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Reduced Basal Levels and Enhanced LPS Response of IL-6 mRNA in Aged Mice

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Senescence is a complex and multifactorial process that may predispose organisms to altered responses to environmental stressors. The cytokine interleukin-6 (IL-6) is expressed by a variety of cells and is one of the earliest mediators of the acute inflammatory response. In this study, the level of IL-6 mRNA in younger (7 months) and old (23 months) mice was determined in the tissue of several organs with or without stimulation with lipopolysaccharide (LPS). Whereas younger animals had a basal expression of IL-6 mRNA in all organs, this was undetectable in the old animals. In contrast, when the mice were injected with LPS, in a majority of the organ tissues there was a robust stimulation of IL-6 mRNA in the old mice whereas the younger animals had a more variable response. These data indicate that the aging process may predispose animals to an exaggerated and potentially harmful inflammatory response.

INTERLEUKIN-6 (IL-6) is a multifunctional cytokine important in homeostasis and as an early marker of acute inflammatory response (1). Its level has been shown to increase in the serum during acute traumatic injury, and this level is positively correlated with the severity of tissue damage (2).

During the aging process, a compromise of the defense mechanism of cells may lead to the accumulation of exogenous and endogenous stress factors that may underlie an increased basal inflammatory response. Indeed, a substantial increase in IL-6 levels in peripheral blood mononuclear cells in elderly subjects has been documented. This is strongly correlated (3) with a parallel increase in levels of the systemic inflammatory marker C-reactive protein. Chronic production of cytokines such as IL-6 can result in cytotoxicity because they recruit and activate macrophages that produce high concentrations of reactive oxygen species (4). Thus, a chronically elevated basal level of inflammatory factors may be deleterious to an organism. This may account for the finding that, despite elevated monocyte function and greater production of cytokines such as IL-6, the older population is nevertheless more susceptible than the young population to viral and bacterial infections (5). An age-related increase in IL-6 has also been correlated with an enhanced potential for future disability in the older population (6).

Not only is the level of IL-6 increased in the aged population, but there is also an alteration in the level of regulatory proteins for this cytokine. It has been demonstrated that the level of plasma IL-6 soluble receptor is significantly lower in aged adults than it is in young adults (7). In contrast to previous studies that demonstrated an increase in IL-6 level in older subjects, Delpedro and his colleagues (8) found a decrease in IL-6 production by lipopolysaccharide-activated monocytes derived from subjects 65 years and older compared with the monocytes purified from 25-year-old donors. There is then some inconsistency as to whether mononuclear cells derived from elderly subjects are hyperactive or rather show a diminished response compared with those derived from the young. However, it is clear that the aging process does alter the responsiveness of blood-derived monocytes, and that changes in the production of cytokines and their receptors may reflect an altered immune response associated with senescence.

Whereas most studies have focused on assessing the level of IL-6 in plasma or the secretion of the cytokine by blood-derived leukocytes, the main purpose of the present study was to determine basal levels and effects of lipopolysaccharide (LPS) stimulation on levels of IL-6 mRNA within several organ tissues. The expression of this mRNA in the lung, liver, spleen, and kidney was quantitated in younger (7 months) and old (23 months) mice with and without stimulation by LPS. There was a major increase in all of the tissues of old animals upon activation. However, the younger animals displayed more variable responses to LPS, generally of a lower magnitude. It is possible that although organ-derived IL-6 may play a fundamental immune-related role in aged animals, in younger animals there is a greater range of functions involving this cytokine.

METHODS

Materials

Murine glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was obtained from Ambion (Austin, TX). LPS and, unless otherwise noted, all other chemicals were obtained from Sigma (St. Louis, MO).

Animal Treatment

Male B/6C3F1 mice, a hybrid between C57BL/6 mice and C3H mice from Harlan Labs (Indianapolis, IN), aged 7

or 23 months, were housed four per cage and were maintained on a 12 hour light–12 hour dark cycle in a temperature controlled (20 ± 1°C) room. Food and water were provided ad lib. Half of the mice were injected intravenously (infraorbitally, as recommended by Waynforth and Flecknell; 9) with 100 μl of LPS (0.1 mg/ml dissolved in sterilized normal saline) 2 hours before sacrifice by cervical dislocation; naïve mice served as controls. The basis of selecting the 2-hour time point was to ensure that the acute early-phase response to LPS was monitored. Most groups consisted of three animals; results reported with fewer animals in a group were due to animal mortality prior to the conclusion of the experiment.

RNA Extraction

Tissues were excised quickly, blotted on absorbant paper to remove blood, and immediately placed in liquid nitrogen and stored at −70°C before processing. Total RNA was extracted by using the TRI REAGENT Kit obtained from Molecular Research Center, Inc. (Cincinnati, OH). RNA concentrations were determined by measuring absorption at a 260-nm wavelength.

Preparation of cDNA Probe

A rat IL-6 cDNA-containing plasmid (gift from Drs. Wolfgang Northemann and Georg Fey, Scripps Research Foundation, La Jolla, CA) was transformed into JM101 cells and then digested with *Pst*I and *Bam*HI restriction enzymes to obtain the insert for hybridization.

Dot Blot Analysis

Five-microgram aliquots of total RNA were denatured with Northern gel loading buffer (2 mM ethylenediamine tetra-acetic acid, 60% glycerol, 0.5% Bromphenol Blue, and 0.5% xylene cyanol). Dot blotting was performed with the Bio-Dot SF apparatus (Bio-Rad Laboratories, Hercules, CA). Zeta-Probe membranes (Bio-Rad Laboratories) were then hybridized with one of the cDNA probes labeled with [^{32}P]dCTP to a specific activity of approximately 10^9 cpm/μg by using the RTS Radprime System (Life Technologies, Gaithersburg, MD). The membranes were autoradiographed at −70°C for periods varying from 8 hours to 7 days, using x-ray film (X-OMAT AR, Kodak, Rochester, NY); blots are displayed in Figure 1. A densitometer (Eagle Eye, Stratagene, San Diego, CA) was used to quantify the bands in arbitrary units that correspond to the relative area-log pixel-density product under each blot. Values were normalized by using mRNA levels of a housekeeper gene (GAPDH) as the baseline.

Statistical Analysis

The differences between groups were assessed by using the heteroscedastic *t* test for unpaired replicates. The acceptance level of significance was $p < .05$, using a two-tailed distribution.

Results

The basal level of IL-6 mRNA in 7-month-old mice was similar in all organ tissues that were analyzed (Table 1). In the 23-month-old animals, the basal expression of the mRNA for this cytokine was much lower and sometimes undetectable. When animals were injected with LPS, the younger animals showed varied responses. The levels of IL-6 mRNA were significantly increased in lung. The mRNA levels were unchanged in spleen and liver, whereas in kidney there was a significant decrease in response to LPS. Unlike the younger animals, older mice expressed a major increase in IL-6 mRNA levels consequent to LPS treatment in virtually all tissues tested (Figure 2).

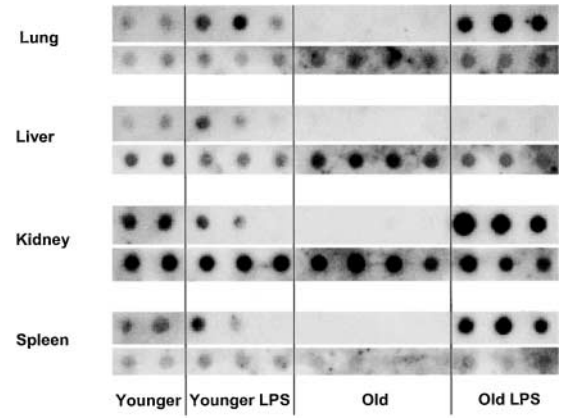

Figure 1. Dot-blot radiographic images of mRNA expression levels for four tissues from 7- (younger) and 23-month-old (old) mice. Interleukin-6 expression is shown on the upper panel for each tissue, glyceraldehyde phosphate dehydrogenase on the lower. Half the animals received an infraorbital injection of lipopolysaccharide (LPS), 2 h prior to sacrifice.

Discussion

The basal levels of IL-6 mRNA were substantially higher in tissues from younger mice compared with those from the older animals. This may be because tissue-derived IL-6 plays a role in the maintenance of homeostasis in the younger mice. Indeed, a role for IL-6 has been established in osteoclastogenesis, and senescence has been shown to disrupt this process (10). This interleukin also has a function in early vascular development of the murine brain as well as in the maintenance of physiological homeostatic conditions (11). Our data showed depressed basal IL-6 mRNA levels in aged animals, and this may be because either the older animals do not require basal expression of the interleukin for

Table 1. Levels of Interleukin-6 mRNA in Tissues From 7- and 23-month-old Mice

Tissue	Younger (Naïve)	Old (Naïve)	Younger (LPS)	Old (LPS)
Lung	0.44 ± 0.07	0.025 ± 0.010	1.41 ± 0.62	1.06 ± 0.27
Liver	0.18 ± 0.07	0.009 ± 0.005	0.44 ± 0.22	0.053 ± 0.009
Kidney	0.329 ± 0.002	*0.008 ± 0.005	0.14 ± 0.08	*0.55 ± 0.06
Spleen	0.51 ± 0.18	0	0.22 ± 0.16	1.06 ± 0.48

Notes: Half the animals received an infraorbital injection of lipopolysaccharide (LPS), 2 h prior to sacrifice. Data were densitometrically quantitated and represent the mean of 2–4 animals ± SE. Levels are relative to glyceraldehyde phosphate dehydrogenase intensities ± SE (n = 2–4).

*Differs significantly from the corresponding value for younger animals ($p < .02$).

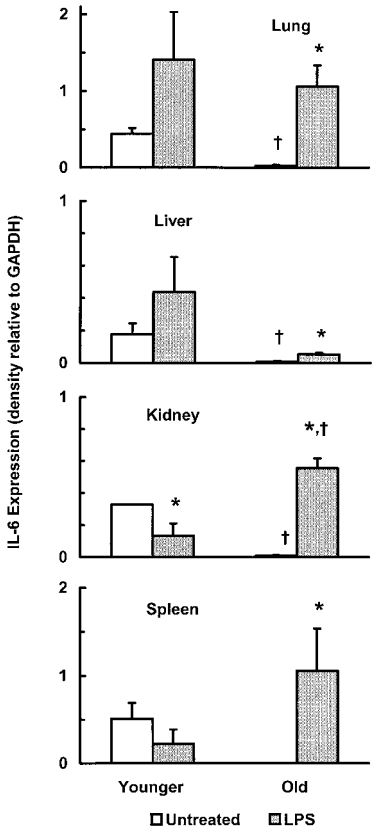

Figure 2. Levels of interleukin-6 (IL-6) mRNA of tissues from 7- and 23-month-old mice. Half the animals of either age received an infraorbital injection of lipopolysaccharide, 2 h prior to sacrifice. Data were densitometrically quantitated and represent the mean of 2–4 animals ± SE. * Value differs from that of corresponding control mice ($p < .02$); † value differs from that of corresponding younger mice ($p < .02$). GAPDH = glyceraldehyde phosphate dehydrogenase.

development and homeostasis, or because aging causes a dysregulation in cytokine expression.

The aging process is associated with a higher responsivity of IL-6 mRNA to exogenous inflammatory stimuli. The intracellular level of T-cell IL-6, stimulated with phorbol 12-myristate 13-acetate, is significantly higher in aged donors than in younger blood donors (12). In the present study, injection of LPS in the 23-month-old mice produced a significant increase in IL-6 mRNA expression in all of the tissues analyzed. In contrast, the LPS-stimulated level of IL-6 mRNA expression varied in the 7-month-old mice with the specific tissue analyzed. To ensure that the changes in IL-6 levels are an acute early-phase response, the animals were sacrificed after 2 hours of exposure. It is possible that if the animals are exposed for longer periods of time, the modulation of IL-6 mRNA may change. Thus, over time, the expression of the cytokine may continue to increase in the younger animals whereas in the older mice it may start to decline.

In an interpretation of whole-organ cytokine mRNA expression, the possible contribution from residual blood cells should be minimized. However, prevention of mRNA degradation is paramount. Thus we did not attempt to perfuse tissue to remove blood cells; rather, we excised the tissue quickly and froze it immediately, blotting the tissue prior to freezing to minimize residual blood. Consequently, contributions to the measured IL-6 expression levels that are due to blood cells' remaining in tissue vasculature should be small.

The present study has focused on the genetic regulation of IL-6 mRNA expression and does not measure tissue levels of the cytokine directly. A future study will augment the results described here by a parallel assay of levels of IL-6 protein.

Although IL-6 may play a role in normal physiological maintenance of different organ systems, this cytokine is also an early marker of an acute inflammatory response (2). Whereas organ system-derived IL-6 may play a fundamentally immune-related role in the aged animals, it is possible that this is not the case in the younger animals and LPS activation might actually in some cases disrupt the basal expression of the cytokine necessary for homeostasis. Alternatively, younger animals may be able to protect themselves against excessive inflammatory responses by restricting the levels of the cytokine mRNA expression, and this control may be missing in the older animals. Thus, although the level of IL-6 mRNA expression in the 7-month-old animals modestly increases in the lung and actually declines in the kidney, the tissues of aged animals are all equally hyperresponsive, and, upon LPS stimulation, all of the tissues analyzed in the 23-month-old mice expressed a manyfold increase in levels of IL-6 mRNA.

The tissue cytokine mRNA responses found here resemble those of cytokines in plasma. Following intracerebroventricular injection of LPS, 2-year-old rats respond with a greater elevation of circulating levels of IL-6 than do 3-month-old rats (13). Similarly, the response of IL-6 and other cytokines to intraperitoneally injected LPS is greater in aged than in young mice (14). An augmented reactivity of periodontal cells, aged by repeated cycles of division, to LPS induction of IL-6 and other cytokines has also been reported (15). This increased reactivity of older cells to immunogenic stimuli may account for the greater susceptibility of old animals to LPS-induced lethality (16). However, such enhanced tissue susceptibility may be concurrent with an age-related depression of effective reactivity in tissues specifically responsible for immune activation, such as the spleen (17). This raises the possibility that the augmented changes found in aged animals are in fact inappropriately targeted and do not represent a physiologically effective response. Recent reports indicate that Toll-like receptor 4 (Tlr4) and the molecule CD14 are important mediators of LPS-invoked innate immunity (18–20). The increased response of the older animals to LPS may be the result of upregulation of CD14 or Tlr4 on the cell surface.

Although the exact mechanisms by which such age-dependent changes in inflammatory responses occur are unknown, the acceleration of processes leading to these age-associated alterations in cytokine profiles may play a role in the onset and progression of chronic age-related inflammatory diseases such as rheumatoid arthritis. Understanding the mechanisms underlying these age-related changes can allow the development of dietary regimens and pharmaceu-

tical interventions that may ameliorate the dysregulation of the immune response and decrease the rate of progression of age-related degenerative diseases.

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