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Interferon-γ and polyunsaturated fatty acids increase the binding of lipopolysaccharide to macrophages

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Summary. We have previously shown that interferon-γ (IFN-γ) increases the polyunsaturated fatty acid content of membrane phospholipids in cells that were sensitive to endotoxin. In this study, IFN-γ was found to stimulate the binding of endotoxin to the murine macrophage cell line J774.2 and the human monocyte cell line U937. Interferon-γ-activated J774.2 cells showed a 66% increase in fluoresceine isothiocyanate (FITC) labelled LPS binding ($P < 0.0005$ *vs* control cells) and a 49% increase in tritium labelled LPS binding ($P < 0.0001$ *vs* control cells). Interferon-γ also induced a 35% increase in binding of FITC-LPS in U937 cells ($P < 0.0001$ *vs* control cells). In contrast, pretreatment of J774.2 cells with interferon-β (IFN-β) had no effect on binding of FITC-LPS. Preincubation with exogenously supplied polyunsaturated fatty acids, linoleic and arachidonic acids, resulted in increases of 74% and 69% in FITC-LPS binding, respectively (both $P < 0.0005$ *vs* control cells). On the other hand, pretreatment with the saturated fatty acid, palmitic acid, had no effect on FITC-LPS binding. We propose that IFN-γ-induced changes in the membrane phospholipid fatty acid composition of macrophage-like cells influence the binding of endotoxin.

Keywords: interferon-γ, arachidonic acid, linoleic acid, macrophage, lipopolysaccharide

Bacterial endotoxin can induce a rapidly fatal illness which has proved exceptionally difficult to combat clinically and which remains a major cause of morbidity and mortality. Despite this, the detailed biochemical and molecular events underlying the toxicity of endotoxin remain unknown and the reason why some patients become hypersensitive to endotoxin has so far eluded explanation.

Variations in the sensitivity of experimental animals to endotoxin (lipopolysaccharide (LPS)) can be induced by infections with various microorganisms, their products, or certain host-derived cytokines (Movat *et al.* 1987). Sensitivity to the lethal effects of LPS can be dramatically increased by infection with mycobacteria (Suter & Kirsanow 1961) which can result in the production of cytokines, especially interferon-γ (IFN-γ), which has also been shown to induce endotoxin sensitivity in an animal model (Billiau *et al.* 1987). Interferon-γ receptors are found on most cells, including macrophages and macrophage-like cells, and many studies have been carried out recently to examine murine macrophage responses to IFN-γ. The precise mechanism by which this cytokine can stimulate these cells to be hyperresponsive to 'triggering' agents, such as LPS, is

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unknown. However, some of our recent studies suggest that increases in the polyunsaturated fatty acid (PUFA) content of murine peritoneal macrophages are linked to increased sensitivity to LPS *in vivo* (Stark *et al.* 1990). We have also shown that IFN-γ induces precisely the same alterations in phospholipid fatty acid profiles in both macrophage and macrophage-like cell lines *in vitro* (Jackson *et al.* 1992). Furthermore, we have shown that treatment with IFN-γ increases membrane fluidity (Darmani *et al.* 1993a) and stimulates an increased turnover of phospholipid acyl groups (Darmani *et al.* 1993b). Changes in plasma membrane lipid composition are known to have significant effects on cellular responses (Yeagle 1989).

In this study we have examined the effect of IFN-γ on the binding of LPS to human and murine monocyte-macrophage cell lines and the effect of phospholipid alterations on this binding.

Materials and methods

Reagents

All reagents were purchased from Sigma Chemical Company Ltd, Poole, UK, unless otherwise stated. ^{3}H-Sodium borohydride (25mCi) was supplied by Amersham International plc, Aylesbury, UK. Human and murine recombinant IFN-γ were purchased from Genzyme Diagnostics, West Malling, Kent, UK.

Growth medium

Cells were cultivated in Dulbecco's modification of Eagle's medium (DMEM) which had been supplemented with 10% foetal calf serum (FCS), 100 units/ml penicillin and 100 μg/ml streptomycin.

Cell lines

The cell lines used in the course of this investigation were the murine macrophage-like DBA/2 lymphoid neoplasm P388D (ICN Biomedicals Ltd, High Wycombe, Bucks, UK), the murine (BALB/C) tumour monocyte-macrophage J774.2 and the human histiocytic lymphoma monocyte-like U937 cell-line (European Collection of Animal Cell Cultures, Salisbury, Wilts, UK). These cells were maintained in DMEM supplemented as described above.

Radiolabelling of LPS

To 10 mg of *Escherichia coli 0111:B4* LPS, 4 ml of 7×10^{-2} M sodium periodate was added and left for 150 minutes at 22°C. To the reaction mixture, 20 ml ice-cold ethanol was then added and left at 4°C in order to precipitate the LPS. After centrifugation at 2000 ***g*** at 4°C for 15 minutes, the supernatant was removed and the contents of the tube mixed and left at 4°C to allow further precipitation of the LPS. Following centrifugation at 4°C for 15 minutes the pellet was resuspended in 1 ml ice-cold water and 40 μl ethylene glycol was added. To this, 25 mCi of ^{3}H-NaB_3H_4 which had previously been diluted in sodium borohydride (2 mg in 0.5 ml) was added and left overnight at 4°C. Sodium borohydride (3 mg in 0.5 ml) was then added. Following incubation at 0°C for 30 minutes, 4 ml ethanol was added and the mixture shaken gently to allow the acetic and boric acids to dissolve. The ethanol was evaporated under a stream of nitrogen and the labelled LPS separated using a small (50 × 15 mm) PD 10 column (Pharmacia Biotech Ltd, St Albans, Hertfordshire, UK). The radiolabelled LPS was eluted close to the void volume.

Pretreatment with cytokines

Exposure of J774.2 cells (1×10^6/ml) to 50 U/ml murine recombinant IFN-γ, murine recombinant IFN-α or murine recombinant TNF-α and U937 cells (1×10^6/ml) to 100 U/ml human recombinant IFN-γ was carried out for 18 hours in a 37°C, humidified 5% CO_2 incubator. At the end of the incubation period the cells were transferred to test-tubes, washed three times with DMEM, collected by centrifugation at 1000 ***g*** for 5 minutes and finally suspended in DMEM.

Pretreatment with fatty acids

To aid solubility of the fatty acids, the ammonium salts were prepared by incubating the fatty acids in 0.2 ml of 2 M ammonia solution at 60–70°C for 30 minutes. The ammonia was blown off under a stream of nitrogen and the resulting ammonium salt was resuspended in a known volume of growth medium. Cells were cultured in the presence of different concentrations of the various fatty acids for 18 hours in a 37°C humidified 5% CO_2 incubator. The cells were then washed twice, collected by centrifugation at 1000 ***g*** for 2 minutes and finally resuspended in growth medium.

Binding of ^{3}H-LPS

Cells were incubated in the presence of 0.5 μCi of the radiolabelled LPS for 2 hours at 37°C in a humidified CO_2 incubator. The cells were centrifuged at 1000 ***g*** for

3 minutes and an aliquot of the supernatant was kept for radioactivity analysis. The cells were then washed with 10 ml of phosphate buffered saline (PBS) three times by centrifugation at 1000 ***g*** for 3 minutes, resuspended in 1 ml deionized water and sonicated for 30 minutes in an ultrasonic water-bath at maximum power until complete cell lysis had been achieved and the radioactivity was determined in the cell suspension.

Binding of FITC-LPS

Control, IFN-γ, linoleate, arachidonate and palmitate-pretreated cells were incubated in the presence of 2 μg FITC-LPS from *Escherichia coli O111:B4* for 30 minutes at 37°C in a humidified CO_2 (5%) incubator. The cells were washed in PBS three times and collected by centrifugation at 1000 ***g*** for 5 minutes and were finally resuspended in 0.5 ml PBS. The level of FITC-LPS bound to the cells was determined using a Becton Dickinson Fluorescence Activated Cell Scanner (FACS). The median fluorescence value for 10 000 cells was obtained on a PC using Lysis software. Two different batches of FITC-LPS were used during the course of these experiments and this would explain any differences in staining between control cells in the different figures.

The number of molecules of LPS bound per cell was determined from standard curves generated with FITC-labelled calibrating beads ranging from 4.1×10^4 to 19×10^5 molecules of FITC. Since the conjugation ratio for FITC-LPS was estimated to be 1 : 20, the final values were multiplied by a factor of 20 in order to convert the values to number of LPS molecules bound per cell.

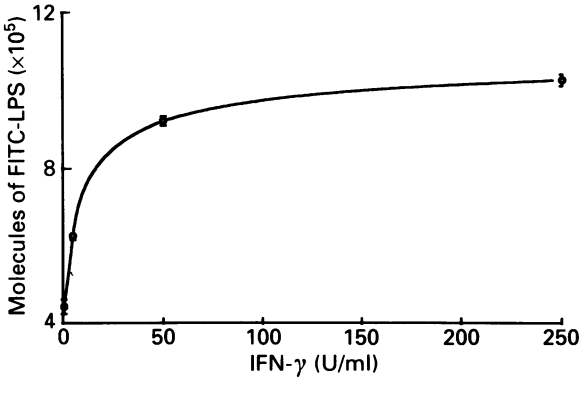

Figure 1. Binding of FITC-LPS from *Escherichia coli 0111:B4* to the cell line J774.2. Cells were incubated for 18 hours with various concentrations (0–250 U/ml) of interferon-γ, washed and incubated with 2 μg/ml FITC-LPS for 30 minutes at 37°C. Values shown are means for 10^4 cells expressed as ± standard error of the means for 5 experiments.

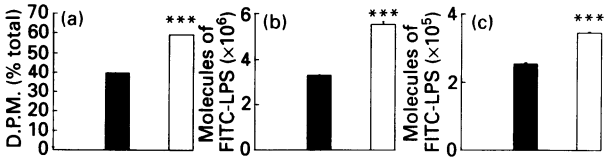

Figure 2. Binding of a, ^{3}H-LPS from *Escherichia coli 0111:B4* to ■, control and □, interferon-γ-pretreated J774.2 (***$P < 0.0001$); b and c, FITC-LPS from *Escherichia coli 0111:B4* to ■, 10^4 control and □, interferon-γ-pretreated J774.2 and U937 cells (***$P < 0.0001$), respectively. J774.2 and U937 cells were pretreated with either 50 U/ml recombinant murine interferon-γ or 100 U/ml recombinant human interferon-γ (respectively) for 18 hours. Cells were then washed and incubated with either 0.5 μCi ^{3}H-LPS for 2 hours at 37°C or with 2 μg/ml FITC-LPS for 30 minutes at 37°C. Values shown are means ± standard error of the means for 5 experiments.

Radioactivity determination

Samples were quantified for radioactivity by liquid scintillation counting using a biodegradable counting scintillant (Amersham International plc). The radioactivity was determined using a Betamatic liquid scintillation counter (Kontron Instruments, Watford, UK) with automatic quench correction by the external channels ratio method.

Results

Figure 1 shows a dose–response curve for FITC-LPS binding to J774.2 cells incubated in the presence of 0–250 U/ml IFN-γ. The curve plateaued after 50 U/ml and this concentration was used throughout the study. Cells were also incubated with IFN-γ for 1, 3, 6 and 18 hours and the results (not shown) indicated that IFN-γ pretreatment for 18 hours was necessary for maximum effect on LPS binding.

Figure 2 shows the binding of (a) radiolabelled (^{3}H)-LPS from *Escherichia coli 0111:B4* to control and IFN-γ-pretreated J774.2 cells, and of fluorescein isothiocyanate labelled (FITC-LPS) *Escherichia coli 0111:B4* endotoxin to (b) control and IFN-γ-pretreated J774.2 and (c) control and IFN-γ-pretreated U937 cells. It can be seen that the effect of IFN-γ is a significant increase in LPS binding to J774.2 and U937 cell membranes. Interferon-γ induced a 49% increase in ^{3}H-LPS binding to J774.2 cells ($P < 0.0001$ *vs* control cells) and a 66% increase in FITC-LPS binding ($P < 0.0005$ *vs* control cells). In controls an average of 3.32×10^6 molecules of FITC-LPS were bound per cell and in IFN-γ-pretreated J774.2 cells an average of 5.52×10^6 molecules of FITC-LPS were bound per cell. When IFN-γ-pretreated cells were

incubated with a 50-fold excess of unlabelled LPS there was a 90% inhibition of FITC-LPS binding indicating that the effects induced by IFN-γ were specific (results not shown).

Interferon-γ also induces a highly significant increase in the binding of FITC-labelled *Escherichia coli 0111:B4* endotoxin to U937 cells ($P < 0.0001$ *vs* control cells). In controls, an average of 2.56×10^5 molecules of FITC-LPS were bound per cell and an average of 3.46×10^5 molecules of FITC-LPS were bound per cell to IFN-γ-pretreated cells. This represents a 35% increase in endotoxin binding to these cells.

These experiments were repeated using FITC-labelled *Salmonella minnesota* endotoxin and, again, IFN-γ-pretreated cells exhibited similar enhanced endotoxin binding (results not shown). Moreover, similar results were also obtained with the murine macrophage-like DBA/2 lymphoid neoplasm P388D cells.

We have previously shown that incubating cells in medium with supplementary fatty acids could modify the phospholipid fatty acid changes induced by IFN-γ under standard conditions (culture medium and calf serum only) (Jackson *et al.* 1992). We therefore incubated cells in media containing different concentrations of the unsaturated fatty acids linoleic and arachidonic acids (which we have suggested to be involved in increased endotoxin sensitivity (Stark *et al.* 1990)) to check for any subsequent effects on FITC-LPS binding. Figure 3 shows the results of preincubation with (a) 10 and 100 μg/ml linoleic acid, (b) 10 and 50 μg arachidonic acid and (c) 10 and 100 μg/ml palmitic acid on the binding of FITC-LPS to J774.2 cells. It can be seen that linoleic acid induces a highly significant increase in FITC-LPS binding to J774.2 cells ($P < 0.005$ *vs* control cells). In controls, 4.38×10^6 molecules of FITC-LPS were bound per cell. Cells pretreated with 10 μg/ml linoleate bound 5.48×10^6 molecules of FITC-LPS per cell showing a 25% increase in the binding of endotoxin

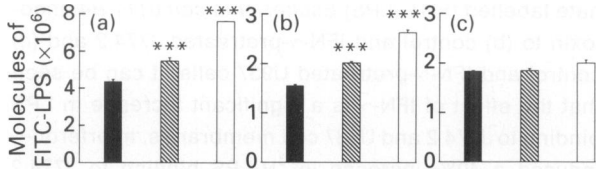

Figure 3. Binding of *Escherichia coli 0111:B4* to J774.2 cells preincubated ■, without and with a, ▨, 10 and □, 100 μg/ml linoleic acid; b, ▨, 10 and □, 50 μg/ml arachidonic acid; and c, ▨, 10 and □, 100 μg/ml palmitic acid for 18 hours, washed and incubated with 2 μg/ml FITC-LPS for 30 minutes at 37°C. Values shown are the means for 10^4 cells ± standard error of the means for 5 experiments. ***$P < 0.0005$.

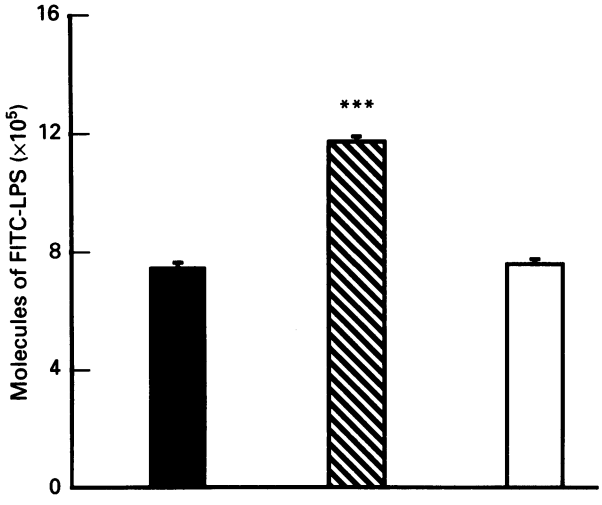

Figure 4. Binding of FITC-LPS from *Escherichia coli 0111:B4* to J774.2 cells preincubated ■, without and with ▨, IFN-γ and □, IFN-β. Cells were incubated for 18 hours with and without 50 U/ml IFN-β at 37°C, washed and incubated with 2 μg/ml FITC-LPS for 30 minutes at 37°C. Values shown are the means for 10^4 cells ± standard error of the means for 5 experiments. ***$P < 0.0001$.

to these cells. When cells had been preincubated with 100 μg/ml linoleic acid there was a 74% increase in LPS binding, which is of the same order of magnitude as the effect of preincubation with IFN-γ (Figure 2b). Cells incubated in the presence of different concentrations of arachidonic acid also showed increases in binding of FITC-LPS. Cells pretreated with 10 μg/ml arachidonic acid, showed a 29% increase in endotoxin binding ($P < 0.0005$ *vs* control cells). Those pretreated with 50 μg/ml arachidonic acid showed a 69% increase in FITC-LPS binding ($P < 0.0005$ *vs* control cells). High concentrations (e.g. 100 μg/ml) of arachidonic acid were found to be detrimental to cell growth.

In contrast to the data with polyunsaturated fatty acids, it is clear from Figure 3(c) that cells pretreated with 10 or 100 μg/ml palmitic acid showed no significant increase in LPS binding.

The effect of IFN-β on FITC-LPS binding is shown in Figure 4. Interestingly, cells preincubated with IFN-β showed no significant increase in FITC-LPS binding. This is in contrast to the effect of preincubation with IFN-γ which results in increased LPS binding ($P < 0.0001$ *vs* control cells). Furthermore, TNF-α-pretreatment did not affect the binding of FITC-LPS to J774.2 cells (results not shown).

Discussion

In this study we have tried to correlate our previous

observation that IFN-γ induces changes in membrane lipids (Jackson *et al.* 1992) with the development of increased endotoxin binding to macrophages. We have found that IFN-γ increases the ability of murine and human macrophage cell lines to bind bacterial endotoxin. The binding of radiolabelled *Escherichia coli 0111:B4* lipopolysaccharide (LPS) is increased significantly by IFN-γ-pretreatment in all three cell types that we tested.

Recently we have shown that IFN-γ can induce increases in the polyunsaturated fatty acid content in macrophages and macrophage-like cell lines *in vitro* (Jackson *et al.* 1992). These alterations are very similar to the changes in fatty acid content seen after BCG-stimulated increases in endotoxin sensitivity *in vivo* (Stark *et al.* 1990). Our present results indicate that polyunsaturated fatty acids, in particular linoleic acid, could also cause a significant increase in LPS binding to macrophage cells in the absence of IFN-γ. The saturated fatty acid, palmitic acid, was not effective in this regard. Combining the results of these and previous experiments (Jackson *et al.* 1992), we can say that IFN-γ increases the content of polyunsaturated fatty acids in the membranes of macrophages and that such alterations in fatty acid content enhance or facilitate the binding of LPS. Thus, an important mechanism by which IFN-γ upregulates responses of macrophages to endotoxin could be by inducing an increase in polyunsaturated fatty acids which allow increased binding of endotoxins.

CD14 antigen has been reported to act as a receptor for complexes of bacterial LPS and LPS-binding protein (LBP) (Wright *et al.* 1990). We have found that IFN-γ-pretreated J774.2 cells bind 5.52×10^6 molecules of FITC-LPS/cell, which is comparable with the value reported for CD14 expression in the THP-1 human monocyte-macrophage cell line after exposure to 1,25-dihydroxyvitamin D_3 (Kitchens *et al.* 1992). Although CD14 plays a key role in the serum dependent response to LPS there is increasing evidence for the existence of one or more mammalian cell receptors for LPS (Morrison *et al.* 1993). The possibility exists, therefore, that changes induced by IFN-γ facilitate the expression of one or more receptor molecules for LPS (S.K. Jackson *et al.*, unpublished). Indeed, recent studies have identified a protein of 70–80 kDa as a dominant LPS target on lymphoreticular cells and there is increasing evidence for at least one additional protein of about 40 kDa which bind LPS (Morrison *et al.* 1993).

Treatment of human fibroblasts in culture with human IFN-β causes inhibition of lateral mobility of concanavalin A cell surface receptors (Pfeffer *et al.* 1980). Interferon-β has also been reported to cause a reduction in the unsaturated fatty acid content of all major cellular phospholipids (Chandrabose *et al.* 1981). An increase in the ratio of saturated to unsaturated fatty acids would be consistent with increased rigidity of the plasma membrane lipid bilayer (Pfeffer *et al.* 1980). Interestingly, in contrast to IFN-γ, which increases membrane lipid bilayer fluidity (Darmani *et al.* 1993a), pretreatment of J774.2 cells with either IFN-β or TNF-α did not have a stimulatory effect on endotoxin binding. These results reflect the importance of phospholipid polyunsaturations in the interaction of macrophage cells with endotoxin.

To further prove the importance of these phospholipid alterations to LPS binding we carried out incubations of J774.2 cells in media containing an increased supply of exogenous polyunsaturated or saturated fatty acids. Preincubation of macrophage cells with exogenous fatty acids results in extensive enrichment with the incubated fatty acids (Shichiri *et al.* 1993). Studies of preincubation of J774.2 cells in media with 100 μg/ml linoleate and 50 μg/ml arachidonate, both polyunsaturated fatty acids, have revealed an increase in LPS binding of the same order of magnitude as that observed with IFN-γ-pretreatment. In contrast to the effect of these polyunsaturated fatty acids, palmitate, a saturated fatty acid, was found to have no significant effect on LPS binding to these cells.

We have previously suggested that IFN-γ increases host susceptibility to endotoxin by increasing the content of polyunsaturated fatty acids in the phospholipids of macrophages which may subsequently be released by phospholipases to form the mediators and signals associated with endotoxaemia (Darmani *et al.* 1993b) and which may result in the expression of molecules which allow enhanced physiological responses of the IFN-γ activated cells to endotoxin. As a result of IFN-γ-induced membrane phospholipid fatty acid changes, associated membrane alterations could directly control the interaction of macrophages with endotoxin and hence lead to enhanced endotoxin binding to these activated cells.

The present study has shown that IFN-γ can play an important role in the interaction of endotoxin with macrophages. The lymphokine alters phospholipid metabolism and turnover and these changes alter membrane properties (Darmani *et al.* 1993a) so as to allow enhanced endotoxin binding. In fact, in the case of lung macrophages, which have been reported to lack binding sites of endotoxin, pretreatment with IFN-γ has been reported to induce binding sites for LPS on the cell surface (Akagawa & Tokunaga 1985).

It is clear from the results of this study that IFN-γ enhances the interaction of macrophage cells with endotoxin. Interferon-γ-induced phospholipid alterations may be essential to binding of endotoxin and research in this area will enhance our understanding of the basic biochemistry of endotoxin action and provide us with the knowledge for developing strategies aimed at reducing or blocking the sensitivity to endotoxin in vulnerable patients.

Acknowledgement

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