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Original Articles

Cellular Penetration of Fluorescently Labeled Superoxide Dismutases of Various Origins

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Abstract

Background: Using fluorescently labeled superoxide dismutase (SOD) and flow cytometry, we have shown previously that the enzyme CuZn SOD (EC 1.15.1.1) from bovine erythrocytes binds rapidly to the cell surface with slow uptake into the cell during the following hours. The degree of labeling was most important for monocytes in comparison to other blood cells (erythrocytes, lymphocytes, and neutrophils) and fibroblasts. In agreement with the flow-cytometric findings, the inhibition of superoxide production was more important for SOD-pretreated monocytes than for neutrophils, as demonstrated with the cytochrome c reduction assay. It was thus of interest to confirm the observed differences between monocytes and neutrophils with confocal laser microscopy, study in greater detail the kinetics of binding, penetration, and intracellular localization of the enzyme, and compare the results obtained with bovine CuZn SOD with those from SODs of other origins and carrying different active sites.

Materials and Methods: Recombinant human (rh), bovine, and equine CuZn SODs, as well as rh and *E. coli* Mn SODs, were studied before use with respect to specific activity and purity (HPLC, SDS-PAGE electrophoresis). Fluorescein isothiocyanate was covalently conjugated to the various SODs for study with high-resolution confocal scanning laser microscopy. Superoxide production by monocytes and neutrophils was measured with the cytochrome *c* assay.

Results: As expected from our experiments with flow cytometry, only rare neutrophils were labeled with FITC-SOD, even with the longest incubation time of 3 hr and the highest dose of 1500 units/ml. In addition, they showed a localized fluorescence pattern that was quite different from the diffuse punctate fluorescence pattern of monocytes. Lymphocytes were not labeled at all. The rapid binding to the cellular surface of monocytes was confirmed, and even after 5 min of preincubation, FITC-SOD was found on a small percentage of monocytes. This was correlated with a reduction in superoxide release after phorbolmyristate acetate (PMA) stimulation by 40%. An interesting finding was the perinuclear accumulation of the penetrated SOD after the longest pretreatment of 3 hr, suggesting a barrier against further progression. Indeed, through confocal microscopy we were able to exclude any fluorescence at the nuclear level. While the fluorescence labeling patterns and the kinetics of penetration were quite similar for bovine, equine, and rh CuZn SOD, the Mn SODs showed poor labeling, correlated with a weak inhibitory effect on cytochrome *c* reduction, which was not statistically significant.

Conclusions: The rapid binding of native CuZn SODs on the surface of monocytes, leading to reduced superoxide release by these cells, explains the observation that beneficial effects of injected SOD lasted for months despite rapid clearance of the enzyme from the bloodstream, according to pharmacodynamic studies. The preferential binding to monocytes, in contrast to neutrophils, may play a role in chronic inflammatory diseases in which the monocytes are in an activated state. The differences in binding capacity between CuZn SODs and Mn SODs, correlated with different inhibitory effects of superoxide production by monocytes, may also have therapeutic significance.

Introduction

Soon after the discovery of superoxide dismutase (SOD, EC 1.15.1.1) in 1969 (1), the enzyme was tested for its therapeutic efficacy in free radical–related disorders (2–5). Its beneficial effects were questioned, however, because of the rapid clearance of the enzyme from the bloodstream. Despite several reports indicating intracellular penetration (6–9), many scientists remained convinced that SOD action was limited to the extracellular space.

Using fluorescently labeled bovine CuZn SOD and flow cytometry, we have shown previously that the enzyme binds rapidly to the cells (10). The degree of labeling was concentration-dependent and most important for monocytes in comparison to other blood cells (erythrocytes, lymphocytes, and neutrophils) and human skin fibroblasts. In agreement with the flow-cytometric findings, the cytochrome *c* assay showed significantly diminished $O_2\cdot^-$ production by monocytes pretreated with SOD and washed thereafter, whereas pretreatment of neutrophils with the same enzyme concentrations and incubation times did not result in significant inhibition of $O_2\cdot^-$ production. Another interesting finding was the persistence of the anticlastogenic effects of CuZn SOD after intensive washing of the cells (10). Removal of all extracellular SOD was ascertained by activity measurements in the cell culture medium. This work also included preliminary studies with confocal laser microscopy in monocytes, which showed that the CuZn SOD binds rapidly (15 min) to the cell surface of monocytes, but that the fluorescence becomes visible in the cytoplasm only after 1 hr. No fluorescence was seen at the nuclear level.

Since all these studies were carried out with CuZn SOD extracted from bovine erythrocytes, it was important to confirm whether the observations described above are also true for SODs of other origins.

Materials and Methods

Enzymes

CuZn SOD from bovine and horse erythrocytes were obtained from Palleau Production (Chateau-Landon, France). Manganese SOD from *E. coli* and bovine CuZn SOD for comparative studies were purchased from Sigma (St. Louis, MO). Recombinant human (rh) CuZn and Mn SOD were a gift from Bio-Technology General (BTG) Ltd (Rehovot, Israel). Catalase from bovine liver was obtained from Sigma.

All SODs were studied before use for specific activity and purity. The activity was assayed through the reduction of cytochrome *c* by superoxide generated with a xanthine-xanthine oxidase reaction (1). The protein content of the samples was measured according to Lowry et al. (11). The purity of the enzymes was studied by high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE).

HPLC

Aliquots containing the same activity of each SOD were injected into an anion exchange column for protein separation (PVDI 31.75 × 100 mm, Société française de chromato-colonnes, Paris, France) using a 30-min NaCl gradient (0–0.15 M) in 20 mM Tris-HCL, pH 7.5 with a flow rate of 1 ml/min. The peaks were registered with a UVA detector at a wavelength of 280 nm, AUFSS = 0.04, chart speed 12 cm/hr.

SDS-PAGE

Electrophoretic separation by SDS-PAGE was performed in a Pharmacia apparatus with a 0.5-mm-thick 7.5% polyacrylamide gel for horizontal electrophoresis as described by Laemmli (12). The gels were silver stained according to Heukeshover and Dernick (13).

Preparation of Fluorescently Labeled Enzymes

Fluorescein isothiocyanate (FITC, Sigma) was covalently conjugated to the various SODs and to catalase by constant stirring in 50 mM Na_2CO_3 at 4°C for 1 hr. The FITC-SODs were purified by Sephadex G-25 chromatography with 10 mM potassium phosphate buffer (pH 7.4) as the eluant. The enzymatic activity of FITC-modified SODs was determined with the cytochrome *c* assay in comparison with the respective unlabeled enzymes.

Cells

Venous blood from healthy blood donors was anticoagulated with heparin. Polymorphonu-

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clear neutrophils (PMN) and mononuclear cells were separated by dextran sedimentation and Ficoll/Hypaque density gradient centrifugation. The percentage of monocytes among mononuclear cells varied according to donors with a mean of 5%. Parallel experiments were therefore carried out with the blood of the same donor, and equal numbers of mononuclear cells were brought to adherence on plastic dishes. After 1 hr, the monocyte monolayer was rinsed with phosphate-buffered saline (PBS) to remove the lymphocytes. The lymphocyte suspension was used for the study of this cell type, whereas the monocytes were incubated with FITC-SODs of various origins. Equal quantities of protein or activity units were used in parallel experiments. To avoid nonspecific binding, the cells were pretreated for 20 min with 5% fetal calf serum (FCS) before exposure to FITC-SODs. After incubation with SOD, the cells were washed three times with PBS to eliminate all residual free FITC-SODs.

High-Resolution Confocal Laser Microscopy

Fixation of cells was realized with 4% formol for 30 min. Nuclei were stained with chromomycin a3 (Sigma) for 30 min at room temperature in the dark. After rinsing with PBS, the cell layer was mounted in Fluoprep (Bio-Mérieux, Marcy l'Etoile, France). Confocal fluorescent images were obtained with a MRC-600 confocal scanning laser microscope (Bio-Rad, Richmond, CA), equipped with a 25-mW multiline argon ion laser (Ion Laser Technology, Salt Lake City, UT), a z-stepping motor, a 80386/87 MS/DOS Nimbus microcomputer (Research Machines, Oxford, U.K.) and SOM software (Bio-Rad). The Optiphot (Nikon, Tokyo) was equipped with a ×60 plan apochromat (Nikon) objective with a 1.4 numerical aperture. Immersion oil with a 1.515 refraction index was used. The (x, y) scanning was usually made with a 1.0 electronic zoom. To reduce noise, a Kalman filter recording an average of 10 images/sec was used for confocal imaging. Excitation at 488 nm and emission at LP 515 nm were the wavelengths for detecting the localization of the FITC-SOD. For imaging of the chromomycin a3–stained DNA of nuclei, the combination of two filters was used: one filter for excitation at 458 nm and a high-pass filter of 550 nm for emission.

Cytochrome c *Assay*

After incubation with native or FITC-SODs for various periods and after three washes, the monolayer of monocytes was incubated in 1 ml of 80 μM ferricytochrome c solution in PBS for 20 or 60 min and stimulated with phorbolmyristate acetate [(PMA) Chemicals for Cancer Research, Eden Prairie, MN; final concentration 30 ng/ml]. The absorbance of the supernatants was measured in a spectrophotometer at 550 nm, and the concentration of reduced cytochrome c was determined by using an extinction coefficient of 21,000 $M^{-1} \cdot cm^{-1}$. The total $O_2 \cdot^-$ production was calculated, assuming that the reduction reaction was stoichiometric (14). Reaction mixtures without cells served as blanks. Monocytes handled in the same way, but not pretreated with SOD, served as controls.

Statistics

Significant differences between groups were assessed with the paired Student's t-test. A p value of <0.05 was considered significant.

Table 1. Intracellular distribution pattern and semiquantitative count of FITC-SOD-labeled monocytes as a function of activity

SODs (specific activity)	1 150 units/ml	2 1500 units/ml
CuZn, rh, BTG	M, C	M, C, PN
(3831 units/mg)	+	+++
CuZn, bovine, Sigma	M, C, PN	M, C, PN
(3590 units/mg)	++	+++
CuZn, bovine, Palleau	M, C, PN	M, C, PN
(4778 units/mg)	++	+++
CuZn, horse, Palleau	M, C, PN	M, C, PN
(4000 units/mg)	++	+++
Mn, rh, BTG	M	M
(3584 units/mg)	±	±
Mn, *E. coli*, Sigma	C	C
(2959 units/mg)	±	+

C, cytoplasm; M, membrane; PN, perinuclear; ±, <1%; +, ≤25%; ++, >25%; +++, >50% labeled cells, respectively.

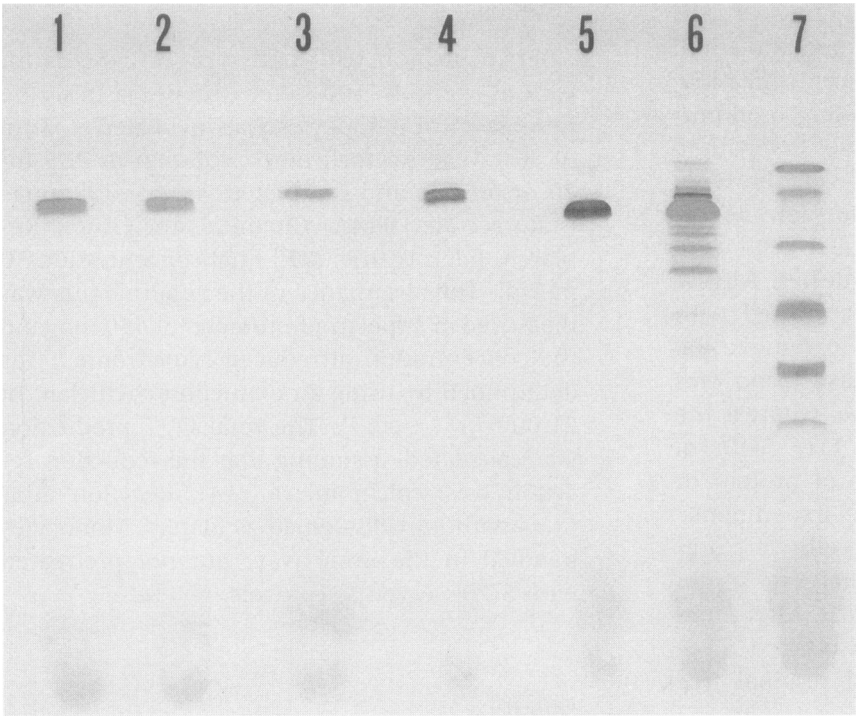

Fig. 1. SDS-PAGE. Each lane (1–4) contains 1 μg of sample protein. (1) Recombinant human CuZn SOD, BTG; (2) CuZn SOD from bovine erythrocytes, Sigma; (3) CuZn SOD from bovine erythrocytes, Palleau Production; (4) CuZn SOD from equine erythrocytes, Palleau Production; (5) recombinant human Mn SOD, BTG; (6) Mn SOD from *E. coli*, Sigma; (7) low-molecular-weight protein markers (mol. wt. 29000–116000).

Results

Specific Activity and Purity of Different SODs Used in This Study

The specific activities of the various SODs are shown in Table 1. Bovine and equine CuZn SOD from Palleau Production had the highest specific activity, followed by rh CuZn SOD from BTG and bovine CuZn SOD from Sigma. The lowest specific activity was noted for the Mn enzymes from BTG and Sigma. The influence of FITC-labeling on enzyme activity was studied in parallel experiments with labeled and native SODs. The reduction in activity varied between 9% and 20% after elution from the Sephadex column under identical conditions. This loss was taken into account for the experiments with FITC-labeled SODs.

With the injection of aliquots containing equal numbers of activity units, HPLC chromatograms were quite similar for all CuZn SODs, with one major peak. The Mn enzymes showed minor peaks corresponding to protein contamination.

Given the same quantity of protein, only one minor additional band for the bovine, human, and horse SODs was detected through electrophoresis. The purity evaluated by scanner was about 98%. The major band corresponded to their molecular weight of 32 kD. The Mn SOD from *E. coli* showed multiple minor bands in addition to the main band corresponding to a molecular weight of 39 kD, purity 77% (Fig. 1).

Detection of FITC-SOD by Confocal Laser Microscopy

COMPARISON OF LYMPHOCYTES, MONOCYTES, AND NEUTROPHILS. In agreement with the results obtained in our previous study by flow cytometry (10), lymphocytes from different donors did not show any fluorescent labeling even after an incubation time of 3 hr with doses of 1500 units/ml. This was true not only for unstimulated lymphocytes but also for lymphoblasts studied after 48 hr of phytohemagglutinin (PHA) stimulation.

Also as expected from our experiments with flow cytometry, only rare neutrophils were labeled with FITC-SOD, despite the use of a maximal incubation time of 3 hr and the highest dose (1500 units/ml). The fluorescence pattern was different from that of monocytes, focalized in a single, very intense spot (Fig. 2).

In contrast to lymphocytes and neutrophils, monocytes regularly had a diffuse fluorescence pattern, as in our previous study. This was seen after FITC-SOD pretreatment only, not in untreated monocyte layers.

COMPARISON OF DIFFERENT SODS ON MONOCYTES. The various types of SOD were compared using identical activity (150 and 1500 units/ml) or identical quantities of protein.

At identical activity, the intracellular distribution of the fluorescently labeled SODs was similar for the two bovine CuZn SODs and the horse SOD, and was visible on the membrane, in the cytoplasm, and around the nucleus as a function of incubation time. As in our previous study, numerous monocytes showed green fluorescence on the surface after an incubation time of only 15 min. Even 5 min of incubation at 1500 units/ml was sufficient to obtain labeled cells at low frequency (about 1 for 1000 cells). The intensity of the labeling and the frequency of labeled cells was less important with the lower dose of 150 units/ml (Table 1 and Fig. 2). As ascertained by focusing through the cell, the fluorescence was never located at the nuclear level. An example of 1-μm-thick confocal sections is shown in Figure 2. For the human enzyme, no perinuclear accumulation was seen after an exposure time of 3 hr with the lower dose of 150 units/ml, whereas fluorescence throughout the cell, including perinuclear localization, was observed with the dose of 1500 units/ml. For both Mn SODs, the number of fluorescent cells was very low. Therefore, the intracellular distribution of fluorescence and its pattern was difficult to determine. In the rare cells labeled with Mn SOD from *E. coli*, the pattern was spotty, similar to the pattern in neutrophils (Fig. 2, panels 4 and 8). For the recombinant human Mn SOD, the rare labeled cells were fluorescent on the surface after 3 hr. No fluorescence was detectable in the cytoplasm.

In another series of experiments, the monocytes were incubated with an equal amount of 30 μg/ml of each protein. The bovine CuZn SOD with the highest specific activity, for which 30 μg/ml corresponded to 150 units/ml, was used as a reference. Fluorescence labeling of the cells could be seen after the incubation time of 3 hr for the two bovine enzymes. The frequency of labeled cells was most important for the reference enzyme. No labeling was seen with rh CuZn SOD and Mn SOD from *E. coli*. Horse SOD and rh MnSOD were not studied. No fluorescence was observed with FITC-labeled catalase on monocytes pretreated for 3 hr with 1500 units/ml.

PMA-Stimulated Superoxide Production by Monocytes

In four independent experiments, equal numbers of monocytes were pretreated with the different SODs at a concentration of 300 units/ml for 2 hr. After removal of all residual extracellular SOD with three washes, the cells were stimulated with PMA, as indicated in Materials and Methods. Superoxide production, measured with the cytochrome *c* assay, was significantly reduced after pretreatment with bovine, equine, and rh CuZn SODs ($p < 0.02$) compared to non-pretreated cells of the same donor. Mn SODs also diminished $O_2{\cdot}^-$ production, but the difference was not statistically significant (see Table 2). No difference in the inhibitory effect was observed between native and FITC-modified SOD at equal concentrations (Table 2).

In three other experiments, superoxide production by monocytes was measured after a short incubation time of 5 min, when no intracellular SOD was detectable with confocal laser microscopy (bovine CuZn SOD 1500 units/ml). The incubation time with cytochrome *c* was reduced to 20 min for these experiments to exclude penetration of the enzyme during the assay period. Cytochrome *c* reduction was inhibited by 46%, 43%, and 31%, respectively for the three assays.

Discussion

The results obtained here through confocal laser microscopy indicate that not only 15 min but even 5 min of incubation was sufficient to detect FITC-SOD on the surface of monocytes. Whereas fluorescence was limited to the membrane for up to 1 hr, FITC-SOD penetrated into the cytoplasm after 2–3 hr, as ascertained by focusing through the cell. Monocytes again showed more labeling than neutrophils, whereas lymphocytes were not labeled at all, even with the highest dose of CuZn FITC-SODs of 1500 units/ml and an incubation time of 3 hr. This was also the case for lymphocytes stimulated with PHA for 48 hr. For neutrophils, the percentage of FITC-SOD labeled cells was very low. They exhibited a peculiar focalized pattern of fluorescence that was quite different from the diffuse punctate distribution pattern in monocytes.

Similar results have been obtained by others with native CuZn SODs, and liposome-encapsulated or chemically modified (acylated) CuZn SODs penetrated into erythrocytes, lymphocytes,

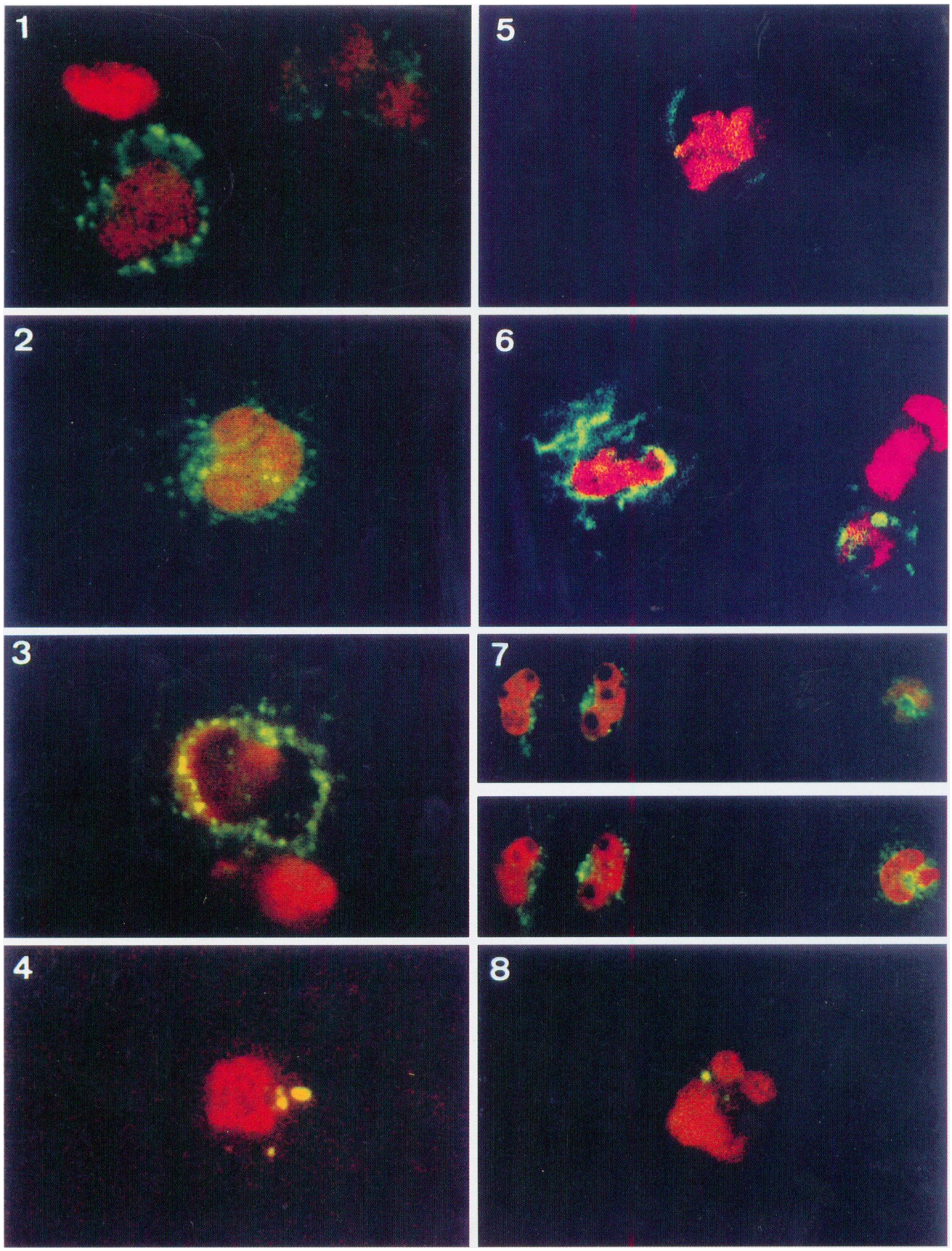
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Table 2. PMA-stimulated superoxide production by monocytes: Inhibitory effect of pretreatment with various SODs

Experiment	CuZn rh BTG	CuZn bovine Sigma	CuZn bovine Palleau	CuZn horse Palleau	Mn rh BTG	Mn *E. coli* Sigma
I	−62.2	−62.2	−75.6	−45.6 (−46.8)	+18.6 (−4.1)	−57.1
II	−39.9	−28.8	−34.2	−51.4 (−42.9)	+20.0 (+20.0)	−15.6
III	−36.1	−30.7	−44.4	−71.0 (−64.3)	−19.0 (−15.9)	−18.7
IV	−42.1	−46.8	−34.7	−70.7 (−73.1)	−29.9 (−29.6)	−20.5
Mean	−45.0*	−42.1*	−48.7*	−59.7 (−56.8)*	−2.6 (−7.4)[a]	−28.0[a]

Pretreatment of cells for 2 hr with 300 units/ml SOD followed by three washes; PMA concentration, 30 ng/ml. The results are expressed as percent inhibition of cytochrome *c* reduction over a 1-hr period. Results in brackets are from parallel experiments with FITC-labeled SOD.

[a]Not significant.

*$p < 0.02$.

and neutrophils (6,7). We could not find any data on monocytes in the literature, except for a study of mouse peritoneal macrophages by Takamura et al. (15) in which radiolabeled rh SOD was used in a mannosylated form. Beckman et al. (16) reported a punctate distribution pattern of FITC-SOD in endothelial cells from porcine thoracic aorta that is very similar to that observed by us in monocytes. Binding and internalization of CuZn SOD gold complexes in cultured hepatocytes were studied with electron microscopy by Dini and Rotilio (8), who also demonstrated intracellular uptake in the liver after intraperitoneal injection of SOD (17). In vivo studies carried out by Sahgal et al. (18) in newborn piglets showed that after intratracheal administration, rh CuZn SOD could be demonstrated by confocal laser microscopy in a variety of cell types of airways, respiratory bronchioles, and alveoli, significantly increasing lung SOD activity.

The mechanisms involved in the uptake of SOD are still subject to debate. Two such mechanisms, receptor-mediated endocytosis and fluid-phase endocytosis, have been considered (9). While uptake via receptor-mediated endocytosis requires the binding of the ligand to a specific cell surface receptor, fluid-phase endocytosis is a nonsaturable receptor-independent process. Inside the cell, the SOD was supposed to be located in endosomes and lysosomes (16,17). Intranuclear localization of exogenous SODs was not reported. Also in the present study, no fluorescence in the nuclear compartment could be detected by confocal laser microscopy, even if a long incubation time of 3 hr at a high concentration of 1500 units/ml was used, perinuclear accumulation of the FITC-SOD was observed with time. The remaining fluorescence on the membrane and in the more peripheral cytoplasmic areas suggests a unidirectional intracellular migration from the cellular to the nuclear membrane, which appears to be a barrier for further progression. The absence of detectable nuclear FITC fluorescence is in agreement with the findings of Petkau et al. (19), who studied uptake of

Fig. 2. Results from incubation with FITC-SOD (1500 U/ml) for 3 hr as shown by confocal laser microscopy. (1) Recombinant human CuZn SOD, BTG; (2) CuZn SOD from bovine erythrocytes, Sigma; (3) CuZn SOD from bovine erythrocytes, Palleau Production; (4) *E. coli* Mn SOD, Sigma; (5) recombinant human Mn SOD, BTG; (6) CuZn SOD from equine erythrocytes, Palleau Production. The four CuZn SODs have the same intracellular diffuse distribution pattern, whereas the Mn SODs have poor labeling after 3 hr. (7) Two focal sections taken at a distance of 1 μm in the middle of the same cell. There is no nuclear labeling. (8) One of the rare FITC-labeled PMN. The single intracellular focal spot shows a distribution pattern in the cytoplasm that is different from that of monocytes.

exogenous radioactive SOD in different cellular fractions and found only minor quantities in the nuclear fraction. However, passive transport across the nuclear membrane is possible for proteins with a molecular weight of 30 kD, as demonstrated recently for the green fluorescence protein (GFP) (20). The SOD may have been degraded after incorporation of the label into larger proteins. This would explain why the label did not appear in the nuclear compartment. However, this hypothesis does not explain the striking perinuclear accumulation of the punctate labeling pattern in the monocytes.

Whether intracellular uptake of SOD is necessary for its protective action is another subject of controversy. While Kyle et al. (9) conclude that intracellular uptake of SOD is required for its protective effects, others think that binding to the membrane is sufficient for protection against free radical attack (7,21,22). Inhibition of superoxide production of membrane-bound SOD may be due to efficient dismutation of superoxide radicals on the outer surface of the cell membrane, since the active site of NADPH oxidase faces the outside of the cell membrane, and there may remain enough SOD at the membrane for dismutation of superoxide, even after 3 hr. Our results obtained with the cytochrome *c* assay indicate diminished superoxide release after an incubation period with SOD of only 5 min, when FITC-SOD was detected only on the membrane, not in the cytoplasm, by confocal laser microscopy. In our opinion, the discussions about whether exogenous SOD is protective extracellularly, intracellularly, or on the membrane level are not the heart of the matter, since any of these localizations of SOD may be protective, depending on the biological site of oxidative stress.

Most studies in the literature concerning cellular penetration of SOD have been done with the CuZn enzymes, as was the case in our previous report (10). The principal aim of the present study was to compare SODs of various origins and active sites in the monocyte model. Bovine CuZn SODs from two different manufacturers, as well as horse CuZn SOD, showed quite similar penetration and intracellular distribution. Minor differences were observed in simultaneous experiments on the same cells with the lowest activity of rh CuZn SOD, where the number of labeled cells was less important. Penetration and intracellular distribution also appeared to be delayed, since there was no perinuclear accumulation.

The most important finding of this study, however, is the difference between CuZn and Mn SODs. Both Mn SOD from *E. coli* and rh Mn SOD showed poor binding capacity in parallel comparative experiments with CuZn SODs. Despite the fact that rh CuZn and rh Mn SOD were prepared by the same manufacturer (BTG), binding and penetration were quite different. The observations under the confocal laser microscope were in agreement with differences in the cytochrome *c* assay, which showed a weak inhibitory effect on superoxide production by the two Mn SODs. This difference was observed with FITC-labeled and native SODs. The reasons for these discrepancies between SODs with different active sites are not clear. Differences in electrostatical charges may be responsible, since it has been shown that SODs with a higher pI have a higher affinity to the membrane than others (23).

The findings reported here are of interest from a medical and therapeutic standpoint. We show that SOD binds to the cell membrane after only 5 min. This may explain the reports of SOD's beneficial effects despite the rapid clearance of the enzyme following intravenous injection. The preferential binding of exogenous CuZn SOD to monocytes compared to neutrophils may explain why therapeutic effects of SOD have been reported mainly for chronic inflammatory diseases, in particular rheumatoid arthritis, in which the monocytes are in an activated state and exhibit increased superoxide production (24). Further studies are necessary to determine whether the differences observed between CuZn SODs and Mn SODs, as well for binding capacity and inhibition of PMA-stimulated superoxide production by monocytes, are also reflected in the therapeutic efficacy of these enzymes.

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