Late Administration of a Lipophilic Tyrosine Kinase Inhibitor Prevents Lipopolysaccharide and *Escherichia coli*–Induced Lethal Toxicity

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Septic shock induced by gram-negative bacteria results primarily from excessive stimulation by lipopolysaccharide (LPS) of macrophages to produce tumor necrosis factor (TNF)-α and interleukin (IL)-1. The cellular effects of LPS, TNF-α, and IL-1 are mediated via tyrosine phosphorylation pathways. A recent report indicated that selective inhibitors of tyrosine kinases, tyrphostins of the AG126 family, protect mice against LPS-induced lethal toxicity in mice. Protection was most effective when the tyrphostin was injected before the LPS. In the present study, tyrphostin AG556, which is more lipophilic than those of the AG126 family, was effective in preventing LPS-induced lethal toxicity when administered 2 h after LPS. AG556 also prevented viable *Escherichia coli*–induced lethal toxicity when given 2 h before and, to a lesser extent, 2 h after the bacterial inoculation. AG556 may block a critical step downstream of the signaling pathway induced by LPS after TNF-α production.

Sepsis and septic shock are heterogeneous clinical syndromes that can be triggered by a variety of microorganisms [1]. Septic shock results from systemic infections that cause hypotension and multiorgan dysfunction. The mortality rate from septic shock is high (25%–75%), and ~100,000 persons die annually of this syndrome in the United States [2]. Septic shock induced by gram-negative bacteria results primarily from excessive stimulation of macrophages by lipopolysaccharide (LPS) that resides on the outer membrane of these bacteria [3]. LPS activates macrophages to produce tumor necrosis factor (TNF)-α and interleukin (IL)-1. These cytokines cause many of the symptoms of septic shock [4–6]. In addition, LPS has a direct effect on endothelial cells and granulocytes, leading to an increase in their adhesive properties [7, 8]. Interaction of granulocytes with endothelial cells facilitates capillary damage, leading to vascular leak syndrome [9, 10]. Acute respiratory distress is one of the severe manifestations of this syndrome and frequently occurs in septic shock [11, 12].

The cellular effects of LPS [13–15], TNF-α [16, 17], and IL-1 [17, 18] are mediated via protein tyrosine phosphorylation pathways. Thus, interception of this pathway by inhibitors of protein tyrosine kinases (PTKs) may provide a double block of the effect of LPS and of its induced cytokines.

Tyrphostins are benzylidene malononitrile derivatives that specifically inhibit PTKs. Most tyrphostins are 100- to 10^4-fold more potent in inhibiting PTKs than protein kinase A, protein kinase C, or Ca^{++}/calmodulin-dependent kinases. Their structures were designed to interfere with the substrate binding site of tyrosine kinases, although some also interfere with the ATP binding site. Tyrphostins exhibit selectivity in their ability to inhibit different PTKs, such as those associated with the EGF receptor, HER2-Neu, the PDGF receptor, and $p210^{Ber\text{-}Abl}$. They inhibit distinct biologic responses, such as proliferative, repair, and inflammatory processes [19–21].

We recently reported [22] that PTK inhibitors of the tyrphostin AG126 family protect mice against LPS-induced lethal toxicity. The protection correlates with the ability of these agents to block LPS-induced production of TNF-α in vitro and in vivo as well as inhibition of TNF-α–induced cytotoxicity in vitro and lethal toxicity in vivo.

AG126 offered almost complete protection against LPS-induced lethality only when injected 2 h before LPS. Simultaneous administration of AG126 and LPS offered partial protection [22], and administration of AG126 2 h after LPS had essentially no protective effect. Here we describe the protective effect of a lipophilic tyrphostin, AG556, against LPS and *E. coli*–induced lethal toxicity when administered long after LPS or viable *E. coli* infection.

Materials and Methods

Materials. Tyrphostins were synthesized as described [19]. Stock solutions (50–150 m*M*) were made in dimethyl sulfoxide (DMSO). Dilutions were made in PBS. LPS from *E. coli* O55:B5 prepared using phenol extraction was obtained from Sigma (St. Louis). Recombinant human TNF-α (5×10^6 U/mg) was obtained from Reprotech (Rocky Hill, NJ). *E. coli* O55:B5 was obtained from the laboratory of the Ministry of Health (Jerusalem). Female

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The experiments were evaluated and approved by the Animal Care and Use Committee, Beilinson Medical Center.

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CD1 mice (ages, 6–8 weeks) for in vivo experiments were obtained from Harlan (Jerusalem).

Tyrphostin protection against TNF-α cytotoxicity in vitro. A TNF-α–sensitive cell line, mouse A9 fibroblasts, was plated in 96-well flat-bottom microtiter plates at 30,000 cells/0.1 mL to establish a dense monolayer. After incubation for 24 h at 37°C in a humidified 5% CO_2 atmosphere, cycloheximide was added to a final concentration of 50 μg/mL. Tyrphostins at different concentrations were added before or after addition of TNF-α at different concentrations. After incubation for an additional 18 h, the supernatants were carefully aspirated, the monolayer was washed twice with PBS, and 200 μL of neutral red solution (0.02%) was added. After incubation for 2 h, cells were washed and the dye that had been absorbed by the live cells was extracted upon addition of 200 μL of Sorenson buffer containing 50% ethanol. The dye concentration was determined in an ELISA autoreader using a 550-nm filter.

Assay of PTK hck. The procedure used was similar to that described for the assay of human $p58^{hck}$ [23], except that anti-hck (N-30) antibody was used for immunoprecipitation of hck, followed by the addition of protein A agarose. In brief, resident peritoneal macrophages were isolated from C57BL mice by adherence of cells from the PBS-peritoneal wash to plastic wells (24 macrowell plates). Macrophages (10^6/mL of RPMI 1640 containing 10% fetal calf serum) were washed with PBS and lysed using lysis buffer. After centrifugation, the lysates were treated with anti-hck antibody (N-10; Santa Cruz Biotechnology, Santa Cruz, CA), followed by the addition of protein A agarose. The immunoprecipitates were incubated 5 min in the presence of [^{32}P]ATP-γ and enolase as a substrate. After incubation was terminated by boiling, electrophoresis was done on a polyacrylamide gel (10%) followed by autoradiography.

Administration of tyrphostins. In all experiments in which tyrphostins were omitted, PBS containing DMSO at the appropriate concentrations was administered.

Controls were studied simultaneously with the tyrphostin-treated animals. The experiments were done unblinded. All animals were analyzed, and survival was an end point.

Statistical analysis. Results of all in vitro experiments are expressed as mean ± SE. Differences between means were determined using Student's test. The survival of different groups of mice was analyzed by χ^2 and Fisher's exact probability tests, depending on the sample size. Statistical significance was inferred for comparison in which $P < .05$.

Results

We searched for tyrphostins that would be effective against LPS toxicity when administered long after LPS. Tyrphostins were screened for their ability to inhibit TNF-α–induced cytotoxicity in vitro and to prevent LPS-induced lethal toxicity in mice long after administration of either TNF-α or LPS. Tyrphostin AG556 (figure 1) fulfilled these requirements. This tyrphostin contains an aliphatic chain terminated in a benzyl moiety, rendering it highly lipophilic, more so than tyrphostins of the AG126 family.

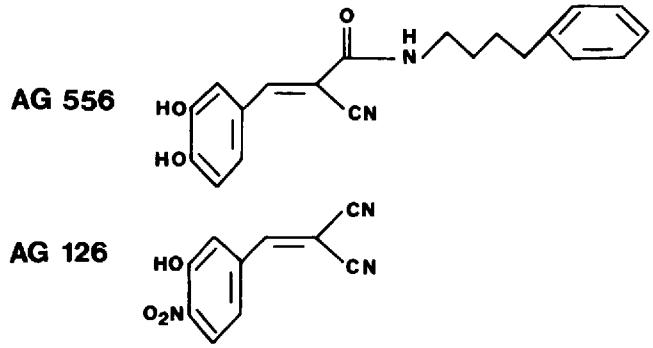

Figure 1. Tyrphostins AG556 and AG126.

Effects of AG556 on TNF-α–induced cytotoxicity in vitro. Recombinant human TNF-α at different concentrations was added to the mouse fibroblastic cell line (A9) and cultured in the presence of cycloheximide. AG556 was added at different concentrations 1 h before the addition of TNF-α. After incubation for 18 h, the viability of cells was tested by dye exclusion, using neutral red. As seen in figure 2, AG556 (in a dose-dependent manner) was effective in preventing TNF-α–induced cytotoxicity. We then studied the protective effect of AG556 against TNF-α toxicity when it was added at different times after TNF-α. As shown in figure 3, AG556 was effective in preventing TNF-α–induced cytotoxicity as late as 4 h after TNF addition. Its protective effect, however, slowly declined with late addition. In contrast with AG556, tyrphostin AG126 was much less effective with late addition (figure 3).

Effect of AG556 on LPS-induced lethal toxicity in vivo. Figure 4 shows the effect of different doses of AG556 on LPS-induced lethal toxicity in mice. A dose of 20 μg/mouse reduced mortality to ~50%, whereas a dose of 100 μg/mouse prevented death. In this experiment AG556 was given 2 h before LPS.

A striking difference between AG126 and AG556 is the ability of the latter to prevent LPS-induced lethal toxicity upon late administration. AG556 (200 μg/mouse) was essentially equally effective ($P > .05$) in preventing LPS-induced lethality when given 2 h before or after LPS (figure 5). Under similar experimental conditions, injection of AG126 2 h after LPS failed to protect the mice against LPS-induced lethal toxicity (figure 6).

AG556 was equally effective ($P > .05$) in prevention of LPS-induced lethality when administered intraperitoneally (ip) or intravenously (data not shown).

The LD_{50} of control mice given the same dose of LPS varied in different experiments (e.g., figures 4, 5). The reason for the differences is not known but may have resulted from uncontrolled variations in growth and maintenance conditions that affected the tolerance of the mice to LPS.

Effect of AG556 on E. coli–induced lethality. AG556 was tested for its ability to protect mice against lethality induced by ip injection of viable *E. coli* O55:B5 (figure 7).

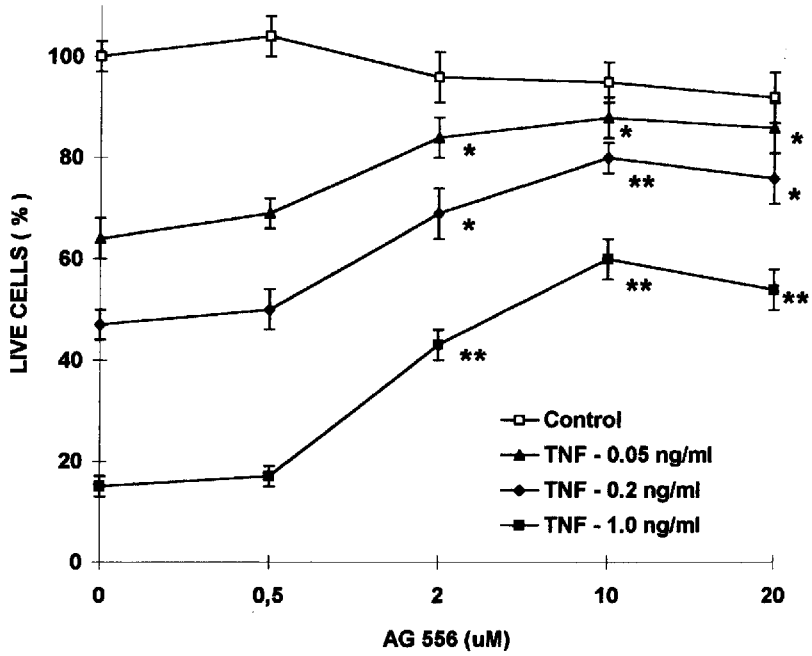

Figure 2. Protective effect of tyrphostin AG556 on tumor necrosis factor (TNF)-α–induced cytotoxicity in vitro. Murine fibroblastic A9 cells were incubated with TNF-α at various concentrations. AG556 at different concentrations was added 1 h before TNF-α. After 18 h, cell viability was determined (see Materials and Methods). Data are mean ± SE of 9 cultures from 3 different experiments (each done in triplicate). Statistically significant differences between tyrphostin-treated and untreated cultures: * $P < .01$, ** $P < .001$.

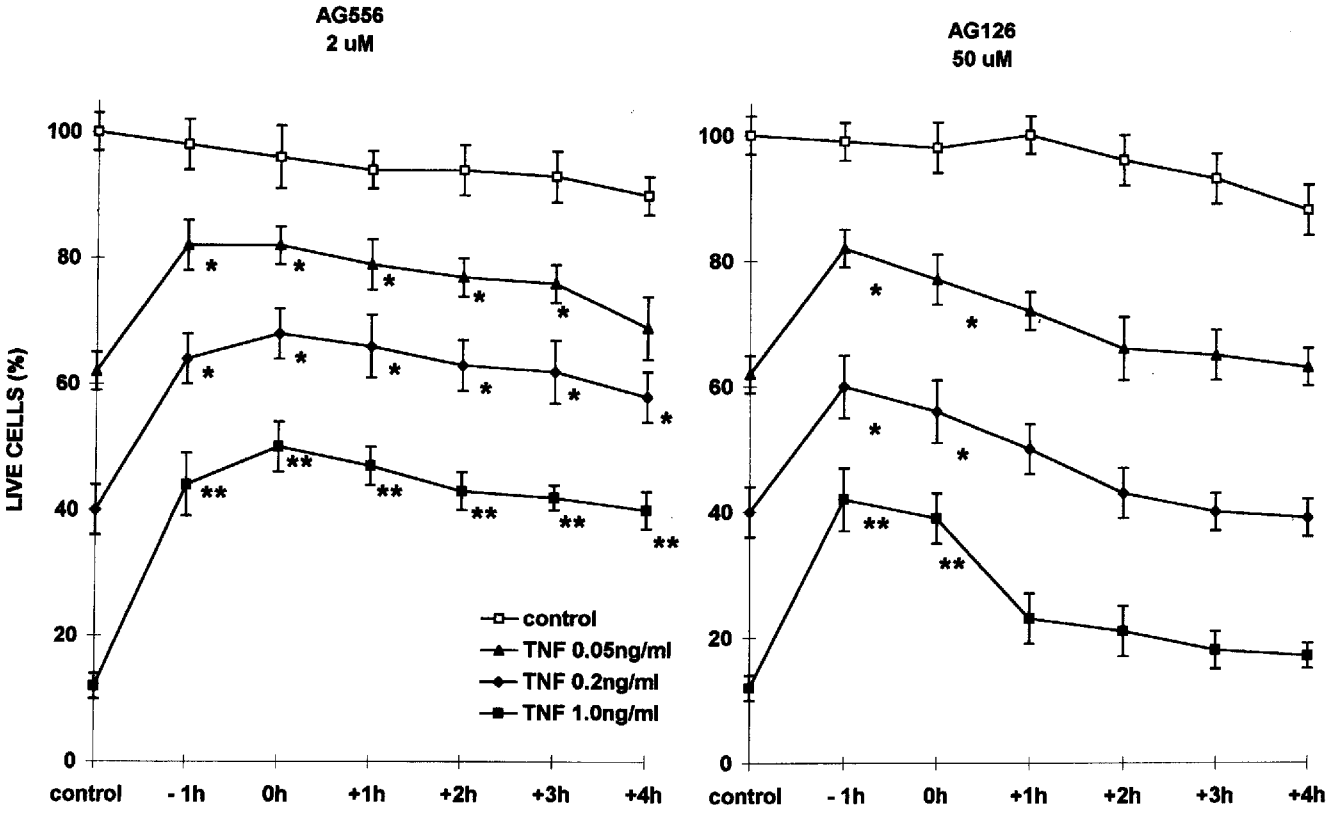

Figure 3. Effect of addition of tyrphostins AG556 and AG126 by time before or after addition of tumor necrosis factor (TNF)-α on TNF-α–induced cytotoxicity in vitro. Experimental design is similar to that described for figure 2. "Control" at origin of abscissa, no tyrphostin added. Statistically significant differences between tyrphostin-treated and untreated cultures at different times: * $P < .01$, ** $P < .001$.

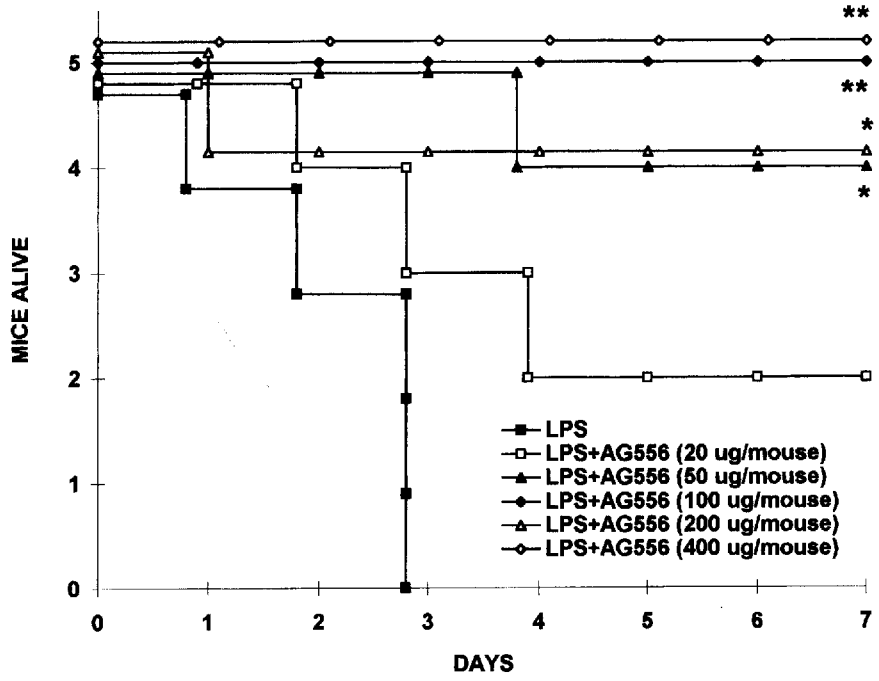

Figure 4. Effect of different doses of tyrphostin AG556 on LPS-induced lethal toxicity. Mice were injected intraperitoneally (ip) with AG556 2 h before ip injection of LPS (1.5 mg/mouse). Controls were injected with PBS containing dimethyl sulfoxide at concentration used for injection of highest dose of AG556 (see Materials and Methods). Mouse mortality was monitored twice daily. Statistically significant differences between mice injected with LPS and AG556 and LPS alone: * $P < .05$, ** $P < .02$.

Injection ip of 2.5 x 10^9 viable *E. coli* resulted in deaths of 23 (92%) of 25 mice after 2 days. Administration of AG556 (200 μg/mouse) 2 h before *E. coli* was given resulted in deaths of 8 (32%) of 25 mice, whereas administration of AG556 2 h after *E. coli* injection resulted in deaths of 17 (68%) of 35 mice.

The mice were not treated with antibiotics. Neither AG556 nor AG126 at a concentration of 50 μM inhibited the growth of *E. coli* O55:B5 in agar (disk assay) or broth.

Effect of AG556 on activation of PTK hck. A previous report indicated that LPS rapidly activated the PTKs $p56^{lyn}$ and $p58^{lck}$ in human peripheral blood monocytes [23]. We have found that LPS rapidly activated the PTK hck in mouse peritoneal macrophages. As shown in figure 8, activation was manifested by an increase in phosphorylation of an exogenous substrate, enolase (50 kDa), by the immunoprecipitated hck, and by increased autophosphorylation of two molecular forms of the enzyme ($p55^{hck}$ and $p60^{hck}$). When the tyrphostins AG556

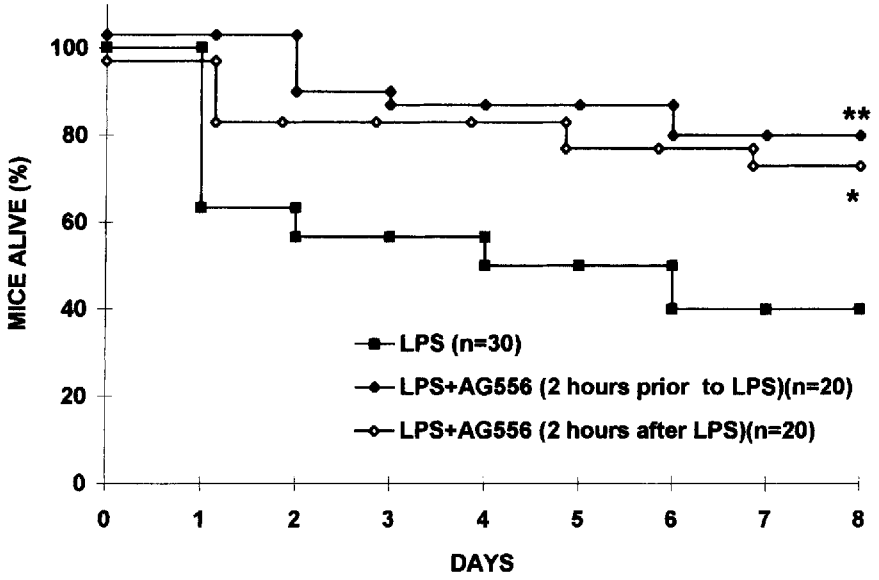

Figure 5. Effect of tyrphostin AG556 injected before or after LPS on LPS-induced lethal toxicity. LPS was injected intraperitoneally (ip) at 1.5 mg/mouse. AG556 was injected ip (200 μg/mouse) 2 h before or after LPS injection. Controls were injected with LPS–dimethyl sulfoxide. Statistically significant differences between mice injected with LPS and AG556 and LPS alone: * $P < .05$, ** $P < .02$.

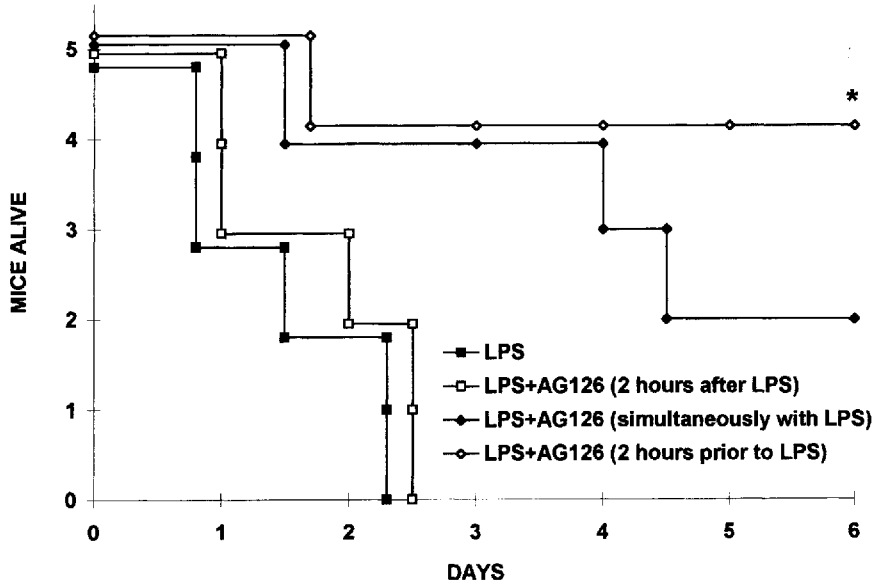

Figure 6. Effect of tyrphostin AG126 injected before or after LPS on LPS-induced lethal toxicity. AG126 (400 μg/mouse) was injected intraperitoneally 2 h before LPS (1.5 mg/mouse), simultaneously with LPS, or 2 h after LPS. Control mice were injected with LPS and PBS–dimethyl sulfoxide. Statistically significant differences between mice injected with LPS and AG126 and LPS alone: * $P < .05$.

or AG126, at a final concentration of 50 μM, were added to the macrophage monolayer before addition of LPS, cell activation was inhibited as assessed by the parameters outlined above.

Discussion

LPS-induced lethal toxicity is a multifactorial phenomenon. LPS can interact directly with target cells and also induce the synthesis of a variety of cytokines that interact with different target cells. The direct effect of LPS as well as the effect of its induced cytokines are mediated by receptor-mediated signal transduction, which is associated with protein tyrosine phosphorylation. We recently reported [22] that blockage of the tyrosine phosphorylation pathway by selective inhibitors of PTKs, tyrphostins, prevented LPS-induced lethal toxicity. Protection against LPS lethality was almost complete upon injection of AG126 2 h before LPS. However, administration of AG126 2 h after LPS injection failed to offer protection [22] (figure 6). Glucocorticoids have been shown to inhibit LPS-induced TNF-α production in mice only when administered before or at the time of LPS challenge and not 20 min later [24]. The protective effect of chlorpromazine on LPS-induced lethal toxicity was observed 30 min before, but not 30 min after, LPS administration [24].

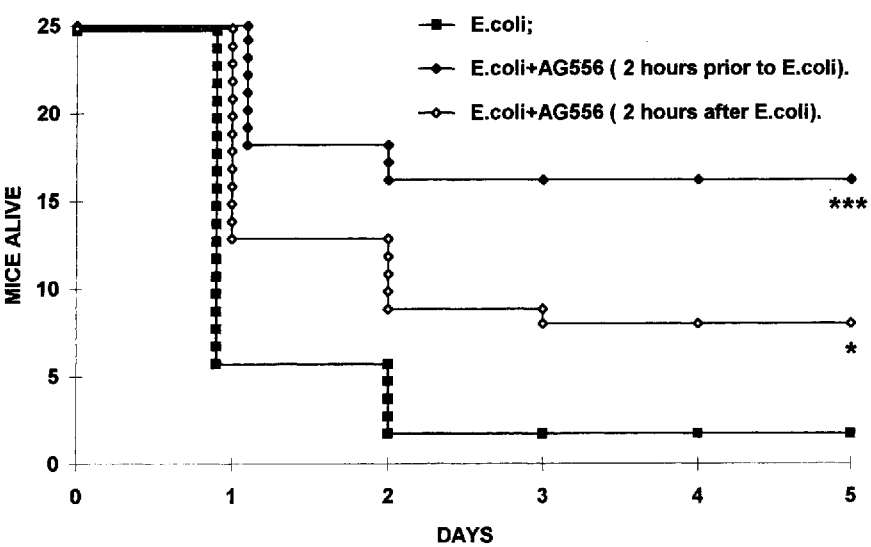

Figure 7. Effect of tyrphostin AG556 on viable *E. coli*–induced lethal toxicity. AG556 (200 μg/mouse) was injected intraperitoneally (ip) 2 h before or 2 h after ip injection of viable *E. coli* (2.5×10^9/mouse). Control animals were injected with *E. coli* and PBS–dimethyl sulfoxide. Statistically significant differences between mice injected with *E. coli* and AG556 and *E. coli* alone: * $P < .05$, *** $P < .01$.

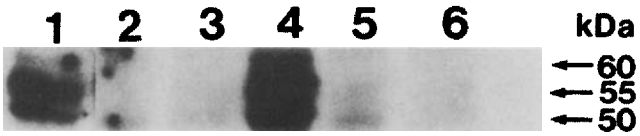

Figure 8. LPS-induced activation of protein tyrosine kinase hck in mouse peritoneal macrophages and prevention by tyrphostins AG126 and AG556. Resident mouse peritoneal macrophages were incubated with AG126 or AG556 (final concentration, 50 μM) for 45 min before addition of LPS (1 μg/mL). After 15 min, cells were washed and lysed, and hck activity and autophosphorylation were measured. Cells by lane: 1, untreated; 2, untreated preincubated with AG126; 3, untreated preincubated with AG556; 4, LPS-treated; 5, LPS-treated preincubated with AG126; 6, LPS-treated preincubated with AG556.

Of more clinical relevance would be the use of tyrphostins that offer protection late after LPS administration. Lipophilic tyrphostin AG556 was equally effective in preventing LPS-induced lethal toxicity in mice when administered either 2 h before or 2 h after LPS. It should be noted that under similar experimental conditions, the dose of AG126 that confers full protection against LPS-induced lethal toxicity is much higher [22]. In addition, tyrphostin AG556 was also effective in protecting TNF-α–induced cytotoxicity in vitro long after addition of TNF (figure 3).

Remick et al. [24] reported that peritoneal cells had peak TNF-α mRNA 30 min after LPS injection, and Gadina et al. [25] found that the in vivo production of TNF-α induced by LPS peaks ~1 h after LPS administration. Thus, it is suggested that the lipophilic tyrphostin AG556 blocks a critical late step after TNF-α production. This notion is further supported by the finding that activation of the tyrosine kinase hck by LPS, an early step in the stimulatory cascade, is similarly inhibited by both AG556 and AG126.

AG556 was also effective in preventing *E. coli*–induced lethality in mice, which is the first demonstration of a protective effect of a tyrosine kinase blocker against sepsis lethality. Administration of AG556 2 h after *E. coli* inoculation was also protective, although less so than when given 2 h before.

The functional properties of the lipophilic tyrphostin AG556 that render it effective late after LPS administration or *E. coli* infection may result from its intracellular distribution. The possibility that it has a selective inhibitory effect on PTK(s) downstream of the signaling pathway should also be considered.

Several investigations have provided evidence that effects of TNF-α are also mediated via activation of the sphingomyelin cycle of signal transduction [26]. Ceramide, the product of this pathway, acts as a second messenger stimulating a ceramide-activated serine/threonine protein kinase [27]. It is not known whether there is "cross talk" between the protein tyrosine phosphorylation pathway and the sphingomyelin cycle of signal transduction. It is possible that some TNF-α effects are protein tyrosine phosphorylation–independent and cannot be completely blocked by protein kinase inhibitors.

At the clinical level, several studies have demonstrated elevated TNF-α levels in serum of septic patients, and some investigators have reported a correlation with the clinical outcome in meningitis and septicemia [28, 29]. These and other investigations have clearly elucidated the major role of TNF-α in the deleterious pathophysiology of sepsis. On the other hand, the effect of TNF-α in infections diseases and sepsis is not necessarily solely detrimental, as TNF-α also mediates antiinflammatory responses that may be protective for the host. In murine models of bacterial peritonitis, TNF-α neutralization has divergent activity [30] or harmful effects [31]. Thus, TNF-α plays a dual role in the pathophysiology of infectious diseases [32] and may be why large-scale clinical trials investigating new treatments directed against the primary endogenous mediators in sepsis have so far been unable to demonstrate a statistically significant effect, using broad entry criteria [33, 34]. Most unsuccessful clinical anticytokine treatments were directed at a single agent. In contrast, blockage of tyrosine phosphorylation by tyrphostins affects the generation and activity of a variety of effector molecules.

As mentioned above, LPS, TNF-α, and IL-1 effects may be mediated by protein tyrosine phosphorylation–dependent and –independent pathways, such as the sphingomyelin cycle of signal transduction. The partial blockage by tyrphostins may be beneficial since it enables the residual cytokines to exert their protective antiinflammatory effects.

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