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RESEARCH REPORT

Comparison of In Vivo Effects of Human Recombinant IL 1 and Human Recombinant IL 6 in Mice

RUTH NETA,[1] STEFANIE N. VOGEL,[2] JEAN D. SIPE,[3] GORDON G. WONG,[4] and RICHARD P. NORDAN[5]

[1]*Armed Forces Radiobiology Research Institute, Bethesda, MD 20814*
[2]*Department of Microbiology, Uniformed Services University for the Health Sciences, Bethesda, MD 20814*
[3]*Thorndike Memorial Laboratory, Boston City Hospital and Arthritis Center, Boston University School of Medicine, Boston, MA 02118*
[4]*Genetics Institute, Cambridge, MA 01890*
[5]*Laboratory of Genetics, National Cancer Institute, Bethesda, MD 20892*

ABSTRACT

IL 1 and IL 6 share a number of biological activities, including induction of fever, neutrophilia and acute phase response, and IL 1 induces IL 6 production by fibroblasts and macrophages. Therefore, it was proposed that IL 6 mediates many of the activities of IL 1. To test this hypothesis in vivo, we assessed induction of IL 6 following IL 1α administration to mice and tested IL 6 for radioprotection and induction of early (CSF) and late (fibrinogen and SAA) acute phase reactants. IL 1α given to mice ip induced, in a dose dependent manner, detectable IL 6 in circulation, with maximal titers at 2-4 hrs. However, unlike IL 1 which is radioprotective when administered in doses above 100 ng/mouse, doses of 10-1000 ng/mouse of human recombinant IL 6 did not result in increased survival of mice following lethal irradiation. In fact, such treatment given 20 hrs before $LD_{50/30}$ doses of radiation resulted in reduced survival of mice. However, IL 6 augmented the radioprotective effect of IL 1. IL 1 in doses above 10 ng/mouse induced within 2 to 6 hrs a dose dependent increase in CSF in circulation, but IL 6 did not induce detectable levels of CSF at 2, 6 and 20 hrs after administration. Administration of IL 6 to mice produced a dose dependent increase in circulating fibrinogen and SAA. Similarly, administration of IL 1 resulted in much greater increases in levels of fibrinogen and SAA. Therefore, IL 1 is a more effective inducer of fibrinogen and SAA in mice than is IL 6. Although definitive conclusions concerning the relative roles for IL 1 and IL 6 in vivo will await availability of anti IL 1 and anti-IL 6 antibodies, our data do not support the suggestion that the above IL 1 effects can be attributed solely to IL 6.

INTRODUCTION

IL 1 is recognized as a key inflammatory mediator as evident from its ability to induce in vivo most of the inflammatory manifestations (see reviews 1,2). The use of recombinant IL 1 has eliminated previous uncertainties regarding the composition of the preparations of natural IL 1 that were used for many years for in vivo studies. Such studies were

plagued with concern that partially purified preparations contain some unidentified cytokines in addition to IL 1. Recent work has shown that administration of nanogram quantities of recombinant IL 1 to mice or rabbits results in fever, neutrophilia (3), induction of the acute phase response (4,5), changes in bone marrow population (6), and CSF release (7).

However, IL 1 is known to be induced coordinately with other cytokines (such as TNF), and to induce the production of a cascade of other cytokines such as IL-2 and CSF (8). Thus, a question can be raised as to whether the effects attributed to IL 1 are based on direct effects or due to the induction of a battery of other cytokines. Recently, IL 1 has been shown to induce the release of IL 6 by several cell types including macrophages, endothelial cells, and fibroblasts (9-11). This cytokine was initially described by a number of independent laboratories and called by different names based on various biological activities such Interferon beta 2 (IFNβ2), B Cell Stimulating Factor 2 (BSF2), and Hybridoma/Plasmocytoma Growth Factor (12-16). Upon realizing that all these activities could be attributed to the same recombinant cytokine, it was renamed IL 6.

IL 6 has been identified recently as having the activity of a hepatocyte stimulating factor (17,18), thymocyte costimulating factor (19,20) and endogenous pyrogen (11). Because of the inductive relationship between IL 1 and IL 6, it has been proposed that IL 6 is the direct mediator of a number of activities previously attributed to IL 1. To test this hypothesis, we have assessed the capacity of IL 1 to induce IL 6 in vivo and compared the systemic effects of direct delivery of IL 6 and IL 1 in radioprotection, in induction of early (CSF) and late (SAA, fibrinogen) acute phase reactants, and in induction of changes in bone marrow cell sizing profile. The rationale for such comparison of systemic administration of the two cytokines was based on previous findings that administration of nanogram quantities of IL 1 reproduced the effects of administration of micrograms of LPS (an IL 1 inducer) (21,22). In this report we show that although IL 1 indeed induces systemic production of IL 6, ip delivery of IL 6, up to 3 microgram quantities, does not mimic the above effects of IL 1.

MATERIALS AND METHODS

Mice. B6D2F1 mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Mice were quarantined on arrival and screened for evidence of disease before being released from quarantine. They were maintained in an AAALAC accredited facility in plastic Micro-isolator cages on hardwood chip contact bedding, provided commercial rodent chow and given acidified (HCl to a pH of 2.5) tap water ad libitum. Animal holding rooms were maintained at 70±2 F with 50±10% relative humidity using at least 10 air changes per hour of 100% conditioned fresh air. The mice were on a 12 hour light-dark full spectrum lighting cycle with no twilight. Mice were 8-12 weeks of age when used. All cage cleaning, handling, and injections were carried out in a laminar flow clean air unit.

Cytokines. Human recombinant IL 1α was generously provided by Dr. Peter Lomedico of Hoffman - La Roche, Nutley, NJ. The preparation, lot 1/87, was supplied in 50 mM potassium phosphate and 0.1 M NaCl (pH 6.5) buffer and used on a weight basis. Human recombinant IL 6 in 20 mM NaOAc pH 5.0, specific activity of 2×10^6 CESS units/mg, lot 1190-130 and batch 9, were received from Genetics Institute, Cambridge, MA. This material was reanalyzed in our laboratory in the hybridoma growth factor (HGF) assay (see below) and was found to possess 1.75×10^6 HGF units/mg. All reagents were diluted to the desired concentration in pyrogen-free saline just prior to a single ip injection of 0.5 ml to mice. The cytokine preparations were assayed for LPS contamination in a LAL assay and determined to contain less than 0.1 ng per maximal concentration of inoculum.

Irradiation. Mice were placed in plexiglass containers and were given whole body irradiation at 40 cGy/min by bilaterally positioned ^{60}Co elements. The number of surviving mice was recorded daily for 30 days.

Measurement of IL 6 in the serum. Mice were bled from the orbital plexus at designated times after administration of IL 1 and serum was collected following clot formation. Serum IL 6 activity was determined using the hybridoma growth factor assay described by Aarden et al. (23). This method incorporated the IL 6 - dependent hybridoma, B9, in a conventional microproliferation assay. Briefly, individual serum samples were initially treated to several 10-fold dilutions in the assay medium (RPMI 1640, 10% FCS, $5x10^{-5}$ M 2ME, and 50 ug/ml gentamycin). These samples were then two-fold serially diluted in 96-well culture plates containing 0.1 ml volume of assay medium per well. Two thousand B9 cells in 0.1 ml volume of assay medium were then added to each well (final volume 0.2 ml). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After 72 hrs the cultures were pulsed with 0.5 μC ^{3}H-thymidine for 4 hrs, harvested onto glass fiber filters and counted in a liquid scintillation counter. One HGF unit was defined as the reciprocal of the dilution which yielded 50% of the maximal ^{3}H-thymidine incorporation.

Measurement of CSF activity in the serum. Mice were bled at 2, 6, and 24 hours after injection and serum was collected after clot formation by centrifugation. CSF activity was measured in pooled serum samples collected from 4-5 mice per treatment group per experiment. The bone marrow colony assay for CSF activity has been described in detail (7). Briefly, C3H/HeJ bone marrow cells were enriched for mononuclear cells by density gradient centrifugation on lymphocyte separation medium (Litton Bionetics, Charleston, SC). The cells collected from the interface of the gradient were washed and resuspended in RPMI 1640, supplemented with antibiotics, glutamine, sodium bicarbonate HEPES buffer, and 15% FCS. Three serial two-fold dilutions of each serum sample (30%, 15%, and 7.5% v/v) were prepared in this medium and 0.2 ml of each dilution was added to each of duplicate wells in a 6 well tissue culture plate. A final cell suspension was prepared in $1x10^5$ cells/ml in complete medium supplemented with 0.3% Bacto-Agar (Difco, Detroit, MI) and maintained at 41°C. One ml per well was added immediately after resuspension of the cells in the agar-medium mixture. Once solidified, the cultures were incubated at 37°C, 6% CO_2, for 6-7 days at which time colonies (more than 25 cells per colony) were counted under a dissecting microscope. Serum CSF activity was expressed as colony forming units (CFU) per ml of serum, based on colony count within the linear part of the dilution curve.

Fibrinogen assay. Assays for fibrinogen in diluted citrated plasma were performed by measuring the rate of conversion of fibrinogen to fibrin in the presence of thrombin excess. The calibration was made using the Sigma Diagnostic Kit (Sigma Chemical Co., St. Louis, MO). Measurements of fibrin clot formation were performed on a fibrometer (Becton-Dickenson, Mountain view, CA). The data are expressed as mg of fibrinogen per 100 ml of plasma.

SAA assay. SAA concentration was measured in the serum using an Elisa assay as described (24), with monoclonal rat anti-SAA antibodies prepared according to the method described by Wood et al. (25). Triplicate 200 μl aliquots of diluted serum were analyzed. SAA is expressed in terms of serum amyloid A equivalents, μg/ml, using SAA-rich LDL as a standard.

Statistical analysis. Statistical evaluation of the results was done using Chi-square analysis and z-test.

RESULTS

Induction of IL 6 with IL 1 in vivo. B6D2F1 mice were given ip injections of 1000 ng IL 1 and their sera were assessed for the presence of IL 6 at 0.5, 1, 2, 4, 8, and 24 hrs after treatment. The results (Fig.

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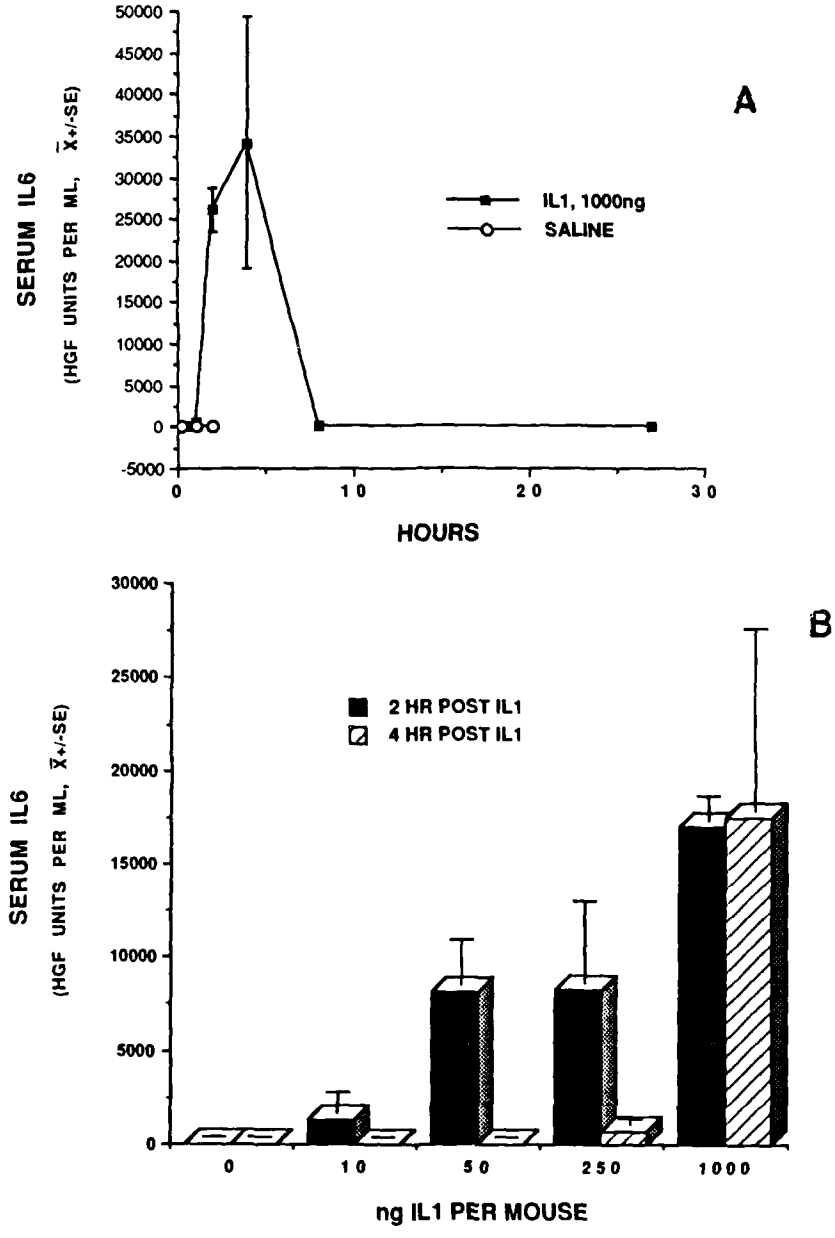

Figure 1. Serum IL 6 levels following IL 1 administration. Time response curves (A) and dose dependence (B) of induction of IL 6 with IL 1 in B6D2F1 mice (n=3 in A and 4 in B).

1) show that IL 1 treatment induced an increase of IL 6 in the circulation beginning at 1 hr, which peaked at 2-4 hrs and declined by 8 hrs after treatment (Fig. 1A). Only 1 out of 4 mice had detectable IL 6 in circulation 2 hrs following administration of 10 ng of IL 1 (Fig. 1B). All 4 mice injected with 50 ng of IL 1 had detectable IL 6 levels at 2 hrs. At these doses of IL 1 (10,50 ng) IL 6 was not detected at 4 hrs. Following 250 ng of IL 1 , low titers of IL 6 were detected at 4 hrs after injection, while 1000 ng of IL 1 resulted in high titers of IL 6 at 2 and

4 hrs. Thus, systemic administration of IL 1 induces dose dependent transient elevation of plasma levels of IL 6 in vivo in mice.

Induction of CSF in circulation. In previous experiments IL 1 in doses as low as 10 ng per mouse induced increases in circulating CSF (7). This induction of CSF by IL 1 was dose dependent, reaching a plateau at 100 to 2000 ng with the maximal titers at 2 to 4 hrs. Presently, high titers of CSF were induced with 1000 ng IL 1 at 2 hrs (6430±233), which declined by 6 hrs (2823±155), but were still detectable at 20 hrs (330±268). In contrast, IL 6 in doses ranging from 100 to 1000 ng did not induce detectable circulating CSF at 2, 6, or 20 hrs. Thus IL 6 and IL 1 differ in their ability to induce circulating CSF.

Effects on bone marrow cell size. Our previous work has shown that IL 1 increases the size distribution of the entire nucleated bone marrow cell population (6). Such increases were detectable with as little as 10 ng IL 1. Presently, 3000 ng of IL 1 ip resulted in increased numbers of large bone marrow cells at 20 and 96 hrs ($p<0.01$)(Fig.2). However, bone marrow cells from mice treated with 3000 ng of IL 6 did not show significant enlargment when compared to saline treated mice. Thus IL 1 and IL 6 differ in their effect on bone marrow cell size distribution.

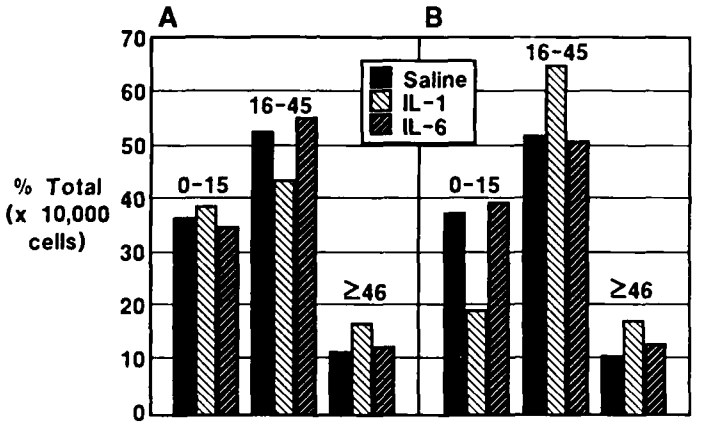

Figure 2. Effect of IL 1 and IL 6 on bone marrow cell size distribution at 20 hrs (A) or 96 hrs (B) after administration. Cells in channel 0-15 were smaller than 160 um^3, in channel 16-45 were 160-320 um^3, and above channel 46 were larger than 320 um^3. (n=3).

Comparison of IL 1 and IL 6 in radioprotection. B6D2F1 mice were given ip injections of IL 1, IL 6, or saline 20 hrs before irradition. Figure 3 summarizes the results of three experiments in which two doses of radiation were used. Following irradiation with 1000 cGy 45% of control, saline treated mice survived. IL 1 protected up to 90% of mice from death, but administration of 10-1000 ng IL 6 resulted in greatly reduced survival (only 5-15%) (Fig. 3A). Similarly, a radiosensitizing effect of IL 6 alone was also observed at 1050 cGy. In this series of three experiments, 15% of control, saline treated mice survived (Fig. 3B). However, suboptimal, non-radioprotective doses of 50 ng IL 1 combined with 1000 ng of IL 6 resulted in synergistic radioprotection ($p<0.05$). Furthermore, even at doses at which IL 1 conferred significant radioprotection (500 ng), combined injection of IL 1 and IL 6 resulted in significantly enhanced ($p<0.05$) survival of mice. Thus, unlike the finding with IL 1, treatment with IL 6 alone did not confer radioprotection. However, combined administration of IL 1 with IL 6 resulted in an enhanced radioprotective effect.

Induction of late acute phase reactants. Our previous work has shown that treatment of mice with IL 1 results in increased SAA and fibrinogen

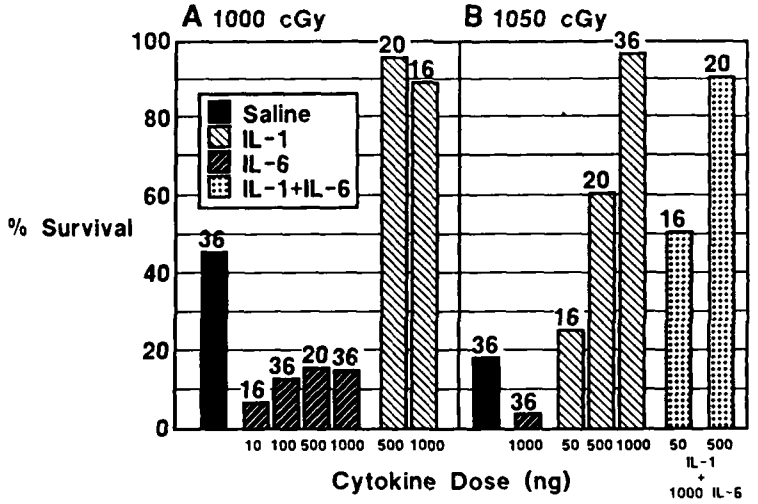

Figure 3. Effect of IL 1α and IL 6 alone and in combination on survival of irradiated mice. B6D2F1 mice were irradiated with 1000 cGy (A) or 1050 cGy (B) 20 hrs following ip administration of IL 1, IL 6, their combination, in the doses indicated, or saline. Numbers on top of the bars indicate the total number of mice/treatment.

in circulation. SAA was detected at 6 and 20 hrs and fibrinogen at 12 and 20 hrs (4,5). When compared on a weight basis with IL 1, IL 6 was a much less potent inducer of fibrinogen than IL 1 (Fig. 4). Similarly IL 6 was a less potent inducer of SAA than IL 1 at 6 and 20 hrs (Table 1).

TABLE 1
In vivo production of SAA in response to IL 1α and IL 6

Treatment	SAA concentration (μg/ml)*		
	2 hrs	6 hrs	20 hrs
Experiment 1			
saline	0.01	0.01	0.01
IL 1, 1000 ng	0.02	260	300
IL 6, 1000 ng	0.11	0.02	0.49
Experiment 2			
saline	0.05		0.05
IL 1, 1000 ng	2.2		490
IL 6, 1000 ng	0.60		1.0

*pool of sera from 3 mice were assayed in each experiment.

DISCUSSION

The foregoing results provide evidence that IL 1 induces IL 6 not only, as previously reported, in cell culture (9-11), but also systemically, as evident by its appearance in circulation. Others have shown increased levels of IL 6, at the time of graft rejection, in the serum and urine of patients receiving kidney transplants (26). Detectable levels of serum IL 6 have also been reported in febrile patients (27). This rise in IL 6 in febrile patients preceded the increase in acute phase response. The induction of release of acute phase proteins, one of the primary manifestations of inflammation, has been previously reported in

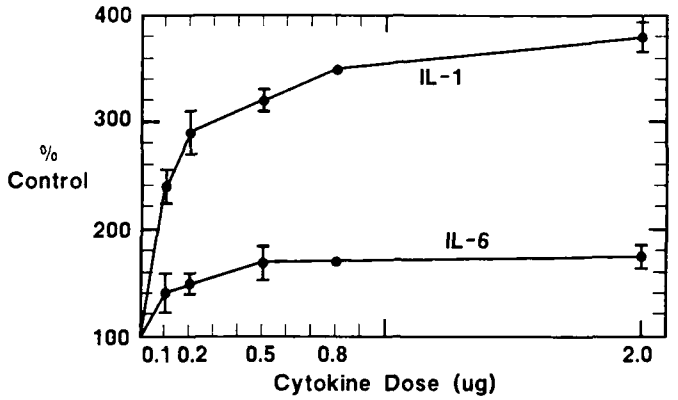

Figure 4. In vivo production of fibrinogen in response to IL 1 and IL 6. B6D2F1 mice were bled 20 hrs following administration of IL 1 or IL 6. The results are the mean of 3 experiments (±SEM), using pooled plasma from 3 mice in each experiment.

vivo (4,5) and in vitro (5,28,29) for IL 1. However, rat primary hepatocyte and human hepatocyte cell lines which produced only restricted spectrum of acute phase reactants in response to IL 1 showed much greater response to IL 6 (18). The induction of IL 6 with IL 1 in several cell types together with identification of IL 6 as a major inducer of acute phase protein synthesis, led to the belief that IL 6, rather than IL 1, may be a direct stimulator of acute phase reactants. Furthermore, based on a broad spectrum of activities of IL 6, some of which overlap with those of IL 1, it was suggested that IL 6 may mediate some of the other activities previously attributed to IL 1.

The experience from this and other laboratories showed that systemic administration of IL 1 reproduced the inflammatory manifestations produced by LPS, an IL 1 inducer. For example, systemical administration of IL 1, like LPS, stimulates the release of CSF (7). Significantly increased levels of CSF were observed after IL 1 doses as low as 10 ng per mouse. The induction of CSF with IL 1 was also demonstrated repeatedly in fibroblasts, endothelial cells, or macrophages in culture (30-33). In the present study, CSF was not detected at 2, 6, or 20 hrs following administration of as much as a 1000 ng dose of IL 6. No evidence exists that IL 6 can induce CSF in cells. Similarly, despite the reported action of IL 6 as Hematopoietin-1 (34), which parallels that attributed to IL 1 (35-37), IL 6 did not induce changes in the size of bone marrow cells characteristic of LPS and IL 1. We are aware that observations on cell size profiles of whole bone marrow populations do not preclude the effect of IL 6 on selected subtypes of such cells. The changes in cell sizes induced with IL 1 in previous studies correlated with increased cell cycling (over 25% increase in population of large cells) and with increase in GM-CFC progenitor cells (6). Thus delivery of 10-100 ng of IL 1 clearly has a much more profound effect on cells in the bone marrow than 1000 ng of IL 6.

In addition, LPS was shown more than 30 years ago to be radioprotective (38,39). Similar radioprotection was reproduced with IL 1 (40). In the present study, however, as much as 1000 ng per mouse of IL 6 did not confer radioprotection. In fact, treatment with IL 6 alone resulted in apparent radiosensitization, i.e. greater numbers of mice died of the radiation syndrome following IL 6 than saline administration. However, this effect was reversed in the presence of 50 ng of IL 1, which alone was not radioprotective, but which synergized with IL 6 in radioprotection. This result indicates that IL 6 in the presence of IL 1 is a radioprotective agent. Induction with IL 1 of bone marrow cell cycling and CSF's may

present some of the necessary prerequisites for interacting with IL 6 in radioprotection. It is difficult at present to explain the radiosensitizing effect of IL 6 in the absence of IL 1. Perhaps, as suggested by Dr. Charles Dinarello (personal communication), IL 6 suppresses endogenous production of IL 1 which may result in enhanced susceptibility to radiation.

IL 6 was also less efficacious than IL 1 in systemic induction of fibrinogen and SAA. Production of fibrinogen, α_1-antichymotrypsin, α_2-macroglobulin, and cysteine proteinase inhibitor was eliminated by the presence of anti-IFN-β2/IL 6 antibody in primary rat hepatocytes and human hepatocyte lines stimulated with supernatants which contained both IL 1 and IL 6 (18). Such treatment, however, did not eliminate α_1-acid glycoprotein or haptoglobin production. Yet the use of anti-IL 1 and anti-IL 6 antibodies in combination abrogated production of all examined acute phase proteins. Similarly, reduction of albumin synthesis was maximally reversed using the anti-IL 1 and anti-IL 6 antibodies in combination. More recent results (Dr. Jack Gauldie, personal communication) indicate that genes for SAA or α_1-acid glycoprotein may be induced with IL 1 alone, while other genes, such as fibrinogen, are not induced with IL 1. Thus our results showing less effective in vivo induction of fibrinogen with IL 6 than IL 1, may indicate that IL 1 may be necessary at the local site to induce sufficient endogenous levels of IL 6, which, in turn, may directly act to induce fibrinogen message. Final resolution of the mechanisms which function in vivo in the induction of various subclasses of acute phase reactants will depend on the selective in vivo elimination of IL 1 and IL 6 with sufficient quantities of specific antibody.

The results presented in this study, and work of others to date, already indicate that IL 1 and IL 6, although induced in sequence have diverse as well as overlapping activities, but the relative contribution of each of these cytokines to the inflammatory processes remain to be established.

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Address reprint requests to:
Dr Ruth Neta
Armed Forces Radiobiology Research Institute
Bethesda, MD 20814