Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 11008–11013, October 1996
Immunology

Functions of interleukin 1 receptor antagonist in gene knockout and overproducing mice

EMMET HIRSCH*†, VERA M. IRIKURA*, SARAH M. PAUL*, AND DAVID HIRSH*‡

Departments of *Biochemistry and Molecular Biophysics and †Obstetrics and Gynecology, College of Physicians and Surgeons of Columbia University, 630 West 168th Street, New York, NY 10032

Communicated by Frederick W. Alt, Children's Hospital, Boston, MA, July 23, 1996 (received for review April 29, 1996)

ABSTRACT **Interleukin 1 receptor antagonist (IL-1ra) is a cytokine whose only known action is competitive inhibition of the binding of interleukin 1 (IL-1) to its receptor. To investigate the physiological roles of endogenously produced IL-1ra, we generated mice that either lack IL-1ra or overproduce it under control of the endogenous promoter. Mice lacking IL-1ra have decreased body mass compared with wild-type controls. They are more susceptible than controls to lethal endotoxemia but are less susceptible to infection with *Listeria monocytogenes*. Conversely, IL-1ra overproducers are protected from the lethal effects of endotoxin but are more susceptible to listeriosis. Serum levels of IL-1 following an endotoxin challenge are decreased in IL-1ra nulls and increased in IL-1ra overproducers in comparison to controls. These data demonstrate critical roles for endogenously produced IL-1ra in growth, responses to infection and inflammation, and regulation of cytokine expression.**

Interleukin 1 (IL-1) is a proinflammatory cytokine that participates in the response to infectious and inflammatory challenges by recruiting and activating neutrophils and macrophages, by producing fever and vascular dilation, and by inducing mediators such as IL-6, acute phase reactants, and prostaglandin E2 (reviewed in ref. 1). These potentially beneficial functions require modulation to avoid causing serious damage to the organism. Examples of IL-1-mediated injury include shock and organ failure associated with sepsis (2), and joint inflammation in rheumatoid arthritis (3). Endogenous modulation of IL-1 activity is achieved via regulation of IL-1 synthesis and processing, release from intracellular and membrane-bound stores, and the expression of cell-surface and soluble receptors (4). A unique mode of IL-1 inhibition is effected by the action of the IL-1 receptor antagonist (IL-1ra). Binding of IL-1ra to IL-1 receptors prevents access by IL-1 and fails to elicit any agonistic activities, including receptor internalization, induction of NF-κB, or binding of the IL-1 receptor accessory protein (5–7).

IL-1ra is encoded by a single-copy gene. There are three known forms of this protein, two intracellular and one secreted. The intracellular variants are generated by differential splicing of alternative 5′ exons I and II (4, 8). All three forms of IL-1ra have comparable abilities to inhibit IL-1 effects when administered to cells in culture. High doses of recombinant IL-1ra given to experimental animals have been shown to improve survival during endotoxemic shock (9, 10) and to reduce inflammation in experimental arthritis (11). However, the role of endogenously produced IL-1ra during inflammation has only recently been addressed. Antibody neutralization of IL-1ra has been shown to lead to exacerbation of inflammation in animal models of formalin-immune complex colitis (12), *Schistosoma mansoni* egg-induced granuloma formation (13), and *Propionibacterium acnes*-induced hepatitis (14). These studies suggest that one role of IL-1ra *in vivo* is to modulate inflammatory events mediated by IL-1. Although the secreted form of IL-1ra performs this modulation by competing for the IL-1 receptor, the function of intracellular IL-1ra is less clear. A recent report suggests that it antagonizes IL-1 by destabilizing IL-1-induced mRNAs (15).

To examine the functions of IL-1ra during infection and inflammation, as well as in development and homeostasis, we have created transgenic mice that either lack IL-1ra or overproduce it under the control of the endogenous promoter. The use of these mutant mice presents several advantages over antibody neutralization of IL-1ra and the administration of exogenous IL-1ra protein: Antibody studies may be limited by nonspecific binding or incomplete neutralization, particularly as IL-1ra levels in disease may exceed 100 ng/ml in tissues. Exogenous IL-1ra protein may not have access to all relevant tissue sites *in vivo*. This was recently demonstrated in a murine model of experimental arthritis, in which IL-1ra expressed by transfected synoviocytes was more potent by four orders of magnitude than systemically administered IL-1ra (16). Neither antibody neutralization nor systemic IL-1ra administration is practical for long-term use *in utero* or over the lifetime of the animal. Furthermore, unlike exogenously administered protein, use of the natural promoter is likely to produce excess IL-1ra with endogenous expression kinetics. This is important because the timing of appearance of IL-1ra in pathophysiology is likely to be critical, given that the cytokine network is complex, redundant, and controlled on multiple levels.

Mice lacking all three forms of IL-1ra were created by gene targeting. Mice producing excess amounts of the secreted form of IL-1ra were generated by pronuclear injection of mouse zygotes with a genomic DNA construct containing the endogenous regulatory sequences. We find that IL-1ra has important functions in the attainment of normal body mass, in survival following challenge with either endotoxin or *Listeria monocytogenes*, and in the regulation of the expression of IL-1.

EXPERIMENTAL PROCEDURES

IL-1ra Gene Targeting and Overexpression Constructs. A 9.0-kb *Hin*dIII/*Eco*RI restriction fragment from a murine strain 129 λ genomic DNA library was used to make overexpression and targeting vectors (Fig. 1*A*, first line). This fragment contains the four exons and three introns encoding the secreted form of IL-1ra, plus approximately 2.0 kb of upstream and 2.5 kb of downstream untranslated regions. Studies with the human gene suggest that the important transcriptional regulatory elements are contained within the 2.0-kb upstream sequence (17). The alternative exon I and exon II of the intracellular forms of IL-1ra lie in a region several kilobases upstream (based on the human sequence), which is not present in the construct. The construct was modified by the site-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: IL-1, interleukin 1; IL-1ra, interleukin 1 receptor antagonist; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α.

‡To whom reprint requests should be addressed. e-mail: dih1@columbia.edu

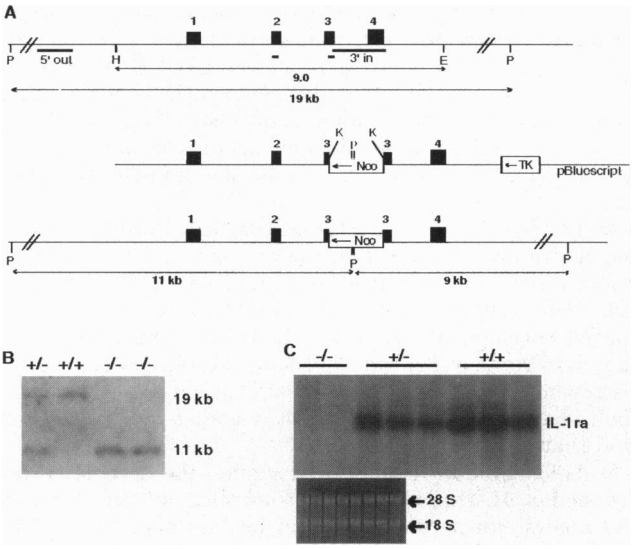

FIG. 1. Targeted disruption of the IL-1ra locus. (*A*) Targeting strategy. The first line shows the endogenous genomic IL-1ra locus, including the four exons (numbered black boxes) and three introns of the secreted form of IL-1ra. The second line depicts the linearized targeting construct, with neomycin resistance (*Neo*) and herpes simplex thymidine kinase (*TK*) genes. The third line represents the targeted IL-1ra locus. Restriction enzyme sites: *H*, *Hin*dIII; *E*, *Eco*RI; *K*, *Kpn*I; *P*, *Pst*I. Short black lines indicate the location of PCR primers. Longer black lines indicate the locations of the probes used in Southern blot analysis (*5′ out*, upstream probe external to the targeting construct; *3′ in*, downstream probe within the construct). Double-arrowed lines and corresponding numbers depict the length of the various fragments in kb. (*B*) Southern blot showing the genotyping of offspring of IL-1ra hemizygote matings. Genomic DNA was digested with *Pst*I and probed with the 5′ out external probe. +/+, wild type; +/−, hemizygously disrupted IL-1ra; −/−, homozygously disrupted IL-1ra. (*C*) Northern blot of total lung RNA from individual mice sacrificed 3 hr after i.p. injection with 20 mg/kg lipopolysaccharide (LPS). The corresponding ethidium bromide-stained formaldehyde gel is reproduced for loading comparison.

directed mutation of guanine 3168 (18) within exon III to adenine (Altered Sites Mutagenesis System, Promega). This modification resulted in the generation of a unique *Kpn*I restriction site, but did not alter the amino acid sequence of the protein. The construct was cloned into the pBluescript II SK(+) vector (Stratagene). The excised *Hin*dIII/*Eco*RI fragment was used to generate IL-1ra-overexpressing transgenic mice by pronuclear injection.

The transgenic construct was further modified for gene targeting by the insertion of a neomycin resistance gene (*Neo*) (pMC1neo Poly A, Stratagene) at the artificially generated *Kpn*I restriction site. A herpes simplex thymidine kinase (*TK*) cassette (pIC-19R/MC1-TK) was added at the 3′ end of the construct to enrich for recovery of targeted clones. The targeting vector was linearized using *Cla*I prior to electroporation (Fig. 1*A*, second line).

Production of IL-1ra Null Mice. Embryonic stem cells from the CCE line, derived from 129/Sv mice, were donated by Elizabeth Robertson (Harvard University). Embryonic stem cells were cultured over an STO cell feeder layer in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, 2 mM glutamine, 0.1 mM 2-mercaptoethanol, 50 ng/ml ciliary neurotrophic factor, and 15% fetal calf serum. Twenty million cells were electroporated with 15 μg of linearized plasmid DNA. Antibiotic selection with G418 (30–350 μg/ml) and gancyclovir (2 μM) were begun after 48 and 120 hr, respectively. After 10 days, surviving clones were transferred to 24-well plates and expanded. When confluent, the majority of cells were frozen in liquid nitrogen for future blastocyst injection. The remaining cells from each clone were expanded for DNA extraction and Southern blot analysis. Clones were screened for homologous recombination by Southern blotting of genomic *Pst*I digests using a 5′ genomic DNA probe external to the end of the DNA construct. A 3′ probe internal to the construct was used to confirm targeting and lack of second site recombination.

Hemizygously disrupted embryonic stem cell clones were microinjected into C57BL/6J blastocysts, which were then transferred to pseudopregnant females, yielding chimeric offspring. These chimeras were mated with C57BL/6J mice. Germ-line transmission was identified by agouti coat color. Genotyping was performed by Southern blot analysis, as noted above, or by PCR. Mice used in the experiments detailed below are homozygous IL-1ra null, hemizygous, and wild-type littermate offspring of hemizygous parents derived from a first or second generation backcross to the C57BL/6J strain.

Production of IL-1ra-Overexpressing Mice. The IL-1ra transgene (lacking the *Neo* and *TK* cassettes) was microinjected into one of the two pronuclei of 1-day-old B6CBAF2 embryos. The embryos were then transferred to pseudopregnant females. Pups were screened for integration of head-to-tail tandem arrays of the transgene by Southern blot analysis. Because the artificially produced *Kpn*I site is unique within the construct, *Kpn*I digestion of genomic DNA extracted from transgenic mice and hybridized with an internal probe yields a 9.0-kb band that is equal to the length of the construct. Transgenic lines were perpetuated by repeated back-crossing to B6CBAF1/J mice.

Oligonucleotide Primers and Probes. All numerical designations of nucleotide positions are based on the published genomic sequence of Zahedi *et al.* (18).

PCR was performed to distinguish the endogenous from the disrupted IL-1ra locus using an exon II sense primer complementary to bases 2149–2172 (AACCAGCTCATTGCTGGGTACTTA) and an exon III antisense primer corresponding to bases 3175–3198 (GCCCAAGAACACACTATGAAGGTC). The reaction was amplified for 30 cycles.

Northern blot detection of IL-1ra mRNA was performed using a 40-mer oligonucleotide complementary to sense nucleotides 188–227 in exon I (TGAAGGCTTGCATCTTGCAGGGTCTTTTCCCAGAAGGGCG). The external 5′-genomic DNA probe used to screen Southern blots of embryonic stem cell clones for homologous recombination events was an 800-bp *Ssp*I/*Eco*RI restriction fragment approximately 4 kb upstream of the transcription start site for the secreted protein. The 3′-internal genomic probe used for Southern blot analysis of genomic IL-1ra DNA was a 1032-bp *Eco*RV/*Stu*I restriction fragment corresponding to bases 3246–4277.

Northern Blot Analysis and Histology. RNA was extracted from murine tissues using the guanidine isothiocyanate method (TRIzol Reagent, GIBCO/BRL). Ten micrograms of total RNA was loaded per lane in formaldehyde agarose gels and transferred to nylon membranes (Hybond-N, Amersham). Hybridization was performed at 65°C with the 40-mer oligonucleotide noted above. Tissue sections for histologic evaluation were stained with either hematoxylin and eosin or with tissue Gram stain (Accustain, Sigma).

LPS Administration. Purified LPS (endotoxin) extracted from *Salmonella typhimirium* (Sigma) was dissolved in sterile phosphate buffered saline (PBS) at 2 mg/ml, then boiled for 5 min prior to freezing in aliquots at −20°C. Animals were administered LPS by peritoneal injection. The data from males and females were combined for analyses of survival and cytokine production following administration of LPS.

***L. monocytogenes* Handling and Inoculation of Mice.** A starting culture of *L. monocytogenes* strain EGD donated by Edward A. Havell (North Carolina State University) was passaged through a mouse, grown in overnight culture, and then frozen in aliquots. All liquid media culturing and dilution

was performed in brain–heart infusion media (Difco). Prior to each experiment an aliquot was thawed, brought to log-phase growth after overnight culture, and diluted to an estimated 5×10^3 colony-forming units per 100 μl of inoculum, which was administered via the tail vein. The inoculum size was confirmed by titering on Luria–Bertani plates. Animals were monitored daily. No deaths were observed after the sixth day of infection. Bacterial titers in liver were determined in a group of mice euthanized on the sixth day. Organs were weighed, homogenized in PBS, and plated in serial log dilutions. Colony-forming units were counted at 48 hr. Bacterial titers are expressed as the number of colony-forming units per gram organ. Tissue samples for histology were obtained postmortem or from surviving animals on the fourth or sixth day after inoculation.

Serum Protein Determinations. Mouse serum was collected by cardiac puncture, frozen at −70°C, and later thawed for protein determinations by ELISA [for IL-1α, Intertest-1αX, Genzyme; for IL-1β, IL-6, and tumor necrosis factor α (TNF-α), Cytoscreen Immunoassay Kit, BioSource International]. IL-1ra ELISAs were performed as follows: 96-well plates (Corning) were coated with 15 μg/ml protein G-purified polyclonal goat anti-mouse IL-1ra antibody. Samples and standards were then captured and sandwiched with 50 μg/ml protein G-purified biotinylated secondary polyclonal goat anti-mouse IL-1ra antibody (materials kindly provided by Lyle Moldawer, University of Florida). Colorimetric detection was performed following incubation with a 1:3000 dilution of streptavidin–horse radish peroxidase (Sigma) and 0.5 mg/ml *o*-phenylenediamine dihydrochloride chromagen (Sigma). Absorbance readings at 490 nm with background correction at 650 nm were used to calculate concentrations in samples in comparison to murine IL-1ra standards. The lower limit of detection of the IL-1ra assay was approximately 5 ng/ml.

Mice. Mice were housed in a conventional animal facility, which was known to harbor mouse hepatitis virus. All animals were handled in accordance with institutional guidelines.

Statistics. Continuous variables with normal distributions (e.g., body mass) were analyzed using Student's *t* test or one-way ANOVA, respectively, for comparisons between two or more than two groups. Continuous variables with nonnormal distributions (serum protein values, listerial titers within harvested organs) were analyzed by the Mann–Whitney *U* test or Kruskal–Wallis one-way ANOVA by rank. Survival curves after LPS injection were compared using the Kaplan–Meier statistic. Survival rates 6 days after inoculation with *L. monocytogenes* were compared using the χ^2 test.

RESULTS

Generation of IL-1ra Null Mice. Of 386 embryonic stem cell clones surviving electroporation and double selection with G418 and gancyclovir, three were found in which the targeting construct had replaced the endogenous locus by homologous recombination. These three clones were injected into C57BL/6J blastocysts, yielding 13 chimeric mice. One of these 13 chimeras transmitted the mutation through its germ line, yielding offspring hemizygous for the disruption construct. Matings between hemizygotes generated wild-type, hemizygous, and IL-1ra null offspring, as depicted in Fig. 1*B*.

Generation of Mice with Extra Copies of the IL-1ra Gene. IL-1ra transgene tandem arrays were found in five separate loci in two founder mice generated by pronuclear injection. These distinct loci segregated from each other during breeding with wild-type mates. Two such loci and corresponding transgenic mouse lines were designated "T14" and "T16." To maximize gene dosage of IL-1ra, the experiments reported here were performed with mice hemizygous at both the T14 and the T16 loci, unless otherwise specified. These mice were generated from crosses of a mouse homozygous for both T14 and T16 with a wild-type B6CBAF1/J mate. Control animals for these experiments were taken from matings of a wild-type sibling of the T14/T16 homozygote with a B6CBAF1/J mate. A Southern blot analysis of the genomic DNA from these mouse lines shows the common endogenous locus and 9.0-kb transgenic tandem array, as well as the distinct flanking regions, which vary depending on the site of integration (Fig. 2*A*).

IL-1ra Null Mice Have a Growth Deficit. Both IL-1ra null and IL-1ra overexpressing mice were born in expected Mendelian ratios and were developmentally normal upon gross examination, demonstrating that IL-1ra is not required for normal development. However, IL-1ra null mice had lower body weights than control littermates (Table 1). The weight discrepancy was apparent by 6 weeks of age and continued into adult life. IL-1ra overexpressors had similar weights as wild-type controls.

IL-1ra Gene Copy Number Determines the Level of IL-1ra Production. IL-1ra expression was undetectable by Northern blot analysis or serum ELISA in healthy mice at rest. To demonstrate that alteration of the number of functional copies of the IL-1ra gene results in alteration of IL-1ra production, mice were stimulated with LPS. IL-1ra mRNA and protein were produced in proportion to the number of copies of the IL-1ra gene present in the genome (Figs. 1*C* and 2*B* and Table 2). Peak levels were reached between 8 and 16 hr after LPS stimulation (see Figs. 4*A* and *B*) and were sustained for at least 24 hr (data not shown).

Endogenously Produced IL-1ra Is Critical for Survival During Endotoxemia. To examine the role of endogenously produced IL-1ra in a murine model of septic shock, IL-1ra mutant mice were monitored for survival after administration of 10 mg/kg LPS by intraperitoneal (i.p.) injection. IL-1ra null animals were more susceptible, and IL-1ra overexpressors

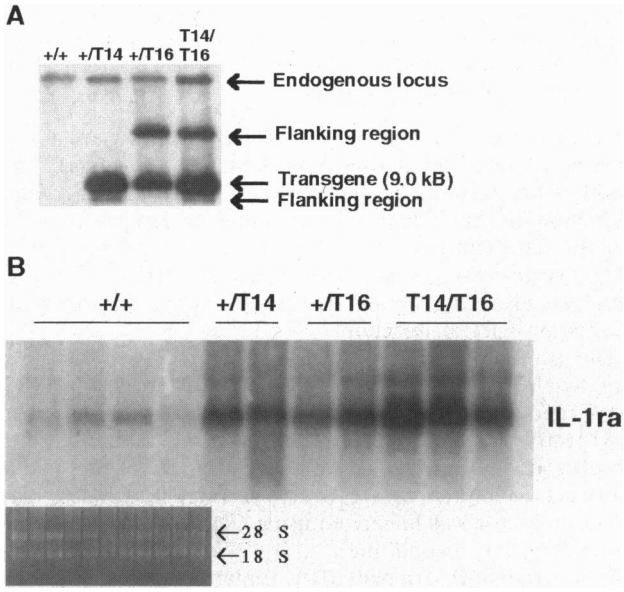

FIG. 2. Overexpression of IL-1ra. A 9-kb genomic IL-1ra DNA *Hin*dIII/*Eco*RI restriction fragment, as depicted in Fig. 1*A*, was modified by a silent point mutation within exon III, generating a unique *Kpn*I restriction site. This fragment was used to generate IL-1ra overexpressing mice by pronuclear injection. (*A*) Southern blot analysis of genomic DNA digested with *Kpn*I, showing differential banding patterns from mice of the following genotypes: wild type (+/+), hemizygous for the transgene in the T14 locus (+/T14), hemizygous for the transgene in the T16 locus (+/T16), and hemizygous for both loci (T14/T16). (*B*) Northern blot analysis of total liver RNA from individual mice sacrificed 3 hr after i.p. injection with 20 mg/kg LPS. The corresponding ethidium bromide-stained formaldehyde gel is reproduced for loading comparison.

Table 1. Weight in grams ± SD of wild-type (IL-1ra$^{+/+}$), IL-1ra hemizygous (IL-1ra$^{+/-}$), and IL-1ra null (IL-1ra$^{-/-}$) littermates at 13 weeks of age

Genotype	Males	Females
IL-1ra$^{+/+}$	32.2 ± 2.9 (n = 9)	24.6 ± 3.3 (n = 7)
IL-1ra$^{+/-}$	32.1 ± 3.8 (n = 11)	24.1 ± 3.5 (n = 14)
IL-1ra$^{-/-}$	22.9 ± 3.0 (n = 5)	20.2 ± 2.5 (n = 12)
P value	<0.001	0.005

were less susceptible than controls to the lethal effects of endotoxin (Fig. 3). IL-1ra hemizygotes had intermediate survival. The protection afforded by IL-1ra overexpression was limited, since it could be overcome by increasing the dose of LPS. At 30 mg/kg LPS, there was no difference in survival between wild-type and overproducing animals (survival = 0/22 for wild type vs. 1/22 for overproducers).

The numbers of circulating blood leukocytes were not significantly different between IL-1ra null and control animals harvested 8 hr after LPS administration (mean ± SEM: IL-1ra nulls = 640 ± 387/mm^3; controls = 867 ± 278/mm^3; n = 3 per group; P = 0.6, Student's t test). Liver sections obtained 16 hr after administration of LPS were either normal or contained mild or nonspecific changes (hepatocytic vacuolization or focal acute necrosis), with no differences among the genotypes.

IL-1ra Acts as a Positive Regulator of IL-1 During Endotoxemia. IL-1 has been shown to induce its own expression in peripheral blood mononuclear cells and endothelial cells *in vitro* and in rabbits *in vivo* (19, 20). This autoinduction can be inhibited by IL-1ra in peripheral blood mononuclear cells (21, 22), suggesting that IL-1ra functions as a negative regulator of IL-1 production. Consequently, we predicted that excess IL-1 antagonism in IL-1ra overexpressors would result in diminished production of IL-1, whereas decreased IL-1 antagonism in IL-1ra null animals would result in increased production of IL-1. We tested this prediction by measuring the levels of cytokines produced in serum following an endotoxin challenge. Surprisingly, we found that IL-1ra acts as a positive regulator of IL-1 in serum during endotoxemia (Fig. 4): serum levels of IL-1α and IL-1β were lower in IL-1ra null animals than in wild-type controls. Conversely, serum IL-1α was higher in IL-1ra overproducers. Peak IL-1β levels were higher in IL-1ra overproducers, and IL-6 levels were higher in IL-1ra null animals than in controls, but these differences were not statistically significant.

There were no significant differences between mutants and their respective controls in serum TNF-α 1 hr after LPS injection (n = 3 per genotype; means ± SEM: IL-1ra overexpressors, 1,960 ± 458 pg/ml; wild-type controls, 2,453 ± 1,420 pg/ml; IL-1ra nulls, 11,223 ± 6,707 pg/ml; wild-type controls, 7,167 ± 4,224 pg/ml). TNF-α was undetectable at 3 hr or later after administration of 10 mg/kg LPS, except in 2 mice among 27 tested. In a separate experiment using a higher LPS dose (20 mg/kg) TNF-α was detectable at 1 and at 3 hr, but again there were no significant differences between mutants and controls.

Endogenously Produced IL-1ra Impairs the Host Response to *L. monocytogenes*. IL-1 has been shown to be a critical factor in the generation of nonspecific immunity during infection with the facultative intracellular bacterium *L. monocytogenes* (23–26). We examined the role of endogenously produced IL-1ra in listeriosis by administering to mice an intravenous inoculum of 4.6–5.6 × 10^3 bacteria. Survival was increased in IL-1ra null animals and was decreased in IL-1ra overproducers compared with their wild-type controls (Table 3). The ability to clear infectious organisms from the liver by the sixth day after inoculation, as evaluated by bacterial titering, also correlated with genotype. Histologic findings in livers ranged from normal to severely affected with multifocal granulomatous necrotizing hepatitis. Findings in the spleen ranged from normal to severe lymphoid depletion with multifocal necrosis and hemorrhage. The severity of the histologic appearance correlated with the severity of infection within the organ, as assessed by bacterial titering and visualization of bacteria on hematoxylin and eosin and tissue Gram stains. No histologic findings were specific for genotype.

DISCUSSION

We report here consequences of loss-of-function and gain-of-function mutations of IL-1ra in mice. The goal of these ongoing studies is to determine the roles of endogenously produced IL-1ra in normal and abnormal physiology. Given its specificity as an IL-1 inhibitor and the fact that its expression accompanies that of IL-1, the simplest hypothesis is that IL-1ra functions *in vivo* to modulate the effects of IL-1. This hypothesis is supported by our demonstration of altered susceptibilities of IL-1ra mutant mice to endotoxemia and listeriosis, two pathophysiological states in which IL-1 activity is central to the host response.

Two unanticipated findings arose in these studies. First, IL-1ra is required for attaining normal body mass. Likely mechanisms underlying this phenomenon include antagonism of IL-1-mediated appetite suppression and catabolism (27).

Table 2. Serum IL-1ra levels after systemic challenge with LPS

Genotype	No. of IL-1ra gene copies	IL-1ra ng/ml: 3 hr after 20 mg/kg LPS	3 hr after 10 mg/kg LPS	8 hr after 10 mg/kg LPS
IL-1ra$^{-/-}$	0	Undetectable	Undetectable	Undetectable
IL-1ra$^{+/-}$	1	38 ± 7	41 ± 17	49 ± 13
IL-1ra$^{+/+}$	2	87 ± 33	93 ± 28	177 ± 50
IL-1ra$^{+/+}$	2	94 ± 27	86 ± 7	97 ± 24
IL-1ra$^{+/T16}$	6	512 ± 198	—	—
IL-1ra$^{+/T14}$	8	960 ± 327	—	—
IL-1ra$^{T14/T16}$	12	1491 ± 88	1189 ± 51	1350 ± 87

Values represent means ± SEM of 2 or 3 animals per group at the 3-hr time points, and 4–9 animals per group at the 8-hr time point. Each sample was run in duplicate. The number of copies of the IL-1ra gene was determined in overexpressing animals by densitometric comparison of the relative intensities on a Southern blot of the IL-1ra signal vs. a control representing a single-copy gene (CNTF) (data not shown). IL-1ra$^{+/+}$, wild type; IL-1ra$^{+/-}$, hemizygously disrupted IL-1ra; IL-1ra$^{-/-}$, homozygously disrupted IL-1ra; IL-1ra$^{+/T16}$, hemizygous for IL-1ra transgene tandem array at locus T16; IL-1ra$^{+/T14}$, hemizygous for IL-1ra transgene tandem array at locus T14; IL-1ra$^{T14/T16}$, hemizygous for IL-1ra transgene tandem array at both loci, T14 and T16.

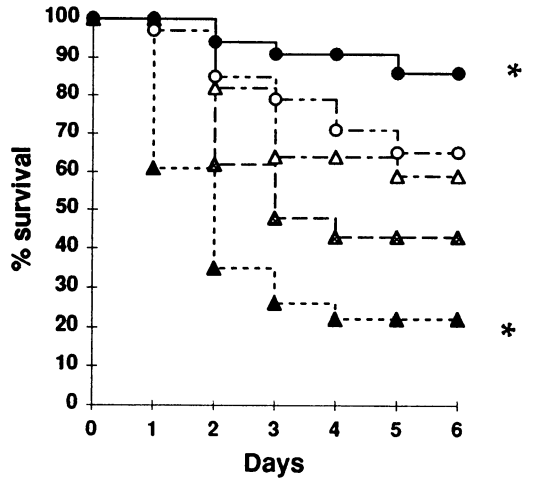

FIG. 3. Survival after i.p. administration of 10 mg/kg LPS. (▲), IL-1ra homozygous nulls (n = 23); (△) IL-1ra hemizygotes (n = 21); (△), wild-type controls (n = 22); (●), IL-1ra overproducers (n = 35); (○), wild-type controls (n = 34). *, $P < 0.05$ vs. wild type, Kaplan–Meier statistic.

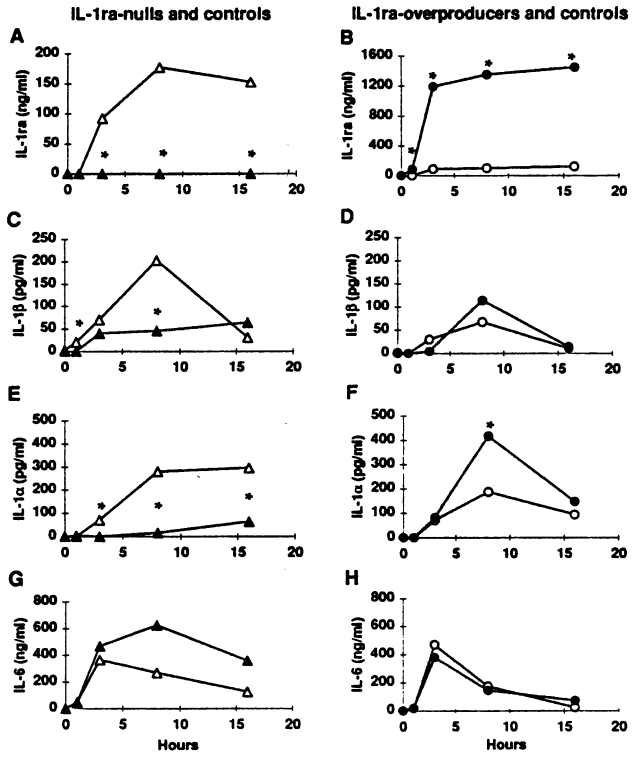

FIG. 4. Expression of IL-1ra (A and B), IL-1β (C and D), IL-1α (E and F), and IL-6 (G and H) in serum after injection of 10 mg/kg LPS. Baseline (time 0), 1- and 3-hr time points were averaged from two to three animals per genotype. Eight- and 16-hr points represent averages from 6 to 10 animals per genotype. All samples were assayed in duplicate. (▲), IL-1ra nulls; (△), wild-type controls; (●), IL-1ra overproducers; (○), wild-type controls; *, $P < 0.05$ at the indicated time points, Mann–Whitney U test.

This is the first demonstration that IL-1ra functions in a normal developmental process in the absence of a specific pathogenic stimulus.

The second unanticipated finding is that altered production of IL-1ra leads to parallel alterations in the production of IL-1, suggesting that IL-1ra acts as a positive regulator of IL-1 levels in serum during endotoxemia. As noted above, prior *in vitro* data suggested that IL-1ra functions as a negative regulator of IL-1 by inhibiting a positive feedback loop in which IL-1 induces its own production (21, 22). We believe that the discrepancy between prior data and our results is a reflection of the complexity of cytokine regulatory systems in the context of an intact organism with multiple cell types. The *in vivo* presence both of inducers of IL-1 synthesis or release, such as complement and TNF-α, and of inhibitors, such as corticosteroids, prostaglandin E2, IL-4, and TGF-β, may impact upon the net effect of a stimulus on IL-1 production. Our data suggest that IL-1ra participates in this multifactorial system *in vivo*, both as an inhibitor of IL-1 activity and as a positive regulator of IL-1 expression.

Parallel regulation of IL-1 and IL-1ra may be part of a mechanism designed to preserve the level of access of IL-1 to its receptor. Absence of IL-1ra in the null mouse increases the availability of the IL-1 receptor and, therefore, the biological effect of a given level of IL-1. By decreasing IL-1 levels in parallel with the decrease in IL-1ra, the net biological effect of IL-1 is shifted back toward baseline. The outcomes of IL-1-mediated processes may be dependent upon the relative quantities of IL-1 and IL-1ra, rather than on the absolute quantity of IL-1 alone. This interpretation is supported in our studies of experimental endotoxemia, in which lethality was

Table 3. Survival and mean bacterial titers within the livers of mice infected with *Listeria monocytogenes*

Genotype	Sex	Survival (%)	P	Mean bacterial titer (cfu per gram of liver)	P
IL-1ra$^{+/+}$	M	10/11 (91)		7.6×10^6 (n = 9)	
IL-1ra$^{+/-}$	M	14/14 (100)		9.2×10^4 (n = 10)	
IL-1ra$^{-/-}$	M	9/9 (100)	0.34	7.6×10^3 (n = 9)	0.052
IL-1ra$^{+/+}$	F	1/7 (14)			
IL-1ra$^{+/-}$	F	2/5 (40)		—	—
IL-1ra$^{-/-}$	F	9/9 (100)	0.002		
IL-1ra$^{+/+}$	M	14/16 (88)		—	—
IL-1ra$^{T14/T16}$	M	4/16 (25)	0.001		

Results from three separate experiments are combined in each case. Note that improved survival of IL-1ra nulls was observed only in females, since wild-type males had nearly 100% survival with the inoculum used. Bacterial titers are reported only in IL-1ra null, hemizygous, and wild-type males because they represent the only group in which survival approached 100%, allowing a complete data set to be obtained. Statistical tests used were χ^2 for survival and Kruskal–Wallis one-way ANOVA by rank for bacterial titers. IL-1ra$^{+/+}$, wild type; IL-1ra$^{+/-}$, hemizygously disrupted IL-1ra; IL-1ra$^{-/-}$, homozygously disrupted IL-1ra; IL-1ra$^{T14/T16}$, hemizygous for IL-1ra transgene tandem array at both loci, T14 and T16. cfu, colony-forming unit.

increased in IL-1ra null mice despite lower absolute levels of IL-1, and was decreased in IL-1ra overexpressors despite higher absolute levels of IL-1. These survival outcomes are to be distinguished from those observed in mice lacking IL-1β converting enzyme (ICE), in which lower IL-1α and IL-1β levels were associated with decreased LPS-induced lethality (28). Our results also suggest the presence of significant physiological overlap between IL-1α and IL-1β. Mice lacking IL-1β were no different from wild-type animals in their susceptibility to high-dose endotoxin and listerial infection (29), whereas animals overexpressing IL-1ra, which inhibits both IL-1α and IL-1β, have significantly altered responses to both of these agents.

Processes that are mediated by IL-1 and that are likely to impact upon responses to challenge with endotoxin or *L. monocytogenes* include the induction of specific inflammatory mediators and the recruitment of leukocytes to sites of inflammation. Among such factors we evaluated, TNF-α appears to be a proximal and transient factor in experimental endotoxemia that is not influenced by IL-1ra. IL-6, a cytokine inducible by IL-1, was higher in IL-1ra null mice than in wild type controls, but not significantly. We observed no characteristic differences between IL-1ra mutants and wild-type animals in the number of circulating leukocytes early in endotoxemia or in the histologic appearance of tissues early in endotoxemia or late in listeriosis.

Responses to inflammatory and infectious challenges should be sufficiently robust to rid the host of an offending agent, yet sufficiently restrained to avoid self-injury. The beneficial and harmful actions of IL-1 are a case in point of this principle. Our experiments using the pathogenic challenges of endotoxin administration and *L. monocytogenes* infection demonstrate an essential role for IL-1ra in maintaining the delicate balance between remedy and harm. Although mice lacking IL-1ra were more susceptible than controls to endotoxin shock, these same mice were protected from a lethal inoculum of *L. monocytogenes*. IL-1ra overexpressors had the opposite responses. Such a dual potential of IL-1ra has also been observed in newborn rats inoculated with live *Klebsiella pneumoniae*, in which survival increased after a moderate dose of IL-1ra but decreased after higher or repeated doses (30). Thus, physiological inhibition of IL-1 by IL-1ra appears to protect the organism from overexuberant responses to infection, but at the risk of impairing the host's ability to eliminate infection. The net outcome of these actions can be unpredictable and may have contributed to the inconsistency of IL-1ra in improving survival in clinical trials of patients with septic shock (31).

We gratefully acknowledge Monica Mendelsohn, Yasushi Saotome, Ichiko Saotome, and Charles Dong for their help with experiments; Lyle Moldawer for reagents and manuscript review; William Dumouchel and Livia Turgeon for assistance with statistics; Heidrun Rotterdam and Steve Brunnert for help with histology specimens; and Leonard Chess for advice on the manuscript. This work was supported in part by National Institutes of Health Grant AI01116 (E.H.) and a National Science Foundation Graduate Fellowship Award (V.M.I.).

1. Dinarello, C. A. (1994) *Eur. Cytokine Network* **5,** 517–531.
2. Waage, A. & Espevik, T. (1988) *J. Exp. Med.* **167,** 1987–1992.
3. Eastgate, J. A., Symons, J. A., Wood, N. C., Grinlinton, F. M., di Giovine, F. S. & Duff, G. W. (1988) *Lancet* **ii,** 706–709.
4. Arend, W. P. (1993) *Adv. Immunol.* **54,** 167–227.
5. Hannum, C. H., Wilcox, C. J., Arend, W. P., Joslin, F. G., Dripps, D. J., Heimdal, P. L., Armes, L. G., Sommer, A., Eisenberg, S. P. & Thompson, R. C. (1990) *Nature (London)* **343,** 336–340.
6. Dripps, D., Brandhuber, B., Thompson, R. & Eisenberg, S. (1991) *J. Biol. Chem.* **266,** 10331–10336.
7. Greenfeder, S. A., Nunes, P., Kwee, L., Labow, M., Chizzonite, R. A. & Ju, G. (1995) *J. Biol. Chem.* **270,** 13757–13765.
8. Muzio, M., Polentarutti, N., Sironi, M., Poli, G., De Gioia, L., Introna, M., Mantovani, A. & Colotta, F. (1995) *J. Exp. Med.* **182,** 623–628.
9. Fischer, E., Marano, M. A., Van Zee, K. J., Rock, C. S., Hawes, A. S., Thompson, W. A., DeForge, L., Kenney, J. S., Remick, D. G., Bloedow, D. C., Thompson, R. C., Lowry, S. F. & Moldawer, L. L. (1992) *J. Clin. Invest.* **89,** 1551–1557.
10. Ohlsson, K., Bjork, P., Bergenfeldt, M., Hageman, R. & Thompson, R. C. (1990) *Nature (London)* **348,** 550–552.
11. Schwab, J. H., Anderle, S. K., Brown, R. R., Dalldorf, F. G. & Thompson, R. C. (1991) *Infect. Immunol.* **59,** 4436–4442.
12. Ferreti, M., Cassini-Raggi, V., Pizarro, T. T., Eisenberg, S. P., Nast, C. C. & Cominelli, F. (1994) *J. Clin. Invest.* **94,** 449–453.
13. Chensue, S. W., Bienkowski, M., Eessalu, T. E., Warmington, K. S., Hershey, S. D., Lukacs, N. W. & Kunkel, S. L. (1993) *J. Immunol.* **151,** 3654–3662.
14. Fujioka, N., Mukaida, N., Harada, A., Akiyama, M., Kasahara, T., Kuno, K., Ooi, A., Mai, M. & Matsushima, K. (1995) *J. Leukocyte Biol.* **58,** 90–98.
15. Watson, J. M., Lofquist, A. K., Rinehart, C. A., Olsen, J. C., Makarov, S. S., Kaufman, D. G. & Haskill, J. S. (1995) *J. Immunol.* **155,** 4467–4475.
16. Makarov, S. S., Olsen, J. C., Johnston, W. N., Anderle, S. K., Brown, R. R., Baldwin, A. S., Haskill, J. S. & Schwab, J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 402–406.
17. Smith, M. F., Eidlen, D., Brewer, M. T., Eisenberg, S. P., Arend, W. P. & Gutierrez-Hartmann, A. (1992) *J. Immunol.* **149,** 2000–2007.
18. Zahedi, K. A., Uhlar, C. M., Rits, M., Prada, A. E. & Whitehead, A. S. (1994) *Cytokine* **6,** 1–9.
19. Dinarello, C. A., Ikejima, T., Warner, S. J. C., Orencole, S. F., Lonnemann, G., Cannon, J. G. & Libby, P. (1987) *J. Immunol.* **139,** 1902–1910.
20. Warner, S. J. C., Auger, K. R. & Libby, P. (1987) *J. Immunol.* **139,** 1911–1917.
21. Granowitz, E. V., Clark, B. D., Vannier, E., Callahan, M. V. & Dinarello, C. A. (1992) *Blood* **79,** 2356–2363.
22. Granowitz, E. V., Vannier, E., Poutsiaka, D. D. & Dinarello, C. A. (1992) *Blood* **79,** 2364–2369.
23. Rogers, H. W., Tripp, C. S., Schreiber, R. D. & Unanue, E. R. (1994) *J. Immunol.* **153,** 2093–2101.
24. Rogers, H. W., Sheehan, K. C. F., Brunt, L. M., Dower, S. K., Unanue, E. R. & Schreiber, R. D. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1011–1015.
25. Havell, E. A., Moldawer, L. L., Helfgott, D., Kilian, P. L. & Sehgal, P. B. (1992) *J. Immunol.* **148,** 1486–1492.
26. Czuprynski, C. J., Brown, J. F., Young, K. M., Cooley, A. J. & Kurtz, R. S. (1988) *J. Immunol.* **140,** 962–968.
27. Pruitt, J. H., Copeland, E. M. & Moldawer, L. L. (1995) *Shock* **3,** 235–251.
28. Li, P., Allen, H., Banerjee, S., Franklin, S., Herzog, L., Johnston, C., McDowell, J., Paskind, M., Rodman, L., Salfeld, J., Towne, E., Tracey, D., Wardwell, S., Wei, F.-Y., Wong, W., Kamen, R. & Seshadri, T. (1995) *Cell* **80,** 401–411.
29. Zheng, H., Fletcher, D., Kozak, W., Jiang, M., Hofmann, K., Conn, C. A., Soszynski, D., Grabiec, C., Trumbauer, M. E., Shaw, A., Kostura, M. J., Stevens, K., Rosen, H., North, R. J., Chen, H. Y., Tocci, M. J., Kluger, M. J. & Van der Ploeg, L. H. T. (1995) *Immunity* **3,** 9–19.
30. Mancilla, J., Garcia, P. & Dinarello, C. (1993) *Infect. Immun.* **61,** 926–932.
31. Fisher, C. J., Jr., Dhainaut, J.-F., Opal, S. M., Pribble, J. P., Balk, R. A., Slotman, G. J., Iberti, T. J., Rackow, E. C., Shapiro, M. J., Greenman, R. L., Reines, D., Shelly, M. P., Thompson, B. W., LaBrecque, J. F., Catalano, M. A., Knaus, W. A. & Sadoff, J. C. (1994) *J. Am. Med. Assoc.* **271,** 1836–1843.