evolution was eliminated. In the presence of iodoacetamide (IAA), which alkylates OSH and GSH in vivo (7), essentially all of the H_2O_2 was metabolized by catalase and O_2 was produced with the expected 1:2 stoichiometry to H_2O_2.

A second means of evaluating the relative contributions of the catalase- and thiol-dependent systems was to measure the clearance of H_2O_2 from seawater by an embryo suspension. A scopoletin assay (5) was used to measure the residual H_2O_2 after removal of the embryos by centrifugation. Fertilized sea urchin eggs (70,000 embryos per milliliter) incubated in 133 μM H_2O_2 (under the conditions of Fig. 2) consumed H_2O_2 in an apparent first-order reaction with a time constant $t_{1/2}$ of 15 min. After preincubation with 5 mM NaN_3 alone, $t_{1/2}$ = 18 min, and after preincubation with 2 mM IAA alone, $t_{1/2}$ = 16 min. However, when eggs were treated with both NaN_3 and IAA, there was no destruction of H_2O_2; $t_{1/2}$ was >4 hours, identical to that found in seawater alone. These data indicate that the catalase- and thiol-dependent systems accounted for all of the H_2O_2 consumption by sea urchin embryos, and that inhibition of either system alone does not significantly change the rate-limiting step in whole eggs (which is likely to be H_2O_2 entry).

The consumption of oxygen by sea urchin eggs at fertilization, discovered 80 years ago (16), is the archetype of metabolic activation. The egg uses the strong oxidant H_2O_2, thereby produced in order to modify its extracellular matrix; it is protected from oxidative stress by the regenerable trap, OSH. The activations of the pentose phosphate pathway (17) and NAD kinase (18) after fertilization are probably required, at least in part, to produce NADPH as substrate for glutathione reductase to allow reaction 4 to continue.

High concentrations of an OSH trap may be preferable to the catalytic GPx system found in mammalian cells, in which H_2O_2 bursts of comparable magnitude are rarely used physiologically. The ovothiol system may also protect marine invertebrate eggs and embryos from the significant concentrations of H_2O_2 generated photochemically in the sea (19, 20). Ovothiols are not restricted to marine invertebrates, for eggs from the rainbow trout *Salmo gairdneri* contain ovothiols A and B (1.7 and 0.34 nmol per milligram of protein, respectively), and similar concentrations exist in Coho salmon eggs (21).

Biological aromatic thiols like OSH have chemical properties distinct from aliphatic thiols like glutathione, including their distinct thiolate pK_a, heightened nucleophilicity (10), and ability to reduce oxygen-centered radicals (13). Because reactive oxygen intermediates have been implicated in many types of cellular dysfunction (22–24) and OSH is not toxic at high intracellular concentrations (at least in these embryonic cells), OSH could have useful applications.

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Induction of Manganous Superoxide Dismutase by Tumor Necrosis Factor: Possible Protective Mechanism

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Manganous superoxide dismutase (MnSOD) scavenges potentially toxic superoxide radicals produced in the mitochondria. Tumor necrosis factor–α (TNF-α) was found to induce the messenger RNA for MnSOD, but not the mRNAs for other antioxidant or mitochondrial enzymes tested. The increase in MnSOD mRNA occurred rapidly and was blocked by actinomycin D, but not by cycloheximide. Induction of MnSOD mRNA was also observed with TNF-β, interleukin-1α (IL-1α), and IL-1β but not with other cytokines or agents tested. TNF-α induced MnSOD mRNA in all cell lines and normal cells examined in vitro and in various organs of mice in vivo. These effects of TNF-α and IL-1 on target cells may contribute to their reported protective activity against radiation as well as their ability to induce resistance to cell killing induced by the combination of TNF-α and cycloheximide.

TUMOR NECROSIS FACTORS (TNFs) are cytotoxic to some tumor cells but not to normal cells (1), and they also mediate many other biological effects (2). The mechanism of TNF cytotoxicity is unclear but can occur in the absence of RNA or protein synthesis (3–5) and may be at least partially mediated through the generation of hydrogen peroxide (H_2O_2) and oxygen-free radicals such as O_2^- (6). Thus, the susceptibility of a cell to killing by TNF-α might be influenced by its content of antioxidant enzymes such as catalase, superoxide dismutase (SOD), and glutathione peroxidase. SOD protects cells from the toxicity of O_2^-, whereas catalase and glutathione peroxidase scavenge H_2O_2 (7). Eukaryotic cells contain copper-zinc SOD, which is found predominantly in the cytosol, and MnSOD, which is found mainly in mitochondria (8). We examined the effect of TNFs on the expression of antioxidant enzyme mRNAs. Unexpectedly, TNF-α was found to induce mRNA for MnSOD but not for Cu-ZnSOD, catalase, or glutathione peroxidase.

The actions of TNF-α and interferon-γ (IFN-γ) on cells are often synergistic (9). We therefore examined the effect of TNF-α and IFN-γ on the expression of antioxidant enzyme mRNAs in the human A549 lung carcinoma cell line. RNA hybridization showed that catalase, Cu-ZnSOD, and glu-

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tathione peroxidase mRNAs were expressed constitutively and were not altered by TNF-α or IFN-γ alone or in combination (Fig. 1). In contrast, the basal level of MnSOD mRNA was undetectable (Fig. 1); however, treatment of cells with TNF-α for 12 hours induced the expression of two distinct species of MnSOD mRNA (Fig. 1) that correspond with known sizes of MnSOD mRNAs (*10*). Specific induction of the two MnSOD transcripts by TNF-α was verified by reprobing the RNA blot with a β-actin probe (Fig. 1). IFN-γ alone had little inducing effect and did not augment the induction of MnSOD mRNA by TNF-α (Fig. 1).

To study the specificity of MnSOD induction, we examined the mRNAs for other mitochondrial enzymes. TNF-α did not alter the levels of mRNAs for cytochrome c oxidase (Fig. 1), pyruvate dehydrogenase, ornithine aminotransferase, or aspartate aminotransferase (*11*), demonstrating that induction of MnSOD by TNF-α is selective.

Studies were carried out to determine the kinetics of induction of mRNAs for antioxidant enzymes. Treatment of A549 cells with TNF-α for 1 to 24 hours did not change the levels of catalase or Cu-ZnSOD mRNAs (*11*). However, 1 hour of such treatment was sufficient to induce substantial levels of both transcripts of MnSOD, which remained high for 24 hours (Fig. 2) to 96 hours (*11*). Dose-response studies showed that maximal induction of MnSOD mRNAs required only 0.1 ng of TNF-α per milliliter (*11*). Treatment with TNF-α at 0.1 to 1000 ng/ml for 12 hours did not alter the levels of mRNAs for catalase, Cu-ZnSOD, or glutathione peroxidase (*11*).

The involvement of protein and RNA synthesis in MnSOD mRNA induction was tested with metabolic inhibitors. Cycloheximide alone had no effect on MnSOD mRNA levels and it did not prevent induction by TNF-α; thus TNF-α action is not dependent on de novo synthesis of polypeptide intermediates. However, it is possible that TNF-α increases production of O_2^-, which in turn stimulates MnSOD mRNA synthesis. The induction of MnSOD mRNA was blocked by actinomycin D (*11*), an indication that the increase in mRNA results from an increase in transcription of the MnSOD gene rather than stabilization of mRNA.

The ability of other mediators to induce MnSOD mRNA in A549 cells was also examined. Dot blot hybridization showed that treatment with TNF-β for 12 hours resulted in a striking increase in MnSOD mRNA. Likewise, IL-1α and IL-1β significantly induced MnSOD mRNA (Fig. 3). However, IFN-αA and IFN-β, IFN-γ, transforming growth factor–β (TGF-β), IL-6, IL-2, H_2O_2, or a combination of phorbol myristate acetate (PMA) and lipopolysaccharide (LPS) did not induce MnSOD mRNA in A549 cells. As observed for TNF-α, IL-1 had no effect on the expression of catalase, Cu-ZnSOD, glutathione peroxidase, and cytochrome c oxidase (*11*). Thus, IL-1s share with TNF the ability to induce MnSOD mRNA selectively in these cells. Furthermore, a dramatic induction of MnSOD by TNFs or IL-1s was also observed at the protein level. MnSOD protein and activity were induced by treatment with either TNF-α (10 ng/ml), TNF-β (10 ng/ml), or IL-1α (100 U/ml) but not by IFN-γ (100 ng/ml) or TGF-β (10 ng/ml) in A549 cells (*12*). MnSOD protein was measured by immunoblot with a polyclonal antibody to MnSOD, whereas MnSOD activity was measured by Nitroblue Tetrazolium reduction in the presence of cyanide, which inhibits Cu-ZnSOD (*13*).

Various cell lines in addition to A549 were tested for MnSOD gene inducibility by TNF-α. The basal levels of MnSOD, unlike those of cytoplasmic Cu-ZnSOD in most tumor cell lines, are low or undetectable as previously reported (*14*). TNF-α induced detectable levels of MnSOD mRNA in human cervical carcinoma ME-180 and murine transformed fibroblast LM cell lines, which are sensitive to killing by TNF-α (Fig. 4A). The human A172 glioblastoma and T24 bladder carcinoma cell lines, which are resistant to TNF killing, constitutively express MnSOD mRNA (Fig. 4A). TNF-α further

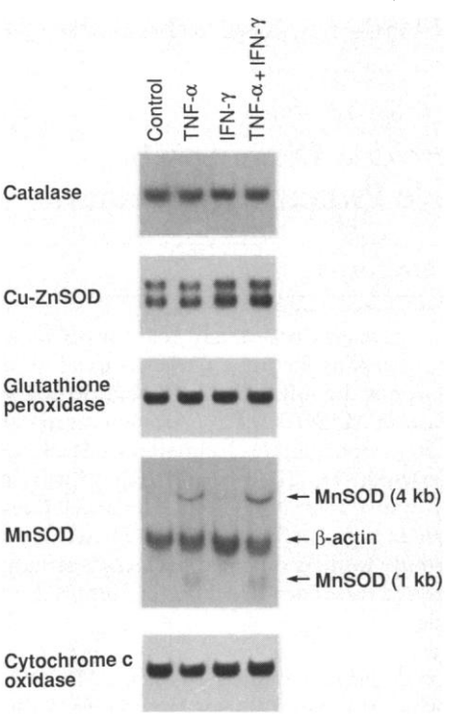

Fig. 1. Effect of TNF-α and IFN-γ on expression of mRNAs for antioxidant enzymes. Confluent A549 cells were treated with TNF-α (0.1 μg/ml), IFN-γ (0.1 μg/ml), or both for 12 hours before RNA isolation. Total cytoplasmic RNA was prepared and polyadenylated [poly(A)$^+$] RNA was enriched as described (*18*). Poly(A)$^+$ RNA was size-fractionated by formaldehyde agarose (1.2%) gel electrophoresis and transferred to nitrocellulose as described (*18*). Filters were hybridized to synthetic DNA probes corresponding to the published cDNA sequences of catalase, Cu-ZnSOD, MnSOD, β-actin, and glutathione peroxidase (*19*) labeled with ^{32}P by random priming. For RNA hybridization, we used 3 μg of poly(A)$^+$ RNA per lane. Recombinant human TNF-α and IFN-γ were produced in *Escherichia coli* (*20*) and purified to homogeneity; their specific activities were 2×10^8 and 4×10^7 U/mg, respectively.

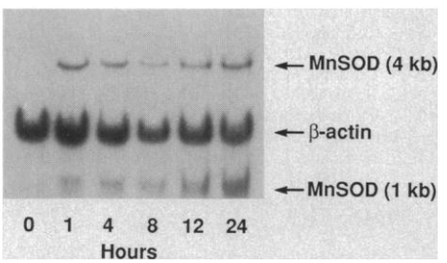

Fig. 2. Kinetic study of MnSOD mRNA induction by TNF-α. Confluent A549 cells were treated with TNF-α (0.1 μg/ml) for 1, 4, 8, 12, and 24 hours before RNA isolation. Poly(A)$^+$ RNA (3 μg per lane) was hybridized with ^{32}P-labeled MnSOD and β-actin probes corresponding to the published sequences (*19*).

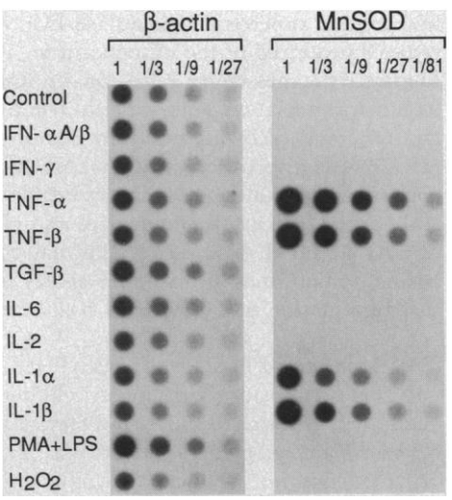

Fig. 3. Effect of various agents on induction of MnSOD mRNAs. Confluent A549 cells were treated with TNF-α (0.1 μg/ml), TNF-β (0.1 μg/ml), IFN-αA and IFN-β (100 U/ml), IFN-γ (0.1 μg/ml), IL-1α (100 U/ml), IL-1β (100 U/ml), IL-6 (100 U/ml), TGF-β (10 ng/ml), PMA (50 ng/ml), and LPS (10 μg/ml) or H_2O_2 (0.03%) for 12 hours. Recombinant human IL-1α, IL-1β, and IL-2 were purchased from Genzyme with specific activities of 10^8 U/mg. Pure recombinant human TGF-β (*20*) was prepared by Genentech. PMA and LPS were obtained from Sigma. Dot blot hybridization with 3 μg of poly(A)$^+$ RNA per sample was carried out as described (*21*).

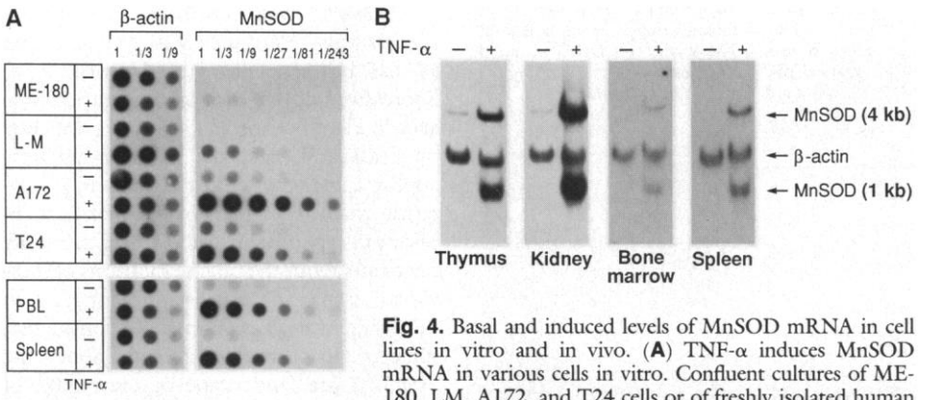

Fig. 4. Basal and induced levels of MnSOD mRNA in cell lines in vitro and in vivo. (**A**) TNF-α induces MnSOD mRNA in various cells in vitro. Confluent cultures of ME-180, LM, A172, and T24 cells or of freshly isolated human peripheral blood lymphocytes (PBL) or murine spleen cells (5×10^5 per milliliter) were treated with TNF-α (0.1 μg/ml) for 8 to 12 hours before RNA extraction. Dot blot hybridization with poly(A)$^+$ RNA (2 μg) was carried out as described (*21*). (**B**) Induction of MnSOD mRNA by TNF-α in vivo. Ten BALB/c mice were given an intravenous injection of either phosphate-buffered saline or murine TNF-α (5 μg per mouse). After 24 hours, RNA was extracted from pooled thymus, kidneys, bone marrows, or spleens, and poly(A)$^+$ RNA (5 μg per lane for thymus and kidney and 2 μg per lane for bone marrow and spleen) was hybridized with ^{32}P-labeled MnSOD and β-actin probes (*19*). Murine TNF-α was produced in *Escherichia coli* (*20*) and purified to homogeneity. The specific activity of murine TNF-α was 8×10^7 U/mg.

increased MnSOD mRNA levels by about 81- and 27-fold in A172 and T24 cells, respectively (Fig. 4A). Induction of MnSOD mRNA was observed in all the cell lines we examined: human cervical carcinoma (HeLa), colon carcinoma (HT-29), oral epidermoid carcinoma (KB), rhabdosarcoma (RD), glioblastoma (U373 and U87MG), normal fetal lung fibroblast (WI-38), the SV40-transformed WI-38 (WI-38 VA13), and breast carcinoma (MCF-7). Murine cell lines we examined were murine fibrosarcoma (WEHI-164) and mast cell (A1), and normal rat kidney fibroblasts (NRK). The inducing effect was not limited to transformed cell lines, but could also be observed in normal cells. Treatment of human peripheral blood lymphocytes and murine spleen cells with TNF-α for 12 hours resulted in at least a ninefold increase in MnSOD mRNA (Fig. 4). Thus, induction of MnSOD mRNA by TNF-α is not cell type–specific. The expression of catalase, Cu-ZnSOD, and glutathione peroxidase mRNAs was constitutive in all cell lines tested (LM, ME-180, A172, and T24), and treatment with TNF-α did not alter the levels of these mRNAs (*11*).

We tested the physiological relevance of the in vitro findings by examining the effect of TNF-α on MnSOD mRNA in vivo. In mice, 24 hours after an intravenous injection of TNF-α, the levels of MnSOD but not of β-actin mRNA were increased in the kidney, thymus, bone marrow, and spleen (Fig. 4B). Intravenous injection of mice with phosphate-buffered saline did not induce MnSOD mRNA.

Our data show that TNF-α treatment selectively and rapidly induces the mRNA for MnSOD both in vitro and in vivo. The induction of MnSOD mRNA appears to be a general phenomenon because it was observed in all cells tested. TNF-β and both IL-1s also induced MnSOD mRNA, whereas other cytokines did not. Since TNF-α and IL-1 protect mice from lethal doses of radiation (*15*), we propose that induction of MnSOD by TNF and IL-1 may contribute to the protection of hematopoietic progenitor and other cells against radiation.

TNF-α actively induces synthesis of new RNA and proteins (*3–5*). Pretreatment of cells with low levels of either TNF or IL-1 can confer resistance to killing by subsequent treatment with TNF-α and cycloheximide in combination (*5, 16*), suggesting that TNF and IL-1 induce synthesis of protective proteins. Because cycloheximide would block the constitutive and induced synthesis of protective proteins, it renders TNF-resistant cells susceptible to TNF-mediated killing (*4, 5*). We propose that MnSOD is one of the proteins involved in protecting cells from the cytotoxic effects of TNF and that the amount of MnSOD induced by TNF or IL-1 may influence the cellular susceptibility to killing by TNF. If MnSOD is indeed one of the proteins responsible for the protection of tumor cells against damage mediated by TNF, then it may well be that the mitochondrion is a site of TNF's cytotoxic actions (*17*). Furthermore, specific measures to block the induction of MnSOD might be used to prevent the emergence of resistant cells during cancer therapy.

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Technical Comments

Toward a Universal Law of Generalization

When sentient organisms make decisions about how to react to novel stimuli or situations, they may do so in accordance with consequences associated with previously learned stimuli or situations. Thus, the probability that a novel stimulus is located perceptually in a consequential region is important in determining the subsequent behavior of the organism. A generalization experiment gives the probability that a response learned to stimulus S_i will be made to stimulus S_j. Shepard (*1*) proposed the basis for a law of generalization that involves two central ideas: first, that the probability that a response learned to stimulus S_i will be made to stimulus S_j is approximately a negative exponential function of the distance between the stimuli in a space of a certain dimensionality; second, that the metric used to define this distance will be Euclidean when the psychological dimensions are correlated and city block when they are not. Some of the examples used by Shepard (*1*) involved similarity, rather than generalization, and he noted that both probabilities arise from the same basic process. As presented, Shepard's theory applies only to experiments in which generalization is tested immediately after a single learning trial with a novel stimulus. The need to invoke this limitation results from the fact that, with highly similar stimuli or with delayed test stimuli, the relation between similarity and distance has been found to be Gaussian in form, and the distance metric appears to be Euclidean for cases in which the theory would predict city block. The work of Nosofsky (*2*) exemplifies this kind of result. Shepard conjectured that perceptual "noise" contributed in some way to these departures from theory but, since he did not have a formal model for dealing with these cases, he treated them as exceptions.

This self-imposed limitation on Shepard's theory can be removed by treating the mental representations of learned and novel stimuli (in generalization experiments) or pairs of stimuli (when estimating similarity) as momentary values from multivariate normal distributions (*3*). In other words, one can formulate Shepard's theory in stochastic terms. If d is the distance between the momentary psychological magnitudes and $g(d) = \exp(-d^{\alpha})$ is a measure of similarity, then $E(g)$ (*4*) is the expected value of the similarity measure. This model was evaluated in two dimensions when the perceptual dimensions are uncorrelated and, consistent with Shepard's theory, g is a negative exponential function ($\alpha = 1$) of city block distance, d. The evaluation revealed that the relation between $E(g)$ and the distance between the means of the distributions of psychological magnitudes (δ) is best described as a modified Gaussian function of Euclidean δ. This result is consistent with Nosofsky's results (*2*) and with several exceptions to Shepard's theory (*1*). The theory as originally discussed by Shepard regarding generalization and similarity was applicable when perceptual "noise" was absent; this extension allows for the possibility of a certain kind of noise (multivariate normal) and consequently extends Shepard's theory to include pairs of perceptually confusable objects.

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4. $$E(g) = \int_{-\infty}^{\infty}\int_{-\infty}^{\infty}\cdots\int_{-\infty}^{\infty} \exp(-d^{\alpha}) \times \frac{\exp\{-0.5(\mathbf{z}-\boldsymbol{\mu})'\,\mathbf{V}^{-1}\,(\mathbf{z}-\boldsymbol{\mu})\}}{(2\pi)^{n/2}\,|\mathbf{V}|^{1/2}} dz_1 dz_2 \ldots dz_n$$

where $\mathbf{V}$ is the variance-covariance matrix of the difference between psychological values, $\mathbf{z}$; $\boldsymbol{\mu}$ is a vector of differences between the means of the momentary psychological values, $\boldsymbol{\mu}_x$ and $\boldsymbol{\mu}_y$.

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Response: I welcome Ennis's demonstration that my recent theory of generalization (*1*) can be reconciled with Nosofsky's (*2*) impressive body of data on human performance in identification tasks. Results obtained by Ennis, by Nosofsky, and by me now appear consistent with the following theoretical characterization. In Nosofsky's identification experiments, subjects are primarily uncertain about the precise locations of individual stimuli in "psychological space." In the generalization experiments I considered, subjects are primarily uncertain about the location, size, and shape of the region of psychological space corresponding to the set of stimuli having the same important consequence as a given training stimulus.

Some of my own earlier results (*3*) had suggested, and Ennis has now more fully and rigorously demonstrated (*4*), how my theory of generalization can be extended to accommodate uncertainty about the locations of stimuli. The generalization theory then yields response probabilities that fall off in an approximately Gaussian manner with Euclidean distance from the training stimulus—the result empirically obtained by Nosofsky (*2*). When the uncertainty is primarily about the disposition of the consequential region, however, probability of response falls off, as I originally deduced (*1*), in close approximation to an exponential decay function of distances of either the Euclidian or "city block" varieties—depending on whether the subject assumes that the possible extensions of the consequential region along the underlying dimensions of the space are correlated or uncorrelated.

Clearly, my theory of generalization need not entail an exponential decay of response probability with distance under every condition. The exponential generalization function is a candidate for a "universal" psychological law in the sense only that the effects of any consequences associated with the *first* encounter with a novel stimulus may decrease exponentially with distance for all sentient organisms—wherever they may have evolved.

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