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IN VIVO STIMULATION AND RESTORATION OF THE IMMUNE RESPONSE BY THE NONINFLAMMATORY FRAGMENT 163-171 OF HUMAN INTERLEUKIN 1β

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IL-1 is a family of closely related proteins deeply involved in the development and maintenance of the inflammatory response. In fact, IL-1 appears identical to the endogenous pyrogen responsible for the increase in basal temperature after infectious events (1, 2); it provokes the changes in plasma levels of divalent cations and acute-phase proteins typical of the inflammatory condition (1, 2); it is involved in the alteration of corticosterone levels and of glucose homeostasis (3, 4) and in the catabolic processes associated with infection (1, 2); it induces neutrophilia and the release of prostaglandins and proteases in different cells (1, 2); and it alters hepatic drug metabolism (5). Besides its important role in stimulating the acute-phase defense response of the host during the infectious event, IL-1 is involved in, and possibly responsible for, the development of chronic inflammatory conditions such as rheumatic diseases and degenerative arthropathies, including rheumatoid arthritis (6, 7).

On the other hand, IL-1 appears to play a key role in the regulation of immune responses. Indeed, IL-1 participates in antigen-induced T cell activation and clonal expansion by triggering IL-2 and IL-4 production and IL-2-R expression (8–10). In addition, IL-1 is one of the factors involved in B cell differentiation and proliferation (11–13) and it has hematopoietic activity for the earliest precursors in the bone marrow (14). Possibly as a consequence of these stimulatory effects, the in vivo administration of IL-1 can enhance the immune response to different antigens (15–18) and it can protect the host from lethal challenges with microorganisms (19, 20) or tumors (21), and from lethal radiation damage (22).

Thus, IL-1 is now considered as a potential candidate for the innovative biopharmacology foreseen for the next decade. With the aim of better defining its clinical use, several attempts have been directed at the identification of minimal biologically active sites of IL-1, which may be responsible for individual effects, among the mul-

tiple activities ascribed to IL-1 (23–28). Indeed, a nine residue-long synthetic peptide, corresponding to the highly hydrophylic fragment 163–171 of human IL-1β (VQGEESNDK), was observed to mimic some of the immunostimulatory activities of the entire IL-1 molecule, while being nonpyrogenic and unable to stimulate prostaglandin production by in vitro cultured fibroblasts (17, 18, 26).

This prompted us to further investigate the potential therapeutic applications of the 163–171 peptide. In the present report, the in vivo activities of the synthetic nonapeptide have been examined in a variety of experimental systems. The IL-1β fragment shows immunostimulatory, immunorestorative, antitumoral, and radioprotective capacities comparable, in several instances, to those of the entire protein. On the other hand, the same peptide could not reproduce any of the inflammation-related and toxic in vivo effects shown by IL-1β.

Materials and Methods

Animals. C3H/HeJ mice were obtained from the The Jackson Laboratory (Bar Harbor, ME) or from Bomholtgaard (Ry, Denmark). C3H/HeN, BALB/c, and CD-1 mice were purchased from Charles River Breeding Laboratories (Calco, Italy). Homozygous *nu/nu* and heterozygous *nu/+* mice (genetic background BALB/c) were provided by Bomholtgaard. Hybrid (C57BL/10 × DBA/2) F_1 mice (B10D2F_1) were bred in the animal facilities of ENEA (Roma, Italy). Mice of both sexes were used between 6 and 12 wk of age, unless otherwise indicated.

IL-1 and Synthetic Peptides. Different preparations of human rIL-1β were used throughout these studies with superimposable results. Fragment 112–269, expressed in *Escherichia coli* and prepared as described elsewhere (3), was kindly provided by Dr. C. A. Dinarello, Tufts University, Boston, MA. The mature recombinant fragment 117–269, expressed in *E. coli*, was either obtained from Biogen (Geneva, Switzerland) or produced at Sclavo (Siena, Italy). All preparations, purified to apparent homogeneity, had a specific activity of 1–2 × 10^7 half-maximal units/mg protein (corresponding to 1.75–3.5 × 10^{14} U/mole) in the murine thymocyte costimulation assay. Endotoxin contamination was measured in the two 117–269 preparations with the LAL chromogenic assay (M. A. Bioproducts, Walkersville, MD) and found to be <2.0 pg/μg IL-1. In selected experiments, two other IL-1 were used with comparable results: a highly purified preparation of natural monocyte-derived IL-1 (mostly β form; generously provided by C. A. Dinarello), and human rIL-1α (mature fragment 113–271, expressed in *E. coli*, sp act 2 × 10^7 U/mg; a kind gift of Dr. P. T. Lomedico, Hoffmann-La Roche, Inc., Nutley, NJ).

The 163–171 peptide of human IL-1β (VQGEESNDK, mol mass 1 kD) was synthesized by the solid-phase method and purified by HPLC as described (26). Two salts of the peptide, 163–171 trifluoroacetate (163–171·TFA)[1] and 163–171 hydrochloride (163–171·HCl) were provided by Novabiochem (Läufelfingen, Switzerland). The 163–171·TFA salt was estimated to contain one-half the equivalent of TFA/peptide mole. Transformations of the TFA salt into HCl salt and vice-versa were performed by ion-exchange chromatography on Amberlite IRA 401 (Cl) resin (BDH Italia, Milan, Italy).

Two other nonapeptides, LKDDKPTLQ (fragment 189–197 of human IL-1β) and FRKDMDKVE (fragment 166–174 of human chorionic somatomammotropin, hCS), corresponding to highly hydrophylic moieties of the proteins, were also synthesized by the solid-phase method.

All peptide preparations were found to be free of endotoxin contamination by the LAL chromogenic assay.

Assays for In Vivo Immunostimulation. Determination of specific antibody-producing cells (PFC) in the spleen of mice was performed as previously described in detail (17). Briefly, mice received a single antigen inoculum (SRBC, 1–2 × 10^8/mouse i. v.; or pneumococcal

[1] *Abbreviations used in this paper:* hCS, human chorionic somatomammotropin; HRBC, horse red blood cells; SAA, serum amyloid A; TFA, trifluoroacetate.

polysaccharide type III, SIII, 0.5 μg/mouse i. p.) or two SRBC inocula 10 d apart (secondary response). At the time of the antigenic challenge, different doses of IL-1 or of peptide preparations were administered to the animals either intravenously, intraperitoneally, or subcutaneously. The numbers of specific PFC in the spleen of mice were determined either 4 d (response to SRBC) or 5 d (response to SIII) later by the Cunningham and Szenberg slide technique (29).

Helper T cell activity was titrated in the spleen of carrier (horse RBC [HRBC])-primed mice as previously described (30). Briefly, mice received an inoculum of 2×10^5 HRBC i.v. and an s.c. injection of either IL-1 or peptide. After 4 d, graded numbers of carrier-primed spleen cells ($5–20 \times 10^4$ cells/culture) were cultured with 10^6 spleen cells from untreated mice and 2×10^5 TNP-HRBC. The anti-TNP-specific PFC response was evaluated in the cultures after 4–5 d by the slide technique. Only PFC numbers of cultures receiving 20×10^4 carrier-primed spleen cells are reported in the results.

Radioprotection. 24 h after inoculation with saline, IL-1β, or the 163–171 peptide, groups of 20 mice were lethally irradiated in a Lucite irradiation chamber with an x-ray dose of 850 rad (dose rate 138.7 rad/min in air) with an x-ray machine (Stabilipan, Siemens Italia, Milan, Italy), operated at 250 kV, 15 mA, 0.5 mm Cu filtration, focus distance 50 cm. Survival was followed for >5 mo, although no further deaths were observed beyond the 27th day after irradiation. Percentage of surviving mice and mean survival time of mice that eventually died were evaluated.

Tumor Rejection. BALB/c mice were challenged subcutaneously in the left inguinal region with 10^3 cells of the poorly immunogenic syngeneic tumor CE-2 (31). Starting 4 h after tumor challenge, mice received in the peritumoral area 10 daily subcutaneous injections of 0.4 ml HBSS alone, or containing IL-1β, the 163–171 peptide of IL-1β, or the 166–174 peptide of hCS. The incidence and growth of tumors were recorded twice weekly and the neoplastic masses were measured with calipers in the two perpendicular diameters. The intervals (in days) between challenge and the observation of tumors larger than 4 and 12 mm were regarded as the latency and survival time, respectively. Mice were then killed for humane reasons (32).

Fever. Increase in basal temperature of endotoxin-resistant C3H/HeJ mice was measured as previously described (26, 33). Mice were kept at room temperature for 1 h before intravenous injection of saline, IL-1, or the peptide. Rectal temperature was monitored every 5 min and expressed as increase compared with the preinjection temperature (ΔT°).

Biochemical Determinations. Serum iron was determined 4 h after intravenous treatments, with a commercially available kit (Boehringer-Mannheim, Mannheim, Federal Republic of Germany).

Fibrinogen was measured in EDTA-treated plasma obtained 24 h after intravenous treatments by the heat-turbidity method of Wycoff (34).

Serum amyloid A (SAA) was evaluated in serum samples by solid-phase competitive RIA (35). Briefly, 25-μl serum samples were heated at 60°C in tightly capped tubes for 1 h, then diluted with 1 ml casein barbital buffer. Triplicate 200-μl aliquots of the diluted samples were analyzed by RIA. SAA was expressed as micrograms of amyloid A equivalents/milliliter.

Serum levels of corticosterone and insulin were determined by RIA, and serum levels of glucose by the hexokinase enzymatic method, as described elsewhere (3, 4). Determinations were performed 2–4 h after intraperitoneal or subcutaneous treatments.

Cytochrome P450-dependent ethoxycoumarin-*o*-deethylase (ED) activity was assessed in liver homogenates of mice treated 24 h earlier, according to Greenlee and Poland (36).

Shock in Adrenalectomized Mice. Determination of IL-1-induced shock and death in adrenalectomized mice was performed as previously described (37). Adrenalectomy was performed under ether anesthesia 10 d before treatment. Adrenalectomized mice were given 1% wt/vol sodium chloride in the drinking water. Mice received an intravenous inoculum of sterile pyrogen-free saline alone or containing IL-1 or the peptide, then monitored for 7 d. All deaths occurred within 24 h.

Statistical Analysis. Data (except those relating to survival) are calculated as the mean ± SEM of values of 3–10 individual animals tested within single representative experiments, or of values from different experiments. Statistical significance was calculated by Student's *t* test.

For the evaluation of helper T cell activity, statistical significance was determined by a two-tailed Student's *t* test on the regression coefficients of straight lines forced through the origin by the least squares method (38) of the titration curves.

Tumor inhibition experiments were performed independently at least three times. Those giving homogeneous results, as determined by the Snedecor and Irwin test (39), were cumulated. The significance of differences in tumor takes was determined by Fisher's exact method, while those in latency and survival time were determined by a one-tailed Student's *t* test.

Survival of treated versus control animals in the experiments of radioprotection and shock after adrenalectomy was evaluated by Fisher's exact method. Differences in the mean survival time of lethally irradiated mice were determined by analysis of variance.

Results

Intravenous administration of the synthetic fragment 163–171 of human IL-1β together with the T-dependent antigen SRBC could greatly increase the primary immune response against the antigen, measured as the number of specific antibody-producing cells (PFC) in the spleen 4 d after immunization (Fig. 1). Two preparations of the peptide were assayed and compared with the mature human rIL-1β. The immunoenhancing effects of human rIL-1β and of the 163–171·HCl preparation were qualitatively and quantitatively similar. In contrast, 10^4-10^6-fold higher doses of the 163–171·TFA peptide preparation were needed to attain maximal immunostimulatory effects. The discrepancy between optimally active doses of the two peptide preparations could be fully ascribed to the presence or absence of TFA. In fact, conversion of the TFA salt of the peptide into HCl salt could significantly increase its adjuvant activity (Fig. 2, *left*). Accordingly, conversion of the HCl salt into TFA salt dramatically lowered the immunostimulatory effectiveness of the peptide (Fig. 2, *middle*). Finally, addition of free TFA to the 163–171·HCl preparation or to human rIL-1β could equally decrease their adjuvant effects (Fig. 2, *right*). TFA was found to interfere with peptide activities in vivo only by increasing the optimal active dosage by several orders of magnitude, without any qualitative effect on the response obtained (data not shown).

Besides its effect on the primary response to a T-dependent antigen, the immunoenhancing activity of the synthetic IL-1β peptide, as compared with the entire molecule, was also evident in the secondary response to SRBC and in the response to the T-independent antigen SIII (Table I). In contrast, neither the nonapeptide nor human rIL-1β could affect the number of spontaneous PFC anti-SRBC in the spleen of nonimmunized mice (Table I).

Administration of the synthetic IL-1β fragment could also restore or significantly augment the immune response in immunodepressed animals (Table II). In most of the immunosuppressive conditions examined (treatment with cyclophosphamide,

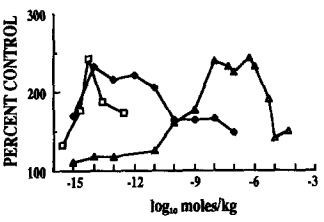

FIGURE 1. In vivo stimulation of the primary response to SRBC by human rIL-1β (□), the 163–171·TFA peptide (△), and the 163–171·HCl peptide (◆). Increasing doses of the different molecules were inoculated intravenously together with the antigen SRBC. The SRBC-specific PFC response in the spleen was evaluated 4 d later and expressed as percent of the control response of animals receiving the antigen alone. Data are the mean values from 45 separate experiments. SEM never exceeded 10% and are thus not shown. Mean anti-SRBC PFC/spleen of control animals in the experiments considered were 37,926 ± 840.

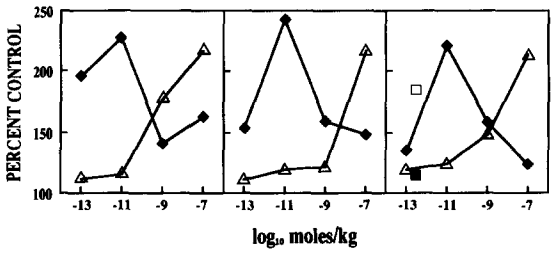

FIGURE 2. Comparison of the immunostimulatory activity of the 163-171·TFA peptide with that of the 163-171·HCl peptide. The primary anti-SRBC PFC response was evaluated in mice immunized with SRBC and inoculated intravenously with increasing doses of the adjuvant peptides. *Left*, 163-171·TFA peptide (△); 163-171·TFA transformed into 163-171·HCl (◆). *Middle*: 163-171·HCl peptide (◆); 163-171·HCl transformed into 163-171·TFA (△). *Right*: 163-171 HCl (◆); 163-171·HCl with addition of one-half equivalent of TFA (△); human rIL-1β (□); human rIL-1β with addition of one-half equivalent of TFA (■). Results are from single experiments representative of six performed and are expressed as percent of the control response of animals receiving the antigen alone. SEM never exceeded 10% and are thus not shown. Mean anti-SRBC PFC/spleen of control animals in the experiments considered were 35,709 ± 1,596.

aging, sublethal irradiation), the immune response of suppressed animals could be restored by inoculation of the nonapeptide to levels comparable to those of the untreated immunocompetent controls. However, in the case of genetically athymic *nu/nu* mice, full restoration of the immune response could not be achieved by administra-

TABLE I

Immunostimulatory Activity of the 163-171 Fragment on Spontaneous, Primary, and Secondary Response In Vivo

Immune response	Antigen	PFC	Specific PFC/spleen (percent control) of mice treated with: Saline	IL-1β	163-171
Spontaneous	SRBC*	Direct	203 (100.0)	227 (112.1)	194 (95.8)
Primary	SRBC‡	Direct	38,357 (100.0)	89,317 (232.9)¶	89,699 (233.9)¶
	SIII§	Direct	1,070 (100.0)	3,500 (327.3)¶	3,116 (291.4)¶
Secondary	SRBC‖	Total	32,160 (100.0)	69,957 (217.5)¶	70,480 (219.2)¶
		Direct	9,397 (100.0)	NT**	16,458 (175.1)¶
		IgG	24,722 (100.0)	NT	54,021 (218.5)¶

Results are the mean of data from 2 to 27 separate experiments, each including three individual mice per experimental group. Both human rIL-1β and the 163-171 peptide were inoculated 4-5 d before assay, at previously determined optimal doses, either intravenously or intraperitoneally. Either C3H/HeN or C3H/HeJ mice were used, with identical results.

* Direct PFC anti-SRBC were measured in the spleen of nonimmunized C3H/HeJ male mice 4 days after administration of hu rIL-1β (6×10^{-12} moles/kg i.p. or 10^{-15} moles/kg i.v.) or the 163-171·TFA peptide (10^{-4} moles/kg i.p. or 10^{-8} moles/kg i.v.).

‡ Mice received either saline or human rIL-1β ($0.1\text{-}3 \times 10^{-14}$ moles/kg) or the 163-171·HCl fragment (10^{-14}-10^{-12} moles/kg) i.v. together with the antigen. Splenic PFC anti-SRBC were measured 4 d later. In other experiments, the 163-171·TFA peptide was active at 10^{-8}-10^{-6} moles/kg by the same route. When administered i.p., human rIL-1β was active at $0.6\text{-}6 \times 10^{-12}$ moles/kg, the 163-171·TFA peptide at $0.3\text{-}1 \times 10^{-4}$ moles/kg, and the 163-171·HCl salt at 10^{-10}-10^{-6} moles/kg.

§ The anti-SIII splenic PFC response was determined 5 d after antigen administration and inoculation of saline, human rIL-1β ($0.5\text{-}1 \times 10^{-12}$ moles/kg i.p.) or 163-171·HCl peptide (10^{-13} moles/kg i.v.). In other experiments, the 163-171·TFA salt was active at 10^{-4} moles/kg when administered i.p.

‖ Direct and total (direct plus IgG) PFC anti-SRBC were measured in the spleen of C3H/HeN mice immunized twice with SRBC, 4 d after the last antigen inoculum. Saline, human rIL-1β ($0.3\text{-}2 \times 10^{-12}$ moles/kg i.p.), or the 163-171·HCl peptide (10^{-11}-10^{-9} moles/kg i.v.) were administered with the second antigen challenge. In other experiments, the 163-171·TFA fragment was active at 10^{-7} moles/kg i.v. and at 10^{-4} moles/kg i.p.

¶ $p < 0.01$ vs. saline control.

** NT, not tested.

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TABLE II

Immmunorestoration by the 163–171 Fragment In Vivo in Immunodepressed Mice

Immunosuppressive condition	Response measured	Response after treatment with: Saline	IL-1β	163–171
Immunosuppressive drugs*	PFC/spleen			
Control		42,170	84,324¶	78,442¶
Cyclophosphamide		26,779	75,335¶	78,525¶
Genetic deficiency‡	PFC/spleen			
nu/+		38,038	115,232¶	121,665¶
nu/nu		693	2,660¶	3,480¶
Age§	PFC/culture			
Young		633	812	1,334¶
Old		162	227¶	501¶
Irradiation‖	PFC/culture			
Control		278	317	473¶
200 rad		126	314¶	485¶

Results are the mean of data from two to five separate experiments. Both human rIL-1β and the 163–171 peptide were inoculated 4 d before assay, at previously determined optimal doses, either intravenously or subcutaneously.

* C3H/HeN male mice received 50 mg/kg i.p. of cyclophosphamide 2 d before inoculation of SRBC. The 163–171·HCl fragment (10^{-11} moles/kg) or human rIL-1β (0.3×10^{-13} moles/kg) were injected intravenously together with the antigen. Splenic PFC anti-SRBC were measured 4 d after. The 163–171·TFA fragment was active at 5×10^{-5} moles/kg i.p. in the same system.

‡ Splenic PFC anti-SRBC were measured in heterozygous (*nu/+*) and athymic (*nu/nu*) BALB/c male mice 4 d after antigen administration. Human rIL-1β (0.6×10^{-14} moles/kg i.v. or 0.6×10^{-11} moles/kg i.p.) and the 163–171·HCl peptide (10^{-11}–10^{-7} moles/kg i.v.) were inoculated together with the antigen. In other experiments, the 163–171·TFA fragment was active at 10^{-7} moles/kg i.v. and at 10^{-5}–10^{-4} moles/kg i.p.

§ Anti-TNP PFC were measured after 4–5 d of culture of 10^6 normal spleen cells with TNP-HRBC and 20×10^4 spleen cells from young (3 mo) or old (15 mo) B10D2F$_1$ male mice inoculated 4 d earlier with HRBC intravenously and with human rIL-1β (10^{-12} moles/kg) or with the 163–171·TFA synthetic fragment (10^{-4} moles/kg) s.c. Mean background anti-TNP response of 10^6 normal spleen cells cultured with TNP-HRBC without added helper cells was of 112 PFC/culture.

‖ Anti-TNP PFC were measured as described above. B10D2F$_1$ male mice were either left untreated or irradiated with 200 rad (x-ray irradiation, Stabilipan) 7 d before intravenous priming with HRBC and subcutaneous administration of human rIL-1β (10^{-12} moles/kg) or the 163–171·TFA peptide (10^{-4} moles/kg). Mean background anti-TNP response in the absence of added helper cells was of 38 PFC/culture.

¶ $p < 0.01$ vs. saline control.

tion of either the synthetic fragment or human rIL-1β, although a highly significant increase in the response could nevertheless be observed (Table II).

Finally, survival of mice undergoing lethal irradiation and tumor engraftment was significantly improved by the 163–171 fragment of human IL-1β, as well as by the entire protein (Table III). In fact, 50 and 75% of mice receiving IL-1β and the nonapeptide, respectively, 24 h before 850 rad irradiation were still alive after >120 d, whereas all control mice died within 16 d. Similarly, significant survival after a lethal tumor challenge was induced in mice by treatment with either the 163–171 fragment or IL-1β, in addition to increasing both the latency time and the survival time of mice developing the tumor (Table III). In another set of experiments, it was found that both IL-1β and 163–171 peptide are nontoxic in vitro to proliferating tumor

TABLE III

Protection by the 163–171 Fragment from Lethal Irradiation and Tumor Graft

In vivo treatment	Survival		Latency time ± SEM	Survival time ± SEM
	Alive/total	Percent surviving		
Radioprotection*				
Saline	0/40	0	–	10.0 ± 0.5
IL-1β	10/20	50§	–	14.8 ± 1.7§
163–171	15/20	75§	–	14.2 ± 3.2
Tumor rejection‡				
Saline	1/20	5	14.5 ± 2.1	25.6 ± 3.2
IL-1β	12/20	60§	25.2 ± 6.3§	36.8 ± 2.7§
163–171	11/20	55§	21.0 ± 3.2§	35.9 ± 3.0§

* $B10D2F_1$ female mice (2 mo old) received an intraperitoneal inoculum of saline alone or containing human rIL-1β (0.6×10^{-9} moles/kg) or the 163–171·TFA peptide (10^{-5} moles/kg) 20 h before irradiation with 850 rad. 40 of 40 saline-injected mice were dead 16 d after irradiation. Mice were followed for up to 5 mo, with no further deaths observed 27 d after irradiation.

‡ BALB/c female mice (8 wk old) were engrafted subcutaneously with 10^3 cells of the poorly immunogenic syngeneic tumor CE-2. Beginning 4 h later, mice received 10 daily injections of saline (HBSS), human rIL-1β (0.6×10^{-16} moles/mouse/day), or 163–171·HCl peptide (10^{-15} moles/mouse/day) subcutaneously in the peritumoral area. Mice receiving the 166–174 peptide of hCS with the same schedule of treatment did not differ from saline-treated controls (surviving/total, 0/20) (data not shown).

§ $p < 0.05$ vs. saline control.

cells and that the capacity to reject the tumor challenge rests fully on their ability to recruit host immunoreactivity (Forni, G., T. Musso, D. Boraschi, C. Jemma, A. Tagliabue, and M. Giovarelli, submitted for publication).

In contrast to the high IL-1-like immunoenhancing and protective effects, the 163–171 fragment could not mimic any of the inflammation-related activities of human IL-1β measured in vivo. In fact, the nonapeptide did not induce a rise in the basal temperature of C3H/HeJ mice (Table IV). In parallel to its lack of pyrogenicity, the nonapeptide could not provoke the acute phase-associated changes induced by IL-1 such as hypoferremia, decrease of blood levels of fibrinogen and glucose, decrease of hepatic P450-dependent metabolism, increase of serum levels of SAA, corticosterone, and insulin (Table IV). Possibly as a consequence of the lack of inflammatory effects in vivo, the 163–171 fragment could not induce shock and death in adrenalectomized mice, in contrast to the potent toxic effect of the entire IL-1 molecule in the same system (Table IV). The lack of inflammatory effects of the 163–171 peptide was evident in each experimental system over a wide range of doses, exceeding up to 10^6-fold the optimal immunostimulatory doses (data not shown). In addition, two other synthetic nonapeptides, corresponding to highly hydrophylic sequences of human IL-1β (LKDDKPTLQ, position 189–197) and of hCS (FRKDMDKVE, position 166–174) did not show either immunostimulatory or inflammatory activities when tested in parallel to the 163–171 fragment of IL-1β (data not shown).

Discussion

Potentiation and/or restoration of the host immune defenses has been attempted for many years in the therapy of several diseases, including immunodeficiencies, infections, and cancer, first by the use of bacterial immunomodulators and more re-

TABLE IV

Lack of Inflammatory and Toxic Effects by the 163–171 Peptide of Human IL-1β in vivo

Response measured	Response after treatment with: Saline	IL-1	163–171
Fever* (ΔT°)	0.15 ± 0.05	1.25 ± 0.25§§	0.15 ± 0.05
Blood levels of:			
Iron‡ (μg/ml)	1.27 ± 0.14	0.77 ± 0.10§§	1.12 ± 0.10
Fibrinogen§ (mg/ml)	1.19 ± 0.17	2.20 ± 0.08§§	1.35 ± 0.21
Glucose‖ (mg/dl)	110.00 ± 5.37	69.01 ± 2.63§§	122.90 ± 3.48
SAA¶ (μg/ml)	3.48 ± 1.23	100.00 ± 39.56§§	2.50 ± 0.65
Corticosterone‖ (μg/100 ml)	3.90 ± 1.36	30.90 ± 2.14§§	2.57 ± 0.40
Insulin‖ (pg/ml)	760.0 ± 103.7	2,033.0 ± 377.0§§	832.0 ± 61.9
Hepatic ED** (nmoles/min/g)	18.59 ± 1.35	12.16 ± 0.63§§	16.03 ± 1.10
Shock after adrenalectomy‡‡ (mortality)	0/10 (0%)	7/10 (70%)§§	0/5 (0%)

Results are the mean ± SEM of values from 4 to 10 mice tested within single representative experiments.

* Increase of basal temperature was measured in C3H/HeJ male mice 25 min after intravenous inoculation of 0.6×10^{-11} moles/kg human IL-1β or $3\text{–}6 \times 10^{-4}$ moles/kg 163–171·TFA peptide.

‡ Serum iron levels were evaluated in CD-1 male mice 4 h after intravenous inoculation of 1.7×10^{-9} moles/kg human rIL-1α or 3×10^{-6} moles/kg 163–171·HCl peptide. In other experiments, human rIL-1β was found to have the same qualitative and quantitative effects as human rIL-1α (not shown).

§ Fibrinogen was measured in plasma of CD-1 male mice 24 h after intravenous injection of 1.7×10^{-9} moles/kg human rIL-1α or 3×10^{-6} moles/kg 163–171·HCl fragment. In other experiments, human rIL-1β was found to be active in the same dose range as human rIL-1α, and the 163–171·TFA peptide did not show any activity at doses up to 10^{-4} moles/kg.

‖ Serum levels of glucose, corticosterone, and insulin were determined in C3H/HeJ male mice 2 h after administration of human rIL-1β (10^{-11} to 2×10^{-10} moles/kg) or of the 163–171·TFA peptide (2 to 4×10^{-4} moles/kg) i.p. or s.c.

¶ SAA was measured in serum of CD-1 male mice that received 6 h earlier an inoculum of human rIL-1β (1.7×10^{-12} moles/kg) or of the 163–171·HCl peptide (3×10^{-8} moles/kg) i.v.

** Cytochrome P450-dependent ED activity was evaluated in the liver of CD-1 male mice 24 h after administration of human rIL-1α (1.7×10^{-9} moles/kg) or of the 163–171·HCl fragment (3×10^{-6} moles/kg) i.v. In other experiments, human rIL-1β was found to be as active as human rIL-1α, and the 163–171·TFA peptide did not have activity at doses up to 10^{-4} moles/kg.

‡‡ Shock in adrenalectomized CD-1 male mice was assessed as mortality (dead/total; percent dead animals) after administration of human rIL-1β (10^{-9} moles/kg) or of the 163–171·HCl peptide (3×10^{-8} moles/kg) i.v. All deaths occurred within 24 h from IL-1 inoculum.

§§ $p < 0.01$ vs. saline control.

cently with synthetic bacterial moieties (40). The increasing knowledge of the biology of the soluble factors involved in cell-to-cell communication has clearly indicated that most of the activities of the classical immunomodulators could be mediated through the induction of interleukins, interferons, and growth factors. These cytokines, now available in large quantities by means of recombinant DNA techniques, are thus beginning to be tested directly in clinical trials. This new generation of immunomodulators represents an improvement in comparison to the previous agents, such as whole bacteria (bacille Calmette-Guérin and *Corynebacterium parvum*) or fragments (levamisole and pyran copolymer). However, the variety of biological effects of each cytokine makes the appropriate clinical exploitation of these factors still difficult.

A further step towards a less empiric immunotherapy may be represented by the identification, within the cytokine molecules, of active sites responsible for certain biological functions. This information is expected to provide the basis for the design of cytokine analogs that are more suitable for pharmaceutical use in terms of stability, biodisposition, and affinity for the receptor.

An example for the feasibility of this approach has come from studies directed to elucidate the relationship between structure and function of IL-1 (17, 26). A short synthetic peptide (VQGEESNDK, corresponding to the fragment 163–171 of human IL-1β), was chosen as possible active site on the basis of its predicted exposure on the surface of the IL-1β protein molecule. Upon a first analysis of its biological activities, the nonapeptide was shown to mimic several of the immunostimulatory effects of IL-1 in vitro and in vivo, although it was not pyrogenic, nor could it induce PGE_2 synthesis in cultured human fibroblasts. This fragment could thus represent one of the active sites of human IL-1β, responsible for its immunomodulatory effects. However, peptide doses up to five to six orders of magnitude higher than those of IL-1 were necessary to reach the biological effect of the entire molecule. The holistic interpretation that other fragments of the molecule are necessary to determine the extremely high activity of IL-1 was proposed to explain the differences in dosage (26). In contrast to this, it was observed in this study that after transformation of the peptide from the TFA salt to the HCl, the immunostimulatory activity in vivo was increased up to 10^6-fold. On the other hand, in vivo administration of IL-1 together with free TFA at a concentration equivalent to that contained in the peptide preparation resulted in a strong reduction of the IL-1 adjuvant activity. This demonstrates that, at least as far as adjuvanticity is concerned, the peptide and the entire IL-1 are equivalent also on a quantitative basis. Thus, the hypothesis that the 163–171 fragment of IL-1 is the active site for immunomodulation gains further experimental support. Conversely, the result that TFA is somehow able to reduce IL-1 functions constitutes a warning that false negative results are likely to be obtained with synthetic peptides of IL-1 obtained by classic procedures, since TFA is a reagent widely used in peptide synthesis and purification.

The analysis of the in vivo immunomodulatory capacity of the 163–171 peptide was extended to the immunocompromised host. The nonapeptide could exert an adjuvant activity at least equivalent to that of the entire IL-1 in animals immunologically depressed by chemotherapy or irradiation as well as in genetically immunodeficient or aged mice. It can therefore be concluded that the nonapeptide is able to restore depressed immunological functions.

The observation that the 163–171 peptide was able to restore immune reactivities after sublethal irradiation, together with previous results of other authors showing the radioprotective capacity of IL-1 (22), prompted us to investigate whether the nonapeptide could also share with IL-1 this latter activity. Indeed, this was the case. Moreover, treatment with the 163–171 peptide can also result in significant protection against a transplanted tumor. In fact, repeated local injection of small amounts of the 163–171 peptide at the tumor challenge site reduces tumor takes of a poorly immunogenic syngeneic sarcoma. Latency and survival times of those peptide-treated mice that eventually develop tumors are extended in comparison to controls. Here, again, peptide activity is equivalent to that of IL-1. Data not reported here show that the tumor growth inhibition is due to the ability of the peptide to trigger and recruit locally

the host immune reactivity, with no direct toxicity on tumor cells (Forni, G., T. Musso, D. Boraschi, C. Jemma, A. Tagliabue, and M. Giovarelli, submitted for publication).

In contrast to its significant effect on the immune response, the synthetic nonapeptide did not show any of several acute-phase and inflammation-related changes caused by IL-1, when injected in vivo at doses up to 10^6-fold higher than the optimal immunostimulatory inocula. Indeed, the 163–171 fragment was nonpyrogenic and failed to induce the typical decrease of plasma iron and fibrinogen, and the increase of the acute-phase protein SAA. Accordingly, the synthetic peptide could not induce the alterations in glucose homeostasis (decrease in blood levels of glucose in parallel to increase of insulin) that occur during sepsis and other inflammatory conditions, and that can be induced by IL-1β. Administration of the peptide was also unable to increase the serum levels of corticosterone, a highly immunosuppressive hormone involved in protein catabolism that is augmented during certain infectious diseases and in response to IL-1 (2, 3). Finally, at variance with the significant effect of IL-1 (5), the 163–171 fragment did not depress the cytochrome P450-dependent hepatic drug metabolism, an event induced by several inflammatory stimuli.

These results confer a broader biological significance to the finding that the 163–171 peptide is an immunostimulatory moiety of IL-1β that is devoid of the inflammatory and toxic effects of the entire protein. Thus, the potential clinical relevance of this molecule is further stressed.

As a corollary to this conclusion, it can be suggested that the ability to activate the immune response, which may in turn affect hematopoiesis by means of induction of soluble growth factors, may be sufficient by itself to induce radioprotection. Therefore, activation of inflammatory pathways would not be necessary to the recovery from radiation damage. Taken together, these data indicate that a further step towards a more rational immunotherapy is possible, by searching for active domains within interleukins.

Summary

The synthetic nonapeptide VQGEESNDK, corresponding to the fragment 163–171 of human IL-1β, showed in vivo immunomodulatory capacities qualitatively and quantitatively comparable to those of the mature human IL-1β protein. In fact, both IL-1β and the 163–171 fragment stimulated the immune response of normal mice and restored immune reactivities of immunocompromised animals. In addition, the synthetic IL-1 peptide was as efficient as the entire protein in inducing tumor rejection and radioprotection. On the other hand, the 163–171 fragment did not cause any of several inflammation-associated metabolic changes inducible by the whole IL-1β molecule in vivo: hypoferremia, hypoglycemia, hyperinsulinemia, increase in circulating corticosterone, SAA and fibrinogen, decrease in hepatic drug-metabolizing enzymes. Furthermore, at variance with IL-1β, the 163–171 peptide did not show the toxic effects causing shock and death in adrenalectomized mice.

Thus, these results confirm our previous in vitro observations that functional domains are identifiable within the multipotent cytokine IL-1β, and demonstrate the biological relevance of this finding in a variety of in vivo systems. The identification of a selectively active fragment of a cytokine may thus represent a significant step towards a better directed and more rational immunotherapeutic approach.

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