Fig 1. Results are expressed as mean ± SEM (N = 10).

incubated with 2.45 × 10^{-2} mmol/L paraquat showed 85.1% ± 10.3% SOD induction, whereas lymphocytes from 30 subjects aged 65 to 79 years incubated with the same concentration of paraquat showed only 8.0% ± 0.9% induction (Table 1, $P < .001$). Similarly SOD induction in neutrophils was significantly higher in the younger adults (28.4% ± 3.6%) than in the elderly subjects (3.5% ± 0.3%; $P < .01$). As shown in Fig 4, SOD induction declined with

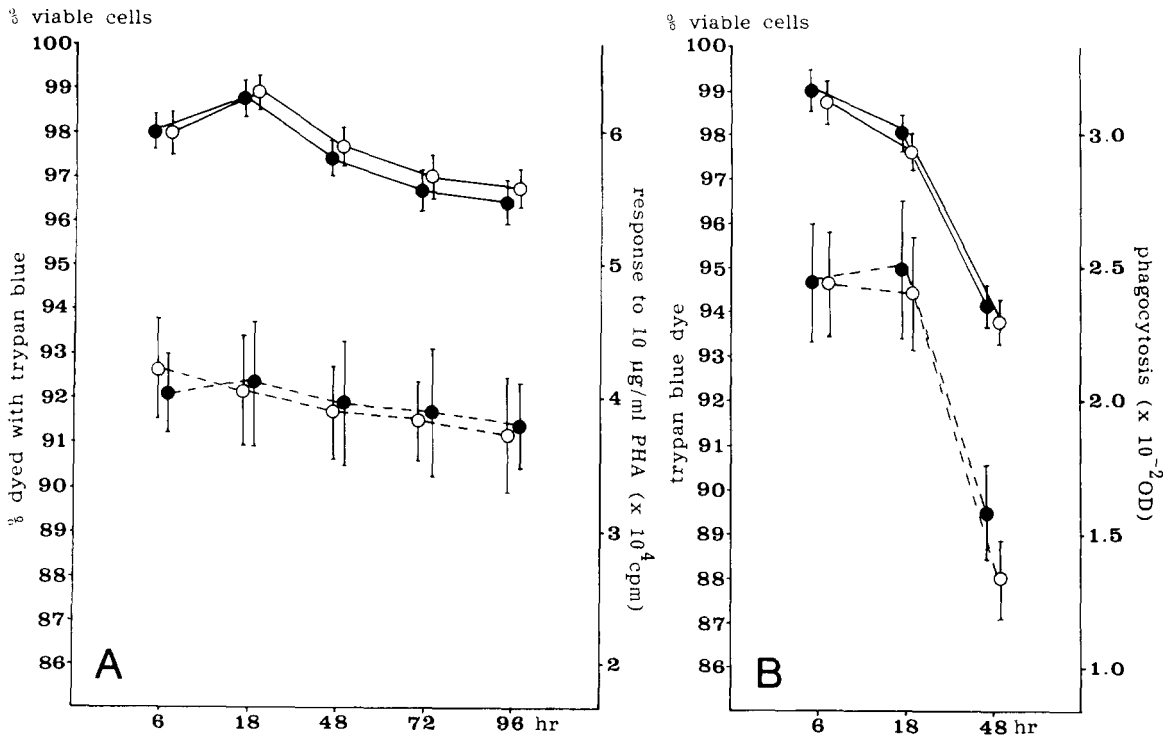

Fig 3. Correlation of incubation time to leukocyte viability (N = 6). (A) Lymphocyte viability after incubation for the indicated periods of time at 37°C with paraquat, 2.45 × 10^{-2} mmol/L (○) or 2.45 × 10^{-3} mmol/L (●). (B) Neutrophil viability after incubation for the indicated periods of time at 37°C with paraquat, 2.45 × 10^{-2} mmol/L (○), or 2.45 × 10^{-3} mmol/L (●). —— and ---- are as defined in legend for Fig 2; donors were as in Fig 1.

Table 1. Percent SOD Induction of Neutrophils and Lymphocytes From Asymptomatic Younger and Aged Individuals

Group/N	Lymphocytes	Neutrophils
Younger adults (20-40 yrs)/36	85.1 ± 10.3	28.4 ± 3.6
Aged individuals (65-79 yrs)/30	8.0 ± 0.9	3.5 ± 0.3

Lymphocytes and neutrophils were incubated at 37°C for 18 hours with 2.45×10^{-2} mmol/L paraquat, respectively, and assessed for SOD induction as described in Methods.

age beginning in the fifth decade, increasing slightly in those over 80 years old.

Forty subjects, 67 to 73 years of age and in good health upon entry into the study, were assessed for SOD induction. Over the next 5 years, 19 of these subjects died, while 21 survived. The 19 nonsurvivors had originally shown markedly lower SOD induction (mean 2.4%) compared with both young adult controls ($P < .0001$) and the 21 survivors (mean 21.1%, $P < .01$; Tables 1 and 2). None of the nonsurvivors showed SOD induction above 7%; in contrast, one third of the 21 survivors (cases 3, 6, 8, 11, 14, 17, 19) showed potent SOD induction (35% to 50%). As shown in Table 3, the number of survivors who showed SOD induction above 10% was far greater than that of nonsurvivor ($P < .001$). Among the nonsurvivors, seven cases died of malignancies (cases 23, 24, 27, 28, 30, 31, 34) and five of cerebrocardiovascular disease (cases 33, 37, 38, 39, 40; two with myocardial infarction, two with strokes and one with Bürger's disease; Table 2). As shown in Table 1 and Figs 1 and 2, SOD induction in neutrophils was qualitatively similar to that in lymphocytes but quantitatively lower.

DISCUSSION

In this study, we describe a reliable technique for assessing the induction of SOD activity in leukocytes subjected to oxygen toxicity caused by the presence of the drug paraquat, which had previously only been reported to induce SOD activity in bacteria.[46] It has been reported that in bacteria and human cancer cell lines, only Mn-SOD is induced under oxidative toxicity or inducers such as TNF or IL1.[47-49] However, in our study not only Cu, Zn-SOD, but also Mn-SOD has been induced to the similar extent (submitted for publication).

Application of this assay of SOD induction using paraquat to normal human subjects demonstrated a marked age-related decline in SOD induction beyond 40 years of age. Interestingly there was a slight increase in SOD induction in those over 80 years of age compared with those in the eighth decade. This finding suggested that preservation of the capacity for SOD induction might be correlated with increased longevity. A similar pattern has been observed in studies of neutrophil chemotaxis in the elderly.[20]

To test the hypothesis that SOD induction might be correlated with survival, we performed a longitudinal study of 40 initially healthy elderly subjects whose SOD induction was assayed upon entry into the study. There was a striking difference with respect to paraquat-induced SOD induction between the 21 subjects who survived 5 years and the 19 subjects who died during this follow-up period. Of the 19 deaths, 13 were due to either malignancies or cardiovascular disease, disease processes for which there may be a pathogenetic role for ROS and lipid peroxides.[5,6,24,25] The results of the 5-year longitudinal study thus are consistent with the earlier cross-sectional study, suggesting that the preservation of SOD induction capacity is correlated with survival to advanced age. Furthermore, these studies raise the possibility that measurement of SOD induction in an individual subject may serve as a predictor of longevity as well as of predisposition to malignancy and cardiovascular disease.

There has been a controversial report[50] that by SOD

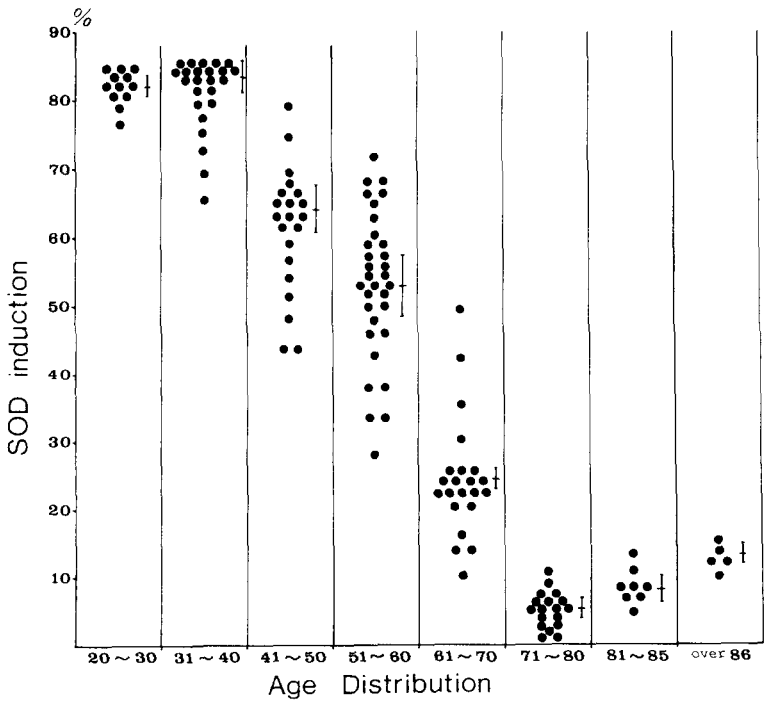

Fig 4. Percent SOD induction of lymphocytes in each age bracket. Lymphocytes were incubated for 18 hours in the presence of 2.45×10^{-2} mmol/L paraquat.

Table 2. SOD Induction of Lymphocytes and Subsequent 5-Year Course of Subjects Aged 67 to 73 Years and Healthy Upon Entry Into the Study

Case No.	Age/Sex	Date Examined	% SOD Induction	Date of Death	Cause of Death
Survivors					
1	68/M	5/83	8.4		
2	71/M	8/83	3.2		
3	73/F	10/83	39.2		
4	67/M	1/84	7.4		
5	70/F	11/83	5.6		
6	72/F	5/83	45.7		
7	72/M	9/83	2.6		
8	73/F	2/84	50.0		
9	70/M	2/84	3.9		
10	73/M	8/83	11.4		
11	70/F	12/83	46.3		
12	67/F	1/84	8.3		
13	69/F	6/83	18.2		
14	70/M	7/83	35.1		
15	72/F	3/83	18.3		
16	68/F	4/83	4.9		
17	68/M	2/84	41.8		
18	70/F	12/83	30.2		
19	72/M	10/83	37.7		
20	72/F	6/83	22.1		
21	73/F	10/83	2.5		
Mean			21.08		
Nonsurvivors					
22	69/M	7/83	1.2	8/87	Sepsis
23	73/M	12/83	0.8	9/86	Lung cancer
24	72/F	11/83	1.4	4/86	Acute myelocytic leukemia
25	69/M	2/84	2.3	1/89	Pneumonia
26	68/F	10/83	1.5	5/85	Urosepsis
27	67/M	5/83	0	2/86	Pharyngial cancer
28	72/M	1/84	2.3	10/88	Lung cancer
29	71/F	4/83	4.5	7/85	Stroke
30	69/M	2/84	1.8	1/88	Brain tumor
31	73/M	10/84	0	6/87	Gastric cancer
32	72/F	4/83	5.6	11/87	Pneumonia
33	71/F	12/85	7.1	2/89	Stroke
34	70/M	6/84	1.4	10/88	Rectal cancer
35	69/M	8/83	3.2	9/88	Pneumonia
36	70/F	4/83	5.4	4/87	Pyelitis
37	72/F	3/85	1.1	11/88	Myocardial infarction
38	70/M	8/83	4.2	12/88	Bürger's disease
39	71/M	11/84	0	10/87	Myocardial infarction
40	70/M	11/84	2.8	10/87	Stroke
Mean			2.45		

enzyme, oxidative damages in the cells and organs will be increased instead of being reduced; increasing the SOD content of erythrocytes predisposed these cells to greater oxidant damage because SOD will push superoxide into hydrogen peroxide, which is more cytotoxic than superoxide. However, one of the main principles of SOD action mechanism is considered to be as follows: superoxide radical that is scavenged by SOD directly inactivates various enzymes, including catalase[51] and glutathione peroxidase.[52] In this connection, in the presence of sufficient amount of SOD, H_2O_2 is also well quenched with catalase (and glutathione peroxidase), which are protected from being degraded or inactivated by superoxide radical.

Table 3. Comparison of the Number of Survivors and Nonsurvivors With SOD Induction Above or Below 10%

SOD Induction	5-yr Survivor	5-yr Nonsurvivor
>10%	12	0
<10%	9	19

$P < .01$ between survivor and nonsurvivor by χ^2. See legend for Table 1.

After many preliminary experiments, paraquat was chosen to induce oxygen stress in preference to direct addition of O_2^-, ROS, or lipid peroxides because the latter additions produced unacceptable reduction in cell viability. The mechanism of SOD induction by paraquat is presumed to be the generation of intracellular O_2^-, with subsequent stimulation of SOD synthesis, perhaps by the action of one or more as yet undetermined intermediate compounds.

Although the lack of induction of SOD activity in response

to paraquat is most likely due to a defect in the steps leading to enzyme synthesis, we have not excluded the possibility that lack of induction of SOD activity in some cell preparations in response to paraquat may be due to an inhibition of enzyme synthesis itself induced by paraquat toxicity. This explanation may be applicable in cases in which induction was seen at a paraquat concentration of 2.45×10^{-3} mmol/L but not at 2.45×10^{-2} mmol/L. Preliminary evidence in support of this hypothesis has been obtained in intrinsic labeling experiments using ^{3}H leucine uptake into the SOD enzyme and other protein[53] in our laboratory (Niwa et al, unpublished data, November 1989).

Alternatively lack of induction may reflect a constitutive level of intracellular SOD sufficient to quench the ROS generated by the concentration of paraquat used. This explanation may be applicable in cases in which induction was seen at a paraquat concentration of 2.45×10^{-2} mmol/L but not at 2.45×10^{-3} mmol/L. Further experiments will be necessary to investigate in more detail the intracellular mechanisms involved in paraquat-induced SOD induction. In addition, it is required to examine in future studies whether paraquat causes a generalized increase in protein synthesis in treated cells. However, regardless of the mechanism, our studies indicate a striking correlation between age and longevity on the one hand and the response of leukocyte SOD activity to paraquat on the other hand. It is well known[25,54] that paraquat mediates O_2^- production, and it is surmised that this leads to OH · production with resultant nuclear chromosome aberration and DNA injury and that long use of this cytotoxic herbicide will be the cause for carcinogenesis and malformation. Investigation of this correlation of aging with SOD induction using paraquat, along with previously reported correlations of aging with neutrophil chemotaxis and serum-lipid peroxide levels,[20] should provide new and important insight into the nature of the aging process and possibly carcinogenesis in humans.

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