Development and application of a radioimmunoassay to detect interleukin-1 in rat peripheral circulation

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Several studies have shown that IL-1β is readily secreted from activated cells of the macrophage lineage (6, 10–12). This observation has favored the view that IL-1β can be considered as a signal molecule to act on distant target cells and to initiate the symptoms of the acute phase response. A variety of bioassays has been employed to study the regulation of IL-1 secretion in vivo and in vitro (18, 24, 29). Bioassays have the disadvantage of being laborious and difficult to standardize, and in addition they may be sensitive to other substances present in plasma. For instance, several IL-1 inhibitors have been characterized and purified and an IL-1 receptor antagonist protein has recently been cloned (15). Data obtained with a bioassay therefore give no real expression of plasma IL-1 levels but, rather, reflect the net effect of IL-1 and its endogenous inhibitors. To overcome these problems we have developed a radioimmunoassay (RIA) that can be used to detect immunoreactive IL-1 in rat plasma and allows us to examine conditions during which IL-1 may circulate in rat plasma. The assay described here is a heterologous assay system that uses a goat polyclonal antiserum raised against recombinant human IL-1β (rhIL-1β) and radiolabeled and nonradiolabeled recombinant rat IL-1β (rrIL-1β) as a tracer and standard, respectively, and that can be used in unextracted rat plasma. The antiserum in combination with rhIL-1β as a tracer and standard, respectively, is also highly suitable to detect IL-1β in unextracted human plasma samples.

MATERIALS AND METHODS

Radioiodination of IL-1β. Several methods for radioiodination of IL-1β were employed. Iodo gen (1,3,4,6-tetrachloro-3a-h-6a-diphenylglycinecaril, Pierce Chemical) labeling was performed according to Salacinsky et al. (34). Briefly, Iodo-gen-coated polyethylene vials (Greiner) containing 5 μg of rrIL-1β or rhIL-1β (Glaxo) dissolved in 10 μl of 0.1 M tris(hydroxymethyl)methanethiol (Tris)-HCl buffer (pH 7.3) were incubated for 15 min at room temperature with 3 mCi Na125I (10 μl of a NaOH solution, pH 7–11, Amersham). Bolton-Hunter labeling (4) was achieved by adding 5 μg of rrIL-1β in 25 μl 0.1 M phosphate-buffered saline (PBS, pH 7.3) and 1 mCi Na125I to Enzymobeads containing immobilized preparations of lactoperoxidase and glucose oxidase (Bio-Rad). The reaction was started by adding 25 μl of a 1% D-glucose (Sigma) solution in water followed by incubation for 20 min at room temperature.

After each labeling procedure the reaction mixture was applied and separated on a Sephadex G-25 medium (Pharmacia) column (15 × 1.5 cm, 13 ml, flow rate 24 ml/h, fraction vol 0.4 ml) equilibrated at room temperature with elution buffer consisting of 0.1 M Tris-HCl (pH 7.3) and 0.1% (wt/vol) gelatin.
The labeled proteins were further purified on Sephadex G-50 fine (Pharmacia) columns (65 x 1.5 cm, 120 ml, flow rate 12 ml/h, fraction vol 2 ml) equilibrated with a solution containing 0.1 M Tris- HCl (pH 7.3), 0.02% (wt/vol) bovine serum albumin (BSA, contain grade, Sigma at 10°C. The labeled rrIL-1β was used for 3-4 wk after labeling.

Antiserum against IL-1β. The antiserum was raised by Dr. J. McKearn (Searle/Monsanto) in a goat by subcutaneous administration of rhIL-1β in Freund’s complete adjuvant and by boosting with Freund’s incomplete adjuvant with intervals of 4 wk. The antiserum used here was obtained from the fourth bleed.

Antibody titration curves. To test the capacity of the antiserum to bind to the rrIL-1β (and rhIL-1β), radiolabeled IL-1β preparations [10,000 counts - min^-1 (cpm) µl^-1] were incubated with twofold serial dilution of the antiserum (100 µl) in RIA buffer [0.1 M Tris-HCl, pH 7.3, containing 0.1% (wt/vol) gelatin, 0.01% (vol/vol) Tween 20 and 2% (vol/vol) aprontinin (Trasylol, 10,000 U/ml, Bayer)]. The next day, bound and free label were separated using solid-phase second-antibody precipitation using donkey anti-goat antibodies (immunoglobulin G class, Saccel, IBD). Briefly, 100 µl of undiluted Saccel was added to each tube and shaken thoroughly. After a 30-min incubation at room temperature, 1 ml RIA buffer containing 2% (vol/vol) horse serum was added followed directly by centrifugation (2,300 g, 4°C, 10 min). After centrifugation, supernatents were aspirated and the radioactivity in the pellets was counted. Displacement curves of serial dilutions of plasma samples obtained from endotoxin-injected rats were in-
radioactivity in first and second peaks was not clearly different for both labeling procedures and varied between 0.3 and 0.2. Although we have successfully used the Bolton-Hunter-labeled rrIL-1β to detect plasma IL-1 levels (12), the characteristics of the Iodo-gen-labeled rrIL-1β appeared to be superior and its use will be described here.

**Antibody titration curves.** First, we tested the antibody binding characteristics of the two peaks obtained by eluting the Sephadex G-25 column purified label on the Sephadex G-50 column. The antibody titration curves in Fig. 1 show lack of binding of the antiserum with the high molecular material. In contrast, antibody concentration-dependent binding was obtained using radioactivity in the second peak. Almost 80% specific binding was reached at an antibody dilution of 1:8,000 and 50% specific binding at a final antibody dilution of 1:32,000. Nonspecific binding was <2% of total binding.

**Displacement curves.** As illustrated in Fig. 2, rrIL-1β effectively displaced binding of the antibody with the Iodo-gen-labeled rrIL-1β. Minimal concentrations to displace varied from 50 pg/ml in RIA buffer (independent t test, \( P < 0.05 \)) to 100 pg/ml in plasma (independent t test, \( P < 0.02 \)) depending on the lifetime of the labeled rrIL-1β and maximal displacement was reached with 100 ng/ml of rrIL-1β. rhIL-1β was 10 times more potent and rmIL-1β was 50 times less potent than rrIL-1β to displace labeled rrIL-1β (Fig. 2). Recombinant preparations of rhTNF (serial dilutions from 25 to 1.55 μg/ml), rhIL-6 (100 to 6.25 μg/ml), human IL-1 receptor antagonist (IL-1ra, 20 μg/ml to 200 pg/ml), rhIL-1α (20 μg/ml to 200 pg/ml), human IL-2 (24 to 3 μg/ml), murine interferon-γ (15 to 1.9 μg/ml), and bacterial LPS (100 to 12.5 μg/ml) did not displace binding of the antibody with Iodo-gen-labeled rrIL-1β.

**In vivo IL-1 administration.** In rats not showing any signs of nonspecific symptoms of sickness (fever or behavioral signs), no circulating immunoreactive IL-1 could be measured above the detection limit of our assay (100 pg/ml). However, intravenous injection of rrIL-1β (6 μg/kg) resulted in measurable concentrations of immunoreactive IL-1, as illustrated in Fig. 3. Immunoreactive

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**Fig. 1.** Antibody titration curves with 2 peaks of Iodo-gen-labeled recombinant rat interleukin-1β (rrIL-1β) eluted from Sephadex G-50 column. ○ Void volume (V₀) peak, ■ 2nd peak (with retardation factor of 0.8).
INTERLEUKIN-1β IN RAT PLASMA

Fig. 4. Time course of effect of LPS on plasma immunoreactive IL-1 concentrations. LPS was administered intravenously (2.5 mg/kg), and plasma was obtained from trunk blood at indicated time intervals. P < 0.05 (Scheffe's F test), 90 min vs. all other time intervals except 120 min. Data illustrate a representative example of 4 different experiments.

by a gradual decrease to 1 ± 0.23 ng/ml at 180 min. As shown in Fig. 5, the increase in circulating immunoreactive IL-1 concentrations was dependent on the dose of LPS administered. At LPS doses that did not induce a change in body temperature, we were unable to detect immunoreactive IL-1 in plasma as reported previously (12). Immunoreactive IL-1 was measurable at intravenous doses of LPS as low as 0.1 mg/kg and reached plateau concentrations at a LPS dose of 1 mg/kg as measured 90 min after LPS administration. It is worth noting that a two- to threefold interexperimental variation in levels of circulating immunoreactive IL-1 in response to LPS could be observed.

Sephadex G-75 column chromatography of plasma. Separation of pooled plasma obtained from LPS-treated (2.5 mg/kg iv) rats containing 8 ng/ml of immunoreactive IL-1 on a Sephadex G-75 column resulted in two peaks of immunoreactive IL-1 (Fig. 6). Approximately 60% of total immunoreactive IL-1 eluted in the void volume, whereas 40% of the immunoreactive IL-1 behaved identically to labeled or cold rrIL-1β and eluted with a Rf of 0.67. The recovery of rrIL-1β or immunoreactive IL-1 in the plasma pool loaded on the column was ~69%.

Macrophage depletion. As shown in Fig. 7, 4 days after administration of C12MDP liposomes, no circulating plasma immunoreactive IL-1 could be detected, whereas control rats showed circulating immunoreactive IL-1 concentrations to levels of 10 ng/ml 2 h after LPS (2.5 mg/kg iv) administration. In the illustrated experiment the C12MDP liposome-induced depletion of macrophages was complete as tested by immunocytochemical and histological techniques on sections of different lymphoid organs as described (12, 32).

DISCUSSION

In the present report we describe a heterologous radioimmunoassay to detect circulating immunoreactive IL-1 concentrations in rats. The antiserum used is raised to rhIL-1β and recognizes rrIL-1β, although with a lower affinity than rhIL-1β (Fig. 2). Despite the considerable homology of 78% between human and rat IL-1β (27), a variety of other antisera raised against human IL-1β were unable to recognize rrIL-1β. This suggest species specific differences in the folding of these recombinant preparations of IL-1β.

Because oxidative labeling procedures have been shown to cause aggregation of rhIL-1β (7), we initially decided to label rrIL-1β using nonoxidizing methods such as Bolton-Hunter. By use of the Bolton-Hunter method, rrIL-1β can be effectively labeled and used to detect circulating immunoreactive IL-1 as described earlier (12). However, a major disadvantage of this labeling was the large variation in specific activity. Moreover, Bolton Hunter is known to incorporate 3-(4-hydroxyphenyl)propionamide.
the rrIL-lp molecule and which may interfere with optimal binding of the antiserum leading to a lower sensitivity of the assay. In the present study we utilized two mild oxidizing labeling methods of which the Iodo-gen method resulted in labels with the highest specific activities. Moreover, minimal aggregation of the labeled rrIL-1β was found compared with using the strong oxidizing agent chloramine-T (data not shown). However, we noted a progressive aggregation during storage of the labeled rrIL-1β. Although we do not know the underlying chemical nature of aggregation, the aggregates appeared to interfere with the sensitivity of the assay despite the fact that the aggregate itself does not bind to the antiserum (Fig. 1).

The specificity and the characteristics of the developed RIA made it possible to detect immunoreactive IL-1 in biological samples obtained from rats. Because IL-1 is considered to act on distant targets such as the brain, we focussed our attention primarily on measurements in the blood compartment. Because serial dilutions of rrIL-1β in plasma and buffer similarly displaced binding of the antiserum to the labeled rrIL-1β, we conclude that plasma components do not interfere with the assay and that we can use our assay in unextracted plasma. Plasma samples of untreated control animals did not show detectable immunoreactive IL-1 concentrations, indicating that circulating immunoreactive IL-1 levels are below 100 pg/ml, the detection limit of the assay. By use of an immunoradiometric assay, it has recently been shown in rats that resting immunoreactive IL-1β concentrations are even under 10 pg/ml (6). In human plasma of healthy subjects circulating IL-1β concentrations have been shown to be below 50 pg/ml (9). The question whether IL-1 circulates under nonpathological conditions or is the result of immune activation by pathogens or injury is therefore difficult to solve with the present sensitivities of the IL-1 assays.

The finding that immunoreactive IL-1 could be measured after administration of rrIL-1β further demonstrates the usefulness of our assay to detect immunoreactive IL-1 in unextracted plasma samples. The half-life of injected rrIL-1β as demonstrated in this study closely resembles that of rhIL-1β or rrIL-1β injected intravenously in rats (3).

LPS has been shown to stimulate the production and release of IL-1 bioactivity from isolated peripheral blood cells in vitro (28). In a recent report comparing the specificity and variability of different bioassays, it has been demonstrated that the most frequently used bioassays are sensitive to other cytokines and unknown biological factors (29). Only a limited amount of data is available using bioassays to detect IL-1 bioactivity in plasma. Moreover, it has been demonstrated that plasma contains marked inhibitory activity (18, 24). The available data using nonbiological assays to detect IL-1 in animal plasma show IL-1 responses with a time course, magnitude, and dose dependence quite similar to that found in our study (6, 8, 16, 17, 39). Our measured immunoreactive IL-1 time course corresponds to that of neuroendocrine changes such as the time-dependent elevation of plasma corticosterone concentrations (12). However, no strict association was seen with the sustained increase in body temperature, since first measurable increases in rectal temperature were noted at ~6 h after LPS administration (Derijk et al., unpublished observation). It is worth noting in this respect that we observed induction of immunoreactive IL-1 in microglia in the central nervous system in response to pyrogenic doses of LPS starting around the 6-h time point (38).

A sustained increase in IL-1 bioactivity in the culture supernatants of human monocytes stimulated with LPS has been shown in vitro (26). In contrast, our in vivo study shows that immunoreactive IL-1 is induced in plasma with an optimum between 90 and 120 min after LPS administration. Although the shorter response in vivo can be explained solely in terms of a much faster elimination or biotransformation of LPS in vivo than in vitro (35), additional regulations of IL-1 production and release, not occurring in vitro, could be involved. In fact, it has been proposed that the activation of the hypothalamic-pituitary-adrenal axis by LPS administration, leading to increased plasma levels of glucocorticosteroids, represents a negative-feedback loop, suppressing the production and release of cytokines (1, 2). This view is supported by the observation that addition of synthetic glucocorticosteroids in vitro to stimulated immune cell preparations inhibits both the induction of mRNA for IL-1 and the release of IL-1 bioactivity (21, 22). It can therefore be argued that the duration of the LPS induced immunoreactive IL-1 response in vivo may be influenced by elevated levels of plasma glucocorticosteroids.

As illustrated in Fig. 5, maximal values of plasma immunoreactive IL-1 concentrations were reached at a dose of 1 mg/kg of LPS. In parallel experiments, we observed no lethality at this dose, whereas after 10 mg/kg ~20% of the animals died within 24 h, confirming the data of others using the same type of LPS (25). The data of our experiments suggest that the magnitude of the immunoreactive IL-1 response does not predict fatal outcome of the action of LPS in rats. In contrast, in humans under septic shock the magnitude of the plasma IL-1 response has been associated with lethality (37).

A large part of plasma immunoreactive IL-1 coelutes with rrIL-1β, demonstrating the presence of a 17-kDa immunoreactive IL-1 form in the circulation of LPS-treated rats. We do not know the nature of the large molecular immunoreactive material, although it has been described that IL-1 can bind to αα-macroglobulin, a large molecular acute-phase protein which is upregulated by endotoxin (5).

Studies involving bioassays have suggested that IL-1 bioactivity can be produced by a variety of different cells, such as endothelial cells, fibroblasts, and T- and/or B-cells (28). Our present data involving the liposome-directed macrophage suicide technique are in support with the notion that plasma immunoreactive IL-1 in the rat predominantly originates from macrophages. Because the liposomes are injected intravenously and intraperitoneally, effective elimination of macrophages from the spleen, liver, lymph nodes, and peritoneum was achieved, indicating their role in the release of immunoreactive...
IL-1 (12). However, toxic liposomes do not affect alveolar,
testis, and synovial macrophages, excluding the possi-

bility that these macrophages contribute to plasma im-
munoreactive IL-1 concentrations after intravenous LPS
administration (31). The importance of macrophages as
main producers of IL-1 in vivo is further supported by
the presence of a specific convertase in macrophages, which
is absent in other cells such as fibroblasts and which is
thought to play an essential role in the active secretion of
bioactive forms of IL-1 (20, 38).

In conclusion, the presence of immunoreactive IL-1 in
plasma after LPS administration supports the view that
IL-1 can act at distant organs and may be important in
the orchestration of the acute phase response.

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