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Expression of TGF-β in attenuated *Salmonella typhimurium*: oral administration leads to the reduction of inflammation, IL-2 and IFN-γ, but enhancement of IL-10, in carrageenin-induced oedema in mice

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SUMMARY

Mice injected with carrageenin in the footpad developed local inflammation which peaked at 48 hr. This was significantly reduced in mice inoculated orally with an attenuated *Salmonella* construct expressing transforming growth factor-β (TGF-β). Administration of the *Salmonella* construct alone had no effect on inflammation. High levels of interleukin-2 (IL-2) and interferon-γ (IFN-γ) were secreted by draining lymph node cells from mice injected with carrageenin following stimulation *in vitro*. Prior inoculation with *Salmonella* enhanced the production of IL-2 and IFN-γ from the draining lymph node cells. Administration of the *Salmonella*–TGF-β construct significantly inhibited the production of these cytokines. In contrast, IL-10 only was secreted from draining lymph node cells of animals inoculated with the *Salmonella*–TGF-β construct. Thus, oral administration of TGF-β can significantly inhibit local inflammation and alter the cytokine secretion pattern of cells from lymph nodes draining the site of inflammation.

INTRODUCTION

Transforming growth factor-β (TGF-β) in a 25 000 MW homodimeric protein which is secreted by various cells, including platelets, lymphocytes and macrophages,[1–4] and which binds to a high-affinity receptor found in most cell types.[5,6] TGF-β can stimulate CD16 expression on monocytes,[7] promote their chemotaxis[8] and augment monocyte synthesis of interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α) and platelet-derived growth factor (PDGF).[9] However, TGF-β is also a powerful immunosuppressive agent, inhibiting IL-1-dependent thymocyte proliferation, IL-2-dependent T-cell proliferation and B-cell proliferation and differentiation.[2,3,10] It can also inhibit the activation of natural killer (NK) cells[11] and the generation of lymphokine-activated killer (LAK) cells and cytotoxic T cells.[12] In addition, TGF-β can down-regulate IFN-γ-induced class II expression on human cell lines,[13] antagonize the effects of IL-1, IL-2 and IL-3,[9] and inhibit both immediate and delayed-type hypersensitivity responses in mice.[14] Finally, it can inhibit cytokine-induced macrophage activation,[15] resulting in the inhibition of both reactive oxygen species[15] and nitric oxide.[16,17]

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Abbreviation: TFG-β, transforming growth factor-β.

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As TGF-β exhibits multiple immunosuppressive properties, its use as a therapeutic agent in inflammatory disease has been investigated. Systemic administration of TGF-β reduced the inflammation and tissue damage observed in animal models of arthritis and relapsing experimental allergic encephalomyelitis.[18–21] In contrast to systemic administration, localized administration of TGF-β enhanced the inflammatory response,[22,23] while local administration of an anti-TGF-β antibody suppressed leucocyte accumulation and joint destruction.[24] Thus, the route of administration can greatly influence the effect of TGF-β *in vivo*. We therefore explored the effect on local inflammation of TGF-β delivered orally, which is perhaps the most convenient and acceptable route of administration of therapeutic agents.

Auxotrophic mutants of *Salmonella typhimurium* are highly attenuated and capable of only limited growth *in vivo* (reviewed in ref. 25). However, they can penetrate the mucosal surface of the intestinal tract, reaching the lymphoid organs, and inducing both humoral and cell-mediated immune responses and protection against highly virulent wild-type organisms. *Salmonella* mutants have now been successfully used as carriers for a number of non-*Salmonella* antigens including streptococcal M protein,[26] the circumsporozoite protein from *Plasmodium*,[27] glycoprotein 63 (gp63) from *Leishmania major*[28] and human IL-1β.[29]

We have now investigated the effect of TGF-β, delivered orally, in a model of acute inflammation. As Th1 cells are responsible for delayed-type hypersensitivity responses[30,31] and have been isolated from the synovium of patients with

rheumatoid or reactive arthritis,[32,33] we also investigated the effect of TGF-β delivered orally on Th1-cell activities. We show here that TGF-β, delivered orally, can reduce carrageenin-induced inflammation. This is accompanied by a marked reduction in IL-2 and IFN-γ levels, but elevation of IL-10 secretion by the draining lymph node cells *in vitro*.

MATERIALS AND METHODS

Mice

Female BALB/c mice, 6–8 weeks of age, were obtained from Harlan Olac Ltd (Bicester, UK). They were housed in temperature-controlled rooms and received food and water *ad libitum*.

Materials

Lambda-carrageenin, concanavalin A (Con A) and lipopolysaccharide (LPS) from *Salmonella enteritidis* were obtained from Sigma (Poole, UK). Culture medium was RPMI-1640 (Gibco, Paisley, UK) containing 10% fetal calf serum (FCS), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-mercaptoethanol (50 μM) and, for cytokine generation, 25 mM HEPES. Mink lung cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco) supplemented with 10% FCS, L-glutamine, penicillin and streptomycin. Paired monoclonal antibodies, purchased from Pharmingen (A.M.S. Biotechnology Ltd, Witney, UK), were used for the detection of IL-2 (JES6-1A12, JES6-5H4), IL-4 (BVD4-1D11, BVD6-24G2), IL-6 (MP5-20F3, MP5-32C11) and IL-10 (JES5-2A5, SXC1) in enzyme-linked immunosorbent assays (ELISA). Interferon-γ (IFN-γ) and TNF-α were detected using a rat anti-murine IFN-γ monoclonal antibody (R46A2) or rat anti-murine TNF-α monoclonal antibody (XT22.11) and polyclonal rabbit antibodies produced in our laboratory by immunization with recombinant IFN-γ or TNF-α. All murine recombinant cytokine standards were purchased from Genzyme (West Malling, UK), except IFN-γ and TNF-α which were the kind gift of Dr G. Adolf (Ernst-Boehringer-Institut fur Arzneimittel-Forschung, Vienna, Austria). Recombinant human TGF-β (rhTGF-β) and a chicken anti-TGF-β antibody were kindly provided by the National Institute of Biological Standard and Control (Potters Bar, UK).

Bacterial strains, media and plasmids

Escherichia coli TG1 was used for plasmid construction and expression studies. *Escherichia coli* and *Salmonella* were routinely grown in Luria broth (Gibco). *Salmonella typhimurium* LB5010[34] was obtained from K. Sanderson (Salmonella Genetic Stock Centre, Calgary, Canada) and the *S. typhimurium aroA*$^-$ *aroD*$^-$ double mutant BRD509[35] from the Medeva Vaccine Group (Imperial College, London, UK). The plasmid pKK233-2 was obtained from Pharmacia (Milton Keynes, UK). High-transducing phage P22HT 105 int[36] was originally from Dr T. Foster (Trinity College, Dublin, Ireland). Murine TGF-β cDNA was the kind gift of the DNAX Research Institute (Palo Alto, CA).

Plasmid construction, gene expression and protein characterization

The leading sequence of murine TGF-β cDNA was deleted and the fragment amplified by polymerase chain reaction (PCR) using primers for TGF-β (sense 5′-CTG-CAG-AGC-TCA-CGG-CAC-CCC-ATG-GCC-CTG-GAT-ACC-AAC-3′; antisense 5′-GGC-GGG-GGC-GGG-GCC-AAG-CTT-GCC-GGG-AGG-GGC-GGG-3′). The resultant fragment was purified and digested with *Nco*I and *Hin*dIII, then inserted into *Nco*I/*Hin*dIII site of pKK233-2. The control pKK233-2 (pKK) and pKK233-2–TGF-β (pKK–TGF-β) were transformed into *E. coli* using previously published procedures.[37] Briefly, competent cells (200 μl) were added to a ligation/DNA reaction tube containing 100 ng DNA in 50 mM $CaCl_2$. The cells were incubated on ice for 60 min followed by heating at 42° for 2 min. To each reaction 1 ml of L-broth was then added. The mixture was incubated at 37° for a further 90 min. Ampicillin-resistant clones were selected by growing an agar containing 100 μg/ml ampicillin and identified by restriction enzyme mapping. Expression of TGF-β was confirmed by analysing bacterial lysates on 15% polyacrylamide gels and transferring to nitrocellulose (Bio-Rad, Hemel Hempstead, UK). Western blot analysis was carried out using a polyclonal rabbit anti-TGF-β antiserum (Genzyme) followed by a goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad) and developed using 3,3′-diaminobenzidine tetrahydrochloride (Sigma).

The plasmid pKK–TGF-β was purified from *E. coli* by the standard procedure of phenol/chloroform extraction followed by precipitation with ethanol and transduced into the *aroA*$^-$ *aroD*$^-$ *S. typhimurium* mutant BRD509, via strain LB5010, using phage P22.[36,38] Transductants were again selected on medium containing ampicillin and expression of TGF-β verified by Western blot as already described. The bioactivity of TGF-β in the bacterial lysate was measured by the inhibition of growth of mink lung cells[39] as described later in the Materials and Methods.

Measurement of plasmid stability in vivo

The *in vivo* stability of the plasmid in *Salmonella* was measured by assaying for viable organisms in the liver and spleen of mice inoculated orally.[28] Briefly, groups of mice were administered orally by a gavage tube with approximately 5×10^9 organisms from an overnight culture of *S. typhimurium* BRD509 alone (BRD509) or BRD509 expressing TGF-β (BRD509–TGF-β). The inoculum dose was confirmed by plating serial dilutions onto agar plates containing 100 μg/ml ampicillin. The spleen and liver were removed at various time-points following inoculation, homogenized and dilutions of the lysates plated onto agar plates with or without ampicillin. The number of colonies in plates with or without ampicillin was then counted.

Induction of inflammation

Groups of mice were inoculated orally with 5×10^9 organisms per mouse from an overnight culture of BRD509 or BRD509–TGF-β in 0·2 ml phosphate-buffered saline (PBS). Controls received PBS alone. Six days after inoculation, an inflammatory response was elicited by injecting each animal with 300 μg carrageenin subcutaneously into one hind paw. Footpad swelling was measured daily using a spring-dial calliper. Results are expressed as the difference (in mm) in the thickness of the carrageenin-injected and saline-injected footpads.

T-cell proliferation assays

Draining lymph node (DLN) cells were removed 48 hr after

administration of carrageenin. Single-cell suspensions were obtained, resuspended at $2{\cdot}5 \times 10^6$ cells/ml in culture medium and dispensed at 100 μl/well in 96-well flat-bottomed plates (Nunc, Roskilde, Denmark). Con A (0·5 or 5 μg/ml) was added to each well and the cultures (total volume of 200 μl) incubated at 37°, in an atmosphere of 5% CO_2, for 48 hr. Cultures, in triplicate, were pulsed with 0·5 μCi/well [^{3}H]thymidine (Amersham International, Amersham, UK) for the final 6 hr of incubation, then harvested and counted in a beta-scintillation counter (Pharmacia). Results are expressed as incorporation of radioactivity (c.p.m. ± 1 SEM).

FACScan analysis

Aliquots of the DLN cells used for proliferation assays were also stained with various antibodies to determine the phenotype of the cell populations, using flow cytometry (Becton Dickinson, Mountain View, CA). The antibodies used were: biotinylated hamster anti-mouse CD3, biotinylated rat anti-mouse CD4, biotinylated rat anti-mouse CD8, biotinylated rat anti-mouse NK cell (all from Pharmingen, Cambridge Bioscience, Cambridge, UK), biotinylated F(ab′)$_2$ anti-mouse immunoglobulin (Dako, High Wycombe, UK) and peroxidase-labelled rat anti-mouse macrophage (Serotec, Kidlington, Oxford, UK). The antibodies were used according to manufacturers' instructions. Binding of the biotinylated antibodies was detected by fluorescein isothiocyanate (FITC)–streptavidin (Vector Laboratories, Peterborough, UK).

Generation of cytokines

DLN cells obtained 48 hr after administration of carrageenin were resuspended at 10^6 cells/ml and dispensed at 1 ml/well in 24-well plates (Corning, Glassworks, Corning, NY). Cultures were stimulated with LPS (10 μg/ml), LPS (1 μg/ml) plus IFN-γ (200 U/ml) or Con A (5 μg/ml) and cell-free supernatants obtained at 24 and 48 hr post-stimulation. Supernatants were stored at −70°.

Measurement of IL-2, IL-4, IL-6, IL-10, IFN-γ and TNF-α

The levels of these cytokines in culture supernatants were measured by ELISA. Flat-bottomed 96-well microtitre plates were coated with the capture monoclonal antibody (1–4 μg/ml, 50 μl/well), and samples or standards dispensed into each well (100 μl). Bound IL-2, IL-4, IL-6 and IL-10 were detected by the addition of biotinylated anti-cytokine monoclonal antibodies (1–4 μg/ml; 100 μg/well) and by 2 μg/ml extravidin–peroxidase (100 μl/well; Sigma). The assay was developed by the addition of TMB (3,3′,5,5′tetramethyl benzidine) substrate (100 μl/well; Kirkgaard & Perry Laboratories Inc., Gaithersburg, MD) and read at 630 nm on a dual-wavelength spectrophotometer (Dynatech, Billinghurst, UK). Bound IFN-γ and TNF-α were detected by the addition of a polyclonal rabbit anti-murine IFN-γ or TNF-α antibody (5 or 10 μg/ml respectively, 100 μl/well). Finally, sheep anti-rabbit alkaline phosphatase IgG was added at a 1:1000 dilution (100 μl/well; Sigma), the assay developed by the addition of *p*-nitrophenyl phosphate (1 mg/ml in 1 M Tris, 3 mM $MgCl_2$, pH 9·6, 100 μl/well) and read at 405 nm. The cytokine content of each sample was read off a standard curve established with the appropriate recombinant cytokine.

Bioassays for IL-1β and TGF-β

IL-1 was measured by a bioassay. Briefly, D10-N4M cells were resuspended at 10^5 cells/ml in RPMI + 10% FCS containing recombinant murine IL-2 (60 U/ml) and Con A (6 μg/ml) and dispensed (100 μl/well) into 96-well flat-bottomed plates containing 100 μl medium plus 50 μl sample. Cultures were pulsed 72 hr later with 1 μCi/well [^{3}H]thymidine for 6 hr, harvested and counted on a β-scintillation counter. The cytokine content of each sample was read off a standard curve established with murine recombinant IL-1β. TGF-β was assayed based on its ability to inhibit the proliferation of a mink lung cell line *in vitro*.[39] Briefly, cells were dispensed at 1×10^5 cells/well in culture medium and incubated overnight at 37°. Cultures were washed to remove non-adherent cells, samples or standards added in a final volume of 100 μl and incubated overnight at 37°. Cultures were washed to remove TGF-β and 200 μl complete medium containing 0·5 μCi/well [^{3}H]thymidine was added. Cultures were incubated for 18 hr before harvesting. The cytokine content of each sample was read off a standard curve established with murine recombinant TGF-β. The incorporation of radioactivity was counted in a beta-counter.

Statistics

Statistical significance ($P < 0{\cdot}05$) was analysed by Student's *t*-test. Results are expressed as mean ± SEM.

RESULTS

Expression of TGF-β by *aroA*$^-$ *aroD*$^-$ *S. typhimurium* BRD509

The TGF-β gene was cloned and expressed in *E. coli* and the plasmid pKK–TGF-β isolated and purified as described in the Materials and Methods. This was transformed first into *S. typhimurium* LB5010 and then transduced into the attenuated *aroA*$^-$ *aroD*$^-$ strain BRD509. A positive clone was selected and TGF-β expression confirmed by Western blot. A band of 12 500 MW was evident in the BRD509–TGF-β track, but not in the BRD509 track (Fig. 1). The bioactivity of the expressed TGF-β was confirmed by bioassay of bacteria lysate (BRD509 8·2 ± 1·2 ng/ml compared with BRD509–TGF-β 45·9 ± 4·7 ng/ml from 1-ml mid-log phase culture, containing approximately 4×10^8 organisms). This activity was completely neutralized by an anti-TGF-β antibody (data not shown).

The stability of the transformed BRD509 was also tested *in vivo*. Groups of mice were administered orally with approximately 5×10^9 organisms and the spleens and livers assayed at various time-points for colony-forming units in the presence or absence of ampicillin. The plasmid-containing bacteria had similar growth curves as the untransformed BRD509 which persisted for >21 days. On day 7, all the transformed BRD509–TGF-β in the spleen were ampicillin resistant [spleen 4·5 ± 0·5; liver 6·3 ± 0·3; mesenteric lymph nodes 3·2 ± 0·9 ($\times 10^3$ bacteria/organ)] and ampicillin-resistant colonies were still detectable at day 14 but were completely absent by day 21 (ref. 28 and data not shown).

Effect of the BRD509–TGF-β construct on carrageenin-induced inflammation

Mice were administered orally with BRD509, BRD509–TGF-β

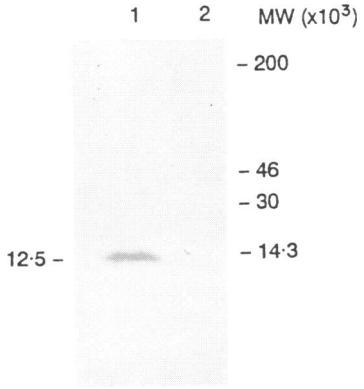

Figure 1. Western blot analysis of TGF-β expression in BRD509–TGF-β. Bacteria were lysed in sample buffer and boiled for 5 min before applying to a 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the gel was transferred to nitrocellulose and probed using a polyclonal rabbit anti-TGF-β antiserum followed by goat anti-rabbit horseradish peroxidase. Lane 1, BRD509–TGF-β; lane 2, BRD509. Molecular weight markers are also shown.

or PBS. Six days after oral inoculation, all animals were injected subcutaneously into one hind footpad with 300 μg carrageenin. Untreated control mice injected with carrageenin developed a significant increase in footpad size by 24 hr, compared to the saline-injected contralateral paw (Fig. 2). This peaked at 48 hr and was still detectable at 120 hr. This swelling was modestly, but not significantly, enhanced at 24 hr by administration of the BRD509 construct alone. In contrast, treatment with BRD509–TGF-β led to a significant decrease in the inflammatory response at all time-points examined (Fig. 2). Oral administration of the construct 6 days before induction of carrageenin-induced inflammation was the optimum time-point for inhibition of this response, as earlier or later time-points were less effective (data not shown). Administration of the

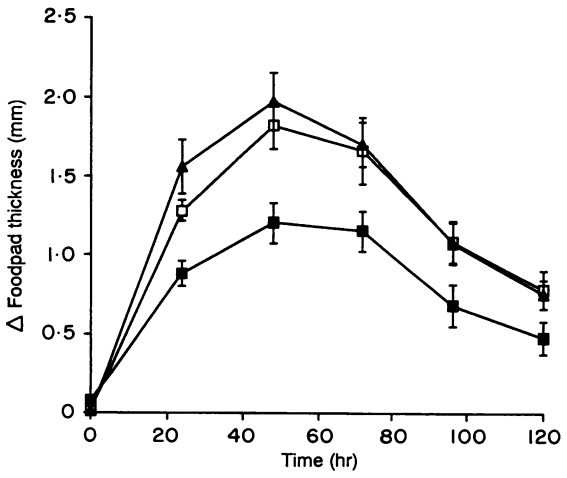

Figure 2. Effect of treatment with BRD509–TGF-β on the inflammatory response induced by carrageenin. Mice were administered orally with BRD509 (▲), BRD509–TGF-β (■) or saline (□). Six days later, all mice were injected with 300 μg carrageenin into the hind paw and swelling measured at regular intervals. Results are expressed as means ± SEM (n = 5) and are representative of three experiments.

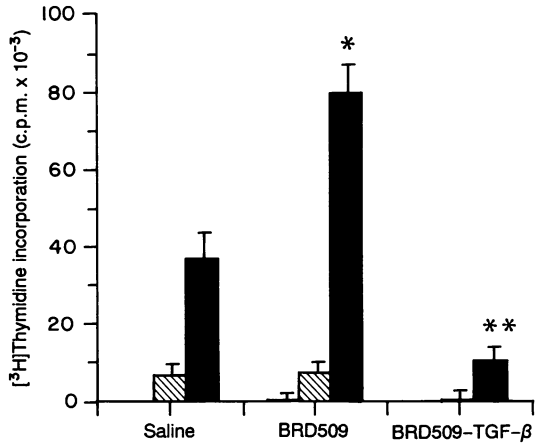

Figure 3. Effect of treatment with BRD509–TGF-β on proliferation by DLN cells. Mice were treated orally with BRD509, BRD509–TGF-β or saline and, 6 days later, injected with 300 μg carrageenin into the hind paw. DLN cells were obtained 48 hr after injection of carrageenin and were either unstimulated, or stimulated with 0·5 μg/ml (▨) or 5 μg/ml Con A (■). Results are expressed as means ± SEM (n = 3) and are representative of three experiments (*P < 0·01 versus saline; **P < 0·001 versus BRD509; P < 0·05, saline versus BRD509–TGF-β). Results are shown for 48-hr cultures; similar results were obtained from cultures incubated for 24 or 72 hr.

construct at the same time or after carrageenin injection was without effect (data not shown).

Effect of the BRD509–TGF-β construct on DLN cell proliferation *in vitro*

Animals were inoculated orally with BRD509, BRD509–TGF-β or PBS and injected s.c. 6 days later in the hind paw with carrageenin. DLN cells were removed 48 hr later, at the peak of the inflammatory response, for phenotype analysis and for restimulation *in vitro* with Con A for 48 hr. DLN cells from control mice treated with PBS, before administration of carrageenin, gave a significant proliferative response to Con A at both 0·5 and 5 μg/ml (Fig. 3). The response to Con A at 5 μg/ml was significantly enhanced in mice administered with BRD509 alone. The reason for this enhancing effect is unclear but may be due to a mitogenic effect of the bacteria. In contrast, cells from mice injected with carrageenin following BRD509–TGF-β treatment produced significantly lower levels of proliferation in response to Con A stimulation (Fig. 3). None of the DLN cells showed a detectable proliferative response to carrageenin *in vitro*. FACScan analysis showed that there was no significant difference in the per cent of $CD3^+$, $CD4^+$, $CD8^+$, B, NK cells and macrophages in the DLN from mice injected with carrageenin and pretreated with PBS, BRD509 or BRD509–TGF-β (data not shown). Oral administration of BRD509 or BRD509–TGF-β had no effect on the size or gross histology of the contralateral lymph node (data not shown).

Effect of the BRD509–TGF-β construct on cytokine production *in vitro*

DLN cells were harvested from mice as described for the T-cell proliferation assay and stimulated *in vitro* with a number of

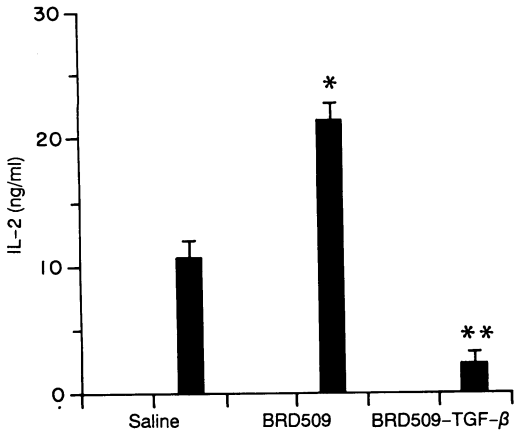

Figure 4. Effect of BRD509–TGF-β treatment on IL-2 production. Mice were inoculated orally with BRD509, BRD509–TGF-β or saline and, 6 days later, injected with 300 μg carrageenin in the hind paw. DLN cells were obtained 48 hr after injection of carrageenin and were either unstimulated, or stimulated with 10 μg/ml LPS, 1 μg/ml LPS plus 200 U/ml IFN-γ or 5 μg/ml Con A (■). Cell-free supernatants were harvested 24 hr later and assayed by ELISA as described in the Materials and Methods. Results are expressed as mean ± SEM (n = 3) and are representative of three experiments (*P < 0·05 versus saline; **P < 0·001 versus BRD509). The same supernatants were used in the data presented in Figs 5 and 6. Similar results were obtained from supernatants collected 48 hr post-stimulation. Cell cultures from mice not injected with carrageenin produced low levels (4 ± 2 ng/ml) of IL-2.

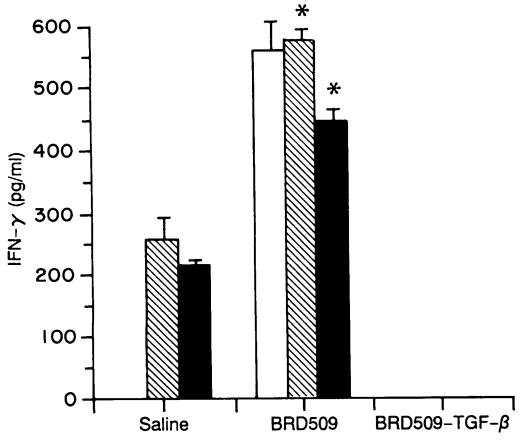

Figure 5. Effect of BRD509–TGF-β treatment on IFN-γ production. Mice were given BRD509, BRD509–TGF-β or saline and, 6 days later, injected with 300 μg carrageenin in the hind paw. DLN cells were obtained 48 hr after injection of carrageenin and were either unstimulated (□), or stimulated with 10 μg/ml LPS (▨) or 5 μg/ml Con A (■). IFN-γ was not used for stimulation as it was to be measured in the culture supernatants. Cell-free supernatants were harvested 24 hr later and assayed by ELISA as described in the Materials and Methods. Results are expressed as means ± SEM (n = 3) and are representative of three experiments (*P < 0·001 versus saline) except for cells from mice treated with BRD509 alone. These cells produced variable amounts of IFN-γ when not stimulated. Similar results were observed at 48 hr post-stimulation. IFN-γ was undetectable in cell culture from mice not given carrageenin.

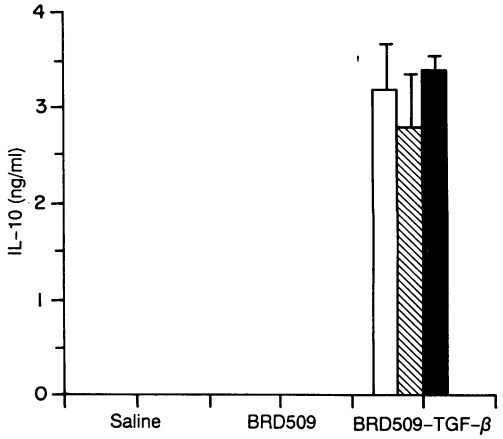

Figure 6. Effect of BRD509–TGF-β treatment on IL-10 production. Mice were given BRD509, BRD509–TGF-β or saline and, 6 days later, injected with 300 μg carrageenin in the hind paw. DLN cells were obtained 48 hr after injection of carrageenin and were either unstimulated (□), or stimulated with 10 μg/ml LPS (▨), 1 μg/ml LPS plus 200 U/ml IFN-γ (■) or 5 μg/ml Con A. Cell-free supernatants were harvested 24 hr later and assayed by ELISA as described in the Materials and Methods. Results are expressed as means ± SEM (n = 3) and are representative of three experiments. Similar results were observed at 48 hr post-stimulation and IL-10 was undetectable in cell cultures from mice not given carrageenin.

reagents known to activate various cell types. Culture supernatants were collected at 24, 48 and 72 hr post-stimulation and assayed for various cytokines.

Only cells activated with Con A produced detectable levels of IL-2 (Fig. 4). DLN cells from mice injected with carrageenin produced a significant amount of IL-2. This was markedly enhanced in animals treated with BRD509 before injection of carrageenin. However, DLN cells from mice given BRD509–TGF-β before carrageenin produced significantly lower levels of IL-2 (Fig. 4).

DLN cells from mice injected with carrageenin alone also produced significant levels of IFN-γ in response to LPS or Con A (Fig. 5). This was again elevated in the culture supernatant of cells from mice treated with BRD509 prior to carrageenin treatment. Occasionally, cells from this group of mice produced high levels of IFN-γ in the absence of mitogen (Fig. 5), suggesting that BRD509 alone induces a state of non-specific activation for IFN-γ secretion. In contrast, no IFN-γ was detected in the culture supernatant of DLN cells from mice injected with carrageenin following administration of BRD509–TGF-β (Fig. 5).

IL-10 was not detected in the culture supernatant of cells from mice injected with carrageenin alone or following BRD509 treatment, when stimulated with LPS, LPS plus IFN-γ or Con A *in vitro* (Fig. 6). In contrast, cells from mice administered BRD509–TGF-β orally and then injected with carrageenin produced significant amounts of IL-10 when cultured in medium alone or with LPS or LPS plus IFN-γ. Interestingly, no IL-10 was detected when the cells were cultured with Con A (Fig. 6). IL-10 was not detected in the culture supernatant of DLN cells from mice not injected with carrageenin (data not shown).

No IL-4, TNF-α or TGF-β was detected in the culture

supernatant of cells from all three groups, whether stimulated with LPS, LPS plus IFN-γ or Con A. The levels of IL-1 and IL-6 in the supernatants were consistently low and not significantly different between the three groups of mice (data not shown).

DISCUSSION

An auxotrophic *Salmonella* mutant has been used to deliver IL-1β in a biologically active form.[29] Here, we demonstrate that TGF-β delivered in a similar way markedly reduced local inflammation following administration of carrageenin in the footpad of mice. This was accompanied by a reduction in the ability of DLN cells to produce IL-2 and IFN-γ, but enhanced production of IL-10. These data therefore suggest that the *Salmonella* carrier system can be used to deliver biologically active TGF-β orally. In addition, TGF-β delivered orally suppressed an ongoing Th1-like response, characterized by the production of IL-2 and IFN-γ, while IL-10 production was enhanced. This finding may indicate a potential immune therapy against inflammatory diseases.

In order to demonstrate the production of a range of cytokines by a heterologous population, such as draining lymph node cells, a number of cellular activating agents were used. These included the B-cell and macrophage mitogen LPS and the T-cell mitogen Con A. Different cytokines appear to have different signalling requirements for their activation. IL-2 was produced in significant amounts only when the cells were activated with Con A, while IL-10, in contrast to Th2 cell clones,[40] was not activated by Con A in our system. Thus, the failure to detect IL-4, TNF-α and TGF-β in the present system may reflect the fact that appropriate stimuli for the activation of these cytokines were not present. DLN cells from mice injected with carrageenin and pretreated with BRD509 produced variable levels of IFN-γ when cultured in the absence of stimuli. This may be owing to a response to bacterial antigens carried by the antigen-presenting cells from residual bacteria in the DLN.

Only cells from mice given BRD509–TGF-β produced IL-10. This was so even when the cells were cultured without additional stimulation *in vitro*. Addition of LPS or LPS plus IFN-γ did not increase the level of IL-10 produced. This may be owing to an optimal IL-10 synthesis activated by BRD509–TGF-β which cannot be elevated further by additional stimuli. In contrast, cells from these animals produced no detectable IFN-γ, regardless of the *in vitro* stimuli used. This suggested that the inability of cells to secrete IL-10 from mice injected with carrageenin alone or carrageenin following treatment with BRD509 was owing to an active inhibitory effect which was abolished by TGF-β. These results are consistent with a previous report that TGF-β can block the effects of IFN-γ,[41] including the down-regulation of IL-10 production by monocytes[42] and the down-regulation of Th2 cell proliferation.[43,44] Conversely, IL-10 can inhibit cytokine synthesis by Th1 cells by down-regulating antigen-presenting cell function and major histocompatibility complex (MHC) class II antigen expression.[40,45,46]

Recent results have demonstrated that TGF-β can exacerbate both *in vivo* and *in vitro* infections with the intracellular protozoan parasites *T. cruzi*, *L. amazonensis* and *L. braziliensis*,[41,47,48] probably by blocking the production of IFN-γ. Indeed, local administration of TGF-β during leishmanial infection was associated with enhanced expression of IL-10 mRNA in the draining lymph nodes.[48] Results presented here demonstrate that oral administration of TGF-β can also enhance the production of IL-10 and down-regulate the Th1 cytokines IL-1 and IFN-γ. However, these *in vivo* findings are different from those obtained *in vitro*, which showed that the presence of TGF-β in cultures of $CD4^+$ precursor T cells led to the development of IL-2 and IFN-γ secreting Th1-like cells.[49]

The way in which orally delivered TGF-β may influence both inflammation and the cytokine secretion pattern of DLN cells is unclear. TGF-β is not secreted by the bacteria *in vitro* but, following a limited cycle of replication *in vivo*, is likely to be released upon the death of the bacteria. Serum of unimmunized mice contained a basal level of TGF-β and this was not elevated by oral treatment with BRD509–TGF-β. Further, TGF-β was not detected in the culture supernatant of DLN cells. It may be that the level of TGF-β delivered was too low to be detected by the assay system used, but was nevertheless sufficient to induce a local regulatory effect on inflammation and the cytokine production profile. Certainly, treatment with TGF-β did not alter the cellular phenotype of the DLN population. In related work, pretreatment with the nitric oxide inhibitor L-NMMA affected the carrageenin-induced inflammatory response and cytokine profile of the DLN cells in a manner similar to that of TGF-β.[50] In that work, inhibition of the inflammatory response was not associated with a change in the histopathology of the paw following carrageenin administration. Thus, as with L-NMMA, TGF-β may act on the induction of the cytokine cascade following carrageenin administration rather than on the cell population itself. This remains to be clarified.

Data presented here also support the importance of Th1 cells in inflammation. Previous reports indicate that Th1 cells predominate in the synovium of patients with rheumatoid or reactive arthritis.[32,33] Here, we show that treatment with BRD509–TGF-β not only down-regulated the secretion of Th1 cytokines, but also suppressed the inflammatory response.

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