cally expressed in the retina.

The isolation of more members of the γ subunit family will help to clarify the relation among the three G protein subunits. To date, G proteins have been distinguished by their α subunits. If there are many diverse members of the γ subunit family, a particular α subunit might be associated with more than one species of γ, and each of these combinations could have a different function. This combinatorial association would increase the diversity of G protein functions.

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16. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. We thank J. K. Northup for the G_i and G_o proteins, R. Miake-Lye for the cDNA library and RNAs, B. W. Birren for the genomic DNA, M. Strathmann for discussions, and T. Amatruda and T. Wilkie for comments on the manuscript.

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Oxygen Radicals in Influenza-Induced Pathogenesis and Treatment with Pyran Polymer-Conjugated SOD

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The pathogenicity of influenza virus infection in the mice involves, at least in part, overreaction of the immune responses of the host rather than a direct effect of virus multiplication. Xanthine oxidase, which is responsible for the generation of oxygen free radicals, was elevated in serum and lung tissue of mice infected with influenza virus. To test the theory that oxygen-free radicals are involved in pathogenesis, free radicals were removed by injecting superoxide dismutase (SOD), a specific superoxide radical scavenger, which was conjugated with a pyran copolymer. The conjugate protected mice against a potentially lethal influenza virus infection if administered 5 to 8 days after infection. These findings indicate that oxygen radicals are important in the pathogenesis of influenza virus infection, and that a polymer-conjugated SOD has therapeutic potential for this virus infection and other diseases associated with free radicals.

A TYPICAL CHARACTERISTIC OF INfluenza virus infection in the mouse lung is the presence of areas of surface consolidation, which under the microscope show extensive hemorrhage, infiltration of lymphoid cells including neutrophils and macrophages, and edema in the alveolar spaces. Several studies have suggested that an overreaction of the host's immune system is involved in pathogenesis of influenza virus infection, and that morbidity and mortality are mediated as immunopathological consequences (*1–5*). Neutrophils and macrophages are known to produce superoxide free radicals (O_2^-) and hydrogen peroxide (H_2O_2), which normally are involved in the killing of ingested or invading microbes. However, the activated oxygen can also cause tissue injuries such as lung damage in adult respiratory distress syndrome and other inflammatory diseases (*6, 7*). Thus, it is possible that complex cellular immunity in influenza virus infection involves oxygen free radicals.

To test the hypothesis that free radicals are involved in influenza pathogenesis, we first studied O_2^- generation by the alveolar phagocytic cells from influenza virus–infected mice (Table 1). On day 8 after virus infection, the O_2^- generating potency of alveolar phagocytic cells was about eight times higher than that on day 0 (immediately after virus inoculation) with or without phorbol myristate acetate (PMA), a potent stimulant of O_2^- generation.

To see whether there was any increase in phagocytic cells in the lung, the population of lung-infiltrated cells was analyzed by broncho-alveolar lavage every other day after infection. On day 0 about 94% of the nucleated cells obtained by broncho-alveolar lavage were macrophages. Granulocytes in-

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creased at an early stage (within 4 days) after infection very rapidly, up to 42%, whereas macrophages decreased to 42% on day 2; granulocytes markedly decreased thereafter (to 13% on day 8). Lymphocyte population increased gradually; we observed 6% on day 0, an increase to 20% on day 4, and an increase to 50% on day 6. Mortality increased after day 6, which paralleled the increased population of lymphoid cells (up to 57% on day 8). Thus, it seems that granulocytes may participate in the early events, as seen in acute-phase inflammation, but lymphocytes appear responsible for the late events, which may be critical. Macrophages, another source of oxygen radicals, are known to be activated during microbial infections, and their production of oxygen radicals also increases (*8*). Although the macrophage population gradually decreased to 35% (day 4) and 30% (day 8) after virus infection, O_2^--generating activity per 10^6 cells of alveolar phagocytic cells significantly increased (Table 1). These results suggested that one source of O_2^- generation was macrophages at the later stage, especially on day 8 or thereafter, whereas a possible association of highly elevated level of lymphoid cells to free radical generation needs to be elucidated.

In addition to these phagocytic cells, another potential source of oxygen radicals is thought to be the xanthine oxidase in plasma and extracellular space. Under normal physiological state the xanthine oxidase activity in many tissues was very low, but it was shown to increase dramatically after ischemia and reperfusion (*9*). Conversion to xanthine oxidase from xanthine dehydrogenase, which is abundant in many tissues (*10, 11*), seems to proceed extensively in such damaged tissue. Furthermore, there is now evidence that xanthine oxidase activity is elevated in adult respiratory distress syndrome (*12*). These findings suggest that similar pathological damage may occur during influenza virus infection if the xanthine oxidase activity is elevated. Therefore, we measured xanthine oxidase activity in the lung and serum of influenza virus–infected mice. We found that xanthine oxidase activity in both the lung and serum increased greatly, particularly 8 days after virus infection, which correlated well with mortality (Fig. 1). Concordantly in a preliminary experiment, allopurinol, a specific inhibitor of xanthine oxidase, exerted a protective effect on the infected mice (*13*). Therefore, O_2^- that originated from xanthine oxidase also seems to be important in influenza virus–induced pathogenesis.

Table 1. Generation of O_2^- by alveolar phagocytic cells obtained from influenza virus–infected mice. Two median lethal doses (LD_{50}) of virus [influenza A/Kumamoto/Y5/67(H_2N_2)] were given as described in the legend to Fig. 2. After virus inoculation, mice in each group ($n = 3$) were killed at 2-day intervals. The infiltrated cells were obtained by broncho-alveolar lavage from 2 ml of Krebs-Ringer phosphate buffer (KRP). Cells were washed once with KRP by centrifugation, and were immediately assayed for O_2^- generation. Release of O_2^- from phagocytic cells was determined spectroscopically on the basis of SOD-inhibitable reduction of ferricytochrome C in the presence or absence of 0.1 μg of PMA per milliliter as a stimulant (*26*). Each value is the average of three determinations ± SE. O_2^- generation is given as nanomoles per 30 min per 1×10^6 cells.

Days after infection	O_2^- generation	
	−PMA	+PMA
0	1.0 ± 0.3	3.3 ± 0.7
2	4.2 ± 1.3	18.6 ± 1.9
4	4.4 ± 1.4	12.5 ± 2.9
6	5.3 ± 1.8	21.0 ± 2.1
8	7.6 ± 1.5	21.7 ± 3.5

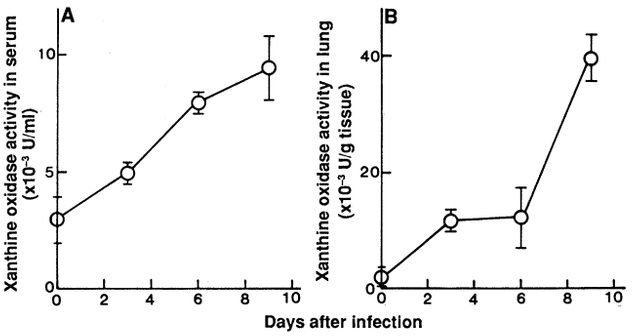

Fig. 1. Xanthine oxidase activity in the serum (**A**) and in the lung tissues (**B**) of influenza virus–infected mice. Blood was obtained by cardiac puncture under light ether anesthesia, and lung tissues from the mice were removed and immediately homogenized with 5 ml of ice-cold 0.01*M* phosphate-buffered 0.15*M* saline, *p*H 7.4, in a microblender (Omnimixer; Ivan Sorvall Inc., Norwalk, Connecticut). The homogenate was centrifuged at 12,000*g* for 10 min at 4°C. The supernatant was assayed for xanthine oxidase activity, which was measured spectrophotometrically as described by Massey *et al.* (*27*), in 0.01*M* phosphate buffer, *p*H 7.4, containing 0.1 m*M* xanthine. One unit of enzyme activity is defined as the amount of enzyme present that caused the formation of 1 μmol of urate from xanthine per minute. This xanthine oxidase activity was completely inhibited by allopurinol, a xanthine oxidase inhibitor. Each point represents the average of three values ± SE.

Damage in tissues and cells induced by activated oxygen species is often prevented by superoxide dismutase (SOD) or catalase, or both (*14*). However, clinical application of SOD as a therapeutic agent is very limited because of shortcomings, such as an extremely rapid plasma clearance time [half-time ($t_{1/2}$) <5 min], instability, and immunogenicity in vivo. We previously prepared a conjugate of styrene maleic acid copolymer (SMA) and neocarzinostatin (NCS), which is a proteinaceous antitumor agent. The conjugate, designated smancs, showed improved pharmacological properties over NCS, which included prolongation (tenfold) of biological half-life in vivo, decreased antigenicity, and enhanced tropism to the lesion or the tumor tissue (*15–17*). Several other examples of conjugation with polymers such as SMA, divinylether maleic acid copolymer (pyran copolymer), and polyethylene glycol also may overcome the shortcomings of protein drugs (*18–20*). Therefore, we conjugated bovine Cu, Zn-SOD with pyran copolymer and examined its efficacy in preventing influenza-induced mortality in mice.

Conjugation of SOD and pyran copolymer (molecular weight ~5600) was carried out in 0.3*M* sodium bicarbonate buffer, *p*H 8.7; several amino groups of SOD were reacted with anhydride residues in this copolymer to form carbamide linkages similar to SMA (*18, 19*). Conjugates (pyran-SOD) were purified by gel filtration on a Sephadex G-100 column. We observed a gradual loss of enzyme activity as more amino groups were modified. Modification of about 10% of the 20 free amino groups in SOD seemed to result in adequate pharmacological properties while retaining about 80% of the enzyme activity. We then tested the pyran-SOD conjugate in vivo and found it to have a very prolonged plasma half-life. When native SOD was injected intravenously (i.v.), there was no measurable activity in the circulation within 30 min; however, concentrations of the pyran-SOD conjugate in plasma remained measurable even after 5 hours.

Daily intravenous injection of the pyran-SOD conjugate at a dose of 200 U per mouse from days 5 to 8 after virus infection dramatically protected mice from death; nine of ten mice survived (Fig. 2). However, native SOD at 200 U had little therapeutic value, perhaps because of its very short plasma half-life (ten of ten mice died). In addition, degeneration of the bronchial epithelium and extensive interstitial infiltration of lymphoid cells were observed in the untreated group, but much milder degenerative morphology was seen in the pyran-SOD–treated group. The influenza virus in the mouse lung was quantified every other day after the virus infection; virus concen-

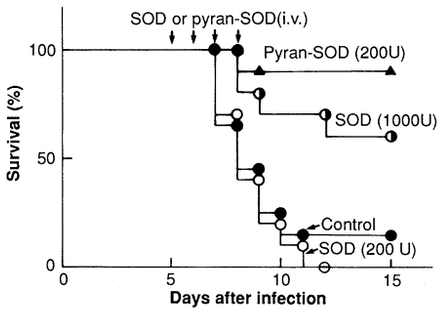

Fig. 2. Therapeutic effect of SOD and pyran-SOD conjugate on influenza virus–infected mice. Male ddY mice (age: 4 to 6 weeks) were used in the experiments. Mice received the influenza virus (Table 1) by inhalation of virus aerosol at twice the LD_{50} dose. Ten mice were used in each treated group and 20 mice in the control group. SOD (200 and 1000 U per mouse) and pyran-SOD conjugate (200 U per mouse) were given intravenous injections once daily for four consecutive days from 5 days after virus infection. ●, Control; ○, SOD (200 U per mouse); ◑, SOD (1000 U per mouse); ▲, pyran-SOD conjugate (200 U per mouse). The mortality rate of mice did not increase after day 15 to day 30. Mice surviving on day 15 were considered as being cured of the virus infection.

trations were maximal on day 4 and decreased to a very low level on day 8 or later (*21*). Treatment on days 1 to 4 at the same dose had no therapeutic effect even though viral production was maximal (*22*). The treatment with pyran-SOD did not affect clearance of the virus from the lung (*23*). This is a contrast to the treatment of cyclophosphamide, which impairs immune response and prolongs survival at most several days but no cure is seen (*1, 3*). These results suggest that oxygen radicals produced by the host's delayed response (day 5 to 8 or later) affect the mortality of virus-infected mice.

Pyran copolymer is a well-known interferon inducer (*24*). Thus, it was possible that the interferon induced by the pyran copolymer exerted the therapeutic effect in these mice. However, the interferon should be effective against viral multiplication (days 1 to 4), but our data showed no effect during these days. Furthermore, no therapeutic effect of pyran copolymer by itself was observed in our experiments (*25*). Thus, it seems most reasonable to attribute the pronounced therapeutic effect of pyran-SOD conjugate to its enzyme activity as an O_2^- scavenger, not as an interferon inducer. These results suggest that activated macrophages and systemic and local xanthine oxidase levels, all generating O_2^-, are important in the pathogenesis of influenza virus infection.

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Categorical Perception of a Natural Stimulus Continuum: Birdsong

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A fundamental issue in perception and communication is how continuously varying stimuli are partitioned into discrete categories. In swamp sparrow songs, note duration is a critical feature distinguishing two note categories with different roles in song construction. Pairs of songs with initial notes from different categories contrast more in their effects on territorial males than song pairs with initial notes differing by the same amount but taken from within one note category. The results indicate categorical perception by wild swamp sparrows.

CATEGORIZATION IS A BASIC PERceptual process by which animals recode variable stimuli into discretely different categories. This recoding is thought to reduce neural information-processing requirements and to increase the speed and accuracy of critical perceptual judgments (*1*). Categorization of visual and auditory stimuli by humans has been widely documented, especially in speech perception where the partitioning process compensates for the variability in our pronunciation of words (*2, 3*). Much animal research has been devoted to identifying vocal features involved in species recognition (4), but little is known about whether animals categorize vocal signals of their own species, as humans do (*5*).

One hallmark of stimulus categorization is the "category boundary effect" (*3*) in which the discriminability of physically equivalent steps along a stimulus continuum is nonuniform. As a result, stimulus pairs astride a category boundary are distinguished more readily than stimulus pairs within a category. Thus, one approach to identifying categorical perception is to measure how well subjects discriminate equal differences along a stimulus continuum (*3*). An alternative approach used here is to assess how stimulus variation influences natural, unconditioned responses. This approach poses a different question by testing which stimulus variants are meaningfully different instead of simply discriminably different. The dependence upon naturally occurring responses rather than arbitrary operant responses serves to reveal the nature of perceptual categories that are meaningful within a communication system.

Research indicated a possible role for categorization in birdsong perception. Songs of the swamp sparrow (*Melospiza*

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