and Use Committee. The auditory stimulus and microelectrode data were digitized at 20 kHz. Single-unit action potential waveforms were digitally discriminated and displayed as rasters (Experimenters Workbench, Datawave). Single-unit responses were quantified by averaging the firing rate during the stimulus period (plus the ensuing 100 ms) and then subtracting the firing rate during the control period (500 ms preceding stimulus onset). This mean firing rate per presentation was used to produce averages, for example, of 10 successive presentations (Fig. 1D) or of the first 50 presentations.

6. Birds that had heard the testing stimulus earlier had been exposed to 200 repetitions thereof at an ISI of 11 s; testing also occurred at an ISI of 11 s.
7. Single units are difficult to "hold" with a recording electrode for periods of more than 30 min. Therefore, most of this study was done with MUA data.
8. The multi-unit response amplitude to each stimulus presentation was calculated by subtracting the root-mean-square (rms) value over the 500-ms preceding stimulus onset from the rms over the period from stimulus onset to offset plus 100 ms. The difference between the two root-mean-square values measures the net response per unit time and corrects for differences in duration between stimuli. Each amplitude was then normalized to the response amplitude on the first presentation (typically the largest) and plotted as a function of stimulus iteration. We used the least-squares method to determine the slope of the straight line that best fitted each set of 100 normalized responses to a stimulus that the bird had or had not heard during an earlier training session. The habituation rate was defined as the absolute value of the slope of the best-fit line (Fig. 2A). Habituation rates were independent of the absolute response level at any given site and so could be used to compare different sites, in different birds, recorded at different intervals after training. We have previously shown that the distributions of habituation rates to novel and familiar songs differ significantly (3). Figure 2A demonstrates, too, that habituation to the familiar song occurred, typically, during the first few repetitions of the stimulus, after which responses stayed at a lower level; it took longer for this lower level to be reached by responses to a novel song.
9. All statistical comparisons were done with the Student's t test ($P < 0.05$, two-tailed).
10. The distribution of entries in Fig. 2B suggests that, even though there was a relatively abrupt change in habituation rate between 46 and 48 hours, a less marked drift toward higher habituation rates occurred between 15 and 46 hours after onset of training. Our stringent criterion for forgetting—habituation rates similar to those induced by presentations of a novel song—did not recognize these changes, which may, however, represent an early forgetting stage that deserves further study.
11. These sounds, which provided a set of conspecific and heterospecific stimuli that the birds had not previously heard, were digitized at 20 kHz (Signal, Engineering Design). The songs and words from human speech were 1.2 to 2.0 s long, and calls were 100 to 400 ms long.
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16. Seventeen birds were trained with 200 iterations (massed) each of two songs, followed by 50 iterations of each of five other songs, and this latter spaced protocol was repeated four times. At an ISI of 11 s, the spaced-training part of this protocol took 3 hours. Another two songs were then played with the massed-training paradigm. The total duration of this protocol was 5.5 hours. In a second version of this protocol, birds ($n = 8$) were trained with 50 iterations (massed training: ISI = 11 s, total training time = 9.2 min) of each of two novel songs; this was then followed by 10 iterations of each of a group of five other novel songs, and the latter sequence was repeated five times (spaced training: ISI = 11 s, total training time = 46 min). We then played another three songs using the massed-training paradigm. Thus, in 1.5 hours, we exposed the bird to 50 iterations of five songs presented in the massed-training paradigm, and 50 iterations of five songs presented in the spaced-training paradigm.
17. When a total of 50 iterations of each song were used, habituation was lost after only 7 hours for songs presented in the massed-training manner (Fig. 4C), but spaced training (*16*) produced habituation that persisted for at least 43 hours (*14*).
18. Five zebra finches that were trained with canary song presented in a spaced paradigm heard four groups of 50 iterations for each of five stimuli, for a total of 200 iterations for each stimulus; this training was compared with 200 massed iterations for a same canary song.
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20. ACT (40 nl, 50 μM) or CYC (40 nl, 1 ng/nl), dissolved in saline, was injected into 46 adult male or female zebra finches into a right- or left-hemisphere NCM recording site that exhibited habituation to novel songs. These injections were made with a glass micropipette (tip outer diameter, 20 μm). As a control, saline vehicle alone was injected into the other hemisphere. The effective sphere of the ACT or CYC injections, as determined by immunocytochemistry, was limited to a subregion of NCM (3). Such injections did not affect auditory responses or the immediate habituation of NCM neurons to playbacks of a novel song. The physiological effect of these RNA and protein synthesis blockers in NCM was reversed in <1 hour, as determined by the loss of long-term habituation for stimuli presented 0.5 hour, but not 1 hour, after injection [S. J. Chew, thesis, Rockefeller University (1966)], allowing for a fairly accurate pinpointing of the time when gene expression or protein synthesis is necessary for the maintenance of long-term habituation. The side of drug injection was alternated in successive experiments to eliminate side-to-side biases in injection or recording technique. Starting 1 hour after drug injection, insulated tungsten microelectrodes were used to record physiological activity at the injection sites. Comparison of simultaneous recordings in the two sides controlled for nonspecific effects or drug diffusion and for the particular songs used in any given experiment. The two sides were previously shown not to differ in habituation rates (*13*). Test stimuli were presented in the same order as during training, starting 1 hour after injection.
21. The presence and duration of the first sensitive period was not established with protocols 2 and 3 because training with a single song with either of these protocols lasted 2.2 and 2.4 hours. The second, third, and fourth sensitive periods during which injections of ACT or CYC in NCM blocked long-term habituation were very similar, regardless of whether we used protocols 1, 2, or 3 . In addition, protocols 2 and 3 revealed a fifth and sixth sensitive period of mnemogenic RNA and protein synthesis, while retaining those that had occurred earlier (Fig. 5).
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Ethylene as a Signal Mediating the Wound Response of Tomato Plants

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Plants respond to physical injury, such as that caused by foraging insects, by synthesizing proteins that function in general defense and tissue repair. In tomato plants, one class of wound-responsive genes encodes proteinase inhibitor (pin) proteins shown to block insect feeding. Application of many different factors will induce or inhibit *pin* gene expression. Ethylene is required in the transduction pathway leading from injury, and ethylene and jasmonates act together to regulate *pin* gene expression during the wound response.

The wound response of tomato plants has been studied for some 25 years and represents a model system for the analysis of cell signaling pathways in plants (*1*). Proteinase inhibitor (*pin*) genes are up-regulated throughout aerial tissues in response to wounding (*2*). *Pin* genes are also responsive to compounds applied experimentally through the transpiration stream of excised leaves and the use of this bioassay has identified a range of positive and negative regulators. Positive regulators (elicitors) include oligogalacturonide fragments of pectin polysaccharides (OGAs) (*3*), an 18mer

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peptide called systemin (*4*), and fatty acid derivatives including jasmonic acid (JA) (*5*). Negative regulators that block wound and elicitor induction of *pin* gene expression include aspirin (ASA) and salicylic acid (6–8). The current model for wound signaling places elevated JA as a common downstream event in the transduction pathways from injury, OGAs and systemin, acting as the sole causal agent of *pin* gene expression (9). We now show that another plant hormone, ethylene, is an absolute requirement for the response. Our evidence leads to a model for wound signaling in which JA and ethylene act together to regulate *pin* gene expression.

Wounding induces ethylene biosynthesis (*10*). Ethylene is made from *S*-adenosylmethionine through the sequential action of two enzymes, 1-aminocylopropane-1-carboxylate synthase (ACS) and 1-aminocylopropane-1-carboxylate oxidase (ACO) (*11*), and transcripts encoding both of these enzymes are known to accumulate transiently in wounded leaves (*12*). Earlier studies investigated a role for ethylene in the induction of wound-responsive *pin* genes by gassing the tomato plants with the hormone (*13*). Since these results proved negative, ethylene was discarded as a causal agent. Subsequently, systemin and JA have both been shown to induce ethylene in suspension-cultured cells of tomato (*14*). Following leaf injury, or application of OGAs, systemin, or JA through the transpiration stream, *pin 2* transcripts become detectable after 2 to 4 hours (Fig. 1). Steady-state levels can remain high for up to 24 hours (*15*). Ethylene is produced rapidly and transiently on challenging the plant with each of the four stimuli, with levels detectable within 30 min and decreasing to near basal levels within 4 hours. These data suggest that the two wound responses, ethylene production and *pin* gene expression, share certain signaling events.

Aspirin (acetyl salicylic acid, ASA) blocks *pin* gene expression in tomato plants. It is a rapid and reversible inhibitor in the bioassay that must be applied prior to wounding or treatment with elicitors such as OGAs and chitosan (6). Its effect could arise from disruption of H^+/K^+ transport at the plasma membrane, a property ascribed to ASA, salicylic acid (SA) and related hydroxybenzoic acids (*16*). Additional sites of inhibition by ASA are at a step leading to JA synthesis (*7*) and at steps downstream of JA in the later events of the wound signal transduction pathway (*8*). ASA pretreatment prevents the induced increase in steady-state levels of *pin 2* transcripts by each of the four stimuli, confirming the presence of a site or sites of inhibition by ASA downstream of JA (Fig. 2). Aspirin also inhibits ethylene production by each of the four stimuli. Thus, there is a correlation between the plant's ability to produce ethylene in response to injury and elicitor application, and the plant's ability to express *pin* genes.

To determine whether there is a causal relationship between ethylene and *pin* gene expression, we used a variety of methods to block the action or synthesis of the hormone. Silver thiosulphate (STS) is thought to act by disrupting the binding of ethylene to its receptor (*17*). STS blocks the induction of *pin* gene expression by wounding or elicitor treatments (Fig. 3). Norbornadiene (NBD), a competitive inhibitor of ethylene (*18*), also affects the induction of *pin* gene expression by each of the four stimuli. The inhibitory effect of NBD could be overcome by excess exogenous ethylene, consistent

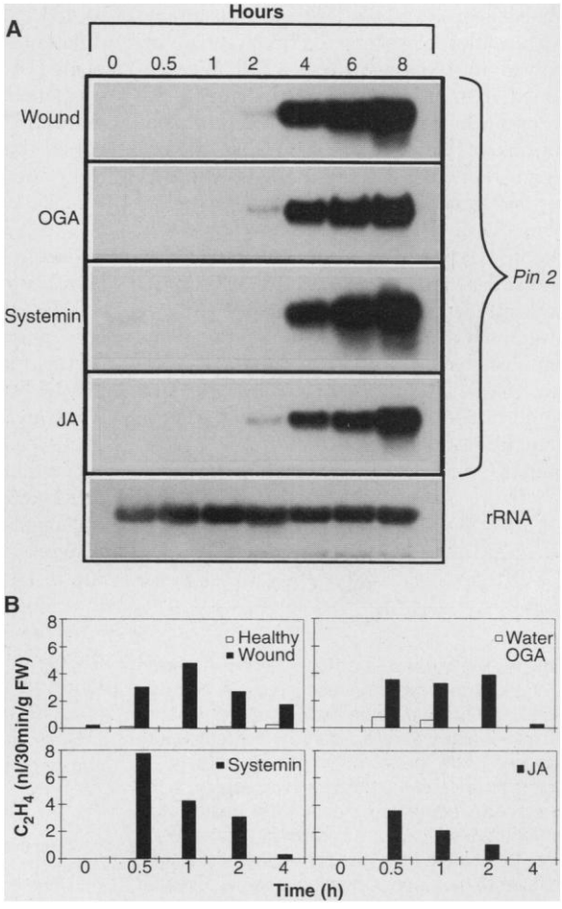

Fig. 1. The effect of wounding and elicitor treatment on amounts of *pin 2* transcripts and ethylene accumulation in tomato plants. Wounding was performed by crushing the terminal leaflets of 21-day-old tomato plants (*Lycopersicon esculentum* Mill. cv Moneymaker) with a pair of tweezers. For elicitor treatments, plants were excised at the base of the stem and incubated for 30 min in OGAs with degree of polymerization 1 through 8 at (1 mg/ml) (*6*), systemin (100 nM) or JA (100 μM) before transfer to water for the remainder of the incubation period. For all treatments plants were maintained under constant light at a temperature of 22°C. Leaf material was harvested at the indicated times. (**A**) Northern blot analysis (*27*). Total RNA was extracted (*28*) and hybridized with a ^{32}P-labeled *pin 2* (*29*) probe. Equal loading was confirmed by re-hybridizing stripped blots with a labeled ribosomal RNA probe. (**B**) Ethylene accumulation was measured as described in (*30*) with the use of a Pye Unicam series 204 gas chromatograph fitted with a flame ionization detector and a packed glass column, 6 foot × 1/4 inch, 4-mm internal diameter, containing alumina F1 80/100 mesh. The flow rate of carrier gas (nitrogen) was 60 ml/min. Harvested leaf material from six plants was placed in 20-ml gas-tight vessels and incubated under constant light at 22°C for 30 min prior to removal of 1 ml of the head space for ethylene determination. All experiments were repeated at least three times and in each figure representative results are shown.

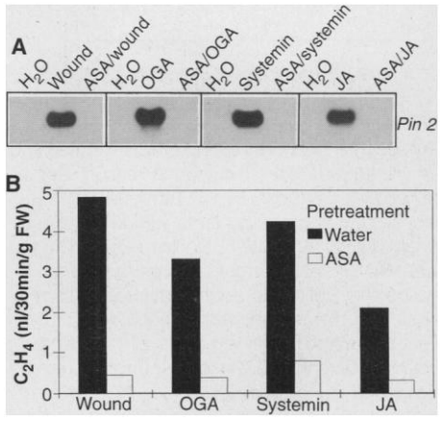

Fig. 2. The effect of aspirin on the accumulation of *pin 2* transcripts and ethylene production in response to wounding or elicitor treatment of tomato plants. Twenty-one-day-old plants were excised at the base of the stem and pretreated in aspirin (2 mM) for 30 min immediately prior to wounding or elicitor treatment. Plant treatments and incubations were as described in Fig. 1 except wounding was performed on excised plants. Control plants were either pretreated in water for 30 min, prior to wounding or elicitor treatment, or maintained in water throughout the incubation period. Leaf material was harvested either at 4 hours and *pin 2* transcript accumulation determined by Northern blot analysis (**A**), or at 1 hour for ethylene quantitation (**B**) as described in Fig. 1.

with its action as a competitive inhibitor. These data suggest that the hormone is required for the wound response. Wound-induced *pin* transcript accumulation is also delayed in the *Never-ripe* (NR) mutant of tomato. The NR mutation results in only a partial loss of ethylene sensitivity (*19*). As further confirmation, we used a reverse genetic approach to block ethylene biosynthesis. On wounding transgenic tomato plants expressing an ACO gene in antisense orientation (*20*), no *pin* transcripts could be detected, establishing an ethylene requirement for *pin* gene expression.

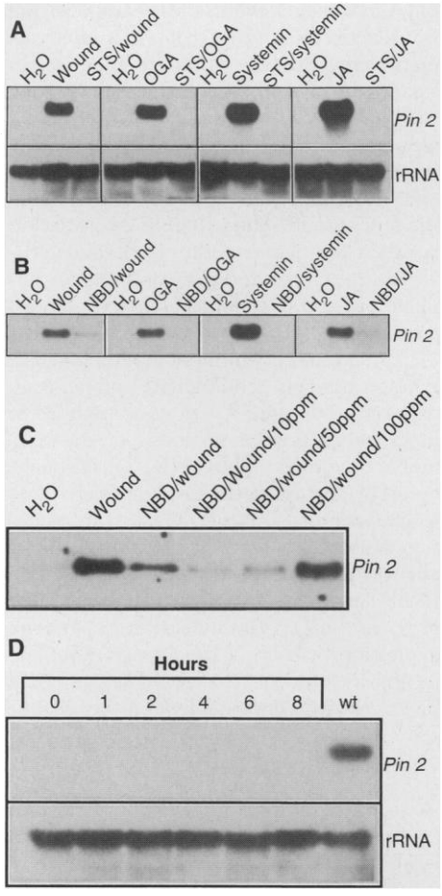

Fig. 3. The effect of inhibiting ethylene action and ethylene biosynthesis on wound- and elicitor-induced *pin 2* gene expression. Ethylene action was blocked by pretreating plants with silver thiosulphate (5 mM) (**A**) and 2,5,-Norbornadiene (2.5 ml/liter) (**B**). STS pretreatment was performed as described in Fig. 2 for aspirin pretreatments. For NBD, plants were allowed to equilibrate in gas-tight chambers containing either air or NBD for 1 hour. Wounding and elicitor treatments were as described in Fig. 2, except in the NBD pretreatments when incubations were performed in gas-tight chambers. Control plants were incubated in water within a NBD atmosphere. (**C**) Wound-induced *pin 2* gene expression can be restored in NBD pretreated plants if ethylene (100 ppm) is present for the first 30 min following wounding. The effect of inhibiting ethylene biosynthesis on *pin 2* gene expression was investigated in tomato plants, (*Lycopersicon esculentum* Mill cv Ailsa Craig), expressing an ACO construct in antisense orientation and driven by the 35*S* CaMV promoter. (**D**) Steady-state levels of *pin 2* transcripts in wounded transformed plants over an 8-hour time course were compared with those in wild-type plants 8 hours after wounding. Total RNA extraction and Northern analysis was performed as described in Fig. 1 using a ^{32}P-labeled *pin 2* probe (*29*).

These results indicate a causal role for ethylene in the regulation of *pin* genes and contrast with earlier conclusions drawn from the lack of effect of ethylene on gassing tomato plants (*13*). The current model for up-regulation of *pin* genes by injury places JA at a common downstream step linking the effects of wounding, OGAs and systemin (*9*). Other factors that affect *pin* gene expression, such as abscisic acid levels in the plant (*21*), and the proteinase-sensitive step inhibited by bestatin (*22*) are thought also to be located on this pathway. JA levels increase following wounding (*7*) and systemin treatment (*15*), and the importance of JA to wound-responsive gene expression has been confirmed by known inhibitors of the octadecanoid pathway (*7*, *22*), and analyses of *Arabidopsis* plants transformed with a chloroplast-targeted lipoxygenase involved in JA biosynthesis (*23*). We reasoned that production of ethylene in the wound response would always occur in conditions of elevated JA, and that if both signals were required simultaneously to trigger *pin* gene expression, direct gassing of plants with ethylene would have no effect. Because ASA blocks ethylene production and at least one site of ASA action is on the pathway leading to elevated JA (*8*), we attempted to rescue ASA pretreated plants with ethylene, JA, and combinations of the two (Fig. 4). Neither ethylene nor JA alone could rescue *pin* gene expression. Rather, a combination of the two hormones is required. The level of expression of *pin* genes in the rescue experiment is much lower than that observed, for example, in the JA control. This may reflect additional consequences of ASA beyond its inhibition of JA and ethylene biosynthesis, such as nonspecific inhibition of kinases and inhibition of any nonheme Fe^{2+}-containing proteins (*24*).

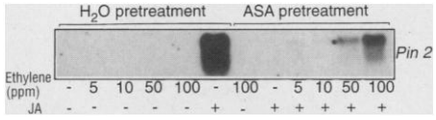

Fig. 4. The expression of *pin* genes in aspirin pretreated plants can be rescued by JA and ethylene. Plants were pretreated in water or ASA for 30 min. Water pretreated plants were treated with ethylene or JA for 30 min in gas-tight chambers before removal of the plants and incubation in the open. ASA pretreated plants were treated with either ethylene, JA, or a combination of ethylene and JA for 30 min in gas-tight chambers before transfer to the open. Control plants were treated with water for the experimental duration. Leaf material was harvested at 4-hour posttreatment, total RNA extracted, and Northern analysis performed using a ^{32}P-labeled *pin 2* probe (*29*).

Exogenous JA induces ethylene, and application of JA is ineffective in the induction of *pin* gene expression in the presence of ethylene action inhibitors, suggesting that ethylene is downstream of JA in the wound transduction pathway. To investigate the events upstream of JA and whether these could also involve ethylene, we measured changes in JA levels in wounded plants. [Leaf material was harvested 30 min post-wounding and used for quantification of JA (*25*). For each determination, the lower leaf from six plants, wild-type or ACO antisense (*20*), was wounded as described in Fig. 1; plant pretreatments using 2 mM ASA, 5 mM STS, or 2.5 ml/liter NBD, were performed as described in Figs. 2 and 3.] In wild-type plants, JA levels were 9499 ± 1796 pmol/g FW at 30 min after wounding. Pretreatment with ASA held JA to 483 ± 263, which was not significantly different ($P > 0.2$) from that of the healthy plant (JA: 252 ± 29 pmol/g FW). In contrast, modifying ethylene action or synthesis reduced JA levels in the wounded plants to values that were not significantly different from one another [silver versus NBD ($P > 0.2$); silver versus wounded ACO antisense ($P > 0.2$); NBD versus wounded ACO antisense ($P > 0.1$)] but were significantly above those in the healthy plant ($P < 0.001$) and below those in wounded wild-type plants ($P < 0.001$). JA levels after pretreatment with silver were 2203 ± 219 pmol/g FW, after NBD, 2319 ± 146 pmol/g FW, and in wounded ACO antisense plants, 1645 ± 191 pmol/g FW.

These data suggest that at least one site of ethylene action in the wound response is the regulation of JA levels in the plant. Whereas ASA pretreatment abolishes any rise in JA, presumably through its multiple inhibitory effects, the specific inhibition of ethylene synthesis or action only reduces the overall level to some 20 to 30% of that found in wild type. This suggests that two processes contribute to the wound-induced increase in JA, only one of which is ethylene-dependent. The data suggest a working

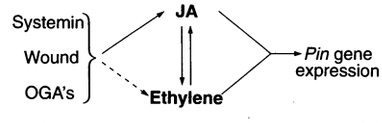

Fig. 5. Model for the function of ethylene and JA in the wound response.

model of the wound signal transduction pathway, in which JA and ethylene are both required for *pin* gene expression (Fig. 5). On wounding, ethylene regulates endogenous JA levels, and application of exogenous JA induces ethylene biosynthesis, which is required to induce a positive effect. As yet we cannot discriminate between parallel events in which wounding induces a small rise in JA, together with a rise in ethylene which triggers an additional rise in JA, and sequential events in which the wound-induced small increase in JA causes ethylene synthesis and its action in turn further amplifies the JA signal.

Jasmonates are much discussed currently for their importance as wound, abiotic stress, and developmental signals (*26*). At least during the wound response, ethylene and JA influence each other's levels in the plant and together act to regulate *pin* gene expression. It will be interesting to determine how many other effects of JA and related fatty acids require ethylene action.

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Immunologic NO Synthase: Elevation in Severe AIDS Dementia and Induction by HIV-1 gp41

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Indirect mechanisms are implicated in the pathogenesis of the dementia associated with human immunodeficiency virus–type 1 (HIV-1) infection. Proinflammatory molecules such as tumor necrosis factor α and eicosanoids are elevated in the central nervous system of patients with HIV-1–related dementia. Nitric oxide (NO) is a potential mediator of neuronal injury, because cytokines may activate the immunologic (type II) isoform of NO synthase (iNOS). The levels of iNOS in severe HIV-1–associated dementia coincided with increased expression of the HIV-1 coat protein gp41. Furthermore, gp41 induced iNOS in primary cultures of mixed rat neuronal and glial cells and killed neurons through a NO-dependent mechanism. Thus, gp41-induced NO formation may contribute to the severe cognitive dysfunction associated with HIV-1 infection.

Neurocognitive deficits are common in HIV-1 infection. Twenty to 30% of patients with acquired immunodeficiency syndrome (AIDS) develop dementia during the course of their illness (*1*). HIV-1 frequently enters the central nervous system (CNS) early in the course of infection and replicates particularly in cells of macrophage origin, including microglia and perivascular macrophages (*2*, *3*). In human brain tissue, HIV-1 has occasionally been detected in astrocytes but rarely if ever in neurons (*2*–*4*). Despite the lack of productive HIV-1 infection in neurons, there is modest neuronal loss in the cortex as well as synaptic loss and dendritic simplification (*5*). The pathological changes of myelin pallor and breakdown of the blood-brain barrier are associated with HIV-1 dementia (*5*, *6*). However, the degree of neuropathologic change may not parallel the severity of neurological symptoms (*6*–*8*), and thus indirect mechanisms are most likely to be involved in the pathogenesis of AIDS (*9*).

Recent studies suggest a possible association between HIV-1 infection of macrophages and the severity of dementia (*2*, *10*). The HIV-1 coat protein gp120, which is shed by the virus, could contribute to neuronal cell death by excitotoxic mechanisms through activation of the *N*-methyl-D-as-

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