

Lipopolysaccharide-Induced Lethality and Cytokine Production in Aged Mice

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Received 21 March 1995/Returned for modification 29 May 1995/Accepted 13 December 1995

This study was designed to define the lipopolysaccharide (LPS) sensitivity of aged mice in terms of lethality and cytokine production and to determine down-regulating responses of corticosterone and interleukin 10 (IL-10). The 50% lethal doses of LPS in young (6- to 7-week-old) and aged (98- to 102-week-old) mice were 601 and 93 μg per mouse (25.6 and 1.6 mg per kg of body weight), respectively. Aged mice were approximately 6.5-fold more sensitive to the lethal toxicity of LPS in micrograms per mouse (16-fold more sensitive in milligrams per kilogram) than young mice. Levels in sera of tumor necrosis factor- α (TNF- α), IL-1 α , and IL-6 after intraperitoneal injection of 100 μg of LPS peaked at 1.5, 3, and 3 h, respectively, and declined thereafter in both groups of mice. However, the peak values of these cytokines were significantly higher in aged than in young mice ($P < 0.05$). Gamma interferon (IFN- γ) was detectable at 3 h, and sustained high levels were still detected after 12 h in both age groups. Although there were no significant differences in levels of IFN- γ in sera from both groups, aged mice showed higher IFN- γ levels throughout the 3- to 12-h study period. Administration of increasing doses of LPS revealed that aged mice had a lower threshold to IL-1 α production than young mice. In addition, aged mice were approximately 4-fold more sensitive to the lethal toxicity of exogenous TNF in units per mouse (10-fold more sensitive in units per kilogram) than young mice. With regard to down-regulating factors, corticosterone amounts were similar at basal levels and no differences in kinetics after the LPS challenge were observed, whereas IL-10 levels in sera were significantly higher in aged mice at 1.5 and 3 h than in young mice ($P < 0.01$). These results indicate that aged mice are more sensitive to the lethal toxicities of LPS and TNF than young mice. We conclude that a relatively activated, or primed, state for LPS-induced cytokine production, in spite of full down-regulating responses by corticosterone and IL-10, may explain at least in part LPS sensitivity in aged mice.

As with many other infections, the incidence of bacterial sepsis increases with age, and mortality due to sepsis remains high, particularly for the elderly (37, 45). It has been reported that gram-negative organisms account for 60% of septic isolates from elderly patients, and septic shock is a life-threatening complication in these individuals (37). Lipopolysaccharide (LPS) is an integral component of the outer membrane of gram-negative bacteria and a major contributing factor in the initiation of a generalized inflammatory process, termed endotoxin shock. This ominous state is principally a macrophage/monocyte-mediated event and is attributable to the excessive production of several cytokines in response to LPS, rather than to LPS toxicity itself. LPS stimulates immune cells to generate proinflammatory cytokines, such as tumor necrosis factor (TNF), interleukin 1 (IL-1), IL-6, and gamma interferon (IFN- γ) (9). The production of these cytokines is an essential event in the development of endotoxin shock, since administration of TNF can induce IL-1 and IL-6, which act synergistically to produce a state of shock and possibly death (9). Furthermore, specific neutralizing antibodies to murine TNF (6, 15) or IFN- γ (15, 24) protect mice against the lethality of LPS.

The underlying mechanism of the vulnerability to infections of the elderly has been ascribed to the breakdown of anatomic barriers, underlying illnesses, or the declining capacity of the immune system (22). Although there is some disagreement on the exact segment of the immune system affected and the degree of involvement, the consensus is that T cells are more

severely affected during the aging process than are B cells and macrophages (47, 49). An age-dependent decrease in IL-2 production in T cells (50) and an increased susceptibility to T-cell-dependent pathogens, such as *Mycobacterium tuberculosis* (39) and *Listeria monocytogenes* (41), in senescent animals have been well documented. In contrast, the LPS sensitivity of aged animals, such as LPS-induced lethality and cytokine production, is not yet fully understood.

The synthesis of cytokines is finely regulated through several processes. Glucocorticoid hormones are the central mediators for the down-regulation of cytokines (5, 7, 33, 42). Bertini et al. (2) have demonstrated that adrenalectomized animals became extremely susceptible to the lethal effect of LPS, suggesting that endogenous glucocorticoid hormones play a critical role in regulating sensitivity to LPS. Since aging is associated with many endocrine alterations, it seems possible that some of these changes will have an impact on cytokine producibility and immune performance. Recently, IL-10 was identified and characterized by its immunoregulatory and cytokine synthesis inhibitory activity (36). Pretreatment with this unique cytokine decreases TNF production in LPS-challenged mice and protects mice from the lethal effect of LPS (23, 27). More recently, an increase in IL-10 production was reported in an in vitro experiment using splenic lymphocytes from aged mice (25).

The purpose of this study was to define the lethal sensitivity of aged mice to LPS and the association of LPS to cytokine production in vivo. Levels of TNF- α , IL-1 α , IL-6, and IFN- γ in sera after the administration of LPS and major down-regulating responses by corticosterone and IL-10 were compared in young and aged mice.

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TABLE 1. LPS sensitivities in young and aged mice

LPS challenge ($\mu\text{g}/\text{mouse}$)	No. of mice (dead/total)	
	Young	Aged
50	NT ^a	0/5
100	NT	3/5
200	0/5	5/5
400	0/5	5/5
600	2/5	NT
800	5/5	NT

^a NT, not tested.

MATERIALS AND METHODS

Animals used. Young (6- to 7-week-old) and aged (98- to 102-week-old) female ICR mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Body weights of young and aged mice were 23.5 ± 0.7 and 58.5 ± 9.3 g, respectively. Mice were housed in groups of five and were allowed food and water ad libitum, and lighting was maintained on a 12-h cycle. All animals were inspected for signs of tumors or infections prior to use in the experiment, and ill-appearing mice were excluded from the study. A postmortem examination that included a careful dissection of the thorax and abdomen was performed on each animal used in the experiment. Data derived from mice with pathologic findings at autopsy, such as tumors in the abdomen and massive ascites containing abnormal cells, were excluded from analysis.

LPS- and TNF-induced lethality. LPS (*Escherichia coli* 055:B5; Difco Laboratories, Detroit, Mich.) was diluted in pyrogen-free saline and given intraperitoneally to young and aged mice. Cumulative mortality was then monitored over the next 4 days. To investigate lethal sensitivity to exogenous TNF, mice were sensitized by intraperitoneal injection of 20 mg of D-galactosamine (Sigma Chemical Co., St. Louis, Mo.) per mouse as described previously (35). Immediately after sensitization, mice were intravenously administered with recombinant human TNF (Dainippon Pharmaceutical Co., Osaka, Japan) and were observed for rates of mortality. In all experiments, LPS and TNF challenges were performed at 10:00 a.m. to exclude any effect of the circadian rhythm of hormone levels on lethal levels of sensitivity.

Levels of cytokines and corticosterone in serum. Young and aged mice were intraperitoneally injected with 100 μg of LPS. Blood samples were collected by cardiac puncture 0, 1.5, 3, 6, and 12 h after the LPS challenge. The blood was

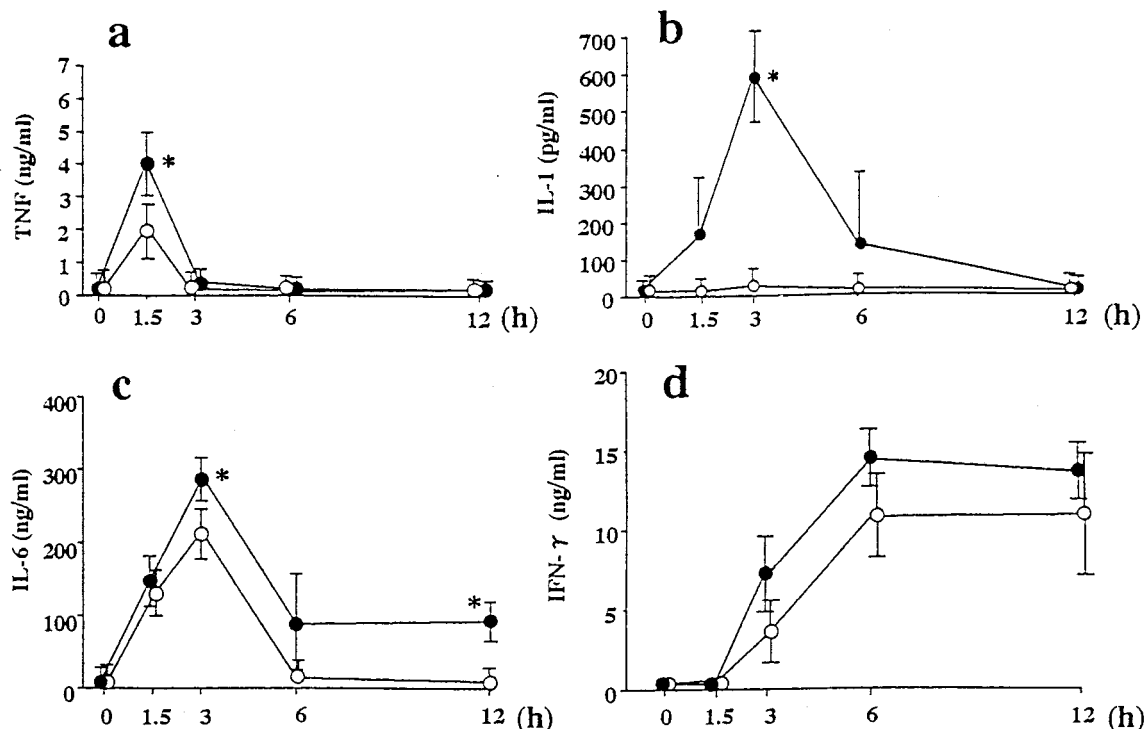
allowed to clot at 4°C for 2 h and centrifuged to obtain serum samples that were stored in aliquots at -80°C before assaying them for cytokines and corticosterone. Levels in sera of TNF- α , IL-1 α , IL-6, IL-10, and IFN- γ were quantified with commercially available enzyme-linked immunosorbent assay kits (TNF- α , Endogen; IL-1 α , Genzyme; IL-6, Endogen; IL-10, Biosource International; IFN- γ , Genzyme). Corticosterone levels in sera were quantitated by radioimmunoassay according to the manufacturer's instructions (ICN Biomedicals Inc., Irvine, Calif.). To determine the threshold of IL-1 α production, young and aged mice were injected intraperitoneally with increasing doses of LPS and levels of IL-1 α in sera at 3 h were determined as described above.

Statistics. Student's *t* test and the chi-square test were used to compare means and survival rates, respectively. A level of 5% was accepted as statistically significant. Fifty percent lethal doses (LD_{50} s) of LPS and TNF were determined by Probit's method.

RESULTS

LPS-induced lethality (Table 1). The results shown in Table 1 reflect the lethality of LPS in young and aged mice challenged with increasing doses of LPS. LD_{50} s of LPS in young and aged mice were calculated to be 601 μg per mouse (95% confidence levels, 419 to 888 μg per mouse) and 93 μg per mouse (95% confidence levels, 47 to 156 μg per mouse) (25.6 and 1.6 mg/kg of body weight), respectively ($P < 0.05$). Aged mice were approximately 6.5-fold more sensitive to the lethal toxicity of LPS in micrograms per mouse (16-fold more sensitive in milligrams per kilogram) than young mice.

Levels of cytokines in sera after LPS challenge (Fig. 1). Levels in serum of TNF- α , IL-1 α , and IL-6 increased after the administration of LPS (100 μg per mouse) and peaked at 1.5, 3, and 3 h, respectively, in both groups of mice. However, the peak level of each cytokine was significantly higher in aged mice than in young mice ($P < 0.05$). In addition, the level of IL-6 in aged mice, which was significantly different from that in young mice, remained detectable at 12 h. On the other hand, IFN- γ levels in aged mice were higher than those of young mice throughout the 3- to 12-h study period, although these differences were not significant.

FIG. 1. Levels in sera of TNF (a), IL-1 (b), IL-6 (c), and IFN- γ (d) after administration of LPS (100 $\mu\text{g}/\text{ml}$) to young (○) and aged mice (●). *, $P < 0.05$.

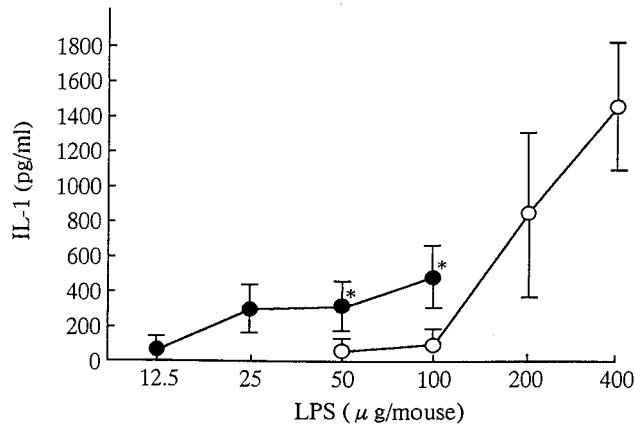


FIG. 2. Levels in sera of IL-1 3 h after the administration of LPS to young (○) and aged mice (●). *, $P < 0.05$, compared with levels found in young mice.

Levels of IL-1 α in sera after administration of increasing doses of LPS (Fig. 2). In preliminary experiments using young, middle-aged, and aged mice, we noticed differences in IL-1 production, especially in the lower stimulation level of LPS (25 and 50 μ g per mouse). Therefore, further experiments were undertaken to compare the thresholds of IL-1 production in young and aged mice. The injection of 25 μ g of LPS induced detectable levels of IL-1 α in sera from aged animals, and levels of IL-1 α elicited by 50 and 100 μ g of LPS in aged mice were significantly higher than those of young mice ($P < 0.05$). Aged mice had a lower threshold of LPS-induced IL-1 α production than young mice. Higher doses of LPS (200 and 400 μ g) induced large amounts of IL-1 α in young mice.

Levels in sera of corticosterone and IL-10 after LPS challenge (Fig. 3). No differences in basal levels of corticosterone in sera were observed between young and aged mice. After the administration of LPS, the levels of corticosterone in sera in both age groups rapidly increased to approximately 700 ng/ml at 1.5 h, and high levels were sustained by 12 h. There were no differences in corticosterone levels between young and aged mice after LPS challenge. Levels of IL-10 in sera increased at 1.5 h after LPS challenge, peaked at 3 h, and then declined in both young and aged mice. However, the levels of IL-10 in aged mice were significantly higher at 1.5 and 3 h than those in young mice ($P < 0.01$).

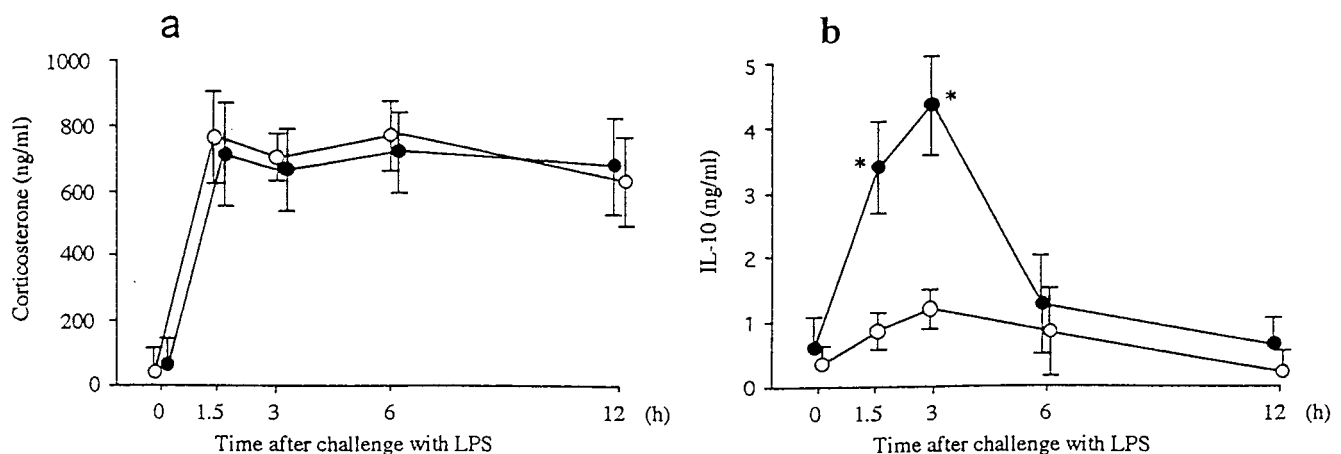


FIG. 3. Levels in sera of corticosterone (a) and IL-10 (b) after the administration of LPS (100 μ g/mouse) to young (○) and aged mice (●). *, $P < 0.01$.

TABLE 2. TNF sensitivities in young and aged mice

TNF challenge (U/mouse)	No. of mice (dead/total)	
	Young	Aged
62.5	0/5	0/5
250	0/5	1/5
1,000	1/5	3/5
4,000	3/5	5/5
16,000	5/5	5/5

TNF-induced lethality in young and aged mice (Table 2). To sensitize mice to recombinant human TNF, mice were intraperitoneally administered 20 mg of D-galactosamine per mouse, as reported previously (35). Since the body weights of aged mice were approximately 2.5-fold those of young mice, it was considered that aged mice were administered a 2.5-fold smaller dose of D-galactosamine (in milligrams per kilogram) than young mice. The LD₅₀s of recombinant human TNF in young and aged mice were calculated to be 2,600 U per mouse (95% confidence levels, 917 to 7,490 U per mouse) and 649 U per mouse (95% confidence levels, 232 to 1,790 U per mouse) (110,638 and 11,094 U/kg, respectively) ($P < 0.05$). Aged mice were approximately 4-fold more sensitive to the lethal toxicity of exogenous TNF in units per mouse (10-fold more sensitive in units per kilogram) than young mice.

DISCUSSION

The major finding of this study is the vulnerability of aged mice to the lethal effect of LPS and TNF. Furthermore, enhanced production of TNF- α , IL-1 α , and IL-6 in response to LPS was observed in these animals. In 1943, Zahl et al. (51) reported for the first time age-related changes in levels of LPS sensitivity; there were no differences in the levels of endotoxin sensitivity in 25-, 50-, and 350-day-old mice, whose absolute amounts of endotoxin (in micrograms per mouse) were compared. Karanfilian et al. (32) have shown that the rate of mortality of 12-month-old mice was greater than that of 3-month-old mice by administering 5 mg of LPS per kg, although LD₅₀s of LPS and LPS-induced cytokines were not determined. Our results clearly indicate that aged mice are hypersensitive to LPS, as evidenced by its level of lethality and the magnitude of the mice's cytokine production.

There have been several conflicting reports of the *in vitro* production of proinflammatory cytokines by macrophages of aged animals; decreases in levels of IL-1 (10, 11, 29), IL-6 (16), and TNF (10, 16) have been reported by some, while increases in levels of IL-1 (19, 43), IL-6 (18, 19), and TNF (19) have been reported by others. Daynes et al. (14) detected the spontaneous production of IL-6 in culture supernatants of lymphoid cells obtained from aged mice but not from mature mice and reported that this was consistently observed in both the spleens and mesenteric lymph nodes of aged mice but not in their peripheral lymph nodes. It has been reported that nonstimulated and complete Freund's adjuvant-elicited macrophages from aged mice produce significantly less IL-1, IL-6, and TNF than those of young mice, whereas levels of production of these cytokines were conversely increased by thioglycolate-elicited macrophages of aged mice (12). On the other hand, with regard to the *in vivo* production of cytokines in aged animals, it has been reported that intraperitoneal challenge with LPS induced significantly greater amounts of TNF in the sera and peritoneal lavage fluids of senescent mice (28) and of TNF and IL-6 in the sera of aged rats (20). We have intraperitoneally administered LPS and have simply observed levels of cytokines in sera and levels of lethality in young and aged mice. Our results are consistent with those results obtained by *in vivo* experiments. *In vitro* production of cytokines may be influenced by several factors, such as the eliciting agents used, culture condition, and source of cells, whereas cytokine levels in serum or peritoneal fluid may be less artificial than those in culture supernatants of macrophages because serum factors and cell-to-cell interactions are left *in situ*. In particular, the LPS-binding protein (46), CD-14 (21), the IL-1 receptor antagonist (1), and/or the soluble TNF receptor (48) have been reported to be important factors affecting LPS sensitivity *in vivo*, and it remains for further investigation to resolve the differences between *in vivo* and *in vitro* cytokine production and LPS sensitivity in aged mice.

The synthesis and activities of cytokines are coordinated by a series of positive- and negative-feedback loops involving multiple-organ systems. The hypothalamic-pituitary-adrenal axis down-regulates cytokine synthesis via endogenous glucocorticoids (7, 42). Moreover, certain cytokines, such as IL-1, IL-6, and TNF, cause a release of adrenocorticotrophic hormone from the pituitary gland to increase corticosterone levels in the blood (3, 4). The importance of feedback circuits between these cytokines and glucocorticoid hormones is well established, because adrenalectomized mice produce high levels of cytokines in response to LPS and become highly sensitive to the lethal effect of LPS (2, 40). Since a disturbance of hormonal homeostasis is the major change accompanying the aging process, it is possible that aged mice may lack a precise down-regulating response of glucocorticoid production, resulting in an excessive production of cytokines and causing an enhanced sensitivity to LPS. For example, it has been reported that levels of dehydroepiandrosterone, which is an abundantly secreted adrenocortical hormone, and its endogenous production decline with advancing age and that this hormone can restore the reduced regulation of IL-6 production in aged mice (14). However, our results indicate no differences in corticosterone levels of young and aged mice following LPS challenge. Glucocorticoid hormones exhibit these cytokine-inhibiting activities by binding specific receptors on target cells and by the subsequent transmission of signals to nuclei. Several investigators have reported age-related changes in glucocorticoid receptor binding and physicochemical properties (30, 31). The lack of a homeostatic control of TNF production in senescent mice was demonstrated in experiments of exogenous glucocor-

ticoids, in which dexamethasone administered before LPS challenge effectively reduced TNF levels in serum samples from mature mice, whereas senescent mice were relatively refractory to down-regulation by dexamethasone (28). It is possible that the low sensitivity of target cells to corticosterone-mediated down-regulation is the underlying mechanism of enhanced cytokine production in aged mice.

Recent evidence suggests that IL-10, a pleiotropic cytokine produced by a variety of activated cell types, plays a major role *in vivo* as a regulator of immune and inflammatory reactions. This study indicates that aged mice produce significantly greater amounts of IL-10 in response to LPS than young mice. Our results are consistent with the report of Hobbs et al. (25), which indicated the age-related increase of IL-10 production and transcription in splenic CD4⁺ cells. It seems paradoxical that aged mice produce more cytokines in the face of a hyper-down-regulating response by IL-10. It has been reported that glucocorticoids inhibit TNF production *in vitro* and *in vivo* if administered prior to LPS stimulation but are rather less effective when applied after the induction of TNF synthesis (5). Similarly, a recent study has demonstrated a diminished effect of IL-10 in LPS-induced mortality after the stimulus, in contrast to a complete protection induced by a simultaneous administration of this cytokine with LPS (27). It is quite likely that the cytokine network is irreversibly engaged in producing a state of shock just after stimulation with LPS, while the down-regulating factors, released during these processes, may not efficiently control cytokine generation in the acute phase.

We could not explain the mechanism of enhanced cytokine production in aged mice by a low level of production of the major down-regulating factors, corticosterone and IL-10, although several other factors possessing cytokine-inhibitory activities, such as transforming growth factor β (8) and prostaglandin E₂ (34), remain to be investigated. An alternative possibility is that macrophages of aged mice may be relatively activated, or primed, for cytokine production by exogenous and/or endogenous stimuli prior to LPS challenge. In this regard, IFN- γ , a macrophage-activating factor, is likely to play a critical role, since this cytokine acts not only as a direct mediator associated with LPS-induced lethality but also as a crucial factor determining the host state for cytokine-producing capacity. We observed higher IFN- γ levels in aged mice throughout the 3- to 12-h study period. These observations are in agreement with the reports of the age-related increase in IFN- γ production (13, 38, 44). More recently, it has been reported that age-associated shifts in the subset composition of lymphocytes underlie the changes in IFN- γ production of aged mice (17, 26). Mice primed by murine recombinant IFN- γ have been shown to produce greater amounts of TNF in response to LPS and become significantly susceptible to the lethal toxicity of LPS (24). Our observations of a lower threshold of IL-1 production and a higher level of lethal sensitivity to exogenous TNF support the hypothesis of a primed state for LPS-induced lethality and cytokine production in aged mice.

We have used D-galactosamine to sensitize mice to TNF because even 100,000 U of recombinant human TNF did not cause the deaths of either young or aged mice. Although aged mice were administered an approximately 2.5-fold smaller dose of D-galactosamine than young mice (in milligrams per kilogram), it remains a possibility that D-galactosamine itself influences the TNF-induced deaths of aged mice. Since D-galactosamine is known to be a hepatotoxic agent, its effects being confined to hepatocytes (35), we have compared the influence of D-galactosamine on liver tissues of young and aged mice. As expected, we observed that 20 mg of D-galactosamine induced slight liver damage in aged mice but not in young mice (data

not shown). These results indicate that D-galactosamine possibly has an impact on the TNF-induced deaths of aged mice. We must be careful in interpreting the data that indicated aged mice are more sensitive to the lethal effect of recombinant human TNF if they were treated with D-galactosamine. Experiments using murine TNF without D-galactosamine were required to exactly define levels of TNF-induced lethal sensitivity in aged mice.

In conclusion, this study characterizes the LPS-induced cytokine-producing capacity of aged mice as a dysregulated hyperproducing state, in spite of full down-regulating responses by corticosterone and IL-10. Imbalances in the cytokine network may lead to nonprotective or inappropriately biased immune responses in aged mice which may play a role in several pathological conditions, such as infections, sclerosis, fibrosis, and dementia, in which these cytokines appear to be involved.

ACKNOWLEDGMENTS

We thank Shogo Kuwahara and Sachiko Goto for their critical readings of the manuscript and helpful suggestions. We also thank Yasuyuki Higashi for his assistance with statistical analyses.

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Editor: R. E. McCallum