Glutamine accelerates interleukin-6 production by rat peritoneal macrophages in culture

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Abstract The effect of glutamine on the production of interleukin-6 (IL-6) was studied in rat peritoneal macrophages in culture. A maximal production of IL-6 was measured at 4 h in lipopolysaccharide (LPS)-stimulated macrophages, and addition of glutamine (5 mM) anticipated this increase by 1 h without any increase in the IL-6 mRNA level. The effect of glutamine required the presence of LPS. Thus, glutamine accelerates IL-6 production from the pre-existing mRNA. The effect of glutamine was not mediated by cell swelling since culture of macrophages in hypoosmotic condition decreased the production of IL-6 in the culture medium with a corresponding decrease in the IL-6 mRNA level.

Key words: Glutamine; Hydration state; Macrophage; Interleukin-6

1. Introduction

Glutamine is a key substrate for the macrophage, not only for energy supply but also for purine and pyrimidine synthesis (for review see [1,2]). Macrophages have the capacity to secrete various compounds such as cytokines among which interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα), the three major cytokines involved in the inflammatory response. In 1992, Wallace and Keast [3] reported that a decrease in glutamine concentration decreased the rate of IL-1 production by lipopolysaccharide (LPS)-stimulated peritoneal macrophages. These results suggested that glutamine might play a key role in the control of IL-1 gene expression. Moreover, glutamine induces cell swelling due to its sodium-dependent transport into various cell types [4,5]. We recently reported that the glutamine-induced cell swelling regulates the expression of the β-actin gene at a transcriptional level in rat hepatocytes [6].

The aim of this work was to study whether (i) glutamine may increase the rate of production of another cytokine (IL-6) by rat peritoneal macrophages, (ii) glutamine acts through a modulation of the expression of the IL-6 gene and (iii) these effects may be related to the glutamine-induced cell swelling.

2. Materials and methods

2.1. Materials

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), LPS (from *Salmonella minnesota*) and glutamine were purchased from Sigma. Recombinant human IL-6 (rh IL-6) was from Boehringer and total RNA isolation reagent 'RNA now' was from Biogentex (Houston, TX, USA). The culture medium was a mixture of medium 199 (25%) and MEM (75%) obtained from Eurobio (Paris, France). Foetal calf serum was from Dutscher (Brumath, France). Hybond-N membranes, multiprime DNA labelling system, [α-^{32}P]dCTP (3000 Ci/mmol) and hyperfilm MP were obtained from Amersham (France). A pGEM-4 plasmid containing the mouse IL-6 cDNA was kindly provided by Dr. J. Doly (UPR 37, Villejuif, France). The rat cDNA for glyceraldehyde-3-phosphate dehydrogenase (GraP-DH) provided by Dr. J.M. Blanchard [7] was used as internal control. Murine hybridoma cell line B9 used for IL-6 assay was kindly provided by Dr. M. Dehoux (INSERM U-408, Paris, France).

2.2. Isolation and culture of peritoneal macrophages

Male Wistar rats (280–300 g) obtained from Charles River (France) were anaesthetized with ether and bleeded. Peritoneal cells enriched macrophages were harvested by 2 peritoneal washes with 40 ml of culture medium containing 3 units/ml heparin. The obtained cell suspension was filtered through sterile gauze and centrifuged (280×g; 7 min). Cells were suspended in the culture medium containing 10% foetal calf serum and 2 mM glutamine. Aliquots of about 8×10^6 cells were plated in 25 cm^2 flasks and cultured at 37°C under a gas phase of air/CO_2 (95:5). After an attachment period of 4 h, the non-adherent cells were removed by shaking. The resulting adherent population consisted of >95% peritoneal macrophages. The culture medium was replaced by fresh medium with 5% foetal calf serum without glutamine for 16 h. Then, macrophages were stimulated by exposure to 1 μg/ml LPS and glutamine was added for different periods of time. Hypotonic and hypertonic media were obtained by decreasing (−50 mM) or increasing (+35 mM) the NaCl concentration of the culture media, respectively. Hypoosmolarity was corrected by raffinose addition (+100 mM).

2.3. Extraction and analysis of cellular RNA

Total RNA was extracted from adherent macrophages with the RNA now kit. RNA (15 μg) was separated on 1.5% agarose/formaldehyde gels and transferred to nylon membrane for Northern hybridization. Membranes were hybridized using random oligonucleotides-primed ^{32}P-labelled insert as described [8]. Filters were washed and exposed to hyperfilm at −80°C using intensifying screens. Relative densities of the hybridization signals were quantified by scanning the films with a Shimadzu densitometer. To correct for differences in RNA loading, all the results were expressed as the ratio of the scanned values for IL-6 mRNA versus those for GraP-DH mRNA (relative level).

2.4. Interleukin-6 bioassay

Cell supernatants were collected, filtered and frozen for the bioassay. IL-6 content was determined using the murine hybridoma cell line B9, as described [9]. Briefly, B9 cells (10^4 cells) were incubated for 4 days in 96 well microtiter plate with the supernatant. Cell proliferation was estimated by a colorimetric determination using the tetrazolium salt (MTT), as described [10]. The test was standardized with rh IL-6. Results are expressed as ng per ml of culture medium.

2.5. Expression of results

The results are expressed as means ± S.E.M. for the observations on the indicated number (*n*) of different cell preparations. Statistical significance of differences was calculated by Student's *t*-test for paired data.

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3. Results

3.1. Glutamine increased the production of IL-6 in the presence of LPS

Rat peritoneal macrophages were cultured for 3 h in the presence of 1 μg/ml LPS with or without 5 mM glutamine. The obtained results showed that glutamine increased about 4-fold the production of IL-6 (12.7 ± 2.8 ng/ml: 100%, control; 401.6 ± 77.5%*, +glutamine; $n = 9$; $P < 0.05$). This effect of glutamine on IL-6 production was dose-dependent and biphasic (Fig. 1). Indeed, a first peak was observed between 0.2 and 1 mM glutamine corresponding to the concentration of this amino acid in blood and a second one was observed at higher concentrations (up to 7 mM). Although macrophages were cultured in a medium containing 5 mM glucose, glutamine might be required for energy supply. Thus, the first peak of IL-6 might correspond to the optimal conditions required for stimulated macrophages, as recently observed for lymphocyte proliferation [11]; the second one might correspond to another effect of glutamine, as previously reported in the studies of the effect of glutamine on the regulation of liver gene expression [6,12].

3.2. Glutamine shortened the time required for maximal IL-6 production induced by LPS

In order to specify the modalities of glutamine action, we studied the time-course of the effect of glutamine on the production of IL-6 in the presence of LPS. As shown in Fig. 2, the inducing effect of LPS on IL-6 production was apparent after 2 h of culture and was maximal at 4 h. In the presence of 5 mM glutamine, maximal IL-6 production was not significantly different from that obtained in the presence of LPS added alone, but was anticipated by 1 h (Fig. 2). Moreover, IL-6 production in the presence of glutamine was significantly

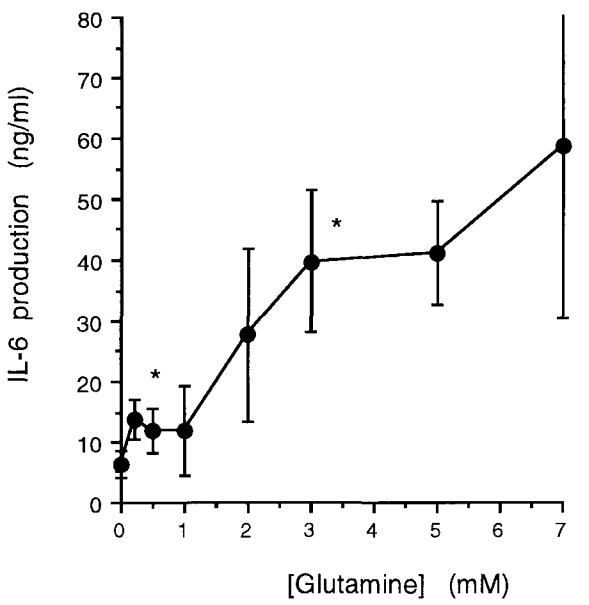

Fig. 1. Dose response of the effect of glutamine on the production of IL-6 by cultured macrophages. Rat macrophages were cultured for 3 h in the presence of different glutamine concentrations with 1 μg/ml LPS. IL-6 concentrations were measured in the culture medium. Results represent the means ± S.E.M. of 3 different cultures, except for 0.2 and 3 mM glutamine ($n = 4$); *significantly different ($P < 0.05$) from the value obtained in the absence of glutamine.

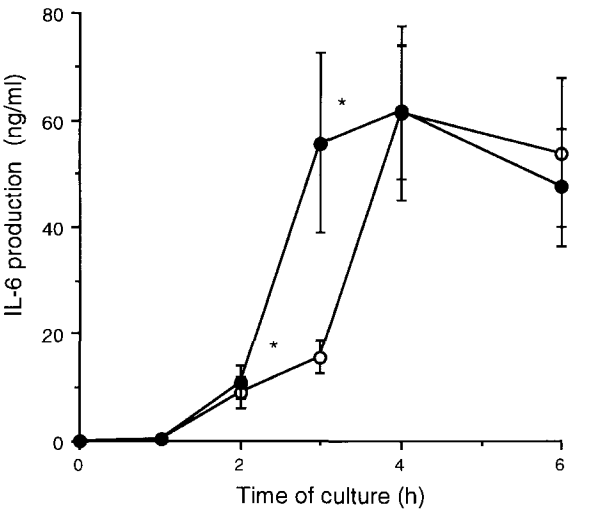

Fig. 2. Time course study of the effect of glutamine on the production of IL-6 by cultured macrophages. Rat macrophages were cultured in the presence of 1 μg/ml LPS with (●) or without (○) 5 mM glutamine for different periods of time. IL-6 concentrations were measured in the culture medium. The results are means ± S.E.M. for 5 different cultures (except for 4 h: $n = 4$); *significantly different ($P < 0.05$) from the corresponding control values.

increased as soon as 2 h of culture (+27.8 ± 7.2%; $n = 5$; $P < 0.05$), as compared with LPS added alone. In the absence of LPS, we were unable to detect any significant effect of glutamine on IL-6 production at 3 h (1.8 ± 1.1 ng/ml, control; 1.0 ± 0.3 ng/ml, +5 mM glutamine; $n = 4$). This demonstrated that (i) glutamine alone was unable to induce the production of IL-6 and (ii) glutamine shortened the time required for maximal IL-6 production induced by LPS.

3.3. Glutamine did not increase the level of IL-6 mRNA

We also tested the influence of glutamine on the level of the IL-6 mRNA in the presence of LPS. Macrophages were cultured for various periods of time in the presence of 1 μg/ml LPS with or without 5 mM glutamine, and the level of IL-6 mRNA was measured. Fig. 3 shows that LPS increased the mRNA level up to 2 h of culture. After this time, the level of mRNA rapidly decreased by about 65% within 2 h. Fig. 3 also shows that this pattern was not altered by glutamine. The comparison of Figs. 2 and 3 shows that a lag-period of 2 h was necessary between the synthesis of the IL-6 mRNA and the maximal production of IL-6 in the absence of glutamine. In the presence of glutamine, this lag-period was shortened by 1 h. Taken together, these results demonstrate that glutamine favoured the production of IL-6 without any changes in IL-6 mRNA. Moreover, the level of the IL-6 mRNA was twofold lower in the presence of glutamine than in its absence after 6 h of culture (0.67 ± 0.22 relative mRNA level, control; 0.30 ± 0.14* relative mRNA level, +glutamine; $n = 6$; $P < 0.05$). This clearly demonstrated that the IL-6 mRNA disappeared more rapidly in the presence of glutamine.

3.4. Hypoosmolarity decreased the production of IL-6 in the presence of LPS

Since glutamine induces cell swelling through its sodium-dependent transport in various cell types, we tested the possibility that the glutamine-induced cell swelling was responsible for its effect on the production of IL-6 in the presence of

LPS. Macrophages were cultured in the presence of 1 μg/ml LPS in hypo- (−50 mM NaCl) or isoosmotic media for 3 h. The results showed that hypoosmolarity decreased the production of IL-6 by about 40% (35.0 ± 14.8 ng/ml: 100%, Iso; 64.2 ± 6.2%*, Hypo; $n = 5$; $P < 0.05$). Because hypoosmolarity induces cell swelling, this result strongly suggested that, unlike glutamine addition, cell swelling might decrease IL-6 production. However, the hypoosmotic medium was obtained by decreasing the NaCl concentration. Thus, an effect of the change in the NaCl concentration could not be excluded to explain the observed modulation of the IL-6 production. To test the effect of osmolarity by itself, we used raffinose to correct hypoosmolarity in the medium. The addition of raffinose (100 mM) to hypoosmotic medium totally blocked the inhibitory effect of hypoosmolarity on IL-6 production (100%, Iso; 60.4 ± 4.1%*, Hypo; 117.3 ± 10.0%, Hypo+raffinose; $n = 4$; $P < 0.05$). This suggested that cell swelling per se might decrease the production of IL-6. Moreover, culture of macrophages in hyperosmotic conditions (+35 mM NaCl) showed that hyperosmolarity significantly increased the production of IL-6 (100%, Iso; 145.2 ± 5.2%*, Hyper; $n = 3$; $P < 0.05$). This demonstrated that changes in cell volume regulate the production of IL-6. In order to specify the level of action of changes in cell volume on the IL-6 production, macrophages were cultured for 3 h with 1 μg/ml LPS in different osmotic conditions, and the level of IL-6 mRNA was measured. As shown in Fig. 4 for a representative experiment, hypoosmolarity inhibited the LPS-induced increase in the mRNA level and, in opposite, hyperosmolarity favoured the stimulatory effect of LPS on the expression of the IL-6 gene (100%, Iso; 54.6 ± 13.8%*, Hypo; 112.7 ± 17.4%, Hypo+raffinose; 127.5 ± 17.1, Hyper). These results demonstrated that osmotic stress regulate the expression of the IL-6 gene.

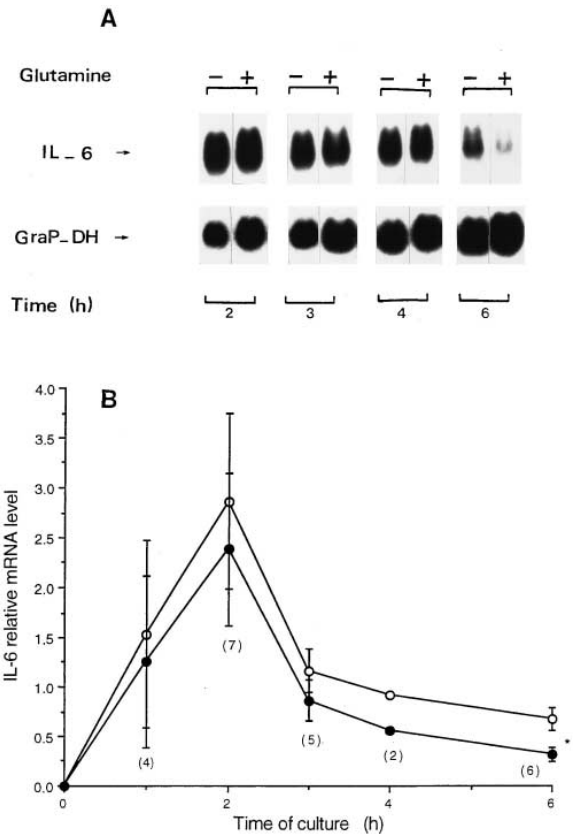

Fig. 3. Effect of glutamine on the level of IL-6 mRNA in cultured macrophages. Rat macrophages were cultured in the presence of 1 μg/ml LPS with (●) or without (○) 5 mM glutamine for different periods of time. Cells were harvested, total cellular RNA was extracted and 15 μg aliquots were subjected to Northern blot analysis. They were probed successively with the IL-6 and GraP-DH cDNAs. (A) Representative autoradiogram. (B) Scanned data of autoradiograms. The results are means ± S.E.M. for the number of experiments indicated in parentheses; *significantly different ($P < 0.05$) from the corresponding control values.

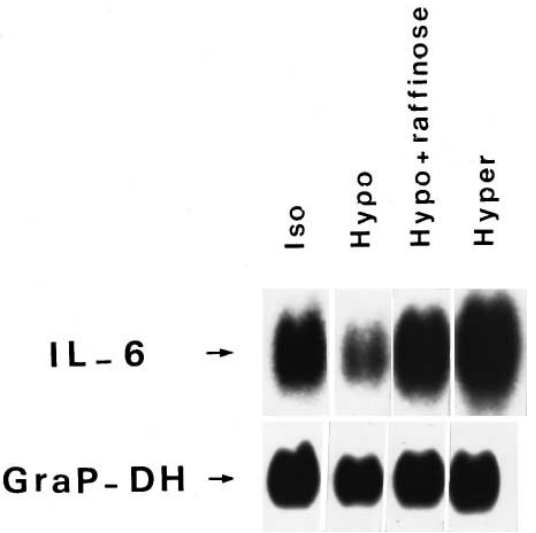

Fig. 4. Effect of anisoosmolarity on the level of IL-6 mRNA in cultured macrophages. Rat macrophages were cultured for 3 h in the presence of 1 μg/ml LPS in isoosmotic medium (Iso) or in different osmotic conditions (Hypo and Hyper). 100 mM raffinose was added to hypoosmotic medium to restore the isoosmotic condition. Total cellular RNA was extracted and 15 μg aliquots analyzed by Northern blot analysis. They were probed successively with the IL-6 and GraP-DH cDNAs.

4. Discussion

The data presented here clearly demonstrate that glutamine exerts an anticipating effect on the production of IL-6 in peritoneal macrophages stimulated by LPS. This underlines the potential role of glutamine in the inflammatory response.

The glutamine effect is not related to an effect of this amino acid on the expression of the IL-6 gene. Indeed glutamine, firstly, was unable to exert any significant effect on the production of IL-6 in unstimulated macrophages and, secondly, did not reinforce the inducing effect of LPS at the mRNA level. We also demonstrate that the IL-6 mRNA disappeared more rapidly in the presence of glutamine. It may therefore be proposed that glutamine accelerates the synthesis of IL-6 from the mRNA pre-existing under the influence of LPS. However, an effect of glutamine on the secretion process cannot be totally excluded. Such an effect of glutamine on cytokine production was previously reported for the production of IL-1 by stimulated murine peritoneal macrophages [3] and for the production of IL-2 and interferon-γ by stimulated human blood mononuclear cells [11]. The mRNA levels of the corresponding cytokines were however not measured. This is there-

fore, to our knowledge, the first demonstration that glutamine increases the production of a specific cytokine without effect on the corresponding gene expression.

Concerning the mechanism by which glutamine may act, we can exclude that the effect of glutamine is mediated by cell swelling. Our results clearly demonstrate that cell swelling has effects opposite to those of glutamine, namely swelling decreased the expression of the IL-6 gene leading to a decrease in the production of IL-6 by LPS-stimulated macrophages. Such an effect of osmotic stress has been reported very recently on the production of TNF-α by Küpffer cells [13] and on the production of IL-1 and IL-8 by human blood mononuclear cells [14]. Our results reinforce the notion that hyperosmotic stress is a stimulant for cytokine production. These results also demonstrated that a dissociation was apparent between the influence of glutamine and cell swelling on the IL-6 production. Interestingly, such a dissociation between the effects of glutamine and cell swelling has also been observed on the phospho*enol*pyruvate carboxykinase gene [15,16], another rapidly induced gene.

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