ACTIVE OXYGEN SPECIES ARE involved in the pathogenesis of a variety of diseases. In many laboratory models and in a few clinical trials, superoxide dismutase (SOD) has proven therapeutically useful in protecting injured tissues from one of these active oxygen species, the superoxide radical (24). The ability of SOD to protect tissues against any particular insult (ischemia, inflammation, hyperoxia, etc.) depends on several parameters such as its rate of plasma clearance (30), ability to equilibrate between extracellular fluid compartments (36), and the ability to closely approach negatively charged cell surfaces (29). Other enzymes that are part of the cell's arsenal against these active oxygen species include catalase and glutathione peroxidase, which eliminate H$_2$O$_2$.

In humans, three isoenzymes of SOD have been extensively characterized: the cytosolic Cu,Zn-SOD or SOD1, a 32-kDa dimer (27); the mitochondrial Mn-SOD or SOD2, an 89-kDa tetramer (26); and an extracellular-SOD or SOD3, a 135-kDa tetrameric glycoprotein (20). SOD3 is also a copper/zinc enzyme, genetically related to SOD1, and is found in a number of tissues but at a much lower concentration than either of the other two enzymes (21, 22). It is, however, the major SOD in extracellular fluids. SOD3 is found as three different forms: SOD3-A with no heparin affinity, SOD3-B with low heparin affinity, and SOD3-C with high heparin affinity. Sandstrom et al. (39) have suggested that forms A and B are generated by proteolytic cleavage of a COOH-terminal "tail" found intact on the C form. The highly hydrophilic, positively charged nature of this COOH-terminal tail imparts the high heparin affinity that allows the enzyme to be largely bound to heparan sulfate on endothelial surfaces (10, 39, 41).

Under normal circumstances, intracellularly generated superoxide is efficiently handled by the cytosolic SOD1 and mitochondrial SOD2. However, under pathological conditions, large amounts of superoxide and its metabolites may be produced (8, 23). In this case, one major site of oxidant attack is endothelial cell surfaces, where membrane perturbation leading to cell death (possibly through apoptosis or programmed cell death) may be induced. Therefore, we propose that the plasma membranes of vascular endothelial cells and parenchymal cells may require additional protection when there is excessive production of these species. Surface-bound SOD3 normally protects endothelial cell surfaces from superoxide attack. However, we have shown that proteases released by inflammatory cells can cleave the SOD3 tail, allowing the enzyme to become soluble and rendering the endothelium susceptible to superoxide attack (28). To protect the endothelium, pharmacological efforts have concentrated on utilizing the cytosolic SOD1, which unfortunately has undesirable pharmacological properties: a short plasma half-life (6–15 min, depending on species) following intravenous injection with rapid clearance by the kidneys and a net negative charge at physiological pH. This net negative charge precludes close contact with cellular surfaces (29) and/or movement into interstitial spaces (36). In contrast, SOD2 is nearly uncharged at physiological pH and has a longer plasma half-life (~4 h) (1). In an isolated, perfused heart...
model, SOD2 is more protective and equilibrates more quickly than SOD1 (36). SOD3 may have a substantial advantage over SOD1 and SOD2 because of its ability to bind to the endothelium. Unfortunately, the SOD3 cDNA has resisted attempts at high-level recombinant expression in bacterial or yeast vectors; it has only been available from an expensive, labor-intensive, low-yield mammalian expression system, Chinese hamster ovary cells (44). A recent study (11) reports expression of human recombinant (hr) SOD3 in E. coli as insoluble inclusion bodies. The solubilized, refolded, purified enzyme was ~30% active from E. coli and only ~7% active from the baculovirus system. Purification yields were not reported, and the products were not tested for heparin affinity. Thus hrSOD3 has not been readily available for study.

We report here the construction of a chimeric fusion SOD that combines the desirable features of both SOD2 and SOD3: the coding sequence from the mature (i.e., minus the mitochondrial targeting signal sequence) human SOD2 followed by the 26-residue COOH-terminal tail from human SOD3. In addition, to obtain high-level expression of this mutant enzyme, we have utilized a novel expression vector designed and constructed for this purpose (7). Characterization of the enzyme and its physiological properties in two laboratory models of inflammation are also described.

MATERIALS AND METHODS

Enzymes and Chemicals

All enzymes used for manipulation of DNA were from New England Biolabs or Stratagene. Sequencing materials were obtained from United States Biochemicals. Purified human recombinant SOD1 and SOD2 were generously provided by Biotechnology General (Iselin, NJ). Human recombinant interleukin-1α (IL-1α) was kindly provided by Hoffmann-La Roche (Nutley, NJ). Ketamine hydrochloride was from Parke-Davis (Morris Plains, NJ), xylazine was from Haver (New York, NY). 125I-labeled bovine serum albumin (125I-BSA) was from ICN Radiochemicals (Irvine, CA), and heparin sulfate was from Eli Lilly (Indianapolis, IN). All other chemicals were from Sigma (St. Louis, MO).

Manipulation of DNA

Isolation of plasmid DNA, preparation of DNA fragments, and DNA ligations were carried out as described by Sambrook et al. (38). Plasmid transformation was performed as described (9). Screening of putative recombinant colonies was done using PCR unless specified otherwise. Restriction enzyme digestions and DNA sequencing were carried out according to manufacturer’s specifications (Perkin Elmer/Cetus).

E. Coli Strains

E. coli UT5600 (New England Biolabs) is a strain deficient in an outer membrane protease that cleaves between sequential basic amino acids (6). E. coli QC774 is deficient in both sodA and sodB. It was originally created by Carlioz and Touati (3) and was a gift of Dr. Bernard Weiss.

pGBI Expression Vector

We have previously described the construction of the expression vector used in these studies (7). It contains the oxygen- and oxidant-sensitive E. coli MnSOD (sodA) promoter as well as both 5’-untranslated and transcriptional termination sequences plus a synthetic linker containing two restriction enzyme cloning sites, NsiI and SacI. The vector also contains the gene for β-lactamase, which confers ampicillin resistance to the host bacterium and provides a selectable marker. High level of expression can be achieved by exposure to the superoxide-generating agent paraquat (methyl viologen) as the inducer. The highest expression is induced by 20 μM paraquat, with the recombinant protein approaching 50% of total soluble protein produced by the host.

Construction of pGB1-sod2

A fragment of the human sod2 cDNA encoding amino acids 25–222 (mature SOD2, minus the mitochondrial transit peptide) was obtained from a human placental cDNA library by PCR amplification and cloned into pBlueScript KS+ (Stratagene). The sequence of the primers used for the PCR amplification was as follows: forward primer, GGAATTCATGCAATTACACAGGCTTCCCGAC; reverse primer, CGAGCCATTACACAGGCGTCTTGCAGCATGTC. The forward primer contains an EcoRI site (single underline) upstream of an NsiI (single underline) restriction endonuclease site. The NsiI sequence contains an ATG start codon. The reverse primer contains a SacI site (single underline) immediately upstream from the translational stop codon. An XmaI site (isoschizomer of PselAI, double underline) was positioned immediately downstream from the stop codon. After amplification and cloning, the SacI and XmaI sites were positioned downstream and upstream, respectively, from the translational stop codon. After corroborating the cloned sod2 cDNA sequence, the recombinant vector was digested with NsiI and SacI restriction endonucleases, and the resultant fragment was recovered from a low-melting point agarose gel (NuSieve, FMC Bioproducts, Rockland, ME). This fragment was then subcloned into SacI/XmaI-digested pGB1. The recombinant plasmid was designated as pGB1-sod2, and its restriction map is shown in Fig. 1. The SOD2 translated from this construct would begin with met-his residues, followed by residues 25–222 of the human SOD2 sequence, followed by pro-gly residues (the first residues of the SOD3 26-residue tail) introduced by the cloning strategy to create an XmaI site to permit subsequent construction of the chimeric sod2/3 gene.

Construction of Chimeric pGB1-sod2/3

To begin construction of the chimeric sod2/3 gene, an ~83-bp cDNA fragment encoding the COOH-terminal 26-amino acids from SOD3 was gel purified after XmaI/SacI double digestion of a sod3 cDNA-containing recombinant plasmid provided by Dr. Y. S. Ho. Plasmid pGB1-sod2 was digested with XmaI and PstI, and the large 3.4-kb resultant fragment was gel purified. In a separate reaction, pGB1-sod2 was digested with SacI and PstI to generate two fragments, a 3.4-kb and a smaller 306-bp fragment. After gel purification, the small SacI/PstI fragment and the large 3.4-kb XmaI/PstI fragment from pGB1-sod2 were ligated to the XmaI/SacI tail fragment from sod3 by incubation overnight at 15°C in the presence of T4 DNA ligase according to the manufacturer’s specifications. The new recombinant plasmid was designated as pGB1-sod2/3.
Figure 1 illustrates the construction scheme for this plasmid. Figure 2 represents the nucleotide and deduced amino acid sequences of the product expressed by pGB1-sod2/3.

Expression and Purification of SOD2/3

After transformation of competent *E. coli* UT5600 (protease-deficient) cells, positive clones were identified and cultured at 37°C for 12 h in LB (Luria-Bertani) medium supplemented with 200 μM MnSO₄ and 20 μM paraquat. After centrifugation, the harvested cells were lysed by sonication in a 0.1 M sodium carbonate, 0.6 M NaCl, pH 10.5 buffer. The cell debris was removed from the cell lysate by centrifugation for 30 min at 10,000 g. The supernatant was collected and subjected to heat treatment at 65°C for 10 min. The sample was cooled immediately in an ice bath and centrifuged for 10 min at 10,000 g to remove precipitated protein. Ultrafiltration with Diaflo PM-30 membrane was used to concentrate the supernatant. The sample was then chromatographed through a 15 × 700-mm column of Sephadryl S-200. Fractions were collected and assayed for SOD activity as described (5, 27). Higher-molecular-weight fractions exhibiting SOD activity were pooled, concentrated by ultrafiltration, and diluted with 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl buffer. This sample was then applied to a heparin-agarose column. Fractions from this column were eluted by using a 0.15–1 M NaCl linear gradient in the buffer described above and subsequently assayed for SOD activity. Pooled fractions were then subjected to ultrafiltration, changing the buffer.

The sequence of chimeric SOD2/3

**Fig. 1.** Construction of plasmid encoding human superoxide dismutase (SOD)2/3. The DNA fragment encoding the human SOD3 COOH-terminal 26-amino acid basic peptide is designated sod3 tail. Amp, ampicillin-resistance gene; Ori, origin of replication; P, promoter sequence; T, termination sequence.
during this process to 10 mM potassium phosphate, 0.15 M NaCl, pH 7.4.

**SDS-PAGE**

Purified hrSOD2, purified hrSOD2/3, and a crude cell lysate of *E. coli* expressing mutant SOD2/3 were subjected to SDS-PAGE using a 15% gel as described (38).

**Gel Filtration Chromatography**

For molecular size determination, a 0.5-ml sample of the purified protein was applied to a Sephacryl S-200 column as described above but using 10 mM potassium phosphate, pH 7.4, 0.15 M NaCl instead. The column was calibrated with the protein standards aldolase (158 kDa), hrSOD2 (89.9 kDa), and hrSOD1 (31.6 kDa). Absorbance at 280 nm was monitored, and *K*ₚ (partition coefficient) of each standard was calculated and plotted vs. its known molecular weight.

**Heparin Affinity Chromatography**

An aliquot of the purified SOD2/3 preparation (500–1,000 units) was applied to a small (0.5- to 1-ml bed vol) column of heparin-agarose (Sigma) in 10 mM Tris-HCl buffer, pH 7.4. The column was washed with this buffer to remove unbound enzyme and then eluted with a gradient of 0–2.0 M NaCl. Fractions were assayed for SOD activity and for conductivity.

**IL-1α-Induced Model of Pulmonary Injury**

Administration of IL-1α and SODs to intact rats. This model was conducted as previously described (19). Male Sprague-Dawley rats weighing 300–350 g were anesthetized with halothane via inhalation inside of a sealed glass jar. Immediately after intravenous injection, the trachea was cut, and the rat was allowed to recover fully from anesthesia. Sham-treated rats received identical anesthesia and surgery but were injected only with sterile saline both intravenously and intratracheally.

Assessment of lung leak index. Four and one-half hours after SOD and IL-1α administration, rats were injected intravenously with 1.0 μCi of 125I-BSA in a volume of 0.5 ml. Twenty minutes later, rats were ventilated by using a Harvard small animal respirator and then subjected to laparotomy, thoracotomy, and right ventricular injection of 200 units of heparin (in 0.2 ml of saline). Thirty minutes after 125I injection, blood samples were obtained, lungs were perfused blood-free with phosphate buffered-saline, and lungs were excised. Right lungs and blood samples were counted in a gamma counter (Beckman, Fullerton, CA). Lung leak index was defined as counts per minute of 125I in the right lung divided by counts per minute in 1.0 ml of blood.

Assessment of lung lavage and blood neutrophils. Saline (3 ml × 2) was slowly injected intratracheally and then withdrawn. Recovered lavage fluid was centrifuged for 5 min. The supernatant was carefully removed and saved. The pellet was then resuspended in 4.0 ml of water and mixed for 30 s to lyse erythrocytes. Immediately thereafter, 2.0 ml of 4 × 10⁻² M Ca²⁺ Mg²⁺-free Hanks’ solution was added and mixed for 5 s. The new mixture was centrifuged again for 5 min, and the supernatant was discarded. The pellet was then resuspended in 1.0 ml of lavage supernatant. Total leukocytes were counted in a hemocytometer, and a cytopsin preparation was Wright stained to determine the percentage of neutrophils.

**Carrageenan-Induced Foot Edema in the Rat**

This model was performed as described by Vinegar et al. (46). Edema was induced by subplantar injection of 0.1 ml of 1.5% carrageenan in saline into the foot pad. The increase in foot volume after 6 h was measured by the water displacement method (45). The saline controls received an injection of vehicle only. Native hrSOD2 or chimeric hrSOD2/3 was administered 10 min before carrageenan by intravenous injection at doses of 0.1 U/g body wt.

**Statistical Analysis**

All values are presented as means ± SE. Data were analyzed using the unpaired Student’s *t*-test.

**RESULTS**

**Expression and Purification of hrSOD2/3**

Because the COOH-terminal SOD3-derived tail portion of the chimeric protein has been shown to be very susceptible to proteolytic degradation, we used *E. coli* UT5600 cells, a protease-deficient strain, as the host for expression of the mutant enzyme. When *E. coli* UT5600 was transformed with pGB1-sod2/3 and cultured as described above, cell lysates contained extremely high levels of SOD activity. The major soluble protein (expressed at >20% of total protein) was of the size expected for the chimeric SOD2/3, as shown by SDS-PAGE in Fig. 3, lane 4. Figure 3 compares the crude lysate containing SOD2/3 (lane 4) with purified preparations of SOD2/3 (lane 3) and SOD2 (lane 2). Purified hrSOD2/3 (95% purity, lane 3) showed a single protein band with an apparent subunit molecular weight of 25–26 kDa, in agreement with the predicted molecular weight of 25,637 Da. Native hrSOD2 (with-
out the tail) was expressed by the pGB1-sod2 vector to slightly higher levels, as previously described (7). The subunit molecular weight of the wild-type SOD2 is shown to be 22.5 kDa (lane 2) for comparison purposes.

A 3-liter culture of the pGB1-sod2/3 transformed cells was subjected to the purification procedure described in MATERIALS AND METHODS, with the results summarized in Table 1. The procedure yielded 63 mg of highly purified SOD2/3, after a purification of 6.6-fold with a yield of 47% of the starting activity. The specific activity of the purified product was 3,200 standard units/milligram of protein, comparable to the specific activity of 3,600 U/mg for SOD2 as originally isolated from human liver (26), especially if one takes into consideration the increase in molecular weight due to the additional amino acid residues comprising the SOD3 COOH-terminal region.

Gel filtration column chromatography of the hrSOD2/3 showed a molecular size of 105 kDa (Fig. 4). This value is very close to the calculated molecular weight of the tetrameric chimeric enzyme (102.6 kDa) and establishes that the tetrameric structure of native SOD2 is preserved in SOD2/3.

**Heparin Binding Ability of SOD2/3**

To test whether the hrSOD2/3 has a SOD3-like affinity for heparin, we performed heparin-agarose affinity chromatography as described in MATERIALS AND METHODS. The results are shown on Fig. 5. The hrSOD2/3 bound to the column, eluting as a single peak at a NaCl concentration of 0.35 M. Under the same conditions, native MnSOD did not bind to the column at all (data not shown).

*Effect of hrSOD2/3 on IL-1-Induced Lung Leak and Neutrophil Accumulation*

We tested the ability of the hrSOD2/3 to protect rat lungs in an in vivo model of acute IL-1-induced lung injury. The model was performed as described in MATERIALS AND METHODS after IV administration of either a saline control, SOD2, or SOD2/3. Lung leak was then quantified as described. The results are shown in Fig. 6. At 0.5 mg, the chimeric enzyme suppressed the IL-1-induced lung leak by ~92% \((P < 0.026)\) compared with the IL-1-treated control. In contrast, 2 mg of native SOD2 suppressed the IL-1-induced lung leak by only 13.8%, which was not statistically significant. Heat-denatured SOD2/3 did not protect at all. SOD2/3 at 2 mg also protected against the damage (79%, \(P < 0.03\)). As an additional index of injury, we measured neutrophils in bronchoalveolar lavage (BAL) fluid after the SOD treatments. The results are shown in Fig. 7. IL-1 administration dramatically increased the numbers of neutrophils in BAL. Although neither native SOD2 (2 mg) nor heat-denatured SOD2/3 prevented

---

**Table 1. Results of the purification of hrSOD2/3 from Escherichia coli UT5600 transformed with pGB1-sod2/3**

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total SOD Activity, units</th>
<th>Total Protein, mg</th>
<th>Specific Activity, U/mg</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell lysate</td>
<td>427,700</td>
<td>881</td>
<td>485</td>
<td>100%</td>
</tr>
<tr>
<td>Supernate</td>
<td>404,800</td>
<td>600</td>
<td>675</td>
<td>95%</td>
</tr>
<tr>
<td>After heat step</td>
<td>345,700</td>
<td>328</td>
<td>1,054</td>
<td>81%</td>
</tr>
<tr>
<td>After gel filtration</td>
<td>284,500</td>
<td>173</td>
<td>1,647</td>
<td>67%</td>
</tr>
<tr>
<td>After heparin affinity chromatography</td>
<td>202,500</td>
<td>63</td>
<td>3,214</td>
<td>47%</td>
</tr>
</tbody>
</table>

SOD, superoxide dismutase.

Fig. 4. Gel filtration chromatography of hrSOD2/3. Samples (0.5 ml) of human recombinant Cu,Zn-SOD (hrCu,Zn-SOD), hrMnSOD, aldolase, and hrSOD2/3 were chromatographed on a Sephacryl S-200 column as described in MATERIALS AND METHODS. Absorbance at 280 nm was monitored, and \(K_{av}\) (partition coefficient) of each sample was calculated according to the following equation: \(K_{av} = V_e/V_t \times (V_v/V_e)^{-1}\). \(V_e\) is the elution volume; \(V_v\) is the void volume; \(V_t\) is the total volume of the gel bed.

Fig. 5. Heparin-agarose column of hrSOD2/3. Chromatography was performed as described in MATERIALS AND METHODS. Fractions were collected, and SOD activity of each fraction was determined and expressed as units/ml of fraction volume. NaCl concentration was determined by conductivity of each fraction. hrSOD2/3 binds to the heparin affinity column and elutes at a NaCl concentration of ~0.35 M.
this increase, SOD2/3 at 0.5 mg suppressed neutrophil accumulation by 91% \( (P < 0.05) \).

**Inhibition by SOD2/3 of Carrageenan-Induced Foot Edema in the Rat**

The model was carried out as described in MATERIALS AND METHODS, with the results shown in Fig. 8. After 6 h, sham (saline)-injected feet showed a volume increase of 0.2 ml, only slightly larger than the actual volume injected \( (n = 3) \). Carrageenan-injected feet receiving no SOD treatment showed an average edema of \( 0.9 \text{ ml} \) \( (n = 15) \). Pretreatment of animals with chimeric SOD2/3 at 0.1 U/g body wt \( (n = 9) \) resulted in a 62% reduction of carrageenan-dependent edema \( (P < 0.003) \). Pretreatment of animals with native SOD2 at the same dosage \( (n = 8) \) produced a 22% reduction of carrageenan-dependent edema that was not statistically significant.

**DISCUSSION**

For more than 30 yr, there have been attempts to use SOD as a therapeutic agent to treat conditions known to involve the production of the superoxide radical, such as inflammation, fibrosis, and ischemia-reperfusion. Although there have been impressive results in laboratory models, the human clinical experience has been less than spectacular. Over time, the reasons for this paradox have become clear. Nearly all the clinical trials have utilized SOD1 (mostly human recombinant or bovine) because of its commercial availability, stability, economy, ease of purification, and low immunogenicity. Despite these attractive features, however, the SOD1 protein displays some unfortunate physiological characteristics that render it poorly suited for therapeutic use. Perhaps the greatest problem is that native SOD1 (molecular radius in the short dimension \( 4.0 \text{ A} \), near neutral charge) has a half-life of \( 4-20 \text{ h} \), depending on species \( (1) \), and SOD3 has a slow clearance, with a half-life of \( 10 \text{ h} \) in the rabbit \( (17) \). (It is actually not possible to measure a true plasma clearance rate for a protein that is not present in the plasma. Both SOD3 and SOD2/3 immediately bind to...
surfaces, so analysis of a plasma sample does not reflect how much of the proteins remain in the body.) We have shown that maintaining an appropriate dose of SOD is critical due to bell-shaped dose-response curves (34, 35) resulting from the facts that superoxide radical can paradoxically both initiate and terminate lipid peroxidation (32) and is involved in a number of cell signaling pathways (25). Hence, a unique concentration of SOD is maximally protective for any given level of oxidative stress, and maintaining this concentration in practice becomes extremely difficult for a protein with a plasma half-life of 10 min. Thus an SOD with a slow plasma clearance should be greatly superior to one that is rapidly cleared, like human SOD1.

In addition, we have shown that the negatively charged human SOD1 (net charge per subunit of −6.1 at pH 7.4) equilibrates rather slowly between vascular and interstitial spaces, whereas the nearly uncharged MnSOD (net charge per subunit of −0.85 at pH 7.4) equilibrates four times faster (36) despite its larger size. Earlier work had revealed that SODs carrying a net positive charge were as much as two orders of magnitude more effective in protecting phagocytosing neutrophils from superoxide-mediated self-destruction than were negatively charged SODs (29). This was thought to be due to the fact that cell surfaces are negatively charged, and an uncharged or positively charged SOD might approach cell surfaces more easily by elimination of electrostatic repulsion forces. In fact, a positively charged SOD might be attracted to such surfaces and might even bind to them. We previously tested this hypothesis by covalently attaching polyllysine polymers to bovine SOD1, such that each subunit, on average, possessed a tail of about a dozen lysine residues. This polyllysyl-SOD1 derivative was ~10-fold better at protecting cells from oxidative stress-induced death than the native SOD1 (29). The subsequent discovery of SOD3 by Marklund and colleagues (13, 20) suggested that nature had used the same design for protecting external cell surfaces. SOD3 subunits possess COOH-terminal tails of ~22 hydrophilic amino acid residues with a strong net positive charge, very much like the polyllysyl-SOD1 that we had synthesized. Also noteworthy is the fact that the SOD3 tail terminates with the sequence -ala-ala-COOH. Any sequence terminating with the basic residues arginine or lysine would be rapidly nibbled away by plasma carboxypeptidases, but the ala-ala cap resists such hydrolysis.

Thus it seemed likely that SOD3 would be a much better therapeutic candidate than SOD1, and we wanted to reexamine the therapeutic potential of SOD in the various physiological models, but using SOD3 instead of SOD1. Attempts to produce large quantities of hrSOD3 in bacterial or yeast hosts were thwarted by the difficulty for these hosts to express the recombinant protein (11). The reason for this is unclear. In any event, we decided to construct a chimeric gene that would produce a hybrid SOD combining the desirable properties gleaned from all the studies described above. The ideal theoretical size might be a molecular radius of 45–50 Å to discourage filtration by the kidney but still allow egress from the vasculature to equilibrate with interstitial fluid. The chimeric enzyme would be based on human SOD2, the least negatively charged of the three SOD isoenzymes. It is tetrameric with a molecular radius of ~40 Å; the addition of the 24-residue tail to each subunit would put SOD2/3 in the desired size range with a radius of 45–50 Å. SOD1 is rather asymmetric, but in its narrow orientation has a molecular radius of only ~15 Å, accounting for its rapid renal clearance. By attaching the COOH-terminal 24 residues of SOD3 to SOD2, a net positive charge would be created along with four binding sites for polyanionic surfaces such as heparin or heparan sulfate, or for collagen fibrils and other components of the extracellular matrix (37). Thus the chimeric enzyme SOD2/3 might actually have more desirable properties from a therapeutic point of view than any of the three naturally occurring forms of SOD. Construction of other chimeric SODs have been described by Inoue et al. (14, 15) and by Stenlund and Tibell (42). Inoue et al. (14, 15) combined the human SOD1 cDNA and that portion of the human SOD3 DNA encoding the final 26 COOH-terminal residue tail. This construct produced an SOD that bound to heparin (HB-SOD) but retained a net negative charge on its surface and an undesirably small molecular radius. Although this enzyme did not quickly appear in the urine, it was largely retained in the kidney (~37% after 20 min) through binding to the apical plasma membrane of tubular cells (14). This HB-SOD possessed better therapeutic properties than native SOD1 (31) but fell short of achieving optimal characteristics. The chimeric enzyme constructed by Stenlund and Tibell consisted of SOD1 with both NH$_2$-terminal and COOH-terminal regions of SOD3. The NH$_2$-terminal sequence produces the tetrameric interfacial surface of SOD3 such that this chimeric enzyme behaves much like true SOD3. There have been no published assessments of its therapeutic characteristics.

A comparison of net surface charges borne by the three wild-type human SODs and for two heparin-binding chimeric SODs at pH 7.4 is shown in Fig. 9. Calculations were made by using PC Gene software and the sequence data for the various proteins. HB-SOD consists of human SOD1 plus the 26 COOH-terminal residues of extracellular-SOD as described by Inoue et al. (15).
data for the various proteins. Of these five SOD preparations, only SOD2/3 bears a net positive surface charge, shown by us to be essential for rapid equilibration between vascular and interstitial compartments (36).

Although native SOD3 clearly has an affinity for vascular endothelial surfaces and may be displaced by heparin (16), SOD3 has been found in much greater amounts bound to components such as type 1 collagen fibrils in the extracellular matrix in lung (37) and the arterial wall interstitium (43) and has been shown to be synthesized and secreted in large amount by arterial smooth muscle cells. These observations raise the question of how diffusible SOD3 is in a normal individual; it may not freely equilibrate between vascular and interstitial spaces or from tissue to tissue. This could be due to size and charge characteristics that impede the equilibration of SOD3 across the plasma-interstitium barrier or it could be due to the very tight binding of SOD3 to polyanionic surfaces producing a very slow equilibration. Native SOD3 elutes from heparin-agarose at a salt concentration of $0.55 \text{ M}$. A common variant form of SOD3 (arg213gly) found in 2–5% of the population binds less tightly due to the replacement of an arginine residue in the tail region of the enzyme. Karlsson et al. (18) have speculated that the reason this mutation is so common may be that it is more mobile than the native SOD3, possibly conveying an advantage under certain pathological circumstances (40). SOD2/3 elutes from a heparin-agarose column at a NaCl concentration of 0.35 M (see Fig. 5), about the same as the mutant form of SOD3. This slightly weaker binding than wild-type SOD3 was not expected because SOD2/3 has a net positive charge and the tail sequences are identical. It may be due to different spatial presentations of the tails in the two proteins. For whatever reasons, this weaker binding, which resulted by happenstance rather than by design, may contribute to making SOD2/3 a better therapeutic agent than SOD3.

As a physiological model of oxidative injury, we used intratracheal IL-1 instillation followed by measurements of lung leak and neutrophil infiltration. We have previously shown (19) that SOD2 at 2 mg/rat can provide modest but significant protection against both end points, although in the present study, statistical significance was not achieved. The chimeric SOD2/3, however, provided essentially complete suppression of both neutrophil migration and the lung leak caused by the IL-1 and was just as effective when the dosage was decreased by 75% (Figs. 6 and 7). The only difference between the mutant and the native enzymes is the presence of the heparin-binding tail. Thus we can conclude that the ability to bind to endothelial cell surfaces and to extracellular matrix components provides a dramatic advantage in protecting against IL-1-induced lung damage.

The second in vivo model tested, the carrageenan-induced foot edema model, is widely used to assess anti-inflammatory agents. Rats were dosed at the remarkably low dose of 0.1 U/g body wt (or 30 $\mu$g/kg), administered intravenously, 10 min before the injection of carrageenan into the footpad. The native SOD2 caused a modest reduction in carrageenan-dependent edema that was not statistically significant. The chimeric SOD2/3, however, caused a 62% reduction in edema ($P < 0.003$), approximately equal to the effects of hydrocortisone or phenylbutazone at dosages of 50 mg/kg (46).

The effectiveness of SOD2/3 in the two models examined, over a 200-fold-dose range, suggests that this engineered enzyme may have great advantages over SOD1, SOD2, and even SOD3, when used in vivo as a therapeutic agent. We have also utilized the chimeric enzyme in a model of ischemia-reperfusion injury in the isolated rabbit heart, finding it $\approx 75$-fold more efficacious than SOD2 in providing recovery of developed ventricular pressure, prevention of lipid peroxidation, and prevention of lactate dehydrogenase release during reperfusion (33). It was similarly protective in a cold ischemia preservation model (33). These isolated organ studies showed rather sharp bell-shaped dose response curves, not suggested in the present studies conducted in vivo. A possible explanation might be the “buffering” of the SOD2/3 concentration by the large binding capacity provided by the rest of the animal’s body. Further dose-response studies are needed to explore this possibility.

The chimeric SOD2/3 has also been shown to effectively prevent the adherence of platelets to vascular endothelial cells in response to bacterial endotoxin (4). This study was performed in vivo in the mouse at an intravenous dose of 2 U/g body wt (600 ng/g body wt). In a mouse model of hepatic ischemia-reperfusion, the chimeric SOD2/3 at an intravenous dose of 1 U/g body wt (300 ng/g body wt) provided complete suppression of serum TNF-α protein expression after 45 or 90 min of ischemia and 6 h of reperfusion, whereas the native SOD2 at the same dosage provided no effect at all (12).

In summary, we believe that this chimeric form of SOD possesses pharmacological properties that are superior to any of the three naturally occurring forms of the human enzyme. By mimicking the ability of extracellular SOD3 to bind to cell surfaces and components of the extracellular matrix, thereby buffering the effective concentration in vivo, the enzyme appears to overcome the major problems that have prevented the translation of superoxide-scavenging therapies from the laboratory to human clinical practice.

This work was supported in part by a Glaxo Cardiovascular Discovery grant and by National Heart, Lung, and Blood Institute Grant 5P50-HL-40784. Present address of J. A. Leff: Amgen, One Amgen Center Drive, Thousand Oaks, CA 91320.

REFERENCES

4. Cerwinka WI, Cooper D, Kriegstein CF, Ross CR, Mc-Cord JM, and Granger DN. Superoxide mediates endotoxin-


