Immunology 1991 **72** 497-501 ADONIS 001928059100084E

Human immunoglobulin preparation for intravenous use induces elevation of cellular cyclic adenosine 3′:5′-monophosphate levels, resulting in suppression of tumour necrosis factor alpha and interleukin-1 production

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Accepted for publication 11 December 1990

SUMMARY

We previously showed that human immunoglobulin preparation for intravenous use (IGIV) suppresses the *in vitro* production of tumour necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1) by rabbit peritoneal exudate cells (PEC) stimulated with lipopolysaccharide (LPS). In this study we investigated the mechanism of the suppression. IGIV treated at pH 4 (pH4-G) was used as IGIV. Fc fragments of pH4-G, as well as untreated pH4-G, suppressed TNF-α and IL-1 production by rabbit PEC stimulated with LPS. The interaction of pH4-G with PEC also resulted in generation of cyclic adenosine 3′:5′-monophosphate (cAMP), known to be an intracellular second messenger. N^6, 2′-0-dibutyryl cAMP (BtcAMP), a lipid-soluble derivative of cAMP, and cholera toxin (CT), an adenylate cyclase activating agent, also suppressed the production of TNF-α and IL-1. Further N-[2-(methylamino) ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8), an inhibitor of cAMP-dependent protein kinases, abrogated the suppression by pH4-G of the productions. These results indicate that the binding of IGIV to PEC via Fc gamma receptors (FcγR) induces the elevation of intracellular cAMP levels, resulting in the suppression of LPS-induced TNF-α and IL-1 productions.

INTRODUCTION

In recent clinical trials of human immunoglobulin preparation for intravenous use (IGIV) therapy in combination with administration of antibiotics, the effectiveness of IGIV against severe bacterial infections was shown to be 60%.[1] Anti-pyretic efficacy, which serves as a major indicator of clinical effectiveness, has also been recorded at a similar level.[1] To elucidate how IGIV reduces fever, we have previously investigated the anti-pyretic activity of IGIV in a pyrogen-inducing fever model of rabbits.[2] The inhibition by IGIV of lipopolysaccharide (LPS)-induced febrile response has been deduced to be mediated through suppression of the production of endogenous pyrogens, because IGIV suppresses the *in vitro* production of interleukin-1 (IL-1) and tumour necrosis factor-alpha (TNF-α), known as endogenous pyrogens,[3] by rabbit peritoneal exudate cells (PEC) stimulated with LPS.[2,4]

Abbreviations: BtcAMP, N^6,2′-0-adenosine 3′:5′-monophosphate; cAMP, cyclic adenosine 3′:5′-monophosphate; CT, cholera toxin; FcγR, Fc gamma receptor; H-8, N-[2-(methylamino)ethyl]-5-isoquinolinesulphonamide dihidrochloride; IGIV, human immunoglobulin preparation for intravenous use; IL-1, interleukin-1; LPS, lipopolysaccharide; PEC, peritoneal exdate cell; PGE_2, prostaglandin E_2; pH4-G, IGIV treated at pH 4; TNF-α, tumour necrosis factor-alpha.

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The mechanism of the suppression by IGIV is, however, unclear. We previously showed that the interaction of IGIV with PEC via Fc gamma receptors (FcγR) may be important for suppression.[3] Therefore, we have postulated that the binding of IGIV to FcγR may result in production of a kind of second mediator, thus affecting the productions of TNF-α and IL-1. In a previous study,[4] we examined whether prostaglandin E_2 (PGE_2), known to suppress productions of TNF-α and IL-1,[5-10] participates in the suppression. We could find no evidence that PGE_2 was involved in the suppression.[4]

In this study, we focused our attention on participation of cyclic adenosine 3′:5′-monophosphate (cAMP), one of the well-known intracellular second messengers, in suppression by IGIV of LPS-induced TNF-α and IL-1 production. We found that the interaction of IGIV with the rabbit PEC via FcγR elevated intracellular cAMP levels, resulting in the suppression of the LPS-induced TNF-α and IL-1 productions.

MATERIALS AND METHODS

Reagents

LPS (from *Escherichia coli*, serotype 026:B6), N^6,2′-0-dibutyryl cAMP (BtcAMP), and cholera toxin (CT) were obtained from Sigma Co., St Louis, MO. MEM and RPMI-1640 were obtained from Gibco Laboratories, Grand Island, NY. RPMI-1640 was supplemented with penicillin (100 U/ml) and streptomycin

(100 μg/ml). N-[2-(methylamino) ethyl]-5-isoquinolinesulphonamide dihydrochloride (H-8) was purchased from Seikagaku Kogyo Co., Ltd, Tokyo, Japan.

IGIV

IGIV treated at pH 4 (pH4-G; lot 2.366.900.0; Sandoglobulin) was provided by the Central Laboratory of the Swiss Red Cross, Berne, Switzerland. The pH4-G was dissolved in MEM to a final concentration of 10%, dialysed against three changes of the medium for 36 hr, and stored in equal samples at −70° until use.

Preparation of Fc and F(ab')$_2$ fragments of pH4-G, and aggregated IgG

Fc fragments were prepared by papain cleavage of pH4-G.[11] A total of 150 mg of pH4-G was incubated at 37° for 16 hr with 1·5 mg of mercuripapain (Sigma Co.) in 0·1 M phosphate-buffer containing 0·01 M cysteine and 0·002 M ethylendiamine tetraacetic acid (pH 7) in a total volume of 5 ml. After stopping the reaction by adding iodoacetamide to a final concentration of 0·01 M, the reaction mixture was dialysed against 0·01 M phosphate buffer. Fc fragments were separated by chromatography on DEAE–Sephacel, as previously described.[11] F(ab')$_2$ fragments were prepared by pepsin cleavage of pH4-G.[11] A total of 150 mg of pH4-G was incubated at 37° for 20 hr with 1·5 mg of pepsin (Sigma Co.) in 0·2 M acetate-buffer (pH 4·5) in a total volume of 10 ml. After adjusting pH to 8·0, F(ab')$_2$ fragments were separated by gel filtration on Sephadex G-150. Aggregated IgG of pH4-G was prepared by dissolving pH4-G in physiological saline to a final concentration of 10%, and then heating it at 60° for 30 min. Aggregated IgG in the heated pH4-G was separated by high-performance liquid chromatography with TSKgel G3000SW column (Tosoh Co., Ltd, Tokyo, Japan).

Animals

Japanese male albino rabbits, weighing 2·5–3·5 kg, were obtained from Shizuoka Experimental Animals Inc., Shizuoka, Japan.

TNF and IL-1 productions by rabbit macrophages

As previously described,[2,4] rabbits were injected intraperitoneally with 50 ml of mineral oil per animal. Four days later, PEC were harvested by washing the peritoneal cavity with sterile physiologic saline. The cells were washed with RPMI-1640 and suspended in the medium. A total of 2×10^6 PEC per well were seeded into each well of a 24-well culture plate (Costar, Cambridge, MA) and reacted with 50 ng of LPS in a total volume of 1 ml at 37° for 24 hr. The plate was centrifuged, and the culture supernatants were harvested from each well and stored at −70° until used for the TNF assay. For IL-1 assay, the culture supernatants were dialysed against MEM at 4° for 24 hr to remove low molecular weight substances that might affect thymocyte proliferation. The dialysed materials were sterilized by filtration and stored at −70° until used for the IL-1 assay.

TNF and IL-1 assays

As previously described,[4] TNF activity was monitored by the lytic assay, using L929 tumourigenic murine fibroblasts, using the method of Carswell *et al.*[12] as modified by Flick & Gifford.[13] One unit of TNF activity was defined as the sample quantity required to achieve 50% cytotoxicity. IL-1 activity was determined by thymocyte proliferation assay,[14] as previously described.[4] IL-1 activity was expressed as means ± standard errors of counts per minute of triplicate wells.

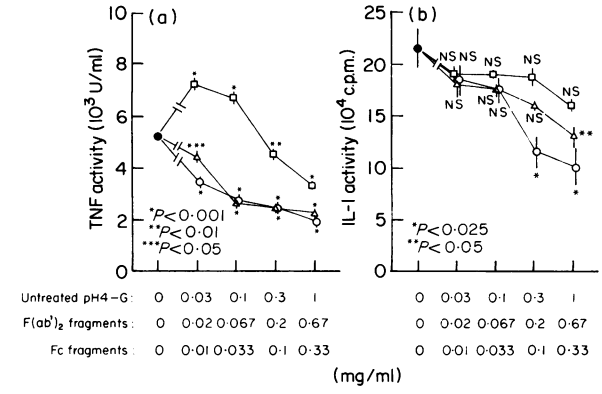

Figure 1. Effect of Fc or F(ab')$_2$ fragments of pH4-G on LPS-induced TNF-α and IL-1 productions. Rabbit PEC (1×10^6) were incubated with 25 ng of LPS in the presence of increasing amounts of untreated pH4-G (○), Fc (△) or F(ab')$_2$ (□) fragments of pH4-G at the final concentrations indicated in a total volume of 0·5 ml for 24 hr. The supernatants were assayed for TNF (a) and IL-1 (b) activities. Data are means ± SEM of TNF and IL-1 activities in triplicate.

cAMP assay

Intracellular cAMP was prepared as previously described.[15] Briefly, 1×10^7 of rabbit PEC were incubated with LPS (25 ng) and/or untreated pH4-G (5 mg) in a total volume of 0·5 ml at 37° for the time indicated in the Results. The cells were centrifuged at 3000 *g*, at 4°, for 1 min. Half a millilitre of 5% trichloroacetic acid was added to the cell pellet. The cells were disrupted by repeated (three times) freezing and thawing. The samples were centrifuged at 3000 *g*, at 4°, for 20 min to remove trichloroacetic acid-precipitable materials. Excess trichloroacetic acid in the aqueous supernatant was removed by extraction with water-saturated ether. The remaining supernatant was used to measure the amount of intracellular cAMP by radioimmunoassay (cAMP[^{125}I] assay system; Amersham Japan Co., Tokyo, Japan).

RESULTS

Suppression of TNF-α and IL-1 productions by Fc fragments of pH4-G

We previously demonstrated that IGIV suppresses LPS-induced TNF-α and IL-1 production.[2,4] We also found that IGIV shows a inhibitory effect on the rosette formation by the interaction of PEC with antibody-sensitized sheep E.[2] These results suggest that the binding of IGIV to PEC via the FcγR may result in the suppression of LPS-induced TNF-α and IL-1 production. We prepared Fc fragments from pH4-G to examine whether the fragments suppress LPS-induced TNF-α and IL-1 production. Untreated pH4-G and the Fc fragments suppressed LPS-induced TNF-α production at any dose used in the experiment, but the F(ab')$_2$ fragments showed a suppressive effect only at high concentration (Fig. 1a). Further untreated pH4-G and the Fc fragments, but not the F(ab')$_2$ fragments, suppressed the production of IL-1 (Fig. 1b). These results

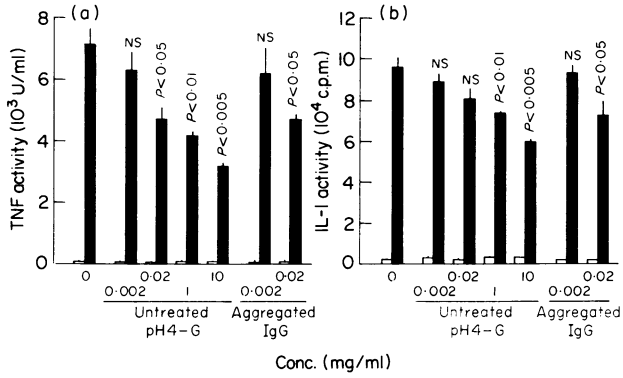

Figure 2. Effect of aggregated IgG on LPS-induced TNF-α and IL-1 production. Rabbit PEC (2×10^6) were incubated with increasing amounts of untreated pH4-G or aggregated IgG at the final concentrations indicated in the presence (■) or absence (□) of 50 ng of LPS in a total volume of 1 ml for 24 hr. The supernatants were assayed for TNF (a) and IL-1 (b) activities. Data are means ± SEM of TNF and IL-1 activities in triplicate.

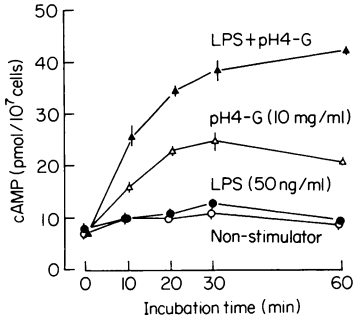

Figure 3. Levels of intracellular cAMP of PEC stimulated with LPS and/or pH4-G. Rabbit PEC (1×10^7) were incubated without or with 25 ng of LPS in the presence or absence of 5 mg of pH4-G in a total volume of 0·5 ml for the time indicated. The amount of cAMP in the sample was measured by radioimmunoassay. Data are means ± SEM of the amount of cAMP in triplicate.

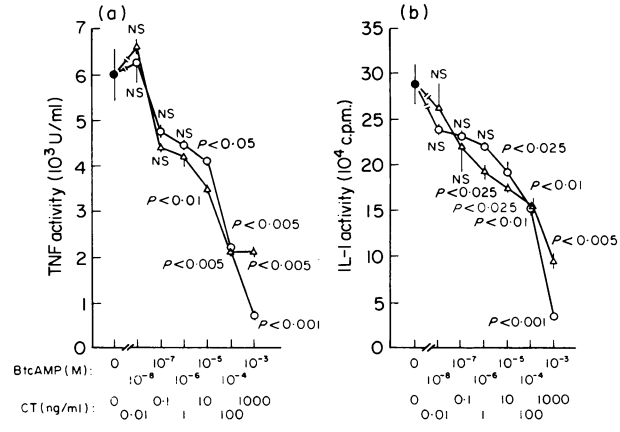

Figure 4. Effect of BtcAMP or CT on LPS-induced TNF-α and IL-1 production. Rabbit PEC (2×10^6) were incubated with increasing amounts of BtcAMP (○) or CT (△) at the final concentrations indicated in the presence of 50 ng of LPS in a total volume of 1 ml for 24 hr. The supernatants were assayed for TNF (a) and IL-1 (b) activities. Data are means ± SEM of TNF and IL-1 activities in triplicate.

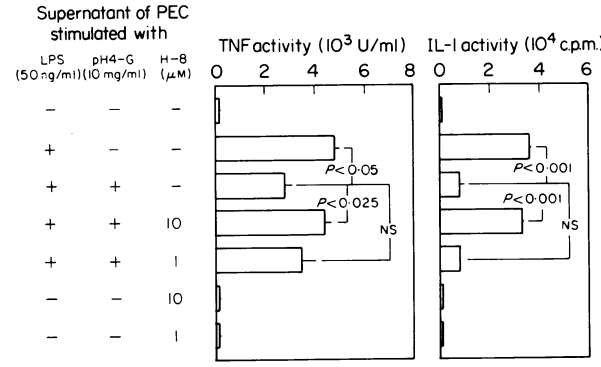

Figure 5. Effect of H-8 on suppression by pH4-G of LPS-induced TNF-α and IL-1 production. Rabbit PEC (2×10^6) were incubated with various combinations of LPS, pH4-G and H-8 at the final concentrations indicated in a total volume of 1 ml for 24 hr. The supernatants were assayed for TNF (a) and IL-1 (b) activities. Data are means ± SEM of TNF and IL-1 activities in triplicate.

suggest that binding of IGIV to PEC via FcγR is required for the suppression by IGIV of LPS-induced TNF-α and IL-1 production.

Suppression of TNF-α and IL-1 productions by aggregated IgG

Aggregated IgG binds to FcγR of macrophages more efficiently than dose monomeric IgG.[16] There is the possibility that the suppression by IGIV of LPS-induced TNF-α and IL-1 production is due to the binding of aggregated IgG contained in the preparation. We determined by high-performance liquid chromatography the content of aggregated IgG in the pH4-G preparation. Indeed, 0·2% of the total IgG was in aggregated form (data not shown). We prepared aggregated IgG by heating the pH4-G preparation, isolated it and examined the effect on LPS-induced TNF-α and IL-1 production. Untreated pH4-G and aggregated IgG suppressed LPS-induced TNF-α production at a concentration of 0·02 mg/ml (Fig. 2a). Untreated pH4-G also suppressed the production of IL-1 at a concentration of 1 mg/ml, as did aggregated IgG at 0·02 mg/ml (Fig. 2b). If the suppression by pH4-G of LPS-induced TNF-α and IL-1 production had been mediated by the aggregated IgG present in the preparation, a 500-fold amount of untreated pH4-G should have been required to suppress the production as strongly as aggregated IgG did. However, only a 50-fold amount of pH4-G showed the same magnitude of suppressive activity against IL-1 production as aggregated IgG did. Both untreated and aggregated IgG exhibited a similar magnitude of activity on TNF-α production at the same concentration. These results indicate that aggregated IgG contained in pH4-G preparation plays little, if any, role in the suppression of TNF-α and IL-1 production.

Increase in cellular cAMP levels of PEC stimulated with LPS and/or untreated pH4-G

The binding of pH4-G to FcγR of macrophages may result in the production of a kind of second messenger in the cells. cAMP is one of the second messengers and its increase in cellular concentration is known to inhibit the production of TNF-α[7,17,18] and IL-1.[19] We examined whether pH4-G elevates intracellular

cAMP levels of PEC. The intracellular cAMP levels of PEC stimulated with LPS showed no more change than did those of unstimulated PEC (Fig. 3). PEC stimulated with pH4-G alone produced a significant amount of intracellular cAMP as early as 10 min after stimulation, and the levels reached the maximum 30 min after stimulation. PEC stimulated with pH4-G in the presence of LPS produced more intracellular cAMP than did PEC stimulated with pH4-G alone.

Suppression of TNF-α and IL-1 productions by cAMP

If cAMP is a second messenger which mediates the suppression of LPS-induced TNF-α and IL-1 production, BtcAMP, a lipid-soluble derivative of cAMP that readily diffuses across the cell membrane, or CT, which increases intracellular cAMP levels by directly stimulating adenylate cyclase, may suppress TNF-α and IL-1 production. Both TNF-α and IL-1 production by PEC stimulated with LPS were dose-dependently suppressed by BtcAMP and CT (Fig. 4).

Elevation of intracellular cAMP levels generally causes the activation of cAMP-dependent protein kinases, resulting in regulation of the initiation process of activation of a receptor-coupling signal transduction system.[7] Next we examined the effect of H-8, an inhibitor of cAMP-dependent protein kinases,[20] on the suppression by pH4-G of LPS-induced TNF-α and IL-1 production. The suppression by pH4-G of TNF-α and IL-1 production was abrogated by H-8 at a concentration of 10 μM (Fig. 5).

DISCUSSION

This report shows that the suppression by IGIV of LPS-induced TNF-α and IL-1 production is mediated by the Fc portion of IgG in the IGIV preparation. Fc fragments of pH4-G suppressed the productions as effectively as untreated pH4-G did. The $F(ab')_2$ had little effect. These results exclude the possibilities that the suppression by IGIV results from the neutralization of LPS activity by anti-LPS antibody contained in IGIV[2] or from the direct action to FcγR of anti-FcγR antibody which might be present in the preparation.[21] We also explored the possibility that the suppression by IGIV is mediated by the Fc portion of aggregated IgG, since pH4-G contains aggregated IgG by 0·2%, which is known to have greater binding activity to macrophages via FcγR than monomeric IgG.[16] We prepared aggregated IgG by heating pH4-G, isolated it and compared the suppressive effect on LPS-induced TNF-α and IL-1 production with that of untreated pH4-G. Both aggregated and untreated IgG showed a similar degree of suppressive activity on TNF-α production and only a 50-fold amount of untreated IgG was required to exhibit as similar a magnitude of activity on IL-1 production as the aggregated IgG dose. Thus we concluded that aggregated IgG present in pH4-G plays little role in the suppression of LPS-induced IL-1 and TNF-α production.

In this study we also demonstrated that the binding of untreated pH4-G to rabbit PEC induces the elevation of intracellular cAMP levels. Increase in intracellular cAMP levels is known to inhibit TNF-α[7,17,22] and IL-1[19] production. Further, we showed that the increased cAMP levels result in the suppression of TNF-α and IL-1 production, using BtcAMP, a cAMP analogue, and CT, an adenylate cyclase-activating agent. Both BtcAMP and CT suppressed LPS-induced TNF-α and IL-1 production. Elevation of intracellular cAMP levels generally causes activation of cAMP-dependent protein kinases that phosphorylate certain specific substrate proteins, resulting in regulation of the initiating process of activation of a receptor-coupling signal transduction system.[7] Furthermore, we showed that H-8, an inhibitor of cAMP-dependent kinases,[20] abrogated the suppression by pH4-G of LPS-induced TNF-α and IL-1 production.

On the basis of these results, we propose the mechanism by which IGIV suppresses LPS-induced TNF-α and IL-1 production to be as follows. IGIV binds to rabbit PEC via FcγR. The binding triggers elevation of intracellular cAMP levels. The elevation causes activation of cAMP-dependent kinases that phosphorylate certain specific proteins, resulting in regulation of the process of LPS-coupling signal transduction for TNF-α and IL-1 production.

We do not have, however, an obvious explanation as to how IGIV increases intracellular cAMP levels of PEC. PGE_2 is known to bind to macrophage surface receptors, resulting in the activation of membrane-associated adenylate cyclase and the elevation of cAMP levels.[23,24] In a preceding study,[4] we found that LPS-induced PGE_2 production was rather suppressed by pH4-G, indicating that PGE_2 is not involved in the elevation of intracellular cAMP levels.

Another interesting finding is that pH4-G together with LPS had a synergistic effect on the levels of intracellular cAMP, although LPS by itself had little effect. Similar synergisms have been observed in the action of cytokines. TNF-α and IL-1 act synergistically in the generation of PGE_2 by fibroblasts.[22] IL-1 and LPS have synergistic inhibitory effects on the growth of a mouse myeloid leukemic cell line.[25] However, the mechanism of these synergisms are unclear.

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