BACTERIAL PATHOGENICITY

Growth hormone modulates IL- α and IFN- γ release by murine splenocytes activated by LPS or porins of *Salmonella* Typhimurium

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The effect of growth hormone (GH) on the release of IL-1 α and IFN- γ from murine splenocytes was investigated. Their release from splenocytes activated by *Salmonella enterica* serovar Typhimurium lipopolysaccharide (LPS) 0.5 μ g/ml was increased by c. 65% in the presence of GH 100 pg/ml. With splenocytes activated by *S. Typhimurium* porins 5 μ g/ml, GH increased the production of both IL-1 α and IFN- γ by c. 56%. Polymyxin treatment abolished the cytokine-releasing activity of LPS but had no effect on the activity of the porin preparation.

Introduction

The potential physiological role of growth hormone (GH) in immunoregulation has been demonstrated by Edwards *et al.* [1]. GH has recently been shown to be synthesised locally, by lymphoid cells in regional lymph nodes [2]. GH, as prolactin (PRL), is a member of the group 1 cytokine family, all of which have an antiparallel 4- α -helical bundle structure [3]. Both LPS and outer-membrane proteins, such as porins, of gram-negative bacteria can stimulate cells involved in the immune response to release cytokines [4] and LPS is known to be a potent stimulus of GH release [5].

The immune response is controlled at several levels. Considerable work has recently been focused on lymphocyte expression of receptors at various stages of the lymphocyte lifespan. Both thymocytes [6] and human lymphocyte cell lines [7] have detectable and saturable specific cell-surface receptors for GH. Whether or not the receptor for GH is present at certain times in the cell cycle has not yet been clarified, but GH could act as an additional signal in the functional specialisation of lymphocytes, providing an opportunity for immune intervention. Besides being an external signal, GH could also act at different levels of internal signal transmission in order to activate DNA transcription.

In the present study, the release of IL-1 α and IFN- γ by murine splenocytes stimulated with GH and with

two components of the outer membrane of gram-negative bacteria—LPS and porins—was investigated.

Materials and methods

Animals

BALB/cByJ male mice weighing 20–22 g were used. The animals were housed at constant temperature (20 $\pm$ 2°C) under a fixed 12-h light-dark cycle with free access to food (Mignini 70K standard diet) and water.

Reagents

Growth hormone (GH) from porcine pituitary glands and polymyxin B were purchased from Sigma. The presence of endotoxin in reagents was checked by the *Limulus* amoebocyte lysate test [8] and all results were negative (i.e., < 0.05 EU of LPS/ml of sample).

Preparation of porins and LPS

Salmonella enterica serovar Typhimurium strain SH5014 [9], kindly provided by Dr M. Nurminen (National Public Health Institute, Helsinki, Finland) was used as a source of the porins [10]. Briefly, 1 g (wet wt) of cell envelopes was suspended in Triton X-100 2% v/v in 0.01 M Tris-HCl, pH 7.5, containing 10 mM EDTA; after the addition of trypsin (10 mg/g of envelopes), the pellet was dissolved in sodium dodecyl sulphate (SDS) buffer (SDS 4% w/v in 0.1 M sodium phosphate, pH 7.2) and applied to an Ultragel AcA34 column equilibrated with SDS (0.25% w/v) buffer. The

fraction containing proteins, identified by absorbance at 280 nm, was dialysed extensively and examined by SDS-PAGE by the method of Laemmli [11]. The protein content of the porin preparation was determined by the method of Lowry *et al.* [12].

LPS was isolated from *S. Typhimurium* SH5014 by the phenol-chloroform-ether method of Galanos *et al.* [13].

Polymyxin treatment

The LPS activity in the porins was neutralised by adding a 1:10 (w:w) ratio of protein:polymyxin B and incubating at room temperature for 1 h. LPS was mixed with polymyxin B at a 1:100 (w:w) ratio (LPS:polymyxin B) [14]. LPS, porins and the mixtures containing polymyxin B were used in pyrogen-free phosphate-buffered saline (PBS), unless otherwise specified.

Assays with murine splenocytes

Splenocytes were prepared according to conventional procedures from aseptically removed mouse spleens. The cells were washed three times in RPMI 1640 medium (Labtek Laboratories, Eurobio, Paris, France) and resuspended in complete RPMI medium (RPMI supplemented with fetal calf serum 10%v/v, 2 mM glutamine, 10 mM HEPES, penicillin 100 U/ml, streptomycin 100 µg/ml) at a final concentration of 3×10^6 cells/ml. Cell viability was evaluated by the trypan blue exclusion test. LPS or porins and GH, in RPMI 1640 medium, were added to the culture at the concentrations indicated in each assay. After 24 h, cell viability was checked and the culture supernates were collected and stored at -20°C until assayed for cytokines. Pilot studies had shown that 24 h was the average cytokine secretion time (data not shown).

RNA isolation and cDNA preparation

Splenocytes (1×10^7), cultured in complete RPMI medium under different experimental conditions, were collected after 5 h and total RNA was extracted by the method of Chomczynski and Sacchi [15].

The RNA pellet was resuspended in ethanol 75% v/v, sedimented, vacuum-dried and dissolved in 15 µl of RNAase-free water. One µg of oligo (dT) (Promega Biotec, Madison, WI, USA) was added to the suspension and the mixture was heated at 65°C for 5 min. After cooling on ice, the mixture was incubated for 2 h at 42°C with 14 µl of the following mixture: 20 mM dithiothreitol (Sigma); 1 mM (each) dATP, dGTP, dCTP and dTTP; RNasin (Promega) 35 U and Moloney murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Eggenstein, Germany) 525 U in reverse transcription buffer.

PCR procedure

PCR primers for murine β actin, interleukin 1 α (IL-1 α), and interferon- γ (IFN- γ) were purchased from Clontech (Palo Alto, CA, USA). cDNA (2 µl) prepared as described above was amplified in the presence of 500 nM (final concentration) 5' and 3' primers, 200 µM (each) dATP, dGTP, dCTP, dTTP and *Taq* DNA polymerase (Promega) 1.25 U made up to a final volume of 50 µl with *Taq* DNA polymerase 10 $\times$ buffer (Promega). The PCR was performed in a Perkin-Elmer thermal cycler for 30 cycles as follows: denaturation, 1 min at 94°C ; annealing, 2 min at 60°C and extension, 3 min at 72°C . The reaction products were visualised by electrophoresis of 25 µl of the reaction mixture at 100 V in an agarose 1.5% gel containing ethidium bromide 1 µg/ml. The gels were then examined on an UV light box and photographed. One µg of *Bgl*I- and

Fig. 1. SDS-PAGE analysis of porins and LPS preparations from *S. Typhimurium* SH5014. **a**, lane A, mol. wt standards (phosphorylase b, 94 000; albumin, 67 000; ovalbumin, 43 000; carbonic anhydrase, 30 000; trypsin inhibitor, 20 100; α -lactalbumin, 14 000); **B**, SH5014 porins. **b**, lane A, SH5014 LPS; **B**, *S. Typhimurium* smooth LPS (Difco).

*Hinf*I-digested pBR328 DNA (Boehringer Mannheim) was run in parallel as mol. wt markers (providing bands at 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 bp).

Cytokine assays

All measurements were performed with monoclonal antibodies. IL-1 α was measured with a Mouse Interleukin-1 α ELISA kit (Genzyme Corporation Cambridge, MA, USA); IFN- γ was measured with a Mouse Interferon- γ ELISA kit (Genzyme).

Statistical analysis

All experiments were performed in triplicate; results were expressed as the mean and SD. Comparisons between tests were by Student's *t* test, with statistical significance considered to be $p < 0.05$.

Results

Porin and LPS preparations

The composition of the porin preparation, as examined by SDS-PAGE, is shown in Fig. 1a. Two polypeptides, of 34 and 36 kDa, were the main components. The LPS concentration in the porin preparation was estimated to be *c.* 0.005% by weight of the protein.

The profile of the LPS preparation, as revealed by SDS-PAGE and staining with silver nitrate [16], showed that the LPS was in the R phase (Fig. 1b).

Cytokine release by splenocytes stimulated with LPS and GH

The release of IL-1 α and IFN- γ from splenocytes (3×10^6 /ml) incubated with different concentrations of LPS with and without GH 100 pg/ml are shown in Fig. 2. With LPS 0.5 μ g/ml alone, splenocytes released

Fig. 2. Effect of GH 100 pg/ml on production of IL-1 α and IFN- γ by murine splenocytes stimulated with different amounts of LPS (0–5 μ g/ml): $\square$, IL-1 α production in the absence of GH; $\square$ with lines, IL-1 α production in the presence of GH 100 pg/ml; $\blacksquare$, IFN- γ production in the absence of GH; $\blacksquare$ with lines, IFN- γ production in the presence of GH 100 pg/ml. Each column represents the mean of three determinations and the bars indicate SD.

285 SD 18 pg of IL-1 α /ml and 1200 SD 60 pg of IFN- γ /ml into the supernate. With GH and LPS 0.5 μ g/ml, splenocytes released 415 SD 20 pg of IL-1 α /ml and 1846 SD 60 pg of IFN- γ /ml into the supernate. With GH and concentrations of LPS $\leq$ 0.5 μ g/ml, cytokine release was greater than the sum of the amounts released by GH and LPS alone. With LPS concentrations $>$ 0.5 μ g/ml, the release was less than the sum of the amounts released by either stimulus alone. GH alone, without prior stimulus with LPS, had only weak cytokine-releasing activity. Controls without LPS and GH did not release significant amounts of cytokines (data not shown). Splenocytes stimulated with LPS mixed with polymyxin B (LPS:polymyxin B 1:100 w:w) did not release significant amounts of cytokines and polymyxin B alone, at the concentration used in the LPS mixture, gave no significant stimulation of cytokine release (data not shown).

With a fixed amount of LPS (0.5 μ g/ml) and different concentrations of GH (10, 100 and 1000 pg/ml), it was seen that both IL-1 α and IFN- γ release were greatest at a GH concentration of 100 pg/ml (Fig. 3).

Cytokine release by splenocytes stimulated with porins and GH

The release of IL-1 α and IFN- γ from splenocytes (3×10^6 /ml) incubated with different concentrations of porins with and without GH 100 pg/ml are shown in Fig. 4. With porins 5 μ g/ml, splenocytes released 120 SD 10 pg of IL-1 α /ml and 600 SD 25 pg of IFN- γ /ml into the supernate. With GH and porins 5 μ g/ml, splenocytes released 257 SD 10 pg of IL-1 α /ml and 936 SD 25 pg of IFN- γ /ml into the supernate. With GH and porin concentrations $\leq$ 5 μ g/ml, cytokine release was greater than the sum of the amounts released by GH and porins alone. With a porin concentration of 10 μ g/ml, the release was less than the sum of the amounts released by either stimulus alone. Again, GH alone, without prior stimulus with porins, had only weak cytokine-releasing activity. Controls without porins and GH did not release significant amounts of cytokines (data not shown).

Splenocytes treated with porin mixed with polymyxin B (porin:polymyxin B, 1:10, w:w) to neutralise the

Fig. 3. Release of IL-1 α and IFN- γ from murine splenocytes stimulated with LPS 0.5 μ g/ml and various amounts of GH (10, 100, 1000 pg/ml): ▨, IL-1 α production in the presence of GH; ■, IFN- γ production in the presence of GH. Each column represents the mean of three determinations and the bars indicate SD.

Fig. 4. Effect of GH 100 pg/ml on production of IL-1 α and IFN- γ by murine splenocytes stimulated with different amounts of porins (0–10 μ g/ml): $\square$, IL-1 α production in the absence of GH; $\square$ with vertical lines, IL-1 α production in the presence of GH 100 pg/ml; $\blacksquare$, IFN- γ production in the absence of GH; $\blacksquare$ with diagonal lines, IFN- γ production in the presence of GH 100 pg/ml. Each column represents the mean of three determinations and the bars indicate SD.

effect of small amounts of LPS in the porin preparation, showed the same results as porins alone (data not shown). This showed that the effect was due to porins and not to LPS. Polymyxin B alone, at the concentration used in the porin mixture, gave no significant stimulation of cytokine release (data not shown).

With a fixed concentration of porins (5 μ g/ml) and different concentrations of GH (10, 100 and 1000 pg/ml) it was seen that both IL-1 α and IFN- γ release was greatest at a GH concentration of 100 pg/ml (Fig. 5).

Patterns of cytokine mRNA expression in cultured splenocytes

The data on the release of IL-1 α and IFN- γ in the supernates were confirmed by studies on specific mRNA in cells treated in the same manner.

The effect of GH 100 pg/ml on cytokine mRNA

expression in splenocytes stimulated with LPS 0.5 μ g/ml and porins 5 μ g/ml is shown in Fig. 6. The intensity of the mRNA bands for IL-1 α and IFN- γ in splenocytes stimulated with either LPS or porins was increased after treatment with GH under the same experimental conditions. IL-1 α and IFN- γ mRNAs were detected only at low levels in splenocytes cultured with GH alone.

Discussion

Bidirectional communication signals occur in the neuroendocrine-immune system interactions. They are important in host resistance to infectious, autoimmune and neoplastic diseases [17]. The concept that GH affects cells of the immune system is demonstrated by the requirement for GH in order to maintain lymphatic tissues populated with lymphocytes, as removal of this hormone results in atrophy of the thymus and secondary lymphoid tissues [18–21]. It has been well established that the immune system has evolved a very elaborate process for the internal regulation of

Fig. 5. Release of IL-1 α and IFN- γ from murine splenocytes stimulated with porins 5 μ g/ml and various amounts of GH (10, 100, 1000 pg/ml): ▨, IL-1 α production in the presence of GH; ■, IFN- γ production in the presence of GH. Each column represents the mean of three determinations and the bars indicate SD.

lymphocyte growth and differentiation; this process involves the release of soluble mediators and the timely appearance of cell-surface receptors specific for these mediators at discrete points during the cell cycle of activated lymphocytes [22, 23].

LPS and porins are surface components of gram-negative bacteria that greatly activate cells involved in the immune response. Activated cells possess the ability to produce a large variety of cytokines; these cytokines can trigger other cell types involved in immune responses.

The data show the remarkable role of GH in regulating the release of IL-1 α and IFN- γ in cells after their stimulation by porins or LPS. GH, at an optimal concentration which probably varies from cell type to cell type, can regulate cytokine release in relation to the intensity of the stimulus to which the cell has been exposed. In the present study, with low levels of LPS or porins cytokine release was enhanced when GH was present. In contrast, at higher

concentrations of LPS or porins, cytokine release in the presence of GH was usually less than the sum of the levels released by either agent alone. The effect of GH on cytokine release from cells stimulated with these bacterial components was confirmed by studies on specific mRNA expression.

The effect of GH on IL-1 α and IFN- γ release and on other cytokines probably involves a series of changes in target cells involved in the immune response. The ability of GH to modulate cytokine release induced by LPS or porins may be correlated with its role in cell metabolism. Raima and Holttä [24] have reported that GH stimulated ornithine decarboxylase (ODC) activity in the isolated perfused rat liver. Kay and Cooke [25] and Klinpel *et al.* [26] observed a marked increase in ODC activity after stimulation of human lymphocytes with various mitogenic lectins. The activity of ODC rises during the early stages of growth in many eukaryotic cells and has been reported to have an unusually short half-life [27]. Enhanced cellular expression of ODC causes an increase in cellular

Fig. 6. Effect of GH on cytokine mRNA expression in splenocytes cultured alone (lane 2) or in the presence of GH (3), LPS (4), LPS + GH (5), porins (6) and porins + GH (7). After 5 h, RNA was extracted and reverse transcribed. The cDNAs obtained were assayed for the presence of specific cytokine mRNA by PCR as described in Materials and methods. Reaction products were run on agarose 1.5% gels in the presence of appropriate mol. wt markers (lane 1). β -Actin was the positive control for transcription.

levels of polyamines [28]; these cationic molecules have been implicated as vital co-factors involved in protein, RNA and DNA synthesis.

Regulation of cytokine synthesis occurs at different transcriptional and post-transcriptional levels [29–31] and a modulatory effect, either directly or indirectly, by GH can occur at either level [17–32]. GH can play a supportive role during the complex series of events that occur during lymphocyte activation by enhancing the uptake of key nutrients, by raising the level of intermediary cellular metabolism, and through the maintenance of high levels of protein synthesis. Presumably GH provides mechanisms that offer another level of cytokine regulation to protect the host. It has been shown that GH is required for protection against the lethal effects of *S. Typhimurium* [33]. The consequences of inflammatory pathway activation during disease are often the result of synergic toxicity between cytokines. The potential pathophysiological role of GH in immunoregulation has been demonstrated by several lines of evidence. GH has been shown to be synthesised locally by lymphoid cells [2]. LPS is a potent stimulus of GH secretion [5] and also of IL-1. *In vivo*, IL-1 causes the release of GH [34]. These findings suggest that the process of infection or inflammation and consequent immune activation can affect neuroendocrine function.

It has also been shown that other hormones may counteract the biological activity of cytokines. Mela-

nocyte-stimulating hormone attenuates the development of inflammatory acute phase responses induced by tumor necrosis factor (TNF) or IL-1 [35]. Glucocorticoid hormones increase the survival of animals given otherwise lethal doses of IL-1 or TNF [36]. These data suggest that the endocrine system may modulate cytokine secretion by preventing the undesired toxic effects resulting from cytokine excess or favouring cytokine activity at low concentrations.

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