



(86) Date de dépôt PCT/PCT Filing Date: 2002/11/20

(87) Date publication PCT/PCT Publication Date: 2003/06/05

(85) Entrée phase nationale/National Entry: 2004/05/21

(86) N° demande PCT/PCT Application No.: GB 2002/005255

(87) N° publication PCT/PCT Publication No.: 2003/046580

(30) Priorité/Priority: 2001/11/21 (0127916.5) GB

(51) Cl.Int.⁷/Int.Cl.⁷ G01N 33/68, A61K 35/74, A61K 31/02, A61P 37/00, A61P 29/00, A61P 1/00, G01N 33/564

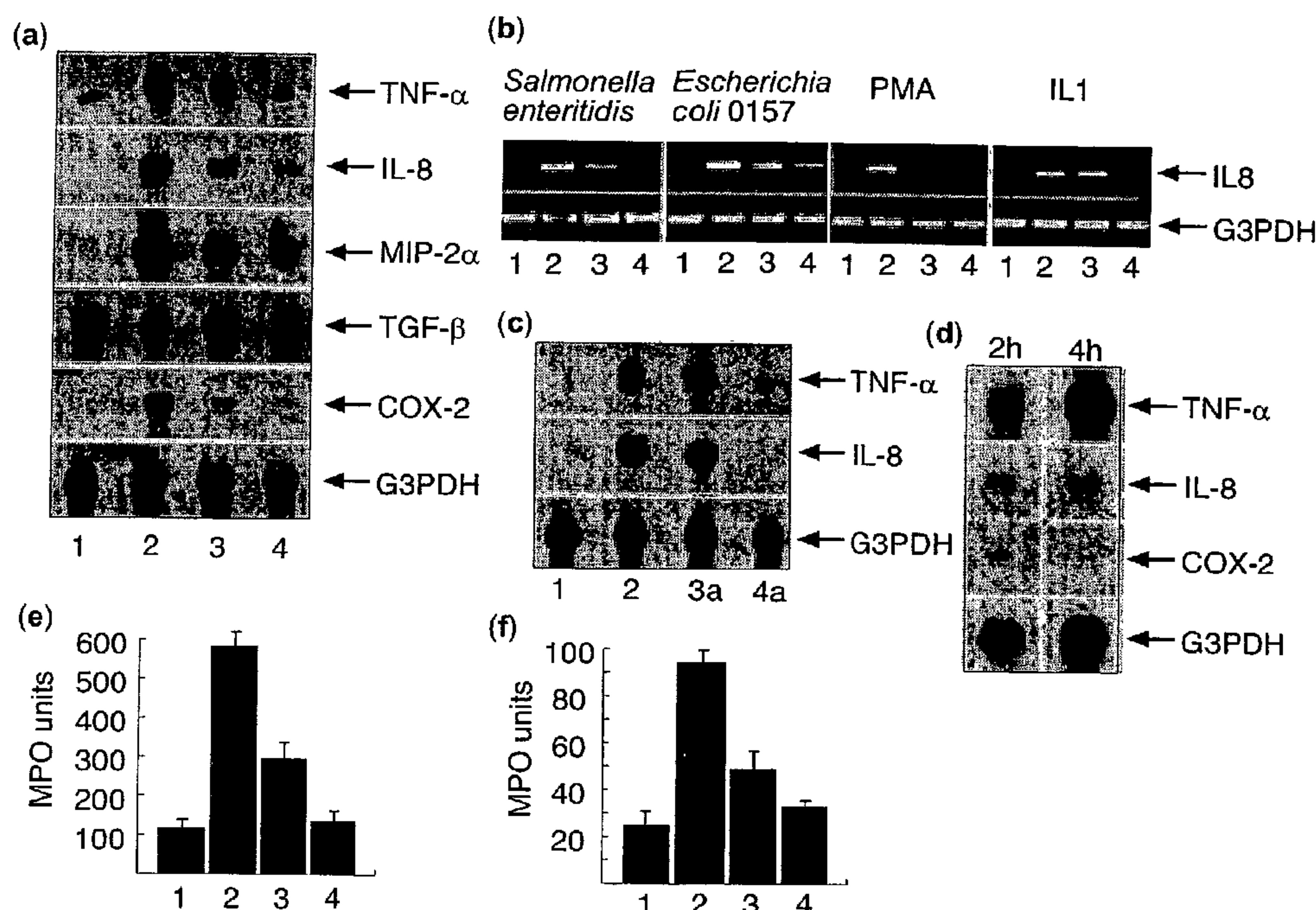
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(54) Titre : TECHNIQUE DE CRIBLAGE DE MEDICAMENTS CANDIDATS POUR LE TRAITEMENT DE MALADIES INFLAMMATOIRES

(54) Title: ASSAY FOR SCREENING CANDIDATE DRUGS FOR THE TREATMENT OF INFLAMMATORY DISEASES



(57) Abrégé/Abstract:

The invention relates to an assay to select a candidate drug for the treatment of inflammatory disease due to cytokine production. The assay comprises: a) exposing said candidate drug to intestinal cells; and b) analysing the effect of the said candidate drug selected from the group consisting of the variation of nuclear export or import of transcription factors from the NF- κ B family the disruption of transcriptional activity of transcription factors from the NF- κ B family, the differential histone acetylation of p65 (RelA); the variation of the amount of PPAR γ /RelA complexes in the cytosol of the cells; and the variation in the levels of one or more cytokines. The invention further relates to methods for the treatment of inflammatory cytokine production associated diseases and to the use of a therapeutically effective dose of *Bacteroides thetaiotaomicron* or a component thereof.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
5 June 2003 (05.06.2003)

PCT

(10) International Publication Number
WO 03/046580 A1

(51) International Patent Classification⁷: **G01N 33/68**,
33/564, A61K 31/02, A61P 1/00, 29/00, 37/00, A61K
35/74

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(21) International Application Number: PCT/GB02/05255

(22) International Filing Date:
20 November 2002 (20.11.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0127916.5 21 November 2001 (21.11.2001) GB

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

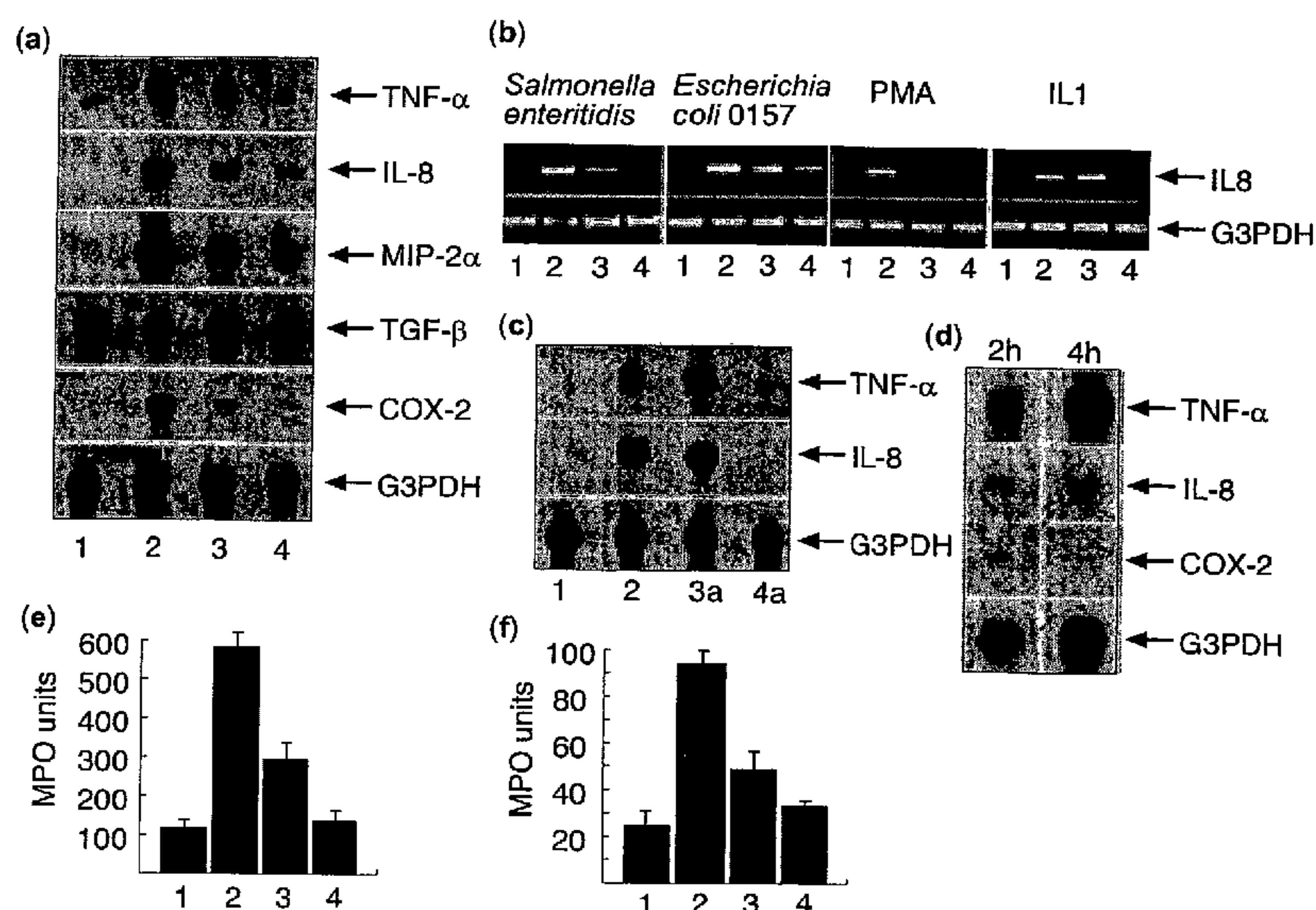
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

[Continued on next page]

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WO 03/046580 A1



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

ASSAY FOR SCREENING CANDIDATE DRUGS

FOR THE TREATMENT OF INFLAMMATORY DISEASES

1

2

3

4

5 **Field of the invention**

6 The present invention is concerned with modulating
7 intestinal responses to infection, in particular
8 through use of the bacterium *Bacteroides*
9 *thetaiotaomicron*.

10

11 **Background of the invention**

12 The mammalian intestine is colonised by several
13 hundred bacterial species whose numbers increase
14 dramatically in the colon, an anatomical site prone
15 to infection, inflammatory disease and cancer.
16 However, the resident flora of the gastrointestinal
17 tract of adult animals conveys a high degree of
18 protection (colonisation resistance) against
19 infection (van der Waaij, 1984; Salminen et al,
20 1998). As a result, most opportunistic pathogens
21 encountered in the environment are unable to
22 establish a foothold and are rapidly excluded (van

1 der Waaïj, 1984; Salminen *et al*, 1998). However,
2 if the commensal flora is compromised,
3 opportunistic pathogens may then persist in the
4 gut. For example, *Pseudomonas aeruginosa* does not
5 usually persist in the murine gut but will colonise
6 and cause very severe infection after disruption of
7 the commensal flora with antibiotics (Pier *et al*,
8 1992). The commensal bacteria also confer some
9 degree of protection against virulent pathogens,
10 such as *Salmonella*. However, these pathogens can
11 overwhelm or circumvent the protective effects of
12 the commensal flora and cause severe infection if
13 they are present in significant numbers.

14
15 Colonisation resistance is due, in part, to
16 competitive exclusion of harmful bacteria, through
17 preferential use of nutrients and substrates or
18 blocking of potential attachment sites on the gut
19 by the commensal flora. However, commensal
20 bacteria also modulate the gut cellular and immune
21 systems of the host (Bry *et al*, 1996; Herias *et al*,
22 1998; 1999; Hooper *et al*, 2000; 2001; Cebra, 1999;
23 Snel *et al*, 1998; Talham *et al*, 1999; Lopez-Boado
24 *et al*, 2000; Shu *et al*, 2000; Campbell *et al*,
25 2001). The flora may alter the gastrointestinal
26 tract to create niche microenvironments that are
27 well suited to them but not to other bacteria.
28 Alternatively, they may modify epithelial cell
29 responses to harmful bacteria (Campbell *et al*,
30 2001) and thereby attenuate changes that would
31 facilitate colonisation and invasion.

32

1 The healthy gut maintains a hyporesponsive tone
2 towards its diverse bacterial load, yet the
3 presence of a threshold level of pathogenic
4 bacteria is sufficient to activate transcriptional
5 systems that rapidly upregulate proinflammatory
6 gene expression in gut tissues. These
7 transcription products then trigger a cascade of
8 responses including the chemotaxis of
9 polymorphonuclear (PMN) cells into the lamina
10 propria of infected intestinal sites. Although
11 these events are essential for bacterial clearance,
12 they also cause tissue pathology that can
13 exacerbate the symptoms of disease. The first
14 point of contact for luminal bacteria is a
15 continuous layer of epithelial cells that both
16 interfaces and segregates the gut immune system.
17 The ability of intestinal epithelial cells to
18 discriminate between pathogenic and non-pathogenic
19 bacteria is crucial in averting harmful
20 inappropriate responses to colonising bacteria and
21 in maintaining gut health. This discriminatory
22 function is imprinted in the systems of bacterial
23 recognition and cell signalling. Recognition of
24 bacterial cell surface structures is, in part, a
25 function of Toll-like receptors expressed on apical
26 and basolateral surfaces of epithelial cells, which
27 trigger Nuclear Factor Kappa B (NF- κ B)- mediated
28 immune activation (Gerwirtz *et al*, 2001).
29 Hitherto, receptor systems linked to immune-
30 suppressor activities have not been identified.
31

Administration of the human commensal bacterium *B. thetaiotaomicron* to germ-free mice triggered expression of key genes linked to intestinal maturation and development of barrier function (Hooper et al, 2001). It also increased intestinal levels of Fuc α 1,2Gal β -glycans (Bry et al, 1996; Hooper et al, 2001) and matrilysin (Lopez-Boado et al, 2000). *B. thetaiotaomicron* was also found to modify the responses of epithelial cells to challenge *in vitro* with *Salmonella* (Campbell et al, 2001). In particular, there was suppression of some pro-inflammatory pathways (Campbell et al, 2001). Despite these potentially protective properties, *B. thetaiotamicron* did not however increase the resistance of ex-germ free mice to infection by *Clostridium perfringens* serotype A (Yurdusev et al, 1989). Nonetheless, the pathogen was cleared from the gut if the mice were treated with *B. thetaiotaomicron* in combination with *Fusobacterium necrogenes* (Yurdusev et al, 1989) and non-pathogenic *Clostridia* strain CI (Yurdusev et al, 1986). This suggests that, although *B. thetaiotaomicron* alone can induce potentially protective changes in the gut, it may need to act in tandem with other commensal strains to significantly enhance overall resistance to infection.

In this invention we define a novel mechanism whereby non-pathogenic bacteria, normally both the nucleocytoplasmic distribution of PPAR γ and the transcriptional activity of (NF- κ B).

1 Further, this invention relates to the use of non-
2 pathogenic bacteria for the modulation of
3 inflammatory responses. Still further, the
4 invention provides evidence for differential
5 histone acetylation of p65 (RelA) affecting I κ B α -
6 mediated export of RelA and for a novel mode of
7 action for peroxisome proliferator-activated
8 receptors (PPAR γ). In particular, the invention
9 describes the attenuation of epithelial
10 inflammatory gene expression by altering both the
11 nucleocytoplasmic distribution of PPAR γ and the
12 transcriptional activity of NF- κ B by use of non-
13 pathogenic bacteria. Further the present invention
14 provides a means of attenuating inflammation for
15 treatment and prevention of disease and
16 inflammatory disorders.

17

18 The novel pathway described in the present
19 invention can be used to screen for novel methods
20 and for novel products to modulate inflammatory
21 cytokine production. Further, the present
22 invention describes the use of a non-pathogenic
23 bacteria in attenuating inflammatory cytokine
24 production and returning the immune system to
25 homeostasis.

26

27 The modulation of inflammatory cytokine production
28 described herein differs from known publications in
29 a number of aspects.

30

31 US 5,925,657 describes an agonist of PPAR γ . This
32 agonist is thiazolidinedione, a chemical compound

1 with a substituted aryl moiety attached to a
2 thiozolidinedione nucleus. In the present
3 invention, a non-pathogenic bacterium is used to
4 attenuate the inflammatory response by directly
5 activating PPAR γ .

6
7 Neish et al, 2000 describe regulation of epithelial
8 responses by inhibition of I κ B α ubiquitination.
9 The present invention differs from Neish et al,
10 2000 by using a different mode of action.

11
12 Evidence is provided for differential histone
13 acetylation of p65 (RelA) affecting I κ B α -mediated
14 export of RelA and for a novel mode of action for
15 peroxisome proliferator-activated receptors
16 (PPAR γ). This novel data provides the potential
17 for devising novel means of, and novel products
18 for, modulating inflammatory cytokine production.
19 *In vivo* validation of the results are also
20 described.

21
22 The present invention provides an assay to select a
23 candidate drug for the treatment of inflammatory
24 disease due to cytokine production. The assay
25 comprises:

26 a) exposing the candidate drug to
27 intestinal cells; and
28 b) analysing the effect of the said
29 candidate drug, said effect being selected
30 from the group consisting of :

- 1 - the variation of nuclear export or
- 2 import of transcription factors from
- 3 the NF- κ B family;
- 4 - the disruption of transcriptional
- 5 activity of transcription factors
- 6 from the NF- κ B family;
- 7 - the differential histone acetylation
- 8 of p65 (RelA);
- 9 - the variation of the amount of
- 10 PPAR γ /RelA complexes in the cytosol
- 11 of the cells; and
- 12 - nucleoplasmic destruction of PPAR γ .

13
14 The assay may further comprise a step of selecting
15 a candidate drug which demonstrates at least one
16 effect selected from the group consisting of:

- 17 - the increase of nuclear export or
- 18 the decrease of nuclear import of
- 19 transcription factors from the NF- κ B
- 20 family;
- 21 - the disruption of transcriptional
- 22 activity of transcription factors
- 23 from the NF- κ B family;
- 24 - the differential histone acetylation
- 25 of p65 (RelA);
- 26 - the increase of the amount of
- 27 PPAR γ /RelA complexes in the cytosol
- 28 of the cells; and
- 29 - nucleoplasmic destruction of PPAR γ .

30
31 According to a preferred embodiment, the effect of

1 the candidate drug which is analysed is the nuclear
2 export of transcription factor from the NF- κ B
3 family or the variation of the amount of PPAR γ /RelA
4 complexes in the cytosol of the cells.

5
6 In another preferred embodiment, the assay further
7 comprises the step of analysing the variation in
8 the level of one or more cytokines selected from
9 the group consisting of TNF- α , IL-8, MIP-2 α and
10 Cox-2; preferably IL-8 and/or MIP-2 α .

11
12 Desirably the intestinal cells used in the assay
13 may be in form of an intestinal cell line
14 maintained in cell culture. Suitable cell lines
15 include Caco-2 cell line. Alternatively the assay
16 could be performed *in vivo* using a suitable animal,
17 like a mammal (eg. mice).

18
19 Optionally the assay may be conducted in the
20 presence of known pathogenic bacteria, for example
21 *Salmonella sp.*

22
23 The present invention also provides a method for
24 the treatment of inflammatory cytokine production
25 associated diseases which includes the step of
26 administering a therapeutically effective dose of
27 an agent capable of modulating:

28 - the nuclear export or the nuclear
29 import of transcription factors of
30 the NF- κ B family;

- 1 - the disruption of transcriptional
- 2 activity of transcription factors
- 3 from the NF- κ B family;
- 4 - the differential histone acetylation
- 5 of p65 (RelA);
- 6 - the amount of PPAR γ /RelA complexes
- 7 in the cytosol of the cells; or
- 8 - nucleoplasmic destruction of PPAR γ .

9

10 Preferably the agent is used for the treatment of

11 an inflammatory disease, especially a disease which

12 is caused, at least in part, by the inflammatory

13 response of intestinal cells. Generally such a

14 disease will involve the production of inflammatory

15 cytokines. Suitably the agent may be *Bacteroides*

16 *thetaiotaomicron* or a component thereof. The term

17 "a component thereof" as used herein refers to any

18 portion (protein, polypeptide, sugar, nucleic acid

19 and the like) normally forming part of *B.*

20 *thetaiotaomicron* and which is able to attenuate an

21 immune response.

22

23 The present invention yet further provides the use

24 of *B. thetaiotaomicron* or a component thereof as a

25 medicament. Preferably *B. thetaiotaomicron* or a

26 component thereof is used for the treatment of an

27 inflammatory disease, particularly an inflammatory

28 disease which is caused, at least in part, by the

29 inflammatory response of intestinal cells. Thus

30 the present invention provides use of *B.*

31 *thetaiotaomicron* for attenuating or preventing or

32 treating inflammatory cytokine production

1 associated diseases or conditions including, but
2 not limited to; inflammatory bowel disease and
3 diseases (particular mention may be made of Crohn's
4 disease and Irritable Bowel Syndrome); rheumatoid
5 arthritis; immunodeficiency syndrome; cachexia;
6 multiple sclerosis, inhibition of proliferation of
7 keratinocytes; inhibition of hyperproliferative and
8 inflammatory disorders of the skin including but
9 not limited to psoriasis and acne vulgaris.

10

11 The present invention yet further provides the use
12 of *B. thetaiotaomicron* in the manufacture of a
13 medicament for the treatment of an inflammatory
14 disease.

15

16 The present invention also provides a method for
17 the treatment of, or to attenuate, the inflammatory
18 response of intestinal cells which comprises
19 administering a therapeutically effective dose of
20 *B. thetaiotaomicron* or a component thereof.

21

22 In more detail *B. thetaiotaomicron* or its
23 components may be used to disrupt the NF-KB pathway
24 and/or to inhibit the p65 (RelA) response and/or
25 act as an excitor or inhibitor of PPAR γ . *B.*
26 *thetaiotaomicron* or its components may therefore be
27 used for the treatment of inflammatory cytokine
28 production associated diseases.

29

30 The *B. thetaiotaomicron* may be administered to a
31 patient live by means of a foodstuff or
32 suppository.

11

1 Thus, this invention describes a method of healing
2 the inflammatory response of intestinal cells which
3 comprises the step of administering a
4 therapeutically effective dose of *B.*
5 *thetaitaomicron* or a component thereof.

6
7 For the prevention and treatment of conditions
8 described herein, *B. thetaiotamicron* is
9 preferentially delivered to the site of action in
10 the gastro-intestinal tract by oral administration
11 in any appropriate formulae or carrier or excipient
12 or diluent or stabilizer. Such delivery mechanisms
13 may be of any formulation including but not limited
14 to solid formulations such as tablets or capsules;
15 liquid solutions such as yoghurt or drinks or
16 suspensions. A preferential delivery mechanism is
17 one that delivers the bacteria orally, preferably
18 without harm through the acid environment of the
19 stomach to the site of action within the intestine.
20 Such bacteria may be given in conjunction with a
21 prebiotic.

22
23 The invention also provides a novel mode of action
24 of PPAR γ and differential histone acetylation of
25 p65 (RelA) affecting IKB α -mediated export of RelA
26 for screening for specific ligands with the
27 potential to modulate, either agonistically or
28 antagonistically, inflammatory cytokine production
29 via the novel pathway described herein.

30
31 The invention further provides a method for the
32 treatment of inflammatory cytokine production

1 associated diseases which includes the step of
2 administering a therapeutically effective dose of a
3 compound capable of altering the transcriptional
4 activity of NF- κ B or capable of increasing the
5 amount of PPAR γ /RelA complexes in the cell cytosol.

6
7 The invention further provide a method of returning
8 and maintaining the immune system of mammals to
9 homeostasis, which includes the step of
10 administering a therapeutically effective dose of a
11 compound capable of altering the transcriptional
12 activity of NF- κ B or capable of increasing the
13 amount of PPAR γ /RelA complexes in the cell cytosol.

14
15 We have found that the anti-inflammatory activity
16 of *B. thetaiotaomicron* involves a totally novel
17 mode of action, defined by accelerated NF- κ B
18 nuclear export and PPAR γ -mediated sequestration in
19 the cytosol. The experimental work (described in
20 detail in the examples) was undertaken in three
21 phases:

22

23 1) Inflammatory cytokine gene expression in Caco-
24 2 cells following exposure to pathogenic/non-
25 pathogenic bacteria. Data was obtained using
26 cDNA macroarray, real-time PCR and Northern
27 hybridisation analysis.

28

29 2) In vitro (Caco-2 transwell cultures) and in
30 vivo (minimal flora rats) validation of the
31 physiological relevance of the anti-
32 inflammatory effects of *B. thetaiotaomicron*.

1 3) Analysis of NF- κ B and AP-1 signal transduction
2 pathways and the relationship between
3 cytosolic RelA and PPAR γ .

4

5 The experimental work performed shows that:

6

7 1) The main target of regulation by *B.*
8 *thetaitaomicron* is NF- κ B and not AP-1.

9

10 2) p65 (RelA) accumulated in cells treated with
11 *B. thetaitaomicron* and *S. enteritidis*
12 (detected up to 30 minutes).

13

14 3) The reduced transcriptional effects of p65 in
15 the presence of *B. thetaitaomicron* are due to
16 an enhanced nuclear clearance (export) of p65,
17 and this export is LMB sensitive (ie. crm -1
18 mediated).

19

20 4) The data correlated totally to the attenuation
21 in inflammatory cytokine/chemokine expression,
22 IKB α expression, PMN recruitment and
23 physiologically lower level of inflammation
24 demonstrated *in vivo* in rats due to *B.*
25 *thetaitaomicron*.

26

27 5. PPAR γ localised to the cytosol in the presence
28 of *B. thetaitaomicron*.

29

30 6. PPAR γ and p65 are physically coupled when co-
31 localised in the cytosol.

32

The present invention will now be illustrated further by reference to the following non-limiting, examples and to the figures in which:

Brief description of the Drawings

Fig. 1

(a) Northern blot hybridisation of cytokine mRNA using ^{32}P -labelled probes specific for TNF- α , IL-8, MIP-2 α , TGF- β , COX-2 and G3PDH of:

- 1) Non-infected Caco-2 cells;
- 2) Caco-2 cells with 10^8 *S. enteritidis*;
- 3) Caco-2 cells with 10^8 *S. enteritidis* and 10^9 *B. thetaiotaomicron*;
- 4) Caco-2 cells with 10^9 *B. thetaiotaomicron*.

(b) Semi-quantitative PCR of IL8 and G3PDH. Caco-2 cells incubated in medium alone (1); with either 10^8 *S. enteritidis*, 10^8 *E. coli* 0157 H7, PMA (300 ng/ml) or IL-1 α/β (20 ng/ml) as indicated (2); as described in (2) but in the presence of 10^9 *B. thetaiotaomicron* (3); or with 10^9 *B. thetaiotaomicron* (4).

(c) Northern blot hybridisation of mRNA using ^{32}P -labelled probes specific for TNF- α , IL-8, MIP-2 α , TGF- β , COX-2 and G3PDH of:

- 1) Non-infected Caco-2 cells;
- 2) Caco-2 cells with 10^8 *S. enteritidis*;

15

1 3) Caco-2 cells with 10^8 *S. enteritidis* and
2 10^9 *B. vulgatus*;

3 4) Caco-2 cells with 10^9 *B. vulgatus*.

4

5 (d) Caco-2 cells were incubated with 10^8 *S.*
6 *enteritidis* and 10^9 *B. thetaiotaomicron* for
7 either 2 or 4 hours as indicated.

8

9 (e) Transepithelial migration of PMN cells through
10 Caco-2 monolayer as determined by MPO Assay of
11 treatment groups (1) to (4) of (a) above.

12

13 (f) MPO Assay of ileal mucosa 6 days post
14 treatment of rats challenged with (1) control;
15 (2) 10^8 *S. enteritidis*; (3) *B.*
16 *thetaiotaomicron* then 10^8 *S. enteritidis*; and
17 (4) *B. thetaiotaomicron* alone. Data are means
18 $\pm$ SD (n=3).

19

20 Fig. 2

21

22 1) Non-infected Caco-2 cells;

23 2) Caco-2 cells incubated with 10^8 *S.*
24 *enteritidis*;

25 3) Caco-2 cells incubated with 10^8 *S. enteritidis*
26 and 10^9 *B. thetaiotaomicron*;

27 4) Caco-2 cells incubated with 10^9 *B.*
28 *thetaiotaomicron*

29

30 and probed with primary antibodies (A-D) anti-RelA;

31 (E-H) anti-PPAR γ ; (I-L) anti-I κ B α ; and (M-P) anti

1 pIk β α . All Scale bars = 25 μ m. Inserts in O and P
2 show details of punctate nuclear label.

3

4 Fig. 3

5

6 (A) Supershift EMSA performed on Caco-2 nuclear
7 extracts using a consensus ³²P-labelled NF- κ B
8 binding sequence oligonucleotide and
9 incubated with anti-RelA antibody on groups
10 (1) to (4) as described above for Fig. 1A.
11 Where indicated the *B. thetaiotaomicron* was
12 heat inactivated at 70°C for 15 minutes.

13

14 (B) Northern hybridisation of mRNA of experimental
15 treatment groups were as described above (1-
16 4), with blots probed with specific ³²P-
17 labelled IkB α and G3PDH probes.

18

19 (C) Western blot of nuclear extracts derived from
20 Caco-2 cells. Treatment groups were as
21 described above (1-4). Immunoblots were
22 probed with specific antibodies to anti-p38
23 and anti-pp38 (New English Biolabs).

24

25 D) Superinduction of c-Fos and c-Jun were
26 determined by Northern hybridisation on total
27 RNA from Caco-2 cells. Treatments were, Caco-
28 2 cells in the absence of cyclohexamide (-C)
29 or as described above (1-4) but in the
30 presence of 10 μ g/ml cyclohexamide. The blots
31 were hybridised with ³²P-labelled probes
32 specific for c-Fos, c-Jun and G3PDH.

1 E, F) Western blots of nuclear extracts derived from
2 Caco-2 cells. Treatment groups were as
3 described above (1-4). Specific antibodies to
4 c-Fos (Santa Cruz), ATF-2 and pATF-2 (New
5 England Biolabs) were used.

6
7 G) Immunoprecipitation (IP) using IKB α and pIKB α .
8 Caco-2 cells following standard culture
9 protocol, treatment groups (1-4). IP analysed
10 by western blotting, NS indicates non-
11 specific, B1 is a control with no cell
12 extract.

13
14 Fig. 4

15
16 A) Northern hybridisation of mRNA from non-
17 infected Caco-2 cells (1), Caco-2 cells
18 following incubation with 10^8 *S. enteritidis*
19 (2), 10^8 *S. enteritidis* and 10^9 *B.*
20 *thetaitaomicron* (3), and 10^9 *B.*
21 *thetaitaomicron* alone (4) analysed by
22 specific ^{32}P -labelled PPAR γ and PPAR α probes.
23 Bacteria were applied for 2 hours.

24
25 B) Treatments (1) and (2) were as described
26 above, or cells incubated with 10^8 *S.*
27 *enteritidis* in the presence of 30 μM 15PG-J₂
28 (3a) or 30 μM fenofibrate (3b) as indicated.
29 Bacteria and drugs were applied for 2 hours.
30 mRNA was analysed by Northern hybridisation
31 with a ^{32}P -labelled PPAR γ specific probe.

18

1 C) mRNA from non-infected Caco-2 cells (1), Caco-
2 2 cells following incubation with 10^8 *S.*
3 *enteritidis* (2), 10^8 *S. enteritidis* in the
4 presence of either 10, 20 or $30\mu\text{M}$ 15PG-J₂
5 (3) (4) (5); or $30\mu\text{M}$ 15PG-J₂ alone (6) was
6 analysed by Northern hybridisation with ^{32}P -
7 labelled TNF- α , IL-8, COX-2 and G3PDH.
8 Bacteria and drugs were applied for 2 hours.

9
10 D) mRNA from non-infected Caco-2 cells (1), Caco-
11 2 cells following incubation with 10^8 *S.*
12 *enteritidis* (2), 10^8 *S. enteritidis* in the
13 presence of 10 or $30\mu\text{M}$ ciglitazone (3) (4) was
14 analysed by Northern hybridisation with ^{32}P -
15 labelled TNF- α , IL-8, COX-2 and G3PDH.
16 Bacteria and drugs were applied for 2 hours.

17
18 E) mRNA from non-infected Caco-2 cells (1), Caco-
19 2 cells following incubation with 10^8 *S.*
20 *enteritidis* (2), 10^8 *S. enteritidis* and $30\mu\text{M}$
21 fenofibrate (3) was analysed by Northern
22 hybridisation with ^{32}P -labelled TNF- α , IL-8,
23 COX-2 and G3PDH. Bacteria and drugs were
24 applied for 2 hours.

25
26 F) Nuclear and cytoplasmic extracts from non-
27 infected Caco-2 cells (1), Caco-2 cells
28 following incubation with 10^8 *S. enteritidis*
29 (2), 10^8 *S. enteritidis* and 10^9 *B.*
30 *thetaitaomicron* (3), and 10^9 *B.*
31 *thetaitaomicron* alone (4) were analysed by

1 Western blotting with anti-PPAR γ (Santa Cruz).
2 bacteria were applied for 2 hours. Caco-2
3 cells prepared as nuclear and cytoplasmic
4 fractions, or detergent soluble (D. soluble)
5 or insoluble (D. insoluble) fractions.

6
7 G) Caco-2 cells co-cultured with 10^8 *S.*
8 *enteritidis* and 10^9 *B. thetaiotaomicron* for 2
9 hours were lysed in PBS containing 1% Nonidet
10 P40 solution and incubated overnight with a
11 polyclonal anti-RelA Sepharose (Santa Cruz).
12 Immuno-precipitates were resolved using SDS-
13 PAGE. Western blots were developed using a
14 monoclonal anti-RelA [NF- κ B p65], anti-PPAR γ
15 antibodies and anti-HDAC3 (Santa Cruz).

16
17 H) Immunofluorescence microscopy of PPAR γ (f-m,o)
18 and RelA (n). Co-localization of PPAR γ and
19 RelA from plates n and o (p). Caco-2 cells
20 were grown in medium alone (t,,j); with 10^8 *S.*
21 *enteritidis* (g,k); with 10^8 *S. enteritidis* and
22 10^9 *B. thetaiotaomicron* (h,l,n-p); or with 10^9
23 *B. thetaiotaomicron* (i,m). Treatments in the
24 absence (t-i, n-p) or presence of 4 μ M TSA (j-
25 m). All Scale bars = 25 μ m.

26
27 Fig. 5

28
29 Mutation of PPAR γ interferes with *B.*
30 *thetaiotaomicron*-mediated attenuation NF- κ B and
31 cytosolic sequestration of RelA.

20

- 1 A) Immuno-detection RelA. Caco-2 cells prepared
2 following standard culture protocol, treatment
3 groups (1-4). Immunoprecipitates prepared
4 using anti-PPAR γ sepharose conjugate.
5
- 6 B) *In vitro* translation showing RelA/PPAR γ
7 association
8
- 9 C) Caco-2 cells were transfected with PPAR γ DN
10 and a NF- κ B luciferase reporter construct
11 (DN), and compared with control cells
12 transfected with NF- κ B luciferase reporter
13 alone (MOCK). Luciferase activity (expressed
14 as % stimulation) was determined in non-
15 infected Caco-2 cells (1), Caco-2 cells
16 following incubation for a further six hours
17 with 10^8 *S. enteritidis* (2), and 10^8 *S.*
18 *enteritidis* in the presence of 10^9 *B.*
19 *thetaitaomicron* (3).
20
- 21 D) Caco-2 cells were transfected with a
22 constitutively expressing green fluroescent
23 protein (GFP) construct (B, C) or PPAR γ DN and
24 GFP (D, E). All cells were incubated with 10^8
25 *S. enteritidis* and 10^9 *B. thetaitaomicron*.
26 Cells were then immuno-stained with anti-RelA
27 (p65) specific antibody (Santa Cruz) and
28 examined by LSCM. (B) and (D) dual channel
29 capture showing combined GFP (green) and RelA
30 (red) images. (B) and (D) same areas as (B)

21

1 and (D) respectively, with single channel
2 capture showing only RelA. Scale bars = 25µM.

3
4 E) Caco-2 cells were transfected with RelA
5 chimeric construct with carboxyl-terminus of
6 yellow fluorescent protein (YFP) and either
7 PPARγ (d-f) or PPARγ DN (g-i), both as
8 chimeras constructs with carboxyl-terminus of
9 cyan fluorescent protein (CFP). Two days post
10 transfection, cells were incubated with 10^8 *S.*
11 *enteritidis* and 10^9 *B. thetaiotaomicron*. CFP
12 fluorescence (d,g) and YFP fluorescence (e,h).
13 Colocalization of CFP-PPARγ and YFP-RelA from
14 plates d and e (f), and CFP-PPARγ (DN) and
15 YFP-RelA from plates g and h (i). All Scale
16 bars = 25 µm. (j,k). Hela cells were
17 transfected with CFP-PPARγ and 2 days post
18 transfection were incubated with 10^8 *S.*
19 *enteritidis* and 10^9 *B. thetaiotaomicron*. Hela
20 cells fixed, permeabilized and immuno-stained
21 for SC35. CFP-PPARγ (j) indirect
22 immunofluorescence of SC35 (k). Scale bars =
23 10 µm.

24
25 Fig. 6

26
27 Fig. 6 shows skeletal muscle (□, wet weight and
28 ■, dry weight) accretion (mg/rat d⁻¹) by control
29 rats or rats orally treated with *Bacterioides*
30 *thetaiotaomicron* (BT), *Salmonella enterica* var.
31 *enteritidis* (SE) or *S. enteritidis* plus *B.*

22

1 *thetaitaomicron* (SE + BT). * shows significant
2 difference to control, BT or SE + BT for dry weight
3 and wet weight respectively ($p \leq 0.05$).

4 Gastrocnemius muscle is taken as representative of
5 skeletal muscle and total skeletal muscle in young
6 Hooded-Lister rats as approximately 47 times the
7 weight of the gastrocnemius muscles (Bardocz et al,
8 1996). Initial weight of gastrocnemius
9 muscles 804 ± 10 mg wet weight [194 ± 7 mg dry weight]
10 per 100 g initial fresh body weight.

11

12 Fig.7

13

14 Fig. 7 shows quantitative data (Northern blot or
15 real time PCR) illustrating effects on
16 chemokine/cytokine mRNA expression. S.e. = *S.*
17 *enteritidis*. B.t. = *B. thetaiotaomicron*.

18

19 Fig. 8

20

21 Fig. 8 shows bacterial counts following a 2 hour
22 incubation with Caco-2 cell monolayer.

23

24 a) Caco-2 cell associated B.t (those remaining
25 after washing cell layer) in the presence and
26 absence of B.t.

27

28 b) Cell associated S.e in the presence and
29 absence of B.t.

30 c) invaded Salmonella (those remaining after cell
31 washing and treatment with gentamycin

1 (100µg/ml for 4 hours) in the presence and
2 absence of B.t. n=6 +/- standard deviation.

3

4 Fig. 9

5

6 Fig. 9A: Shows NF-κB activation in Caco-2 cells in
7 response to *S. enteritidis* using EMSA.

8

9 Fig. 9B: Analysis of cytokine response by Northern
10 hybridisation.

11

12 a) mRNA from Caco-2 cells following 2 hour
13 exposure to 10^8 and 10^{10} cfu of S.e was
14 analysed for induction of proinflammatory
15 cytokines and normalised against G3PDH.

16

17 b) mRNA from Caco-2 cells following exposure to
18 10^9 S.e for variable time periods was analysed
19 for induction of pro-inflammatory cytokines
20 and normalised against G3PDH. Data for up to
21 2 and 4 hours (Figure 9D) is also shown.

22

23 c) mRNA from Caco-2 cells following 2 hour
24 exposure to 10^7 to 10^{10} B.t. was analysed for
25 pro-inflammatory cytokines and normalised
26 against G3PDH.

27

28 d) mRNA from Caco-2 cells analysed for
29 inflammatory cytokine expression (COX-2 and
30 IL-8), illustrating the suppressive effects of
31 B.t. at 2 hours and 4 hours.

32

24

1 Fig. 9C: Time course of Rel (p65) activation
2 following exposure of Caco-2 cells to *S.*
3 *enteritidis* and *B. thetaiotaomicron* using
4 immunocytochemical detection. Caco-2 cells
5 exposed to S.e. +/- B.t. for 2 hours were
6 fixed in 4% paraformaldehyde, permeabilised
7 with Triton X-100 and immunolabelled with
8 anti-p65 (RelA) (Santa Cruz). Secondary
9 detection was with Alexa Fluor 488 anti-rabbit
10 IgG (Molecular Probes). Images were digitally
11 captured using a Zeiss AxioCam on an Axiovert
12 2000 Microscope.

13
14 Figure 9D: Time course of PPAR γ effects following
15 exposure of Caco-2 cells to *S. enteritidis* and *B.*
16 *thetaiotaomicron* using immunocytochemical
17 detection.

18
19 Caco-2 cells exposed to S.e. +/- B.t. for 2 hours
20 were fixed in 4% paraformaldehyde, permeabilised
21 with Triton X-100 and immunolabelled with anti-
22 PPAR γ (Santa Cruz). Secondary detection was with
23 Alexa Fluor 568 anti-goat IgG (Molecular probes).
24 Images were digitally captured using a Zeiss
25 AxioCam on an Axiovert 200 Microscope.

26

27 Fig. 9E

28

29 Shows co-labelling of NF-KB p65 and PPAR γ in Caco-2
30 cells following 2 hour exposure to *B.*
31 *thetaiotaomicron* and *S. enteritidis*.

32

1 Fig. 10

2

3 Skeletal muscle (Gastrocnemius muscle) (□, wet
4 weight; ■, dry weight) accretion (mg/rat d⁻¹) by
5 control rats or rats orally treated with *B.*
6 [B.t], *Salmonella enterica* var.
7 enteritidis [S.e] or S.e + B.t n=6, +/- Standard
8 Error.

9

10 Fig. 11

11

12 Shows p65 immunoprecipitates, separated using SDS-
13 PAGE, electroblotted and immunostained with
14 acetylated lysine monoclonal antibody.

15

16 **Detailed description of the invention**

17

18 **Example 1: Inflammatory responses of Caco-2 cells**
19 **exposed to *S. enteritidis* in the presence and**
20 **absence of *B. thetaiotaomicron* and *B. vulgatus*.**

21 The evidence for bacterial regulation of host
22 inflammatory response was derived from studies
23 investigating inflammatory gene expression
24 following short-term exposure of intestinal Caco-2
25 cells to *Salmonella enteritidis* co-cultured in the
26 presence and absence of *Bacteroides*
27 . Using cDNA macro-array
28 technology (CLONTECH Atlas human cytokine/receptor
29 array system) we identified several genes including
30 TNF- α , IL-8, MIP-2 α and COX-2, whose induction
31 following *S. enteritidis* exposure was attenuated by
32 the presence of *B. thetaiotaomicron*. These results

1 were confirmed using Northern hybridization and
2 real-time PCR (Fig. 1a, f). We also investigated
3 the anti-inflammatory activity of *B.*
4 *thetaiotaomicron* against other inflammatory
5 mediators including IL-1 α , IL1- β , TNF- α , PMA, LPS
6 and enterohaemorrhagic *E.coli* 0157 H7 (Fig 1F). Of
7 those ligands that induced IL-8 expression in Caco-
8 2 cells, only the PMA, *S. enteritidis* and *E.coli*
9 0157 H7 effects were attenuated by *B.*
10 *thetaiotaomicron*.

11
12 In setting the experimental conditions for these
13 studies, many optimisation experiments (including
14 time course, bacterial dose/growth phase, Caco-2
15 cell passage/confluence studies) were undertaken.
16 Importantly we established that the growth,
17 attachment and invasion of bacteria were unaffected
18 by culture/co-culture conditions, thereby ruling
19 out the possibility that the data could be
20 attributed to differential attachment/invasion (see
21 Fig. 8).

22
23 Inflammatory gene expression was determined in non-
24 infected Caco-2 cells (1), cells incubated with 10⁸
25 *S. enteritidis* alone (2), cells incubated with 10⁸
26 *S. enteritidis* and 10⁹ *B. thetaiotaomicron* (3) or
27 cells incubated with 10⁹ *B. thetaiotaomicron* alone
28 (4). Bacteria were applied for 2 hours. Cells
29 were washed and harvested for mRNA isolation and
30 5 μ g of mRNA was analysed by Northern hybridisation
31 with ³²P-labelled probes specific for TNF- α , IL-8,
32 MIP-2 α , TGF- β , COX-2 and G3PDH.

1 Some of the results describing the effects of *B.*
2 *thetaiotaomicron* are consistent with the recent
3 molecular analysis reporting by Hooper *et al*, 2001.
4 Our results were confirmed using Northern
5 hybridisation and real-time PCR. Levels of TNF- α ,
6 IL-8, MIP-2 α and COX-2 were significantly increased
7 in response to *S. enteritidis* challenge (Fig. 1a).
8 In the presence of *B. thetaiotaomicron* however, the
9 level of expression of these genes were all
10 attenuated with the exception of TFG- β , which was
11 decreased by *S. enteritidis* yet maintained at
12 control values by the presence of *B.*
13 *thetaiotaomicron*. The viability and growth rates
14 of both bacteria and the numbers of *S. enteritidis*
15 adhering and invading epithelial cells did not
16 differ between the treatments and were unaffected
17 by the culture procedure.
18
19 The quantitative data shown in Fig. 7 confirms
20 these results and was obtained using mRNA purified
21 from Caco-2 cells following a 2 hour incubation
22 with vehicle (control), 5×10^8 *Salmonella*
23 *enteritidis* (S.e), 5×10^8 S.e and 1.5×10^9 *B.*
24 *thetaiotaomicron* (B.t) or B.t alone. Northern
25 blots were quantified by densitometry and
26 normalised to G3PDH levels. Real time PCR was
27 performed using ABI Taqman and normalised to 18S
28 rRNA levels. n=4 +/- standard deviation.
29
30 To investigate whether the attenuation of
31 inflammatory cytokine expression was specific to *B.*

1 *thetaiotaomicron* a related aerotolerant strain, *B.*
2 *vulgatus*, was also studied.

3

4 Treatments were as described above for (1) and (2),
5 cells incubated with 10^8 *S. enteritidis* and 10^9 *B.*
6 *vulgatus* (3a) or cells incubated with 10^9 *B.*
7 *vulgatus* alone (4a). Bacteria were applied for 2
8 hours.

9

10 *B. vulgatus* was negative for the biological
11 activity (see Fig. 1b).

12

13 In the results obtained the small induction of
14 inflammatory gene expression triggered by both of
15 the commensal strains, which may be related to the
16 fact that late log phase bacteria were tested; was
17 noteworthy. The application of early log phase
18 bacteria devoid of bacterial cell debris, may
19 completely abrogate this response. A further
20 experiment was therefore conducted in which Caco-2
21 cells were incubated with 10^8 *S. enteritidis* and
22 10^9 *B. thetaiotaomicron* for either 2 or 4 hours.
23 The inhibitory effect of *B. thetaiotaomicron* on IL-
24 8 expression was sustained for prolonged periods
25 (Fig. 1d), which contrasted with TNF- α , suggesting
26 that the mechanisms regulating the expression of
27 these genes are differentially influenced by *B.*
28 *thetaiotaomicron*.

29

30 The physiological relevance of the data showing the
31 suppression of inflammatory cytokines by *B.*
32 *thetaiotaomicron* was verified using both a

1 functional *in vitro* model of PMN recruitment and an
2 *in vivo S. enteritidis* rat infection model. PMN
3 recruitment *in vitro* and *in vivo* was monitored
4 using myeloperoxidase (MPO) activity.

5
6 Caco-2 cells were seeded onto inverted Transwells
7 (Corning) and various combinations of bacteria
8 applied to the apical surfaces of the cells.
9 Freshly isolated human PMNs were applied to the
10 basolateral compartment and their transepithelial
11 migration determined by MPO assay (Neish *et al*,
12 2000). Treatment groups (1-4) were as described
13 above. Cells were incubated for 2 hours and then
14 bacteria were removed and fresh media applied.
15 Cells were incubated for a further 2 hours and then
16 washed in HBSS. Both cells, and media derived from
17 the apical compartment, were solubilised in a 1%
18 Triton C-100 and MPO determined.

19
20 The results derived from the *in vitro* model of
21 polymorphonuclear leukocyte (PMN) recruitment assay
22 confirmed the anti-inflammatory activity of *B.*
23 *thetaitaomicron* (Fig. 1e).

24
25 It is noteworthy that *S. enteritidis* infection of
26 the rat intestine is mainly in the ileum, the
27 natural habitat for *B. thetaitaomicron*.

28
29 Newly weaned (21 d) minimal flora rats (fed on
30 normal laboratory diets) were split into 2 groups
31 and one group additionally fed with anaerobically
32 prepared jelly (0.5 g/d) containing 10^8 cfu of *B.*

1 *thetaiotaomicron* for 19 days. Half of the rats in
2 each group were then orally challenged with 10^8 *S.*
3 *enteritidis*. The severity of the inflammatory
4 response in all animals was assessed by determining
5 MPO levels in ileal mucosa at 6 days post *S.*
6 *enteritidis* infection. Treatments were, no *B.*
7 *thetaiotaomicron* and no *S. enteritidis* (1), no *B.*
8 *thetaiotaomicron* then 10^8 *S. enteritidis* (2), *B.*
9 *thetaiotaomicron* then 10^8 *S. enteritidis* (3), and
10 *B. thetaiotaomicron* alone (4). Experiments were
11 undertaken at least 3 times with similar results.
12

13 The level of MPO in the ileal mucosa of rats
14 challenged with *S. enteritidis* increased ($P < 0.005$),
15 but was significantly attenuated by prior oral
16 inoculation and stabilisation of *B.*
17 *thetaiotaomicron* within the flora ($P < 0.001$) (Fig.
18 1f). Furthermore, from the PMN recruitment
19 experiments we found that *S. enteritidis* induced
20 400pg/ml IL-8 protein in culture supernatants over
21 4 h whereas when co-cultured in the presence of *B.*
22 *thetaiotaomicron* the concentration was
23 significantly lower at 230 pg/ml. Colonisation by
24 *B. thetaiotaomicron* was confirmed by specific
25 primer amplification of intestinal tissues. As IL-
26 8 and MIP-2 α are essential chemokines for PMN
27 transepithelial migration (McCormick et al, 1993;
28 Hang et al, 1999), the effects of *B.*
29 *thetaiotaomicron* are therefore due to decreased IL-
30 8 and MIP-2 α transcription, which then impacts on
31 the translation and secretion of active protein.
32 Consistent with Neish et al, 2000 we have

1 demonstrated, using a novel model system, that non-
2 pathogenic bacteria can exert immune suppressive
3 effects by subverting host systems that regulate
4 gene expression. Importantly, we have also
5 provided *in vivo* validation of our findings.

6
7 In addition to MPO data discussed above Fig. 10
8 illustrates further evidence of the protective
9 effects of *B. thetaiotaomicron* on muscle biology
10 during the inflammatory response induced by *S.*
11 *enteritidis*.

12
13 **Example 2: *B. thetaiotaomicron* attenuates**
14 **inflammation by altering the cellular distribution**
15 **and activation state of NF- κ B and PPAR γ proteins**
16 **and involves targeted disruption of the NF- κ B**
17 **signal transduction pathway.**

18
19 The NF- κ B family of transcription factors plays a
20 central role in regulating inflammatory responses.
21 These proteins share a highly conserved NH₂-
22 terminal sequence referred to as the Rel homology
23 domain, which is required for their subunit
24 dimerisation, DNA binding and interaction with the
25 inhibitory I κ B proteins. Signals that induce NF- κ B
26 lead to phosphorylation, of the inhibitory I κ B
27 proteins (I κ B α at Ser-32 and Ser-36) which are then
28 targeted for ubiquitination and proteasome-
29 mediated degradation. Rel proteins, including
30 RelA (p65), are thereby released to translocate to
31 the nucleus and bind DNA.

32

1 Non-infected Caco-2 cells (1) were incubated with
2 10^8 *S. enteritidis* alone (2), 10^8 *S. enteritidis*
3 and 10^9 *B. thetaiotaomicron* (3), and 10^9 *B.*
4 *thetaiotaomicron* alone (4) for 2 hours. Cells were
5 fixed in 4% paraformaldehyde for 30 minutes at room
6 temperature, permeabilised with 0.2% Triton-X 1000
7 at 4°C and examined by indirect immunofluorescence
8 microscopy. Primary antibodies (Santa Cruz) (A-D)
9 anti-RelA [NF- κ B p65], (E-H) anti-PPAR γ , (I-L)
10 anti-I κ B α and (M-P) anti-p κ B α . Secondary
11 antibodies: Alexa Fluor species-specific anti-IgG
12 (Molecular Probes).

13
14 We found that exposure of Caco-2 cells to *S.*
15 *enteritidis* triggered a cascade of events leading
16 to enhanced translocation of RelA to the nucleus
17 (Fig. 2B).

18 Significantly, *B. thetaiotaomicron* was found to
19 abolish the translocation of RelA to the nucleus
20 with virtually all RelA localised to the cytosol
21 following 2 hours of exposure to this bacterium
22 (Fig. 2C, D).

23 Phosphorylation of I κ B α and, importantly, its
24 degradation was observed following exposure to *S.*
25 *enteritidis* in the presence and absence of *B.*
26 *thetaiotaomicron* (Fig 2G). Following 2 h exposure,
27 both the level of I κ B α mRNA (Fig. 3B) and protein
28 (Fig. 2J, K) were enhanced, particularly in cells
29 exposed to *S. enteritidis* alone and *S. enteritidis*
30 /*B. thetaiotaomicron*, indicating the presence of
31 transcriptionally active NF- κ B (Cheng et al. 1994;
32 Chiao et al. 1994).

1 **Viable *B. thetaiotaomicron* selectively interferes**
2 **with the transcriptional activity of NF- κ B proteins**
3 **but does not influence the synthesis or**
4 **phosphorylation of AP-1 proteins.**

5
6 Supershift EMSA performed on nuclear extracts,
7 incubated with RelA [NF- κ B p65] specific antibody
8 (Santa Cruz), from non-infected Caco-2 cells (1)
9 following incubation with 10^8 *S. enteritidis* (2),
10 10^8 *S. enteritidis* and 10^9 *B. thetaiotaomicron* (3),
11 and 10^9 *B. thetaiotaomicron* alone (4) for 2 hours.
12 Where indicated *B. thetaiotaomicron* was heat
13 inactivated at 70°C for 15 minutes prior to its
14 addition to the Caco-2 cells.

15
16 Using electrophoretic mobility shift assays (EMSA)
17 we confirmed that RelA was the major subunit
18 activated by *S. enteritidis* (Fig. 3A), although a
19 lower level activation of p52 was also apparent
20 (data not shown). We then hypothesised that *B.*
21 *thetaiotaomicron* attenuated the production of
22 immune response mediators, triggered by *Salmonella*,
23 by disrupting the NF- κ B signal transduction
24 pathway.

25
26 A similar experiment is illustrated in Fig. 9A
27 which demonstrates EMSA supershifts showing peak
28 activation of p65 at 2 hours following exposure to
29 *S. enteritidis* in Caco-2 cells. Lane 1, no
30 protein, lanes 2, 5, 8, 11 nuclear extracts from
31 control cells; lanes 3, 6, 9, 12 nuclear extracts
32 from cells infected with S.e.; lanes 4, 7, 10, 13

1 nuclear extracts for cells infected with S.e. and
2 supershifted with p65 (RelA) antibody.

3

4 We also showed that viable but not heat-inactivated
5 *B. thetaiotaomicron* inhibited the RelA response
6 (Fig. 3A). Similarly, neither bacterial culture
7 supernatant nor conditioned culture media (media
8 from Caco-2 cells exposed to *B. thetaiotaomicron*)
9 possessed biological activity (data not shown).

10 This suggests that epithelial cell contact is
11 essential for the anti-inflammatory activity of *B.*
12 *thetaiotaomicron*. Within 20 minutes of exposure to
13 *S. enteritidis*, in the presence and absence of *B.*
14 *thetaiotaomicron*, IKB α phosphorylation and
15 degradation was observed (data not shown).
16 Furthermore, the viability, growth, attachment and
17 invasion of the bacterial strains studied were
18 unaffected by culture treatments (data not shown)
19 and hence the observed effects cannot be ascribed
20 to differences in receptor recognition and
21 activation.

22

23 Using the same experimental groups (1-4) as
24 described above, bacteria were applied for 2 hours
25 before mRNA was analysed by Northern hybridisation
26 and blots probed with specific ³²P-labelled IKB α
27 and G3PDH probes. Both the levels of IKB α mRNA
28 (Fig. 3B) and IKB α protein (Fig. 2J, K) were
29 enhanced. This increase could be ascribed to the
30 transcriptional activity of RelA which induces neo-
31 synthesis of IKB α protein (Cheng et al, 1994).
32 IKB α then enters the nucleus and associates with

1 RelA removing it from DNA promoter sites (Arenzana-
2 Seisdedos et al, 1995). Interestingly, PPAR α -
3 mediated stimulation of IKB α synthesis has also
4 been reported (Delerive et al, 2000). To further
5 investigate if a transient RelA translocation does
6 occur in *B. thetaiotaomicron* treated cells
7 experiments were undertaken using leptomycin B
8 (LMB), a specific inhibitor of CRM-1-dependent
9 nuclear export. The rationale was that the
10 cytoplasmic location of Rel proteins, including
11 p65, is maintained by CRM-1-dependent export of
12 RelA/IKB α complexes out of the nucleus (Huang et
13 al, 2000; Tam et al, 2000). We found that LMB
14 caused an accumulation of RelA in nuclei of cells
15 co-cultured with both *S. enteritidis* and *B.*
16 *thetaiotaomicron* (results not shown) indicating
17 that, independent of the presence of *B.*
18 *thetaiotaomicron*, transient RelA translocation to
19 the nucleus does occur. This result is consistent
20 with the phosphorylation and ubiquitination of the
21 cytosolic IKB α /RelA complex and the activation of
22 IKB α gene expression following initial exposure of
23 intestinal cells to bacteria.

24

25 **Example 3: *B. thetaiotaomicron* induces**
26 **nucleocytoplasmic shuttling of PPAR γ and sequesters**
27 **RelA.**

28

29 A recent report of immune-suppression by non-
30 pathogenic bacteria attributed the mechanism to
31 inhibition of both ubiquitination and degradation

1 of I κ B α (Neish et al, 2000), which clearly does not
2 apply to our model system. The predominant
3 cytosolic location of NF- κ B complexes in cells co-
4 cultured with both *S. enteritidis* and *B.*
5 *thetaitaomicron* could then be explained by a
6 greater efficiency of the I κ B α -mediated nuclear
7 export over nuclear localisation. The levels of
8 I κ B α protein are moderately higher in the nuclei of
9 cells treated with both *S. enteritidis* and *B.*
10 *thetaitaomicron* than *S. enteritidis* alone, which
11 could potentially accelerate the deactivation of
12 NF- κ B and contribute to the overall anti-
13 inflammatory properties of *B. thetaitaomicron*.
14 However, a more likely explanation is the
15 differential deacetylation of RelA. It has
16 recently been reported that the duration of nuclear
17 RelA activation is determined by reversible
18 acetylation (Chen et al, 2001). The acetylated
19 form of RelA has a low affinity for I κ B α whereas
20 the deacetylation of RelA by histone deacetylase 3
21 (HDAC3) promotes the binding to I κ B α and the CRM-1-
22 mediated nuclear export of RelA. We showed that *B.*
23 *thetaitaomicron* promoted HDAC3 association with
24 RelA (Fig. 4G). Experiments using trichostatin A
25 (TSA), a specific inhibitor of histone
26 deacetylases, were undertaken. Results showed that
27 inhibition of HDACs, in response to TSA treatment
28 (800nM for 4 hours), resulting in partial p65
29 accumulation but importantly an accelerated exit of
30 PPAR γ to cytosol in cells treated with *S.*
31 *enteritidis* alone. The data presented supports the

1 role of acetylation/deacetylation as an important
2 mechanism facilitating nuclear export of p65 and
3 PPAR γ in *B. thetaiotaomicron* treated cells (see
4 Fig.11).

5
6 Furthermore, both RelA and peroxisome proliferator-
7 activated receptor gamma (PPAR γ) proteins appeared
8 to co-localise within the same cellular compartment
9 (Fig. 2C, G) and we also hypothesised that an
10 important mechanism limiting further RelA nuclear
11 translocation in *B. thetaiotaomicron* treated cells
12 involves cytosolic sequestration of RelA by PPAR γ .
13 The supporting experimental evidence is presented
14 below.

15
16 AP-1 complexes play an important role in the
17 regulation of inflammatory gene expression and are
18 rapidly activated by a variety of extracellular
19 stimuli including growth factors, cytokines and
20 bacteria (Meyer-ter-Vehn et al, 2000). We
21 investigated whether the inhibitory effects of *B.*
22 *thetaiotaomicron*, extended to this signalling
23 pathway. AP-1 activity is regulated by two main
24 mechanisms, involving enhanced expression and
25 phosphorylation of AP-1 subunits consisting of
26 homo- and hetero-dimers of the protooncogene
27 families Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun
28 (JunB, c-Jun and JunD) and ATF (ATF2, ATF3/LRF2 and
29 B-ATF), all members of the leucine zipper family of
30 DNA binding proteins. These proteins are mainly
31 controlled by three related kinases, collectively
32 called mitogen-activated protein (MAP) kinases. We

1 investigated whether the level of protein
2 phosphorylation of p42 and p44 (extracellular
3 signal-regulated kinase; ERK) kinases, the c-Jun N-
4 terminal kinases (JNK)/stress-activated protein
5 (SAP) kinases and the p38 kinases were altered in
6 response to *S. enteritidis* and *B. thetaiotaomicron*.
7 We found that *S. enteritidis* activated p38 MAP
8 kinase (Fig. 3C) but not ERK or JNK during the time
9 period studied (results not shown). This finding
10 is consistent with the fact that p38 is rapidly
11 activated by inflammatory stimuli (Raingeaud et al,
12 1996), whereas the activation of JNK can occur at a
13 later time point (Kujime et al, 2000). Activation
14 of p38 leads to phosphorylation of elk-1, which in
15 conjunction with serum response factor, binds to
16 the serum response element in the *c-fos* promoter,
17 to increase *c-fos* transcription and translation, as
18 shown by changes in the gene and protein levels
19 (Fig. 3D, E). We showed that the p38
20 phosphorylation induced by *Salmonella* was not
21 modified by co-incubation with *B. thetaiotaomicron*.
22 Message and protein levels were both increased in
23 response to *S. enteritidis* and *B. thetaiotaomicron*
24 and the effects on *c-fos* gene expression appeared
25 to be additive. *De Novo* c-Fos synthesis leads to
26 the formation of Jun-Fos hetero-dimers, which have
27 a 10-fold higher DNA binding affinity, resulting in
28 increased AP-1 activity (Musti et al, 1997; Smeal
29 et al, 1991). ATF-2 is also a target of the p38
30 MAP kinase and JNK signal transduction pathways.
31 The transcription factor ATF-2 is phosphorylated by
32 p38 MAP kinase on Thr-69 and Thr-71. JNK however,

1 phosphorylates and activates both ATF-2 and c-Jun.
2 ATF-2 and c-Jun are therefore, differentially
3 regulated by p38 and JNK signal transduction
4 pathways (Raingeaud et al, 1996). In our study
5 neither an increase in c-Jun protein level nor its
6 phosphorylation state was observed in response to
7 either bacterial strain, indicating that the
8 phosphorylation of ATF-2 in response to *S.*
9 *enteritidis* (Fig. 3F) is likely to be induced by
10 p38 MAP kinase and not JNK. These data are
11 consistent with the direct effect of *S. enteritidis*
12 on the p38 MAP kinase activity. It is likely that
13 ATF-2 activation triggers the formation of c-
14 Jun/ATF-2 hetero-dimers that then stimulate *c-jun*
15 gene transcription in response to *Salmonella* (Fig.
16 3D). The anti-inflammatory effects of *B.*
17 *thetaitaomicron* appear to target the NF- κ B pathway
18 selectively and this may explain why genes such as
19 IL-8 that have an absolute requirement for NF- κ B
20 (Mukaida et al, 1994; Elliott et al, 2001) are
21 particularly sensitive to inhibition by *B.*
22 *thetaitaomicron*.

23

24 **Example 4**

25

26 To further investigate the mechanism of immune
27 suppression by non-pathogenic bacteria we initially
28 studied the anti-inflammatory cytokines, IL-10 and
29 TGF- β . IL-10 gene expression was not affected by
30 treatment with *B. thetaitaomicron* (data not shown)
31 although this does not preclude the possibility
32 that constitutive protein may be involved. There

1 was some suggestion from the data that the contra-
2 inflammatory cytokine TGF- β may be involved.
3 However, if this cytokine is acting to down-
4 regulate inflammatory responses, it is more likely
5 to be involved in the longer-term anti-inflammatory
6 effects, as *de novo* cytokine synthesis would not
7 have been significant over the time course of these
8 acute studies.

9
10 The PPARs are emerging as important modulators of
11 inflammatory processes (Nakajima *et al*, 2001).
12 PPARs are ligand activated transcription factors
13 that regulate gene expression by binding with
14 retinoid X receptor (RXR), as heterodimeric
15 partners, to specific DNA sequence elements termed
16 PPAR response elements (PPRE). Recent work
17 however, suggests that ligand activation of PPAR γ
18 may be important in modulating AP-1 and NF- κ B-
19 mediated gene expression (Su *et al*, 1999). We
20 investigated the role of PPARs in the regulation of
21 NF- κ B by *B. thetaiotaomicron*. Consistent with
22 receptor activation we were able to show that the
23 mRNA for PPAR α and PPAR γ decreased in cells exposed
24 to both *S. enteritidis* and *B. thetaiotaomicron*
25 (Fig. 4A) and also following exposure to a specific
26 PPAR γ ligand, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-
27 PGJ₂) but not with fenofibrate, a specific PPAR α
28 ligand (Fig. 5B). PPAR γ activation has previously
29 been associated with down-regulation of PPAR γ mRNA
30 and protein in 3T3-L1 adipocytes (Camp *et al*,
31 1999). Using 15d-PGJ₂ and ciglitazone we also

1 demonstrated that the activation of inflammatory
2 cytokine expression by *S. enteritidis* could be
3 attenuated over a concentration range considered to
4 be physiological (Fig. 4C, D). Two different
5 ligands for PPAR γ were tested as 15d-PGJ₂ can
6 inhibit I KappaB Kinase directly (Straus *et al*,
7 2000). Both 15d-PGJ₂ and ciglitazone have been
8 reported to inhibit AP-1 and COX-2 induction in
9 human intestinal epithelial cells (Subbaramaiah *et*
10 *al*, 2001) and may also have therapeutic benefit in
11 the treatment of colitis (Su *et al*, 1999). We
12 found that PPAR γ and PPAR α ligands were able to
13 attenuate *Salmonella*-mediated cytokine induction
14 (Fig. 4C, D, E). This is consistent with recent
15 reports on PPAR γ agonists, which have been shown to
16 attenuate NK-KB and IL-8 expression in *H. pylori*
17 treated gastric epithelial cells (Gupta *et al*,
18 2001), and also inhibit the infiltration of PMN in
19 ischemia reperfusion-induced intestinal injury
20 (Nakajima *et al*, 2001).

21

22 Although we observed effects with PPAR γ and PPAR α
23 agonists, in subsequent experiments we concentrated
24 on PPAR γ as it has been shown to be expressed at
25 much higher levels than PPAR α in the colonic mucosa
26 (Mansen *et al*, 1996; Fajas *et al*, 1997).

27

28 In addition to regulating gene transcription via
29 PPRE, PPARs have recently been shown to inhibit
30 gene transcription by interfering with other
31 transcription factor pathways through a mechanism

1 independent of DNA binding (Delerive et al, 1999).
2 PPAR γ 2 can be found in both cytosolic and nuclear
3 compartments (Thuillier et al, 1998), although the
4 physiological relevance of cytosolic PPAR γ is
5 currently unknown. Using immunocytochemical
6 localisation on fixed Caco-2 cells we found that *S.*
7 *enteritidis* induced nuclear accumulation of PPAR γ
8 (Fig. 2F). It is important to note that
9 phosphorylation of the NH₂-terminal domain of PPAR γ
10 (at Ser-122) by MAP kinase, reduces ligand binding
11 affinity and negatively regulates the
12 transcriptional and biological functions of PPAR γ
13 (Shao et al, 1998). This would explain why the
14 nuclear accumulation of PPAR γ , during the early
15 stages of an inflammatory response to *Salmonella*,
16 is ineffective in quenching inflammatory gene
17 transcription. Following co-culture of *S.*
18 *enteritidis* with *B. thetaiotaomicron*, PPAR γ
19 redistributed to the cytosol (Fig. 2G, H). The
20 differential distribution of PPAR γ within Caco-2
21 cells exposed to *S. enteritidis* and in the context
22 of *B. thetaiotaomicron* was also demonstrated by
23 western blotting using nuclear and cytoplasmic
24 extracts (Fig. 4F).
25
26 All published data relating to PPAR γ have focused
27 however, on its nuclear site of action. Using
28 immunocytochemistry we found that *S. enteritidis*
29 induced nuclear accumulation of PPAR γ in Caco-2
30 cells (Fig. 4H(g)). In contrast, PPAR γ
31 redistributed to the cytosol following co-culture

1 with *S. enteritidis* and *B. thetaiotaomicron* (Fig.
2 4H(h,i)). Time course studies indicated that this
3 process was clearly evident at 60 min following
4 exposure to bacteria and was virtually complete by
5 2 h (results not shown). The differential
6 distribution of PPAR γ in Caco-2 cells exposed to *S.*
7 *enteritidis* and *B. thetaiotaomicron* was also
8 demonstrated by western blotting of nuclear and
9 cytoplasmic extracts (Fig. 4F).

10
11 The *Salmonella*-induced PPAR γ protein formed a
12 heterodimeric complex with RXR α (demonstrated by
13 IP, results not shown) and partitioned to the
14 detergent insoluble cell fraction whereas that
15 induced by *B. thetaiotaomicron* was detergent
16 soluble (Fig. 4F). The nucleocytoplasmic shuttling
17 of PPAR γ , induced by co-culture of *S. enteritidis*
18 and *B. thetaiotaomicron*, was not blocked by
19 leptomycin B (LMB) treatment (results not shown)
20 and hence not facilitated by the export receptor
21 crm-1 analogous to other nuclear receptors (Bunn et
22 al. 2001). Chilling and metabolic inhibitors did
23 however significantly reduced nuclear export
24 (results not shown). Other biological inhibitors
25 such as TSA (histone deacetylase inhibitor) (Fig.
26 4H(j-m)) and SB (p38 MAP kinase inhibitor) (data
27 not shown) were also applied to *S. enteritidis*-
28 treated Caco-2 cells and were shown to induce
29 punctate cytosolic labeling and export of PPAR γ
30 from the nucleus (Fig. 4H(k)), thus mimicking the
31 *B. thetaiotaomicron* effect. AP-1 signaling
32 pathways, including p38, were not inhibited by *B.*

1 *thetaitaomicron* (results not shown), indicating
2 that acetylation/deacetylation reactions are
3 potentially relevant to the PPAR γ nuclear export
4 mechanism.

5
6 Furthermore, even though PPAR γ and PPAR α ligands
7 attenuate the *Salmonella* mediated cytokine
8 induction (results not shown), the PPAR ligands
9 tested in these experiments did not mimic the *B.*
10 *thetaitaomicron* effect on PPAR γ cellular re-
11 localisation, suggesting a novel endogenous ligand
12 or mechanism of regulation for PPAR γ .

13
14 Using dual label immunocytochemistry we found that
15 much of the PPAR γ protein co-localized with RelA
16 (Fig. 4H n-p). We hypothesise that physical
17 coupling between PPAR γ and RelA is an important
18 factor facilitating cytoplasmic localisation in
19 intestinal cells, exposed to *S. enteritidis* and *B.*
20 *thetaitaomicron*

21
22 It has been suggested that nuclear PPAR γ and NF- κ B
23 can form an inactive complex (Ricote et al, 1999).
24 Furthermore, glutathione S-transferase pull-down
25 experiments demonstrate that PPAR α physically
26 interacts with c-Jun and RelA p65 (Delerive et al,
27 1999). In our study, using immuno-purification
28 (IP) protocols we show that isolation of PPAR γ from
29 cells treated with *S. enteritidis* in the presence
30 of *B. thetaitaomicron* resulted in the co-
31 purification of RelA (Fig. 4G) suggesting that the

1 proteins are physically associated, possibly as
2 components of a layer multi-protein complex.
3 Direct interaction of PPAR γ and RelA was confirmed
4 by in vitro translation and IP (Fig. 4b).
5

6 **Example 5**

7
8 To further investigate the importance of PPAR γ in
9 the regulation of RelA we utilised a dominant
10 negative (DN) form of the receptor (gifted by
11 Professor Chatterjee, University of Cambridge, UK)
12 (Gurnell *et al*, 2000). Within the PPAR receptors
13 there is a striking conservation of both leucine
14 and glutamic acid. These residues are essential
15 for ligand binding and recruitment of nuclear co-
16 activators. Mutation of these residues generates a
17 DN form of the receptor, which impairs its ability
18 to recruit co-activators and release two co-
19 repressors, silencing mediator of retinoid and
20 thyroid receptors (SMRT) and nuclear co-repressor
21 (NcoR) (Gurnell *et al*, 2000). Co-
22 immunoprecipitation experiments with DN PPAR γ
23 indicate that the SMRT interacts with the receptor
24 *in vivo* and that the mutated PPAR γ is a potent
25 transcriptional repressor. Chimeric fluorescent
26 protein constructs of human PPAR γ and the dominant
27 negative form of PPAR γ containing a cyan
28 fluorescent protein (CFP) on the carboxyl-terminal
29 domain were prepared by PCR amplification from
30 previously reported clones (Gurnell *et al*.2000).
31 Chimeric RelA linked to YFP was gifted from Dr J
32 Schmid (Schmid *et al*. 2000). Confirmation of

1 successful expression was performed by Western blot
2 analysis of transiently transfected Hela cells
3 using anti-human PPAR γ and anti-human RelA
4 antibodies.

5
6 Double transfection studies with PPAR γ DN and an
7 NF- κ B luciferase reporter (gifted by Dr Israel,
8 Pasteur Institute, Paris, France) were performed.
9 Consistent with a recent report we found that cells
10 exposed for long periods to pathogenic *S.*
11 *enteritidis* inactivated luciferase activity
12 (Savkovic et al, 2000). To avoid this we
13 stimulated the cells for 2 hours and then removed
14 the bacteria by washing and determined luciferase
15 protein production after 8 hours. We found both in
16 the control and PPAR γ DN transfected cells, that *S.*
17 *enteritidis* induced NF- κ B activation and luciferase
18 protein synthesis ($P < 0.001$) (Fig. 5C). In the
19 PPAR γ wild type (NT) transfected cells (luciferase
20 alone) this response was attenuated by *B.*
21 *thetaitaomicron* ($P < 0.028$), but not in dominant
22 negative (DN) transfected cells (Fig. 5C).
23 Similarly, there was modification of the cellular
24 distribution of RelA, as determined using
25 immunocytochemistry and laser scanning confocal
26 microscopy (LSCM), in cells transfected with green
27 fluorescent protein (GFP) with and without PPAR γ
28 DN. In the control cells (GFP alone) the
29 distribution of RelA was predominantly cytosolic as
30 described above for non-transfected cells (Fig. 5D
31 (insert B,C)). In PPAR γ DN transfected cells, the

1 distribution was irregular and RelA localised to
2 both the cytosolic and nuclear compartments (Fig.
3 5D, (insert D, E)). These data demonstrate that
4 PPAR γ is essential in sequestering RelA and in
5 preventing nuclear translocation.

6
7 We further investigated the cellular distribution
8 of PPAR γ and RelA using fluorescent microscopy
9 performed in Caco-2 cells and Hela cells
10 transfected with chimeric constructs of WT and DN
11 PPAR γ and CFP and chimeric YFP and RelA. Much of
12 the expressed PPAR γ /RelA protein was localized
13 within the nuclei of transfected cells as
14 previously reported (Schmid et al. 2000).
15 Similarly, in cells transfected with PPAR γ WT and
16 RelA the predominant expression was nuclear but
17 there was also very clear evidence of punctate
18 cytosolic labeling and co-localization of PPAR γ and
19 RelA following incubation with *B. thetaiotaomicron*
20 (Fig. 5E(f)). In PPAR γ DN transfected cells
21 however, RelA was absent from the cytosolic
22 compartment (Fig. 5E(i)). These results indicate
23 that nuclear export of the DN PPAR γ , and co-
24 associated RelA, was impaired and it is concluded
25 that PPAR γ is essential for both the nuclear export
26 and cytosolic distribution of RelA induced in
27 intestinal cells by *B. thetaiotaomicron*. In both
28 Caco-2 and Hela cell transfection experiments a
29 significant nuclear pool of expressed PPAR γ and
30 RelA protein was observed. In this scenario,
31 irrespective of WT or DN status, aggregates of

1 PPAR γ and RelA were clearly visible in the nucleus
2 of cells cultured in the presence of *S. enteritidis*
3 and *B. thetaiotaomicron* (also confirmed by IP,
4 results not shown) proving that the PPAR γ and RelA
5 interaction occurs within the nuclear compartment.
6 We noted that the distribution of PPAR and RelA
7 within the nucleus was similar to that of splicing
8 factors that occur within nuclear speckles or
9 spliceosomes. In co-localization studies, we found
10 that PPAR γ protein co-localized with SC35, a
11 specific pre-mRNA splicing factor, (Fig. 5E(k))
12 indicating that the PPAR γ /RelA complex may also be
13 capable of interacting with the mRNA machinery of
14 the cell. Evidence for the interaction between
15 nuclear receptors and splicing compartments was
16 published during preparation of this manuscript
17 (Zhao et al. 2002). Clearly, in Caco-2 cells the
18 presence of *B. thetaiotaomicron* induces enhanced
19 nuclear export of PPAR γ and, in a piggy-back
20 fashion the export of transcriptionally active
21 RelA, thereby preventing further nuclear import and
22 RelA-mediated transcription during sustained
23 inflammation. By this mechanism *B.*
24 *thetaiotaomicron* exerts a potent anti-inflammatory
25 effect.

26
27 The existence of bacteria within the
28 gastrointestinal tract that actively suppress
29 inflammation seems certain. A shift in the balance
30 between enteric microorganisms that suppress
31 inflammation and those that support inflammatory
32 responses may be directly relevant to the etiology

1 of inflammatory diseases of the gastrointestinal
2 tract. Hence it is plausible that inflammatory
3 bowel disease is related to the loss of bacterial
4 strains that actively contribute to immune
5 homeostasis and this may also explain why some
6 patients respond to probiotic therapy (Madsen et
7 al, 2001). Equally, dysfunction of PPARs, due to
8 inadequate levels of receptor protein or due to
9 expression of specific allelic variants that
10 enhance susceptibility to inflammatory conditions
11 in the gut, may be highly relevant. This view is
12 further supported by recent published data
13 indicating that heterozygous PPAR γ -deficient mice
14 are significantly more susceptible to ischemia-
15 reperfusion-induced intestinal inflammation and
16 injury (Nakajima et al, 2001).

17

18 **METHODS**

19

20 **Reagents**

21 Tissue culture reagents were obtained from Sigma
22 and Invitrogen, Antibodies obtained from Santa
23 Cruz, Molecular probes, New England Biolabs and,
24 molecular reagents obtained from Promega,
25 Invitrogen and Amersham.

26

27 ***S. enteritidis*/B. *thetaitaomicron* co-culture**

28 **models**

29 Caco-2 and Hela cells were routinely cultured in 35
30 mm culture dishes. Typically experiments required
31 incubation of cells with the following four
32 treatments: cells in medium alone (1); cells
33 incubated with 10^8 *S. enteritidis* (2); cells

50

1 incubated with 10^8 *S. enteritidis* and 10^9
2 *Bacteriodes thetaiotaomicron* (3); and cells
3 incubated with 10^9 *B. thetaiotaomicron* (4).
4 Bacteria were applied for either 2 h or 4 h and
5 removed by extensive washing. Other bacteria and
6 ligands tested included *E. coli* 0157 H7, *B.*
7 *vulgatus*, PMA, IL-1 α / β and TNF α . All experiments
8 were optimised and protocols were based on detailed
9 dose responses and time courses. Transepithelial
10 migration of PMN cells through Caco-2 cell
11 monolayers was determined by MPO assays (Parkos et
12 al.1991). The Caco-2 cells were incubated for 2 h
13 before the bacteria were removed and replaced with
14 fresh media. Thereafter cells were then incubated
15 for a further 2 h. Both cells and media derived
16 from the apical compartment were then solubilised
17 in a 1% Triton X-100 and MPO determined.

18

19 **Cytokine Analyses**

20 Cytokine analyses were undertaken using Clontech
21 macroarray, Northern hybridizations and real-time
22 PCR of RNA isolated from Caco-2 cells. Total RNA
23 and mRNA were isolated, cDNA produced and PCRs were
24 performed under standard conditions. IL-8 protein
25 concentration was determined by ELISA.

26

27 **EMSA analysis of NF-KB and AP-1**

28 Nuclear extracts were incubated with single
29 stranded ^{32} P-labelled oligonucleotide probes
30 containing consensus binding sequences for NF-KB
31 (5'-AGT TGA GGG GAC TTT CCC AGG C-3') or AP-1 (5'-
32 CGC TTG ATG AGT CAG CCG GAA-3'), separated by

1 electrophoresis and visualized by autoradiography.
2 EMSA supershifts were performed using specific NF-
3 κ B subunit antibodies. Effects of *B.*
4 *thetaitaomicron* on NF κ B and AP-1 signaling were
5 determined by EMSA, western blotting (protein and
6 phosphoprotein), promoter-specific reporter
7 analysis and target gene expression (eg. *fos* and
8 *jun*).

10 **Immunofluorescence analysis**

11 Following experimental treatments Caco2 or Hela
12 cells grown on 35mm culture dishes were fixed in 4%
13 paraformaldehyde and permeabilised in 0.2% Triton
14 X-100/PBS. Cells were incubated for 1 h at room
15 temperature with primary antibodies (1 μ g/ml)
16 diluted in PBS containing 1% serum from the species
17 in which the secondary antibody was raised.
18 Secondary antibodies (1 μ /ml) were either Alexa
19 Fluor 594 donkey anti-goat or Alexa Fluor 488 goat
20 anti-rabbit IgG (Molecular Probes) as appropriate.
21 Labelled cells were mounted with Vectorshield
22 (Vector) and examined on a Zeiss Axioskop 50
23 widefield fluorescence microscope or on a Bio-Rad
24 Radiance2100 laser scanning microscope.
25 Representative digital images were imported into
26 Adobe Photoshop 6.0 for final arrangement.

28 **Construction of Fluorescently-tagged PPAR α**

29 The coding sequence of PPAR γ , and a dominant
30 negative mutant of PPAR γ (Gurnell et al.2000), were
31 modified during PCR amplifications to add a *Xho* I
32 recognition sequence at the 5' end and a *Sac* II

1 recognition sequence at the 5' end and a *Sac* II
2 sequence at the 3' end of the products. The
3 amplifications were performed using *Pfu* DNA
4 polymerase. Products were restricted and cloned
5 into both pECFP-C1 and pEYFP-C1 (Promega).
6 Successful construction was verified by DNA
7 sequencing. Human p65 cloned into pEYFP-C1 was as
8 previously reported (Schmid et al.2000).

9 Caco2 or Hela cells were seeded on 35mm culture
10 dishes grown to 90% confluence and transfected
11 using standard lipofectamine-mediated methods
12 (Invitrogen). After 48 h, bacterial incubations
13 were undertaken as previously described; cells were
14 fixed for 30 min at room temperature in 4%
15 paraformaldehyde in 0.1M sodium phosphate buffer pH
16 7.4, washed in PBS and examined on a Zeiss Axioskop
17 50 widefield fluorescence microscope equipped with
18 custom filters (Omega Optical) for CFP and YFP.
19 Representative images were recorded with a Zeiss
20 AxioCam digital camera, processed with Zeiss
21 AxioVision 3.0 software and imported into Adobe
22 Photoshop 6.0 for final arrangement. In some
23 experiments transfected cells were subjected to the
24 immunofluorescence analyses described above.

25

26 **Example 6 : In vivo rat study**

27

28 The following example was conducted to establish if
29 *B. thetaiotaomicron* could alter the gut function of
30 specific pathogen-free rats and increase their
31 resistance to a pathogen challenge. *Bacteroides*
32 generally appear in the small intestine at the

suckling-weaning transition (Chang et al, 1994; Hooper et al, 2001). The bacterium was therefore given daily to specific pathogen free Hooded Lister rats from weaning (19 d) through to maturity (34-40 days) to ensure that its presence in high numbers during this period of major gut and immune development. At 34 days of age, the rats were orally challenged with *Salmonella enterica* var. Enteritidis S1400.

METHODS

Culture of bacteria:

B. thetaiotaomicron, from Deutsche Sammlung von Microorganismen und GmbH (Braunschweig, Germany) was maintained frozen in Wilkins-Chalgren anaerobic agar. It was subcultured into Wilkins-Chalgren anaerobic broth or M10 medium [10 ml in Hungate tubes (Bryant, 1972)] and grown at 37°C for 48 hours. A sample (0.5 ml) was transferred to fresh media [10 ml in Hungate tubes] and grown at 37°C for 24 hours. This culture contained around 10^8 - 10^9 CFU ml⁻¹.

Ten ml of *B. thetaiotaomicron* culture was centrifuged (2000g, 12 min), the pellet was washed with 0.05M phosphate buffered saline pH 7.2 and then resuspended in commercial jelly [10 ml, 37°C, prepared under anaerobic conditions]. The jelly was poured into a sterile petri dish under CO₂ and allowed to set at 4°C. Approximately 1 hour later, the jelly was cut into weighed amounts (0.5g, ~ 10^8

1 CFU *B. thetaiotaomicron*) and fed to the rats. The
2 bacterium remained viable for at least 6 hours
3 under these conditions. However, usually all jelly
4 was eaten within a few minutes of being placed in
5 the cage.

6
7 *Salmonella enterica* serovar Enteritidis S 1400 was
8 originally isolated from poultry infection and has
9 been characterised (Allen-Vercoe and Woodward,
10 1999). Stocks, maintained on Dorset egg slopes at
11 4°C, were sub-cultured on to Luria-Bertani agar
12 plates and grown at 37°C overnight. Five-ten
13 colonies from the plate were inoculated into 10 mls
14 of Luria-Bertani broth and incubated with agitation
15 at 37°C overnight to give approximately 1×10^9 CFU
16 ml⁻¹.

17
18 **Animal studies:** The Rowett Research Institute is
19 licensed under the UK Animals (Scientific
20 Procedures) Act 1986. The ethical review committee
21 and the animal welfare unit of the institute and
22 the appropriate governmental inspectorate monitor
23 and review all animal studies. The management and
24 experimental procedures undertaken were approved by
25 the ethical committee and done in strict accordance
26 with the requirements of the Act by staff licensed
27 to carry out such procedures.

28
29 Twenty-four male specific pathogen-free Hooded
30 Lister rats (40 g), bred in the small animal unit
31 of the Rowett Research Institute, were weaned at 19
32 days and immediately transferred to a Class II

1 facility. They were housed individually in
2 metabolism cages (Techniplast, Kettering, UK)
3 within flexi-film isolators (Moredun, Animal
4 Health, Penicuik, UK) for the duration of the study
5 (21 days). Nesting tunnels were provided in each
6 metabolism cage and cages were arranged within the
7 isolators to allow sight of but not contact with
8 other animals. Sterile distilled water was
9 available at all times. Rats were weighed daily
10 and faeces was collected throughout the study.
11 Experiments were undertaken at least 3 times with
12 similar results. Data are the means $\pm$ SD (n=3).
13 The rats were initially given free access to a high
14 quality semi-synthetic (100 g protein kg⁻¹)
15 lactalbumin-based diet (Grant et al, 2000). When
16 they reached approximately 80 g (around 30 days
17 old) their food intake was gradually reduced over a
18 3-4 day period to 7g rat⁻¹ day⁻¹, given as two feeds
19 over the day, and then maintained at this level for
20 the remainder of the study. This was the average
21 daily free intake observed for rats of the same age
22 after oral infection with *Salmonella enterica* var.
23 Enteritidis and around 70% of the free intake of
24 this diet by non-infected animals. The rats were
25 housed and managed in this manner from weaning to
26 reduce environmental exposure to bacteria and
27 cross-contamination post-weaning (Grant, 1996).
28
29 Twelve of the rats were given *B. thetaiotaomicron*
30 (approximately 10⁸ CFU) once daily from weaning
31 (19d) through to completion of the study. At 34
32 days of age, six rats pre-treated with *B.*

1 *thetaitaomicron* and six controls were given, by
2 gavage, a single dose of 0.8 ml of *Salmonella*
3 serovar Enteritidis S1400 culture (approximately
4 10⁹ CFU). The remaining rats were given the
5 equivalent amount of culture medium. All rats were
6 returned to their cages and fed lactalbumin-based
7 diet (7g rat⁻¹ day⁻¹) for a further 6 days. On the
8 final day, the rats were fed 2 g of diet and were
9 killed by halothane (Rhone Merieux, Essex, UK)
10 overdose and exsanguination exactly two hours
11 later. The abdomen was opened and tissues were
12 aseptically removed.

13 The stomach and the small intestine were flushed
14 with phosphate buffered saline (PBS, pH 7.2) to
15 remove contents and non-adherent bacteria. Ten cm
16 of jejunum (5-15 cm from the pylorus) and 10 cm of
17 ileum (5-15 cm from the ileo-caecal junction) were
18 removed. These and the stomach tissue, caecum plus
19 contents, colon plus contents, mesenteric lymph
20 node, a representative proportion of the liver and
21 spleen (200-400mg) and one kidney were processed
22 for viable counts.

23
24 Further pieces of jejunum and ileum (15-25 cm from
25 the pylorus and 15-25 cm from the ileo-caecal
26 junction respectively) were collected and frozen in
27 liquid nitrogen for biochemical analysis, as was
28 the remaining small intestine tissue (~ 40 cm),
29 liver, spleen, kidney, thymus, lungs, heart and
30 gastrocnemius hind-limb muscles. These were
31 weighed, freeze-dried and reweighed.

32

1 Viable bacteria in rat tissues

2

3 Tissue samples were weighed and then homogenised in
4 Maximum Recovery Diluent (MRD, Fisher Scientific,
5 UK) using a Janke-Kunkel Ultra-Turrax T25 tissue
6 homogeniser at 20,000 rev min⁻¹ for 30 seconds. Up
7 to six sequential dilutions (1:10 v/v) of the
8 primary homogenate were made in MRD. Samples of
9 each dilution were plated onto the surface of
10 well-dried XLD agar (Fisher Scientific, UK) and
11 MacConkey agar No. 3 (Fisher Scientific, UK) plates
12 and incubated overnight at 37°C. Viable counts
13 were estimated by the method of Miles and Misra
14 (1938) or by a spread plate method (Collins and
15 Lyme, 1989).

16

17 PCR

18

19 DNA was extracted and purified using a QIAamp DNA
20 Stool Mini Kit (Qiagen Ltd, Crawley, UK). The PCR
21 was based on the method of Teng *et al.* (2000). The
22 primer pair: 5'-TGGAGTTTTACTTTGAATGGAC-3' (BTH-F)
23 and 5'-CTGCCCTTTTACAATGGG-3' (BTH-R), identified by
24 Teng *et al.* (2000), were purchased from
25 Sigma-Genosys Ltd (Cambridge, UK). The reaction
26 mixture (50 µl), based on reagents from a Taq PCR
27 Core Kit (Qiagen Ltd, Crawley, UK), contained 50
28 pmol of both primers, 10 nmol dNTP mixture, 5 µl of
29 10X Qiagen PCR buffer, 10 µl 5X Q-Solution, 10 µl
30 sample and 1.25U Taq DNA Polymerase. A hot-start
31 PCR program was used. Reaction mix without Taq DNA
32 Polymerase was heated at 94°C for 15 seconds and

58

1 through 35 cycles of 94°C for 10 seconds, 55°C for
2 30 seconds and 74°C for 1 minute, followed by 1
3 cycle of 74°C for 2 minutes and 45°C for 2 seconds.
4 Amplicons were separated on an agarose gel (10g/l)
5 containing ethidium bromide (1 µg/ml gel). A
6 single amplicon (721 bp) was obtained with DNA from
7 faeces and *B. thetaiotaomicron*. The sequences of
8 these amplicons appeared identical to each other
9 and to the published sequence (Teng et al, 2000).

10

11 MPO

12

13 Tissue samples were homogenised (1:80 w/v) in
14 ice-cold 5mM potassium phosphate buffer pH 6.0
15 using a Janke-Kunkel Ultra-Turrax T25 tissue
16 homogeniser at 20,000 rev min⁻¹ for 30 seconds and
17 centrifuged (3000g x 30 min, 4°C). The pellet
18 (1:20 w/v) was sonicated (3 x 5 sec) in ice-cold
19 0.5M potassium phosphate pH 6.0 containing
20 hexadecyltrimethyl-ammonium bromide (HETAB, 5 g/l)
21 and ethylenediaminetetraacetic acid (EDTA,
22 3.72g/l), left on ice for 30 min and centrifuged
23 (3000g x 30 min, 4°C) [Stucchi et al, 2000]. The
24 supernatant was frozen until assayed.

25 Myeloperoxidase (MPO) activity was determined by
26 monitoring H₂O₂ dependent oxidation of
27 3,3',5,5'-tetramethylbenzidine (TMB, Dynex
28 Technologies, Ashford, UK) in 50mM potassium
29 phosphate buffer pH 6.0 (Zimmerman and Granger,
30 1990). Absorbance at 450 nm was measured after
31 termination of the reaction with 0.18M H₂SO₄.

32 Human myeloperoxidase (Calbiochem, UK) was used as

59

1 Human myeloperoxidase (Calbiochem, UK) was used as
2 a standard and values were expressed as MPO
3 equivalents.

4

5 Immunoreactive MPO in intestinal contents or faeces
6 was determined by a competitive ELISA method.

7 Microtiter plates (Immulon 4, Dynex Technologies,
8 Ashford, UK) were coated [10ng/well] with human

9 myeloperoxidase in 10mM phosphate buffered saline
10 pH 7.4 [PBS] overnight at 4°C. After washing [PBS

11 containing Tween-20 (1ml/l), plates were blocked

12 with PBS containing bovine serum albumin (BSA,

13 10g/l) for 1 hour at room temperature. Plates were

14 washed and samples or standards added [50µl/well]

15 and serially diluted in PBS containing BSA (1g/l),

16 Tween-20 (1ml/l) and leupeptin (1mg/l). Rabbit

17 anti-human myeloperoxidase antibody [Calbiochem,

18 UK, 50µl/well of 1:4000 dilution] was also added.

19 The plates were incubated for 1 hour, washed and

20 then reacted for 1 hour with biotinylated

21 anti-rabbit IgG followed by Extravidin/peroxidase

22 (EXTRA-3 kit, Sigma-Aldrich, Poole, UK). After

23 washing, TMB reagent (7µl/well) was added and

24 incubated in the dark for up to 2 hours. The

25 reaction was stopped by addition (50µl/well) of

26 0.18M H₂SO₄ and the absorbance read at 450nm.

27 Values were expressed as immunoreactive MPO

28 equivalents.

29

30

31

32

1 IgG

2

3 IgG was determined by competitive ELISA.

4 Microtiter plates were coated [1µg/well] with rat

5 IgG (Sigma-Aldrich, Poole, UK) in PBS overnight at

6 4°C. After blocking and washing of the plates,

7 samples or rat IgG standards [50µl/well] were added

8 and serially diluted in PBS containing BSA (1g/l)

9 and Tween-20 (1ml/l). Biotinylated anti-rat IgG

10 antibodies (Sigma-Aldrich, Poole, UK; 50µl/well of

11 1:1000 dilution) were also added and the plates

12 incubated for 1 hour. Plates were then reacted

13 with Extravidin/peroxidase and TMB reagent as per

14 MPO immunoassay. Values were expressed as IgG

15 equivalents.

16

17 IgA

18

19 IgA was determined by capture ELISA. Microtiter

20 plates were coated with goat anti-rat IgA

21 (Sigma-Aldrich, Poole, UK; 100µl/well of a 1:100

22 dilution in PBS) overnight at 4°C. After blocking

23 and washing of the plates, samples or standards

24 [100µl/well] were added, diluted in PBS containing

25 BSA (1g/l), Tween-20 (1ml/l) and leupeptin (1 mg/l)

26 and incubated for 1 hour. After washing, plates

27 were incubated with mouse anti-rat IgA antibodies

28 (Sigma-Aldrich, Poole, UK; 1:500 dilution) for 1

29 hour, rewashed and incubated with biotinylated

30 anti-mouse IgG antibodies (1:1000 dilution) for a

31 further 1 hour. Plates were then processed as per

61

1 MPO immunoassay. Values were expressed as IgA
2 equivalents.

3

4 **LPS-specific IgG and IgA**

5

6 Microtiter plates were coated with
7 lipopolysaccharide (LPS; 10µg/well) from *Salmonella*
8 *enteritidis* (Sigma Aldrich, Poole, Dorset). After
9 blocking and washing, samples or standards
10 [100µl/well] were added. Plates were washed
11 one-hour later, biotinylated anti-rat IgG or
12 anti-rat IgA was added and the plates were then
13 processed as above. One unit of LPS-specific
14 antibodies was defined as that contained in the
15 volume (µl) giving an absorbance of at least 0.2 in
16 the ELISA. The positive control was sera collected
17 from rats 22 days after infection with SE: LPS-IgA,
18 3.2×10^4 units/ml; LPS-IgG, 1.7×10^5 units/ml.

19

20 **Elastase**

21

22 Freeze dried faeces or intestine contents were
23 extracted (1:10 w/v) in ice-cold 0.5M potassium
24 phosphate pH 6.0 containing HETAB, EDTA and NaN_3
25 (1g/l), left on ice for 30 min, centrifuged (3000g,
26 30 min, 4°C) and the supernatants were frozen.

27

28 Sample or leukocyte-elastase (Sigma-Aldrich, Poole,
29 UK) was serially diluted in 0.2M Tris HCl pH 8.0
30 containing 1 M NaCl and leupeptin (1 mg/l) on a
31 microtiter plate. Substrate
32 (N-succinyl-ala-ala-val-p-nitroanilide, 0.2g/l,

1 Sigma-Aldrich, Poole, UK) was added and the
2 absorbance at 405 nm monitored immediately and at
3 intervals up to 20 hours during incubation at 37°C.
4 Values were expressed as leukocyte-elastase
5 equivalents.

6

7 **DNA, RNA, protein**

8

9 DNA, RNA, protein were determined as before by the
10 diphenylamine, orcinol and modified Lowry methods
11 (Grant *et al*, 2000). Salmon testes DNA, yeast RNA
12 and bovine serum albumin were used as standards.

13

14

15 **Statistical analysis**

16

17 Data were assessed by one way analysis of variance
18 (ANOVA) in combination with the Tukey multiple
19 comparison test using the Instat Statistical
20 Package (GraphPad Software Inc., San Diego, USA).

21

22 **RESULTS**

23

24 *Bacteroides thetaiotaomicron* (BT) was detected in
25 faeces samples collected from BT-treated rats. BT
26 was also found in faeces of control rats. However,
27 the levels (equivalent of approximately 10^4 CFU/g
28 faeces) were below those found in faeces from rats
29 treated daily with BT (equivalent of approximately
30 10^6 CFU/g faeces).

31

1 Body weight gains, major organ weights and small
2 intestine and liver compositions for BT rats were
3 similar to those for controls (Tables 1-4). The
4 general distribution of lactose fermentors and
5 non-lactose fermentors in the gastrointestinal
6 tract seemed to be unaffected and, as with
7 controls, no lactose fermentors or non-lactose
8 fermentors bacteria were detected in mesenteric
9 lymph node, liver, spleen or kidney samples. In
10 addition, the levels of non-specific IgA, elastase,
11 and immunoreactive MPO in intestinal contents and
12 faeces, MPO in intestine tissue and non-specific
13 IgG and IgA in serum were comparable to those
14 samples collected from control animals.

Table 1. Bacterial numbers (Log₁₀ CFU/g) associated with tissues taken from control rats or rats orally treated with *Bacteroides thetaiotaomicron* [BT], *Salmonella enterica* var. enteritidis [SE] or *S. enteritidis* plus *B. thetaiotaomicron* [SE+BT].

	Stomach	Jejunum	Ileum	Caecum	Colon	MLN	Liver	Spleen	Kidneys
Salmonella¹									
SE	4.90±0.27	5.04±0.54	5.87±0.90	6.84±0.78	6.45±1.03	5.43±0.21	3.08±0.46	3.77±0.25	ND
SE+BT	5.06±0.33	5.15±0.54	6.23±0.12	8.06±0.81*	7.04±0.87	5.24±0.44	2.34±0.36*	3.04±0.32*	ND
Lactose fermentors									
Control	4.97±1.18	3.27±0.66	4.90±0.97	8.08±0.63	7.68±0.58	ND ^a	ND ^a	ND ^a	ND
BT	3.90±0.84	2.78±0.19	4.15±1.13	7.14±1.40	7.15±1.14	ND ^a	ND ^a	ND ^a	ND
SE	3.88±0.69	3.13±0.55	4.01±1.00	7.26±0.64	6.71±0.35	3.49±0.91 ^b	1.85±0.85 ^b	1.96±1.07 ^b	ND
SE+BT	4.16±0.66	3.11±0.63	4.39±1.34	8.32±0.68	7.42±0.95	3.73±0.90 ^b	1.41±0.20 ^b	1.83±0.56 ^b	ND
Non-lactose fermentors									
Control	3.92±1.12 ^a	3.15±0.57 ^a	3.88±0.98 ^a	6.73±1.06	6.54±0.74	ND ^a	ND ^a	ND ^a	ND
BT	3.80±0.20 ^a	2.82±0.29 ^a	4.46±0.85 ^a	6.69±0.95	6.63±0.66	ND ^a	ND ^a	ND ^a	ND
SE	5.02±0.42 ^b	4.73±0.59 ^b	6.21±0.36 ^b	7.10±0.56	6.48±0.66	5.22±0.30 ^b	3.16±0.58 ^b	3.81±0.22 ^b	ND
SE+BT	5.12±0.23 ^b	4.98±0.72 ^b	6.24±0.85 ^b	8.01±0.62*	7.26±0.90	5.41±0.29 ^b	2.47±0.23 ^{c*}	3.27±0.23 ^{c*}	ND

Means ±SD, n=6, samples collected 6d post-infection with *S. enteritidis*. *SE+BT differs significantly from SE (p≤0.05). For lactose fermentors and non-lactose fermentors values in a column with distinct superscripts differ significantly (p≤0.05). ND, not detected. The limit of detection was 2.7 except for liver spleen and kidneys for which it was 1.3. ¹No salmonella, lactose fermentors or non-lactose fermentors were detected in MLN, liver, spleen or kidneys of control or BT rats.

1 *S. enteritidis* [SE] was found throughout the
2 gastrointestinal tract six days after oral challenge
3 (Table 1). In addition, they were detectable in the
4 mesenteric lymph node, liver and spleen. Total
5 non-lactose fermentors had a similar tissue
6 distribution in these rats (Table 1). The numbers
7 of lactose fermentors in the gut did not appear to
8 be affected by salmonella infection. However,
9 significant numbers of lactose fermentors did appear
10 in the mesenteric lymph nodes, liver and spleen of
11 SE-infected rats (Table 1).

12

13 The numbers of viable salmonella detected in the
14 liver and spleen after challenge with salmonella
15 were greatly reduced if rats had also been treated
16 with *B. thetaiotaomicron* [SE+BT] (Table 1). Total
17 non-lactose fermentors in these tissues were also
18 lowered but lactose fermentor numbers were
19 unchanged. The levels of salmonella, total
20 non-lactose fermentors and lactose fermentors
21 throughout the gastrointestinal tract and in the
22 mesenteric lymph node were similar for SE+BT and SE
23 (Table 1). In addition, faecal excretion of
24 salmonella over the 6 day experiment occurred at
25 comparable rates in pathogen-infected rats (SE+BT,
26 $7.0 \pm 0.8 \text{ Log}_{10}\text{CFU/g d}^{-1}$, SE, $6.2 \pm 0.6 \text{ Log}_{10}\text{CFU/g d}^{-1}$).

27

28

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Table 2. Weights (mg wet weight/100g fresh body weight) of tissues taken from control rats or rats orally treated with *Bacteroides thetaiotaomicron* [BT], *Salmonella enterica* var. *enteritidis* [SE] or *S. enteritidis* plus *B. thetaiotaomicron* [SE+BT].

	Control	BT	SE	SE+BT
Intake (g/d)	7.0	7.0	7.0	7.0
Initial weight (g)	83±2	83±3	86±3	88±2
Final weight (g)	93±3	94±2	98±4	100±3
Stomach	887±64	831±82	965±74	846±149
Jejunum (20cm)	705±133 ^a	759±78 ^a	1035±224 ^b	966±121 ^b
Ileum (20cm)	540±46 ^a	576±66 ^a	1139±251 ^b	999±168 ^b
Small intestine	2979±255 ^a	3074±282 ^a	5021±420 ^b	4425±415 ^{c*}
MLN	105±48 ^a	100±40 ^a	484±153 ^b	325±73 ^b
Spleen	237±38 ^a	228±31 ^a	315±26 ^b	325±50 ^b
Liver	3773±72 ^a	3778±239 ^a	4409±266 ^b	4353±166 ^b
Kidneys	886±35	906±28	875±29	926±62
Thymus	337±32	324±39	355±63	339±29
Lungs	744±43	698±97	767±65	756±82
Heart	482±73	442±18	437±27	450±44
Gastrocnemius muscles	814±24 ^a	834±22 ^a	753±35 ^b	838±47 ^{a*}

Means ±SD, n=6, samples collected 6d post-infection with *S. enteritidis*. MLN, mesenteric lymph nodes. Values in a row with distinct superscripts differ significantly ($p \leq 0.05$).

*SE+BT differs significantly from SE ($p \leq 0.05$)

Table 3 Weight and composition (mg) of tissues taken from control rats or rats orally treated with *Bacteroides thetaiotaomicron* [BT], *Salmonella enterica* var. enteritidis [SE] or *S. enteritidis* plus *B. thetaiotaomicron* [SE+BT].

	Control	BT	SE	SE+BT
Jejunum (20 cm)				
Wet weight	650±99 ^a	712±68 ^a	1006±198 ^b	956±89 ^b
Water	526±76 ^a	588±66 ^a	824±178 ^b	748±86 ^b
Dry weight	124±34 ^a	136±24 ^a	182±24 ^b	208±32 ^b
MPO (µg)	20.7±5.8	29.3±5.2	24.7±9.4	34.4±10.9
Ileum (20 cm)				
Wet weight	500±47 ^a	540±51 ^a	1104±196 ^b	991±159 ^b
Water	425±57 ^a	458±83 ^a	903±74 ^b	785±70 ^{c*}
Dry weight	74±26 ^a	82±42 ^a	201±26 ^b	206±45 ^b
MPO (µg)	24.7±4.2 ^a	33.1±6.2 ^a	94.4±9.5 ^b	48.7±14.9 ^{c*}
Total small intestine				
Wet weight	2756±139 ^a	2883±89 ^a	4885±309 ^b	4391±389 ^b
Water	2098±121 ^a	2150±129 ^a	3884±212 ^b	3273±350 ^{c*}
Dry weight	658±29 ^a	734±50 ^a	1002±79 ^b	1117±76 ^b
DNA	3.4±0.8 ^a	3.9±0.4 ^a	5.5±0.5 ^b	6.5±0.9 ^b
RNA	17.8±2.1 ^a	23.6±3.7 ^a	30.9±1.2 ^b	35.7±5.8 ^b
Protein	268±29 ^a	293±59 ^a	480±56 ^b	530±84 ^b
Liver				
Wet weight	3520±107 ^a	3546±269 ^a	4295±268 ^b	4344±284 ^b
Dry weight	1022±59 ^a	992±78 ^a	1117±94 ^a	1078±89 ^a
Spleen				
Wet weight	223±33 ^a	207±6 ^a	311±35 ^b	299±39 ^b
Dry weight	68±23 ^a	59±11 ^a	63±14 ^a	74±17 ^a
Mesenteric lymph node				
Wet weight	99.0±47.84 ^a	93.5±37.1 ^a		476.2±165.5 ^b
	323.4±68.9 ^b			
Water	63.5±21.6 ^a	66.5±26.0 ^a		304.1±115.2 ^b
	208.5±40.0 ^b			
Dry weight	35.5±29.3 ^a	27.0±16.6 ^a		72.1±72.7 ^b
	114.9±34.0 ^b			

Means ±SD, n=6, samples collected 6d post-infection with *S. enteritidis*. Values in a row with distinct superscripts differ significantly (p≤0.05). *differs significantly from SE (p≤0.05). ND, not detected.

68

1 The weight of the small intestine was increased as a
2 result of salmonella infection (Tables 2,3). This was
3 most marked in the ileal tissue and appeared to be due
4 to accumulation of water and increases in the protein,
5 RNA and DNA content of the tissue (Table 3). Treatment
6 with BT slightly modified the response to infection.
7 Thus, the water content of small intestine from SE+BT
8 [748±22 mg/g] was considerably lower than that in
9 tissue from SE rats [799±17 mg/g]. Indeed, it was
10 similar to the levels in tissue from non-infected rats
11 [BT, 745±24 mg/g controls, 760±10 mg/g]. In contrast,
12 there was no significant difference between SE and
13 SE+BT rats in their small intestine dry weights or DNA,
14 RNA and protein contents.

Table 4. Composition of serum, intestine contents or faeces collected from control rats or rats orally treated with *Bacteroides thetaiotaomicron* [BT], *Salmonella enterica* var. enteritidis [SE] or *S. enteritidis* plus *B. thetaiotaomicron* [SE+BT].

	Control	BT	SE	SE+BT
Serum				
IgA (mg/ml)	0.19±0.02 ^a	0.18±0.02 ^a	0.35±0.06 ^b	0.28±0.04 ^b
IgG (mg/ml)	0.91±0.13	0.83±0.17	0.97±0.10	0.89±0.17
LPS-IgA (units/ml)	ND	ND	ND-800	ND-800
LPS-IgG (units/ml)	ND	ND	ND	ND
Intestinal contents				
Protein (mg)	7.01±1.75 ^a	5.32±1.50 ^a	11.61±3.15 ^b	12.89±3.03 ^b
IgA (mg)	0.38±0.18 ^a	0.23±0.14 ^a	1.19±0.41 ^b	1.42±0.53 ^b
LPS-IgA (units)	ND ^a	ND ^a	162±58 ^b	221±35 ^b
Elastase (µg)	1.43±0.29 ^a	210±0.38 ^a	4.20±0.73 ^b	3.10±0.59 ^c
MPO (µg)	1.86±1.31 ^a	270±1.29 ^a	6.56±2.20 ^b	3.91±1.40 ^a
Faeces				
Dry matter (mg/d)	369±37 ^a	359±37 ^a	532±44 ^b	553±46 ^b
Water (mg/d)	183±46 ^a	213±41 ^a	416±119 ^b	412±84 ^b
Protein (mg/d)	85±15 ^a	97±12 ^a	162±27 ^b	179±17 ^b
IgA (µg/d)	505±202	475±168	1412±773	1022±638
Elastase (µg /d)	6.4±0.9 ^a	5.7±1.0 ^a	12.5±1.5 ^b	8.9±1.1 ^c
MPO (µg /d)	2.5±1.2 ^a	2.6±1.0 ^a	5.5±1.6 ^b	2.9±1.0 ^a

Means ±SD, n=6, samples during 6d post-infection with *S. enteritidis*. Values in a row with distinct superscripts differ significantly ($p \leq 0.05$) ND, not detected.

1 Myeloperoxidase (MPO) activity was significantly
2 elevated in ileal tissue collected from SE rats
3 (Table 4). In addition, immunoreactive-MPO and
4 elastase activity in intestinal contents and faeces
5 were also greatly increased (Table 4). In contrast,
6 the levels of these enzymes in equivalent samples
7 from SE+BT rats were similar to or only slightly
8 higher than control samples.

9
10 Immunoreactive non-specific IgA in intestine
11 contents, faeces and serum was increased as a result
12 of salmonella infection (Table 4). There were
13 however no differences between SE+BT and SE in these
14 IgA responses. Small amounts of LPS-specific IgA
15 were also detected in the blood and intestine
16 contents. Again, there were no significant
17 differences between SE+BT and SE, although there was
18 a tendency for LPS-specific IgA levels in the
19 intestine to be higher for SE+BT rats.

20
21 Gastrocnemius hind-limb muscle weights were
22 significantly lower in SE rats than in controls
23 (Table 2). This was not evident in SE+BT rats and
24 appeared to be a result of a reduced muscle
25 accretion. SE rats deposited approximately 6.6 ± 5.7
26 mg wet weight [1.4 ± 1.2 mg dry weight] of
27 gastrocnemius muscle per day whereas with SE+BT the
28 accretion rate was 17.1 ± 4.1 mg wet weight [4.2 ± 1.0
29 mg dry weight] per day. Deposition of this muscle
30 in controls was 15.5 ± 2.9 mg wet weight [3.8 ± 0.7 mg
31 dry weight] per day and 17.0 ± 4.2 mg wet weight
32 [4.2 ± 1.0 mg dry weight] per day in BT rats. This

1 suggests that the SE rats deposited an average of
2 0.3g (wet weight) total skeletal muscle daily whilst
3 with SE+BT, BT or control rats the levels were
4 around 0.7-0.8 g daily (Figure 6).
5

6 Liver, spleen and mesenteric lymph node wet weights
7 were significantly elevated in salmonella-infected
8 rates (Table 3). This was due primarily to
9 accumulation of water in the liver and spleen and an
10 increase in both water and dry matter in the
11 mesenteric lymph node (Table 4). Peroxidase
12 activity in the mesenteric lymph node of infected
13 rats was also elevated [SE, 31.0 ± 25.0 ; SE+BT,
14 37.7 ± 23.8 ; BT, 0.9 ± 0.9 ; control, $0.4 \pm 0.3 \mu\text{g}$]. There
15 were however no significant differences between the
16 SE and SE+BT groups in these tissue parameters.
17

18 **DISCUSSION**

19

20 **Salmonellosis:** *Salmonella enterica* var. Enteritidis
21 S1400 colonised the whole gastrointestinal tract of
22 rats and translocated to the mesenteric lymph node,
23 liver and spleen. In addition, the wet and dry
24 weight of the small intestine was greatly increased
25 as a result of infection. This was most marked in
26 the ileum and was linked to accumulation of water
27 and higher levels of protein, RNA and DNA in the
28 tissue. The basic characteristics of the infection
29 were therefore similar to those seen for other
30 Enteritidis or *S. enterica* var. Typhimurium strains
31 in the rat models (Naughton et al, 1996; 2001; Ewen

1 *et al*, 1997; Bovee-Oudenhoven *et al*, 1999; Islam *et*
2 *al*, 2000; Havelaar *et al*, 2001).

3 Enteritidis and Typhimurium cause a self-limiting
4 infection in rats, that is localised primarily to
5 the gastrointestinal tract with invasion occurring
6 via the ileum and only limited systemic spread being
7 evidence (Naughton *et al*, 1996; Bovee-Oudenhoven *et*
8 *al*, 1997; 1999; Islam *et al*, 2000). Furthermore,
9 severe bacteremia and death is rare, unless the
10 health status or gut integrity of the rats has been
11 compromised by other factors prior to infection.
12 This contrasts starkly with salmonellosis in some
13 mouse models in which these strains elicit a severe
14 typhoid-like illness with a high incidence of
15 mortality (Lu *et al*, 1999; Kingsley and Baumler,
16 2000; Schechter and Lee, 2000). Thus, salmonellosis
17 in the rat has strong similarities to the
18 self-limiting gastroenteritis-type infections common
19 in humans and domesticated animals infected by
20 Enteritidis or Typhimurium.

21
22 In the present study, salmonella infection was found
23 to provoke a strong inflammatory response in the
24 distal but not the proximal small intestine. Thus,
25 the activity of myeloperoxidase (MPO, a neutrophil
26 marker) in ileal tissue was significantly elevated.
27 In addition, levels of immunoreactive-MPO and
28 leukocyte elastase activity in intestinal contents
29 and faeces were increased. This is compatible with
30 infiltration of polymorphonuclear leukocytes and
31 other inflammatory cells into ileal tissue and the
32 exvasion of inflammatory cells into the intestinal

1 lumen, as previously observed in animal models of
2 salmonellosis (Naughton *et al*, 1995; 1996;
3 Vassiloyanakopoulos *et al*, 1998; Darwin and Miller,
4 1999; Henderson *et al*, 1999). In addition, it is
5 consistent with the ileum being the primary site of
6 colonisation and invasion by salmonella (Carter and
7 Collins, 1974; Naughton *et al*, 1996).

8
9 Non-specific IgA in serum, intestine contents and
10 faeces was significantly increased as a result of
11 salmonella infection. Furthermore, there were
12 indications of development of an LPS-specific IgA
13 response by 6 days post-infection. In addition,
14 faecal dry matter, water and protein outputs were
15 elevated, as were the levels of protein in the
16 intestinal contents. Liver and spleen weights were
17 also increased as a result of the infection.

18
19 Salmonellosis was also found to impair skeletal
20 muscle metabolism in rats. The infected animals
21 continued to deposit skeletal muscle but the daily
22 accretion rates were about 40-50% of those observed
23 in controls, despite both sets of rats having the
24 same dry matter (7g/rat d⁻¹) and protein (0.7g/rat
25 d⁻¹) intake. This may have been due to diversion and
26 utilisation of nutrients to support defensive
27 responses against infection (Klasing and Calvert,
28 1999) and/or the actions of endotoxin or other
29 bacterial factors on muscle protein synthesis
30 (Friman *et al*, 1984; Lang *et al*, 2000).

31

1 ***B. thetaiotaomicron***: In the present study, rats were
2 exposed to high levels of exogenous *B.*
3 *thetaiotaomicron* throughout a period of rapid growth
4 and maturation of the gut and development of the
5 immune system. Some *Bacteroides* strains are
6 opportunistic pathogens and would be likely to have
7 deleterious effects during this developmental
8 period. However, as in studies with mature
9 ex-germ-free animals monocontaminated with *B.*
10 *thetaiotaomicron* (Hooper et al, 1999; 2001; Noack et
11 al, 2000), there were no indications that *B.*
12 *thetaiotaomicron* adversely affected gut or systemic
13 metabolism of the specific pathogen-free rats.
14 Indeed, all the parameters monitored in rats treated
15 with *B. thetaiotaomicron* alone, including
16 inflammatory markers and immunoglobulin levels, were
17 similar to those obtained for controls.
18
19 ***B. thetaiotaomicron* and salmonellosis:** *B.*
20 *thetaiotaomicron* modified the nature of the
21 infection caused by Enteritidis S 1400 in rats and
22 reduced its overall severity. In particular,
23 inflammatory responses in the small intestine were
24 limited, the numbers of viable salmonella found in
25 the liver and spleen were greatly reduced and
26 skeletal muscle accretion rates were restored to
27 around control levels. *B. thetaiotaomicron* thus had
28 effects both locally in the gut and at remote
29 systemic tissues.
30
31 Some aspects of salmonellosis were however
32 unaffected by *B. thetaiotaomicron*. Enteritidis

1 numbers in the gastrointestinal tract and mesenteric
2 lymph nodes and faecal excretion of the pathogen
3 were unaltered. In addition, the enlargement of the
4 small intestine (increase in dry weight and DNA, RNA
5 and protein content) associated with Enteritidis
6 infection was also evident in rats given *B.*
7 *thetaitaomicron* and Enteritidis. This suggests
8 that *B. thetaitaomicron* does not interfere directly
9 with salmonella itself or block its general effects
10 on host metabolism. Instead, the bacterium may
11 selectively modulate host-responses against
12 infection, possibly targetting those that are
13 potentially detrimental to gut integrity.

14
15 A number of bacterial strains have recently been
16 shown to give partial protection against Enteritidis
17 or Typhimurium infection. Some outcompete the
18 pathogen for attachment sites or nutrients in the
19 gut or produce bactericidal compounds. As a result,
20 they reduce the numbers of salmonella found in the
21 intestine and reaching the mesenteric lymph nodes,
22 liver and spleen (Bernet-Camard *et al*, 1997; Hudault
23 *et al*, 1997; 2001; Hendriksson and Conway, 2001).
24 In contrast, many have little or no effect on the
25 numbers of salmonella in the gut. However, as with
26 *B. thetaitaomicron*, they still significantly
27 ameliorate the pathogenic infection (Silva *et al*,
28 1999; Filho-Lima *et al*, 2000; Schu *et al*, 2000;
29 Hendriksson and Conway, 2001; Maia *et al*, 2001).
30 Protection given by the latter strains is thus by
31 mechanisms other than competitive exclusion or
32 secretion of bactericidal compounds.

1 Salmonella breach the intestine epithelium by
2 interfering with cell metabolism, cell-cell
3 interactions and host-response mechanisms (Darwin
4 and Miller, 1999; Netea *et al*, 2000; Eaves-Pyles,
5 2001; Gewirtz *et al*, 2001; Lu and Walker, 2001; Ohl
6 and Miller, 2001). In particular, they trigger
7 rapid infiltration of polymorphonuclear leukocytes
8 into the tissue and provoke acute inflammation and
9 severe disruption of the gut (Madara, 1997; Ohl and
10 Miller, 2001). Neutrophil infiltration into the gut
11 is mediated by chemoattractant chemokines, such as
12 IL-8, that are secreted by epithelial cells in
13 response to pathogenic infection (McCormick *et al*,
14 1995; Madara, 1997; Darwin and Miller, 1999;
15 Fleckstein and Kopecko, 2001). Release of this
16 chemoattractant was high if epithelial cells were
17 cultured with salmonella *in vitro* (McCormick *et al*,
18 1995; Campbell *et al*, 2001). In contrast, its
19 output was greatly reduced if the epithelial cells
20 were cultured with salmonella and *B.*
21 *thetaiotaomicron* in combination (Campbell *et al*,
22 2001).

23

24 Myeloperoxidase levels in ileal tissue and intestine
25 contents of rats given *B. thetaiotaomicron* and
26 Enteritidis were much lower than in comparable
27 samples from animals dosed with salmonella alone.
28 Thus, the inflammatory responses normally triggered
29 in the intestine by salmonella, were apparently
30 attenuated if animals had also been treated with *B.*
31 *thetaiotaomicron*. The bacterium may, as found *in*
32 *vitro* (Campbell *et al*, 2001), block

1 salmonella-linked production of chemoattractant
2 chemokines by epithelial enterocytes and thus
3 prevent the recruitment of neutrophils into the
4 tissue. By modulating this host-response *in vivo*,
5 *B. thetaiotaomicron* may limit the degree of gut
6 inflammation and damage that occurs as a result of
7 infection and thereby preserve gut integrity. This
8 would also reduce the demands of the gut for
9 nutrients to support repair (Klasing and Calvert,
10 1999) and allow more to go to other tissues, such as
11 the skeletal muscle.

12

13 Salmonella drain to the mesenteric lymph nodes, once
14 they pass through the intestinal epithelium
15 (Kingsley and Baulmer, 2000). They may then be
16 cleared from there by macrophages or alternatively
17 break out from the mesenteric lymph nodes and spread
18 to the blood, liver and spleen (Kingsley and
19 Baulmer, 2000; Lu and Walker, 2001). Significant
20 numbers of Enteritidis were detected in the
21 mesenteric lymph nodes, liver and spleen of infected
22 rats. Treatment of animals with *B. thetaiotaomicron*
23 greatly reduced the levels of viable pathogen found
24 in the liver and spleen. However, it did not affect
25 the Enteritidis numbers in the mesenteric lymph
26 nodes. Therefore, *B. thetaiotaomicron* did not
27 appear to limit invasion and drainage of salmonella
28 to this site, despite its major effects on
29 intestinal metabolism. Since pathogen levels were
30 reduced in the systemic tissues, this may indicate
31 that the main protective effects of *B.*

1 *thetaiotaomicron* arise through changes in systemic
2 metabolism.

3

4 Commensal bacteria are potent immuno-modulators
5 (Herias et al, 1999; Talham et al, 1999; Scharek et
6 al, 2000; Isolauri et al, 2001; Lu and Walker,
7 2001). In particular, treatment of mice with
8 *Bifidobacterium lactis* appeared to enhance their
9 immune function and responsiveness against
10 salmonella (Schu et al, 2000). Phagocytotic
11 activity in blood and peritoneal cells, lymphocyte
12 mitogenic responsiveness and secretion of
13 Typhimurium-specific antibodies were elevated. *B.*
14 *thetaiotaomicron* may have similar effects on
15 systemic metabolism. The lower numbers of
16 salmonella in the liver and spleen of *B.*
17 *thetaiotaomicron*-treated rats might therefore be the
18 result of enhanced clearance of the pathogen from
19 the blood and internal organs.

20

21 Lactose fermentors (*E. coli*), albeit in low numbers,
22 were also found in the liver and spleen of
23 Enteritidis-infected rats. However, unlike
24 salmonella, the levels of these bacteria were not
25 affected by *B. thetaiotaomicron*. There may
26 therefore be some selectivity in the bacterial
27 clearance promoted by *B. thetaiotaomicron*.
28 Alternatively, because the *E. coli* are derived from
29 the normal flora of the rats, they may not be
30 readily recognised as potentially harmful, even
31 although they are present at inappropriate tissue
32 sites.

1 *B. thetaiotaomicron* may however reduce systemic
2 spread of salmonella by acting locally on the gut.
3 It is generally accepted that salmonella attach to
4 the gut epithelium, invade through the tissue, drain
5 to the mesenteric lymph nodes and may then spread to
6 the liver and spleen (Kingsley and Baulmer, 2000).
7 However, recent work suggests that systemic spread
8 of the pathogen can occur by an alternative route.
9 Pathogens sampled luminally by dendritic cells or in
10 the subepithelium by CD18-expressing phagocytes may
11 be transferred directly to the liver and spleen,
12 without drainage through the lymphatic system
13 (Varquez-Torres et al, 1999; Isberg and Barnes,
14 2000; Rescigno et al, 2001). As a result,
15 salmonella found in the liver and spleen are likely
16 to be derived from the two uptake routes.
17 Recruitment of these phagocytes will probably be
18 mediated by chemoattractant chemokines produced by
19 epithelial cells (Izadpanah et al, 2001; Kellennan
20 and McEvoy, 2001). Since *B. thetaiotaomicron*
21 appears to limit neutrophil recruitment and
22 inflammation in the gut by preventing secretion of
23 the necessary chemoattractant chemokines (Campbell
24 et al, 2001), it may also suppress the chemokines
25 required to recruit dendritic cells or
26 CD18-expressing phagocytes and as a result prevent
27 or limit uptake of salmonella by this system.

28

29 **Summary**

30

31 Orally administered *B. thetaiotaomicron* reduced the
32 severity of infection caused by Enteritidis in rats.

80

1 The numbers of viable salmonella in the liver and
2 spleen were greatly reduced, skeletal muscle
3 accretion rates were restored to normal and
4 inflammatory responses in the small intestine were
5 attenuated. This was due possibly to enhanced
6 immune responsiveness and rapid clearance of
7 salmonella at remote sites, such as the liver and
8 spleen. However, it was also, at least in part, a
9 result of local effects of the bacterium on the gut.
10 Neutrophil recruitment and inflammation in the
11 intestine was prevented or delayed by the action of
12 *B. thetaiotaomicron*. It may also have blocked
13 direct uptake of salmonella in phagocytes to the
14 liver and spleen.

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1 Claims

2

3 1. An assay to select a candidate drug for the
4 treatment of inflammatory disease due to
5 cytokine production, said assay comprising:

6 a) exposing said candidate drug to
7 intestinal cells; and

8 b) analysing the effect of said candidate
9 drug, said effect being selected from the
10 group consisting of:

- 11 - the variation of nuclear export or
- 12 import of transcription factors of
- 13 the NF- κ B family;
- 14 - the disruption of transcriptional
- 15 activity of transcription factors
- 16 from the NF- κ B family;
- 17 - the differential histone acetylation
- 18 of p65 (RelA);
- 19 - the variation of the amount of
- 20 PPAR γ /RelA complexes in the cytosol
- 21 of the cells; and
- 22 - nucleoplasmic destruction of PPAR γ .

23

24 2. The assay of claim 1, which further comprises
25 the step of selecting a candidate drug which
26 demonstrates at least one effect selected from
27 the group consisting of:

- 28 - the increase of nuclear export or
- 29 the decrease of nuclear import of
- 30 transcription factors of the NF- κ B
- 31 family;

- 1 - the disruption of transcriptional
2 activity of transcription factors
3 from the NF- κ B family;
4 - the differential histone acetylation
5 of p65 (RelA);
6 - the increase of the amount of
7 PPAR γ /RelA complexes in the cytosol
8 of the cells; and
9 - nucleoplasmic destruction of PPAR γ .
10
- 11 3. The assay of claim 1 or 2, wherein the effect
12 of the candidate drug analysed is the nuclear
13 export of transcription factor from the NF- κ B
14 family.
15
- 16 4. The assay of claim 1 or 2, wherein the effect
17 of the candidate drug analysed is the
18 variation of the amount of PPAR γ /RelA
19 complexes in the cytosol of the cells.
20
- 21 5. The assay of claim 1 or 2, further comprising
22 the step of analysing the effect of the
23 candidate drug on the level of one or more
24 cytokines selected from the group consisting
25 of TNF- α , IL-8, MIP-2 α and Cox-2.
26
- 27 6. The assay of claim 5, wherein the effect of
28 the candidate drug analysed is the variation
29 in the level of IL-8 and/or MIP-2 α .
30

- 1 7. The assay of any one of claims 1 to 6, wherein
2 the intestinal cells are obtained from a cell
3 line maintained in cell culture.
4
- 5 8. The assay of any one of claims 1 to 7, wherein
6 said cell line is a Caco-2 cell line.
7
- 8 9. The assay of any one of claims 1 to 6, wherein
9 said assay is performed *in vivo* on an animal.
10
- 11 10. The assay of Claim 9, wherein said animal is a
12 mammal.
13
- 14 11. The assay of any one of claims 1 to 10,
15 wherein the assay is conducted in the presence
16 of known pathogenic bacteria.
17
- 18 12. The assay of Claim 11, wherein said known
19 pathogenic bacteria are *Salmonella sp.*
20
- 21 13. A method for the treatment of inflammatory
22 cytokine production associated diseases which
23 includes the step of administering a
24 therapeutically effective dose of a compound
25 capable of modulating:
- 26 - the nuclear export or the nuclear
27 import of transcription factors of
28 the NF- κ B family;
 - 29 - the disruption of transcriptional
30 activity of transcription factors
31 from the NF- κ B family;

- 1 - the differential histone acetylation
- 2 of p65 (RelA);
- 3 - the amount of PPAR γ /RelA complexes
- 4 in the cytosol of the cells; or
- 5 - nucleoplasmic destruction of PPAR γ .
- 6
- 7 14. The method of claim 13 wherein the compound is
- 8 capable of altering the transcriptional
- 9 activity of NF- κ B.
- 10
- 11 15. The method of claim 13 wherein the compound is
- 12 capable of increasing the amount of PPAR γ /RelA
- 13 complexes in the cell cytosol.
- 14
- 15 16. The method of any one of claims 13 to 15
- 16 wherein the immune system of a mammal is
- 17 returned to and maintained at homeostasis.
- 18
- 19 17. Use of an agent for the treatment of an
- 20 inflammatory disease, said agent being
- 21 selected according to the assay of any one of
- 22 claims 1 to 12.
- 23
- 24 18. Use of an agent as claimed in claim 17 wherein
- 25 the inflammatory disease is caused, at least
- 26 in part, by the inflammatory response of
- 27 intestinal cells.
- 28
- 29 19. Use of an agent as claimed in claim 17 or 18
- 30 wherein the inflammatory disease is associated
- 31 with the production of an inflammatory
- 32 cytokine.

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20. Use of an agent as claimed in any one of claims 17 to 19 wherein the inflammatory disease is selected from the group consisting of inflammatory bowel disease; Crohn's disease; Irritable Bowel Syndrome; rheumatoid arthritis; immunodeficiency syndrome; cachexia; multiple sclerosis, proliferation of keratinocytes; hyperproliferative and inflammatory disorders of the skin; psoriasis and acne vulgaris.
21. Use of *Bacteroides thetaiotaomicron* or a component thereof as a medicament.
22. Use of *Bacteroides thetaiotaomicron* or a component thereof as claimed in claim 21 for the treatment of an inflammatory disease.
23. Use of *Bacteroides thetaiotaomicron* or a component thereof as claimed in claim 21 or 22 for the treatment of an inflammatory disease which is caused, at least in part, by the inflammatory response of intestinal cells.
24. Use of *Bacteroides thetaiotaomicron* or a component thereof as claimed in any one of claims 21 to 23 for the treatment of an inflammatory disease which is selected from the group consisting of inflammatory bowel disease; Crohn's disease; Irritable Bowel Syndrome; rheumatoid arthritis;

1 immunodeficiency syndrome; cachexia; multiple
2 sclerosis, proliferation of keratinocytes;
3 hyperproliferative and inflammatory disorders
4 of the skin; psoriasis and acne vulgaris.
5

6 25. Use of *Bacteroides thetaiotaomicron* or a
7 component thereof as claimed in any one of
8 claims 21 to 24 wherein *Bacteroides*
9 *thetaiotaomicron* is administered to a patient
10 by means of a foodstuff or suppository.
11

12 26. Use of *Bacteroides thetaiotaomicron* or a
13 component thereof as claimed in any one of
14 claims 21 to 25 wherein *Bacteroides*
15 *thetaiotaomicron* is delivered orally.
16

17 27. Use of *Bacteroides thetaiotaomicron* or a
18 component thereof as claimed in any one of
19 claims 21 to 26 wherein *Bacteroides*
20 *thetaiotaomicron* is administered live.
21

22 28. Use of *Bacteroides thetaiotaomicron* or a
23 component thereof in the manufacture of a
24 medicament for the treatment of an
25 inflammatory disease.
26

27 29. A method of treating the inflammatory response
28 of intestinal cells, said method comprising
29 the step of administering a therapeutically
30 effective dose of *Bacteroides thetaiotaomicron*
31 or a component thereof.
32

1 30. The method of claim 29 wherein *Bacteroides*
2 *thetaitaomicron* is administered live to a
3 patient by means of a foodstuff or
4 suppository.

5
6 31. The method of claim 29 or 30 for the treatment
7 of inflammatory cytokine production associated
8 diseases.

9
10 32. The method of any one of claims 29 to 31 for
11 the treatment of a disease selected from the
12 group consisting of inflammatory bowel
13 disease; Crohn's disease; Irritable Bowel
14 Syndrome; rheumatoid arthritis;
15 immunodeficiency syndrome; cachexia; multiple
16 sclerosis, proliferation of keratinocytes;
17 hyperproliferative and inflammatory disorders
18 of the skin; psoriasis and acne vulgaris.

19
20 33. The method of any one of claims 29 to 32
21 wherein the therapeutically effective dose of
22 *Bacteroides thetaiotaomicron* or a component
23 thereof is delivered orally.

Fig. 1

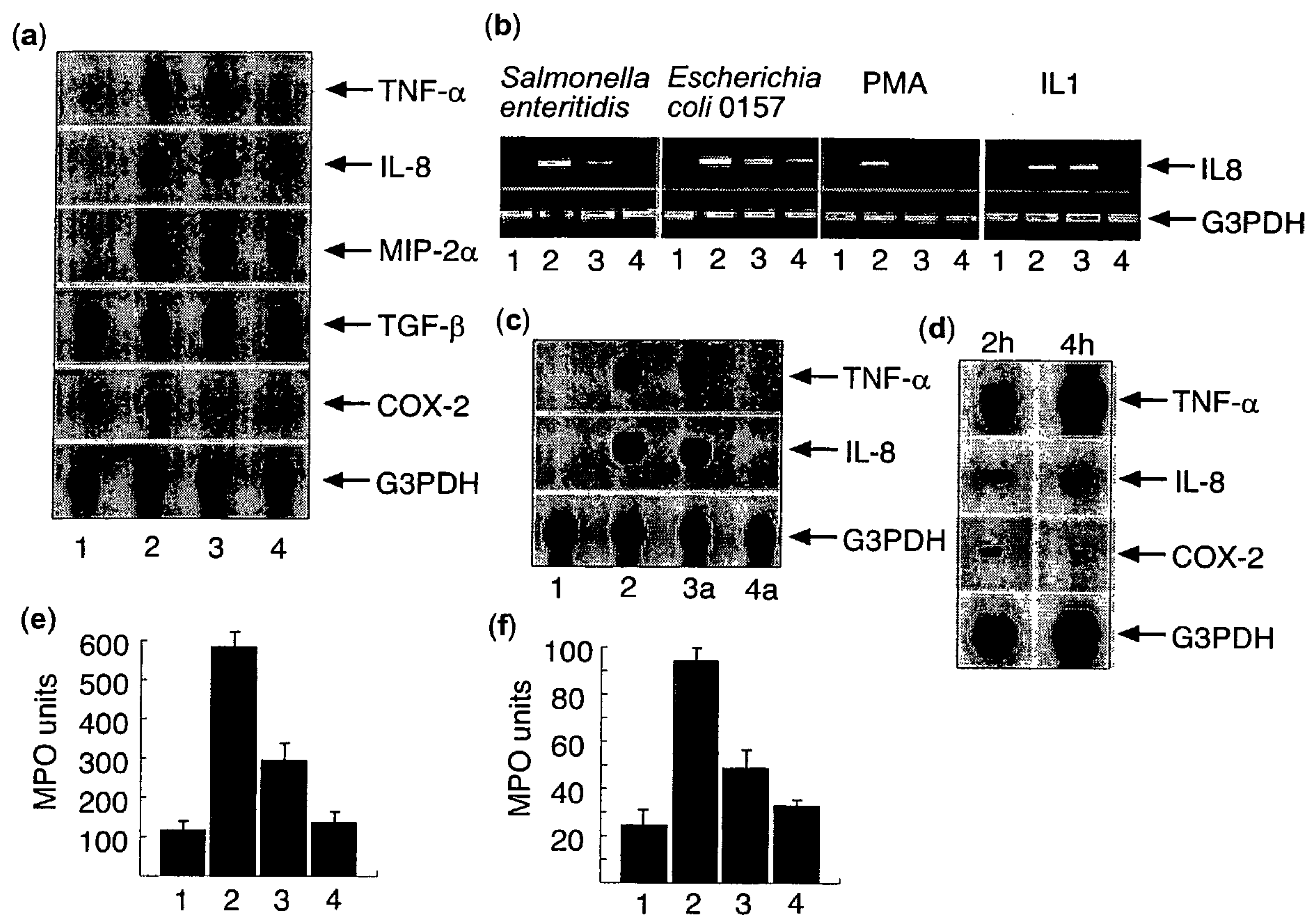
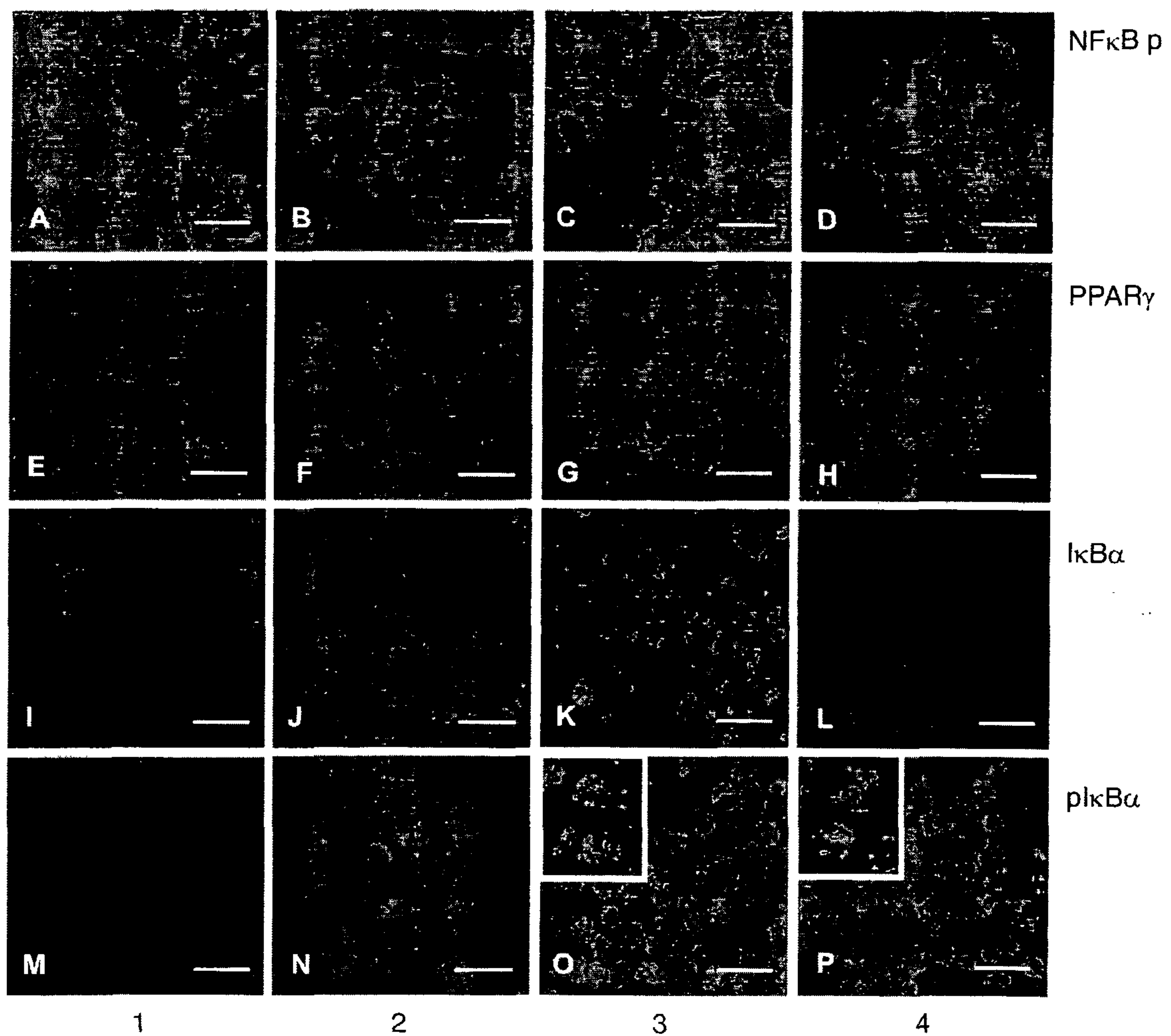


Fig. 2

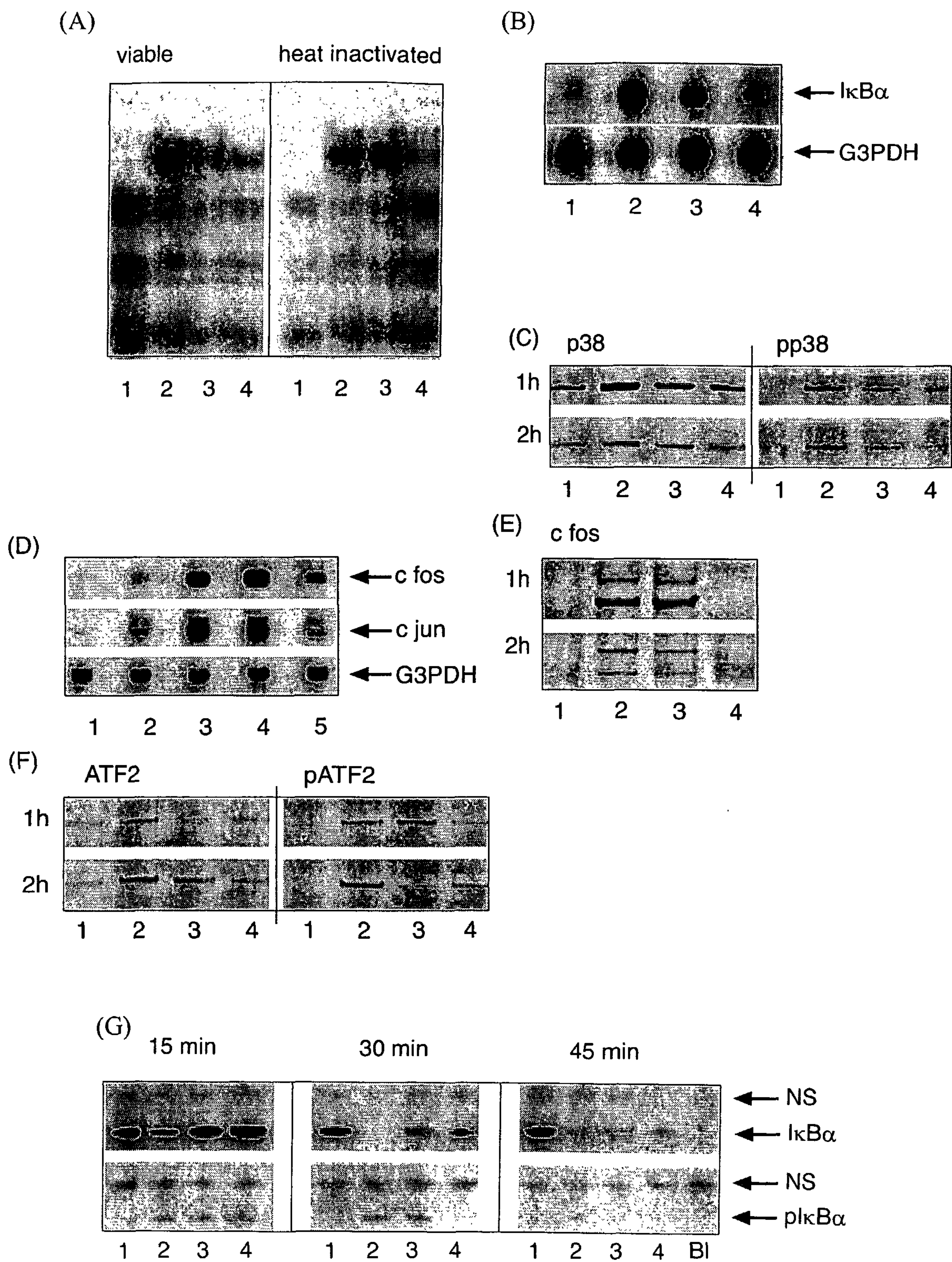


Fig. 3

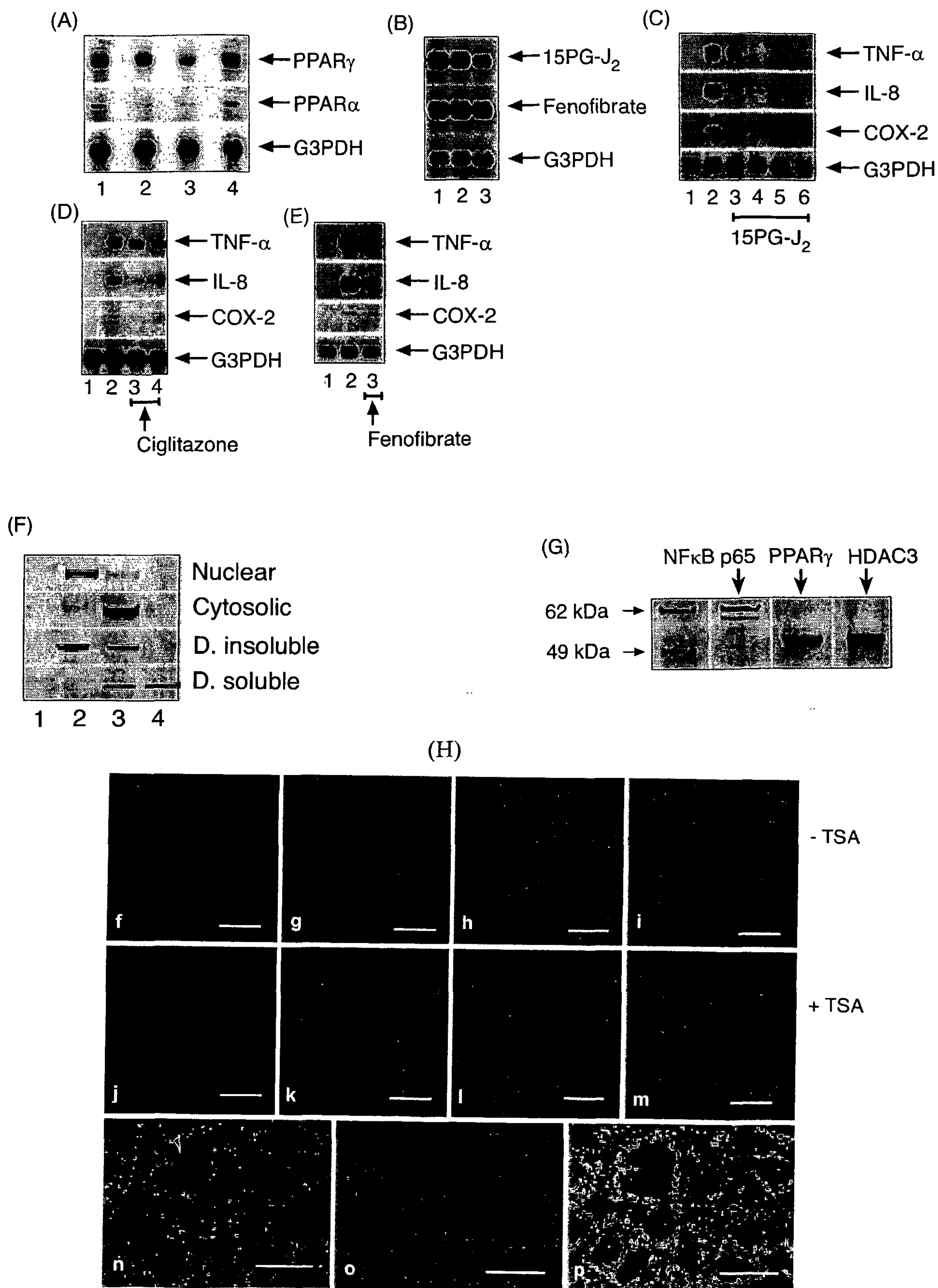


Fig. 4

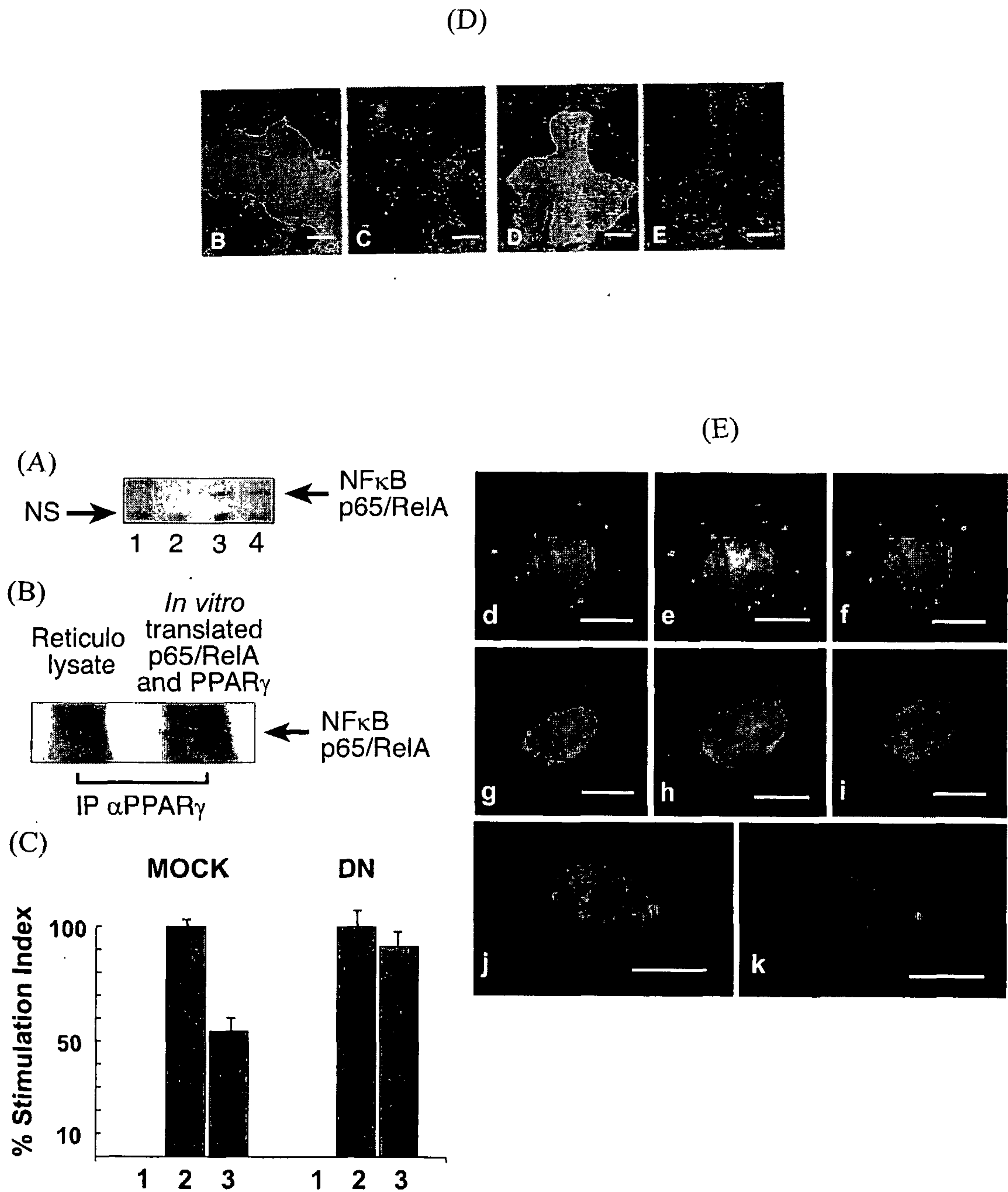
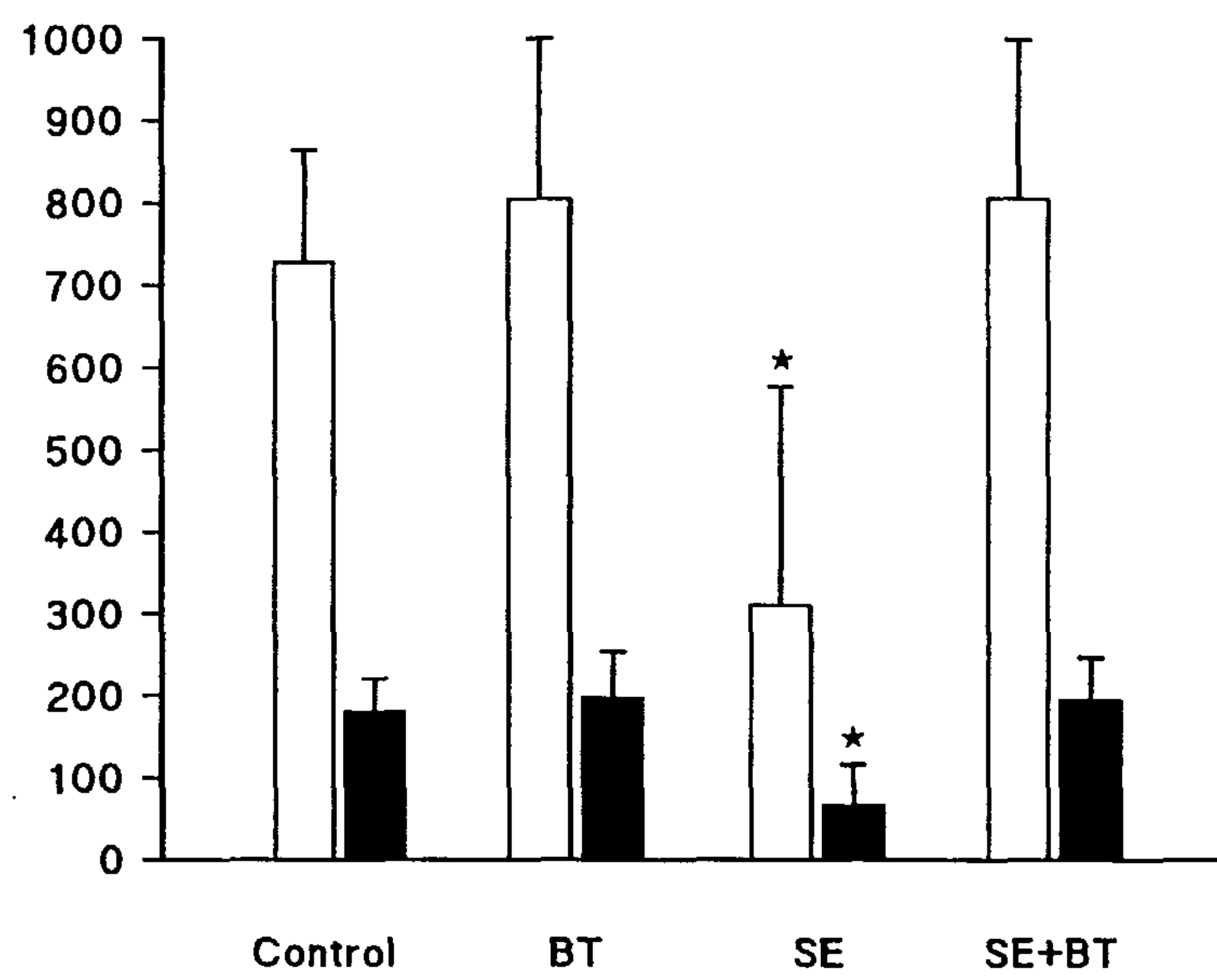
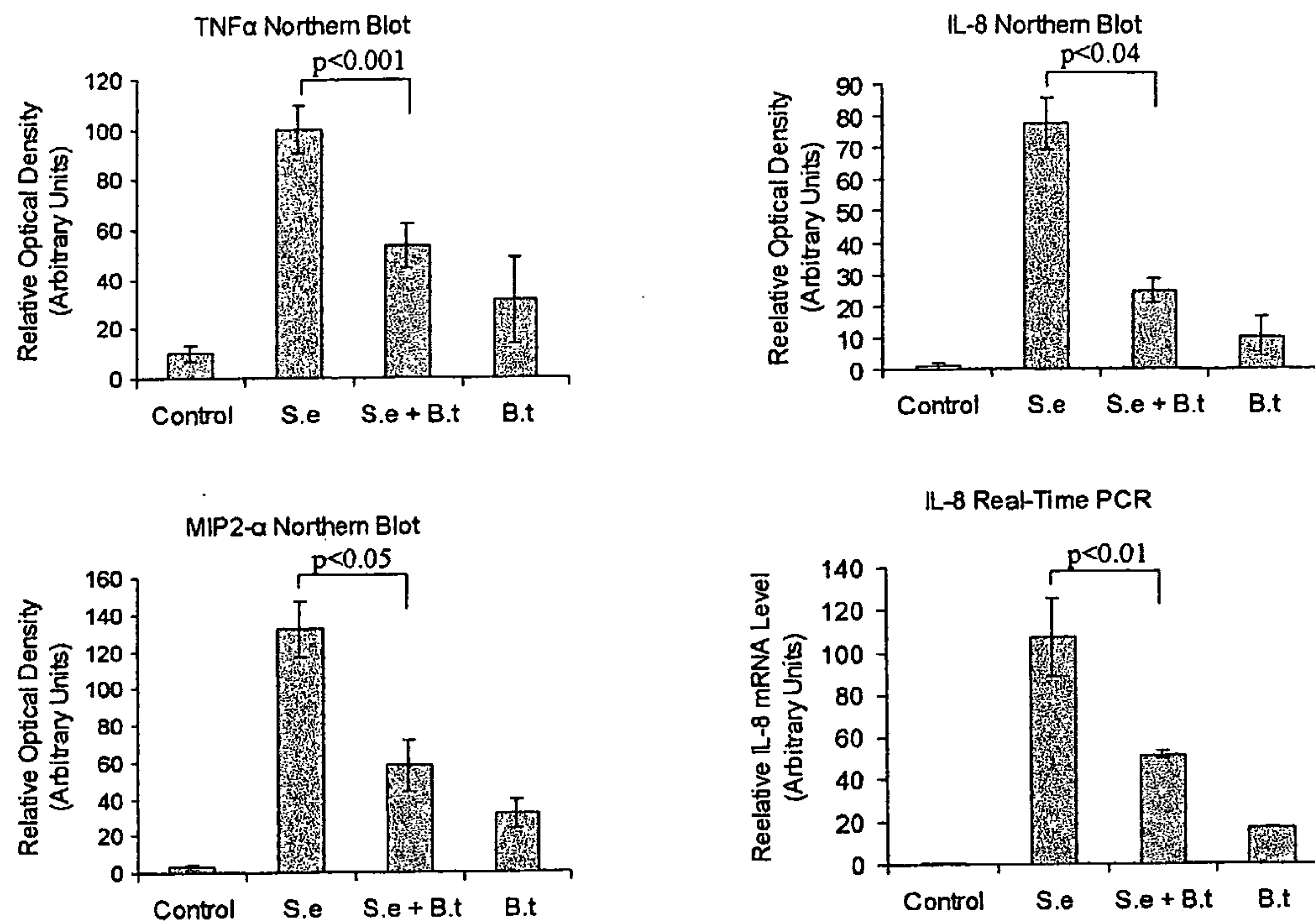
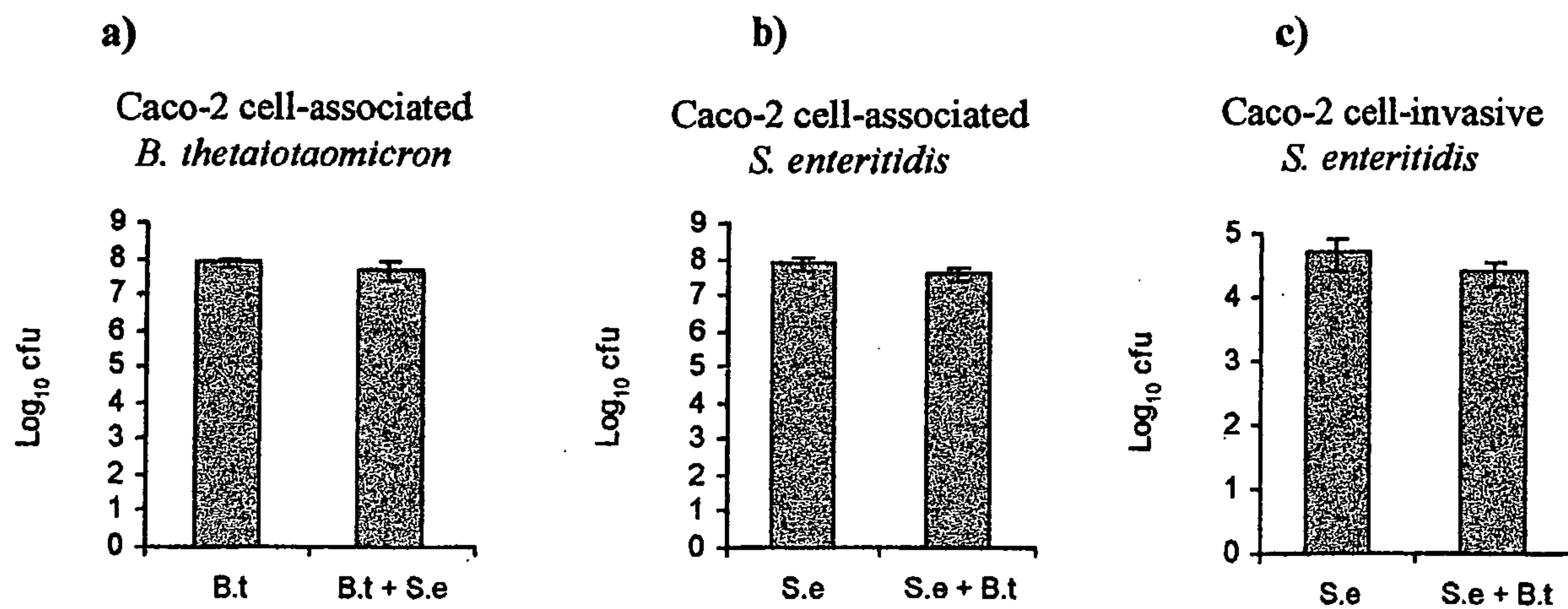


Fig. 5

**Fig. 6**

**Fig. 7****Fig. 8**

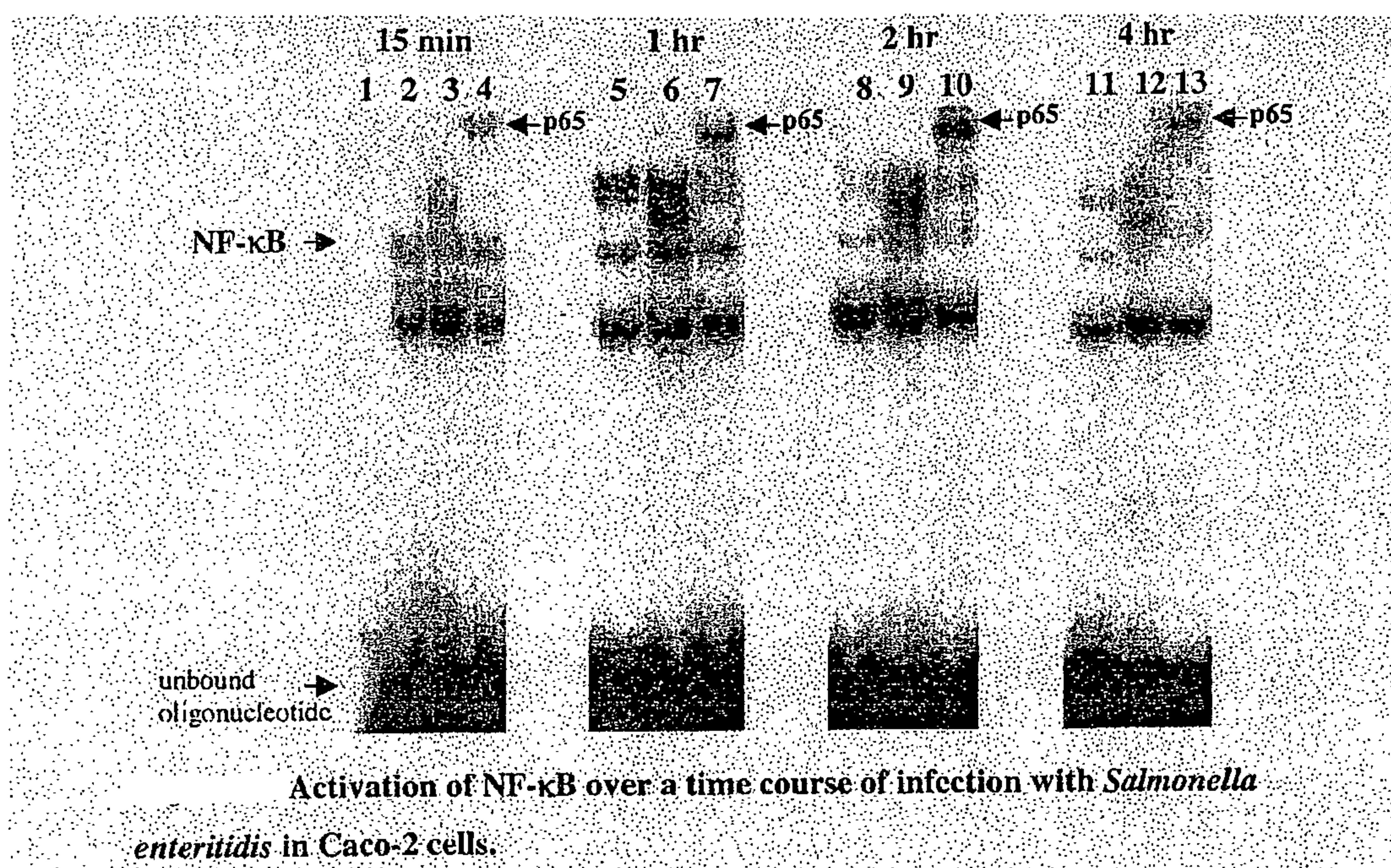


Fig. 9A

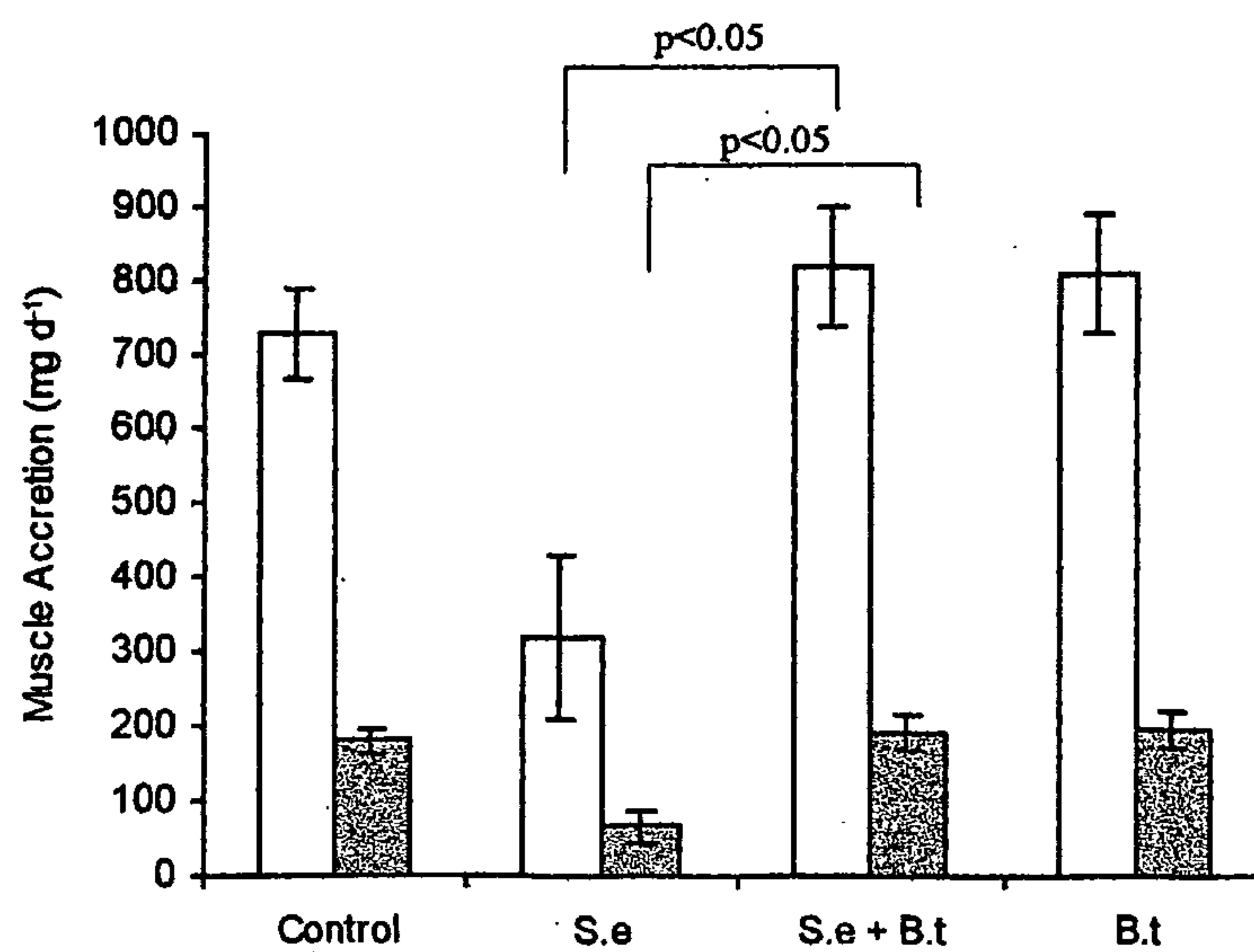


Fig. 10

Fig. 9B

Northern blots
(example dose and time course data)

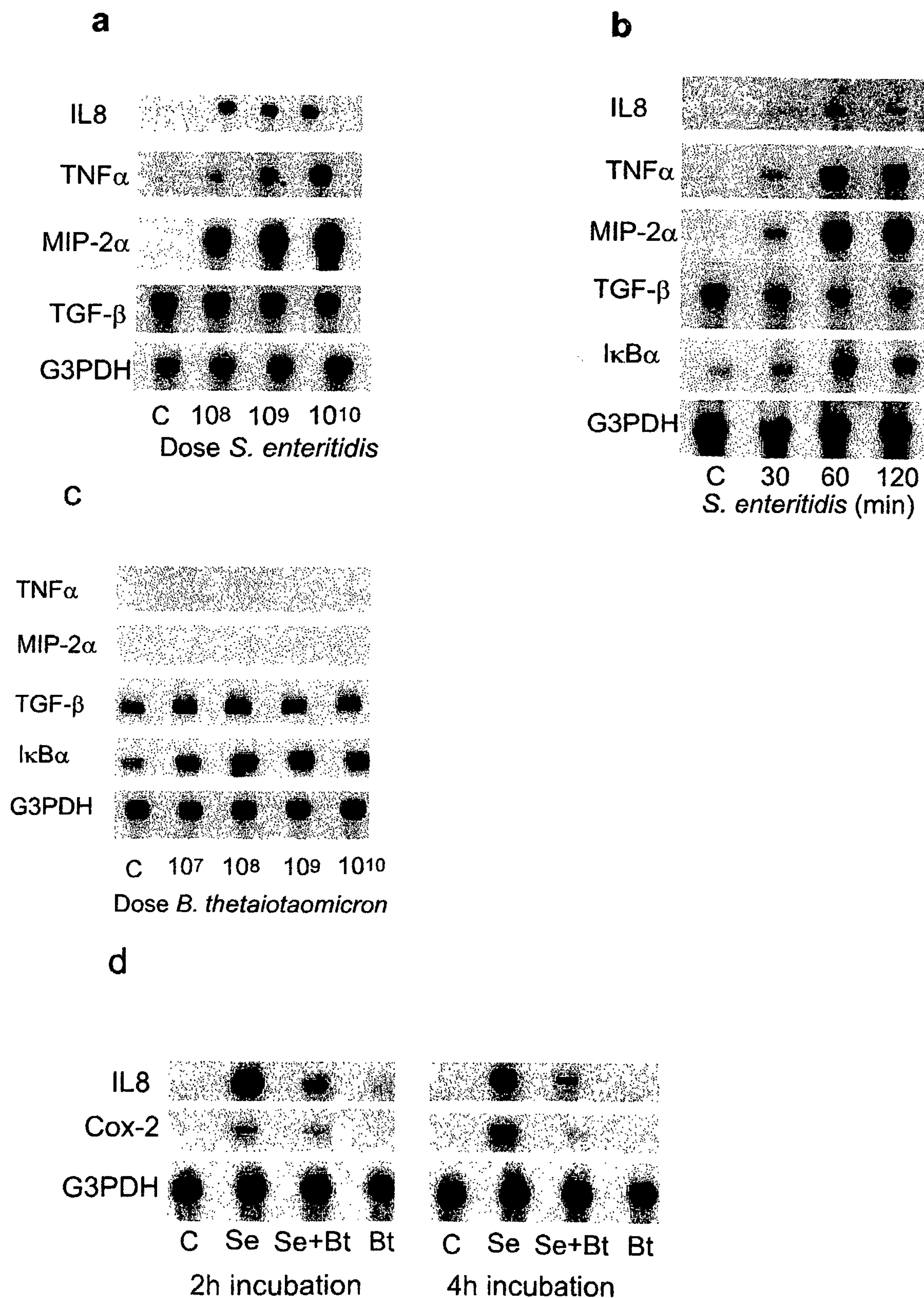
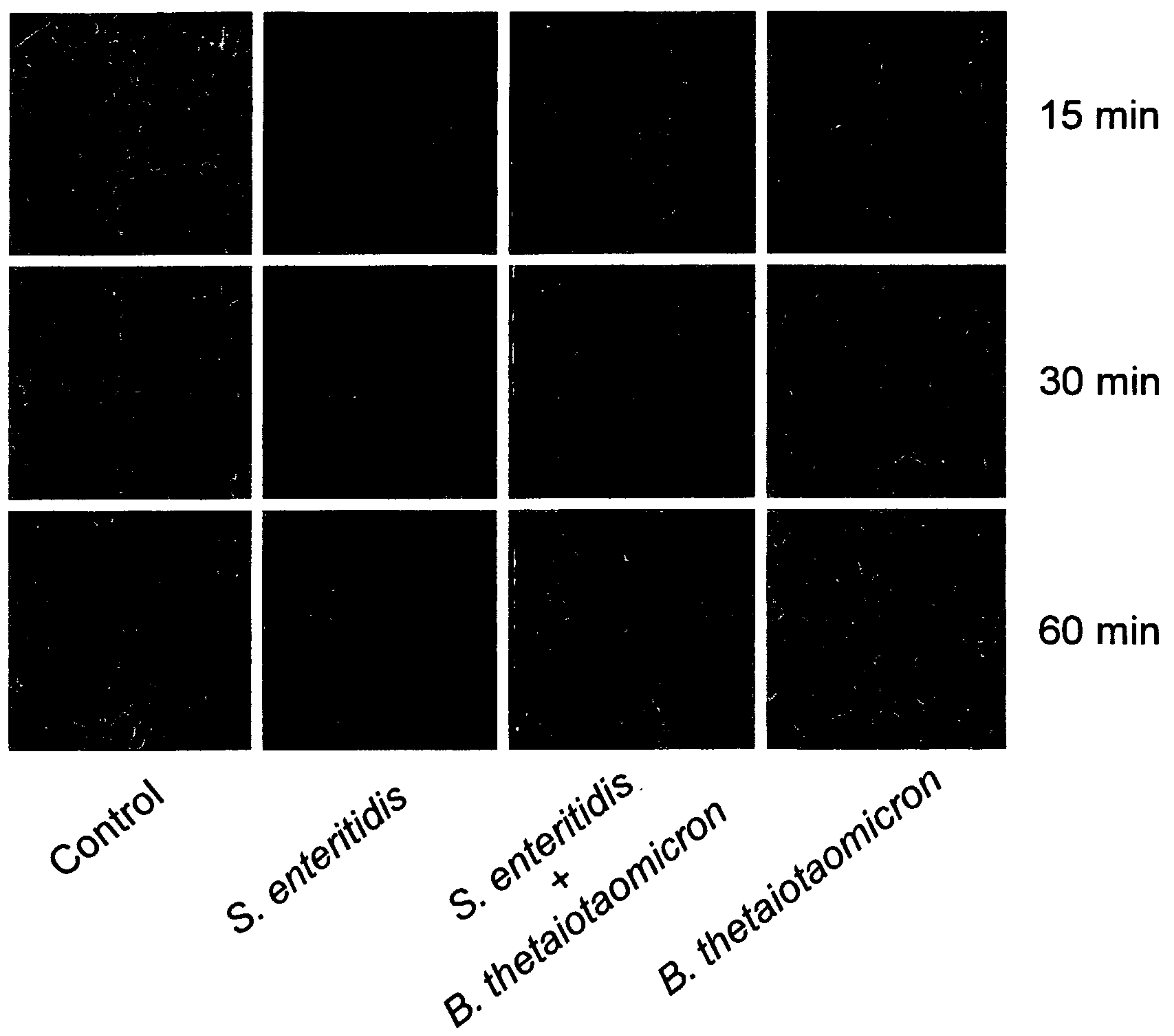
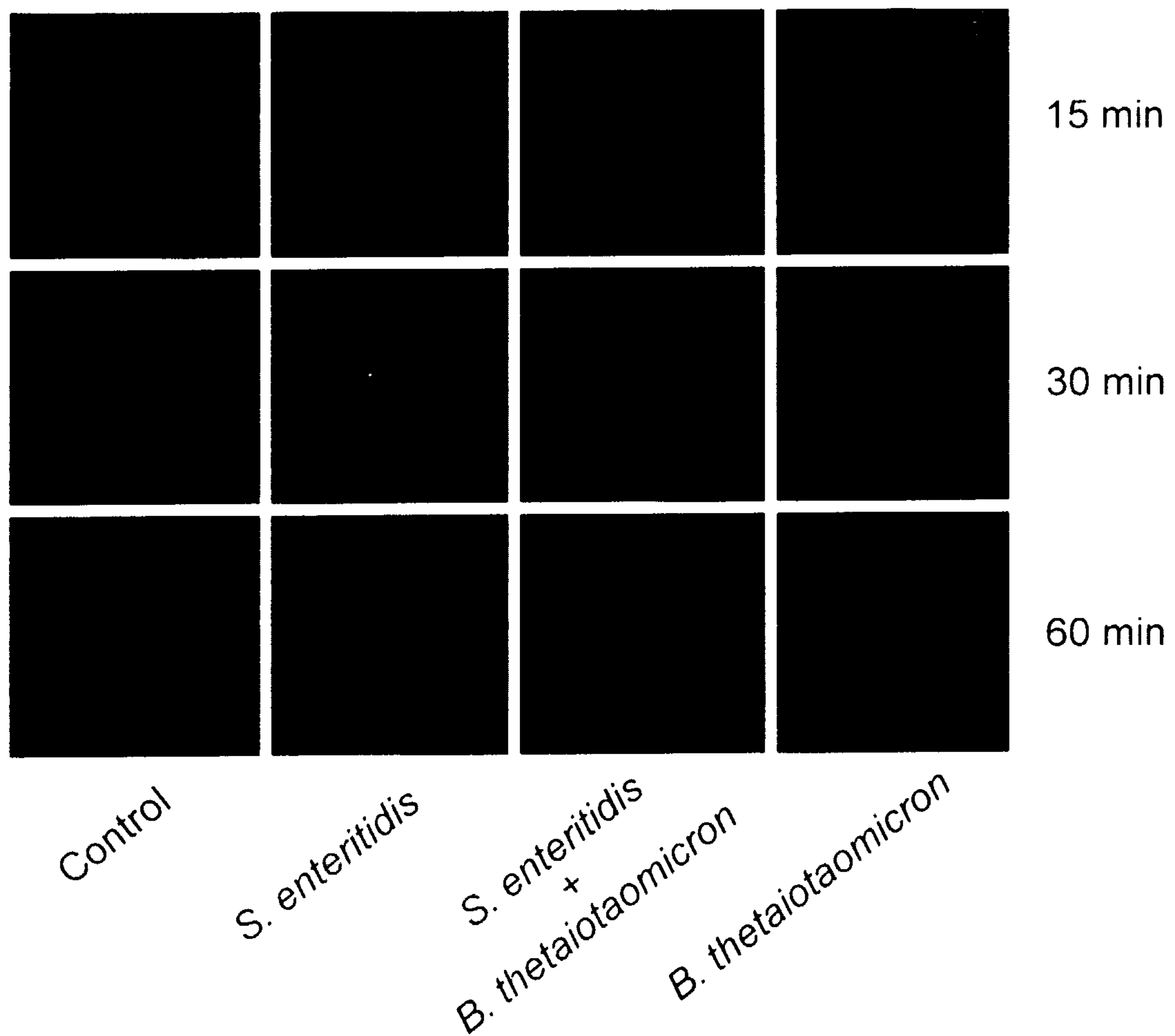


Fig. 9C**NF κ B p65**

Note: 2 hour data illustrated in manuscript

Fig. 9D

PPAR γ 

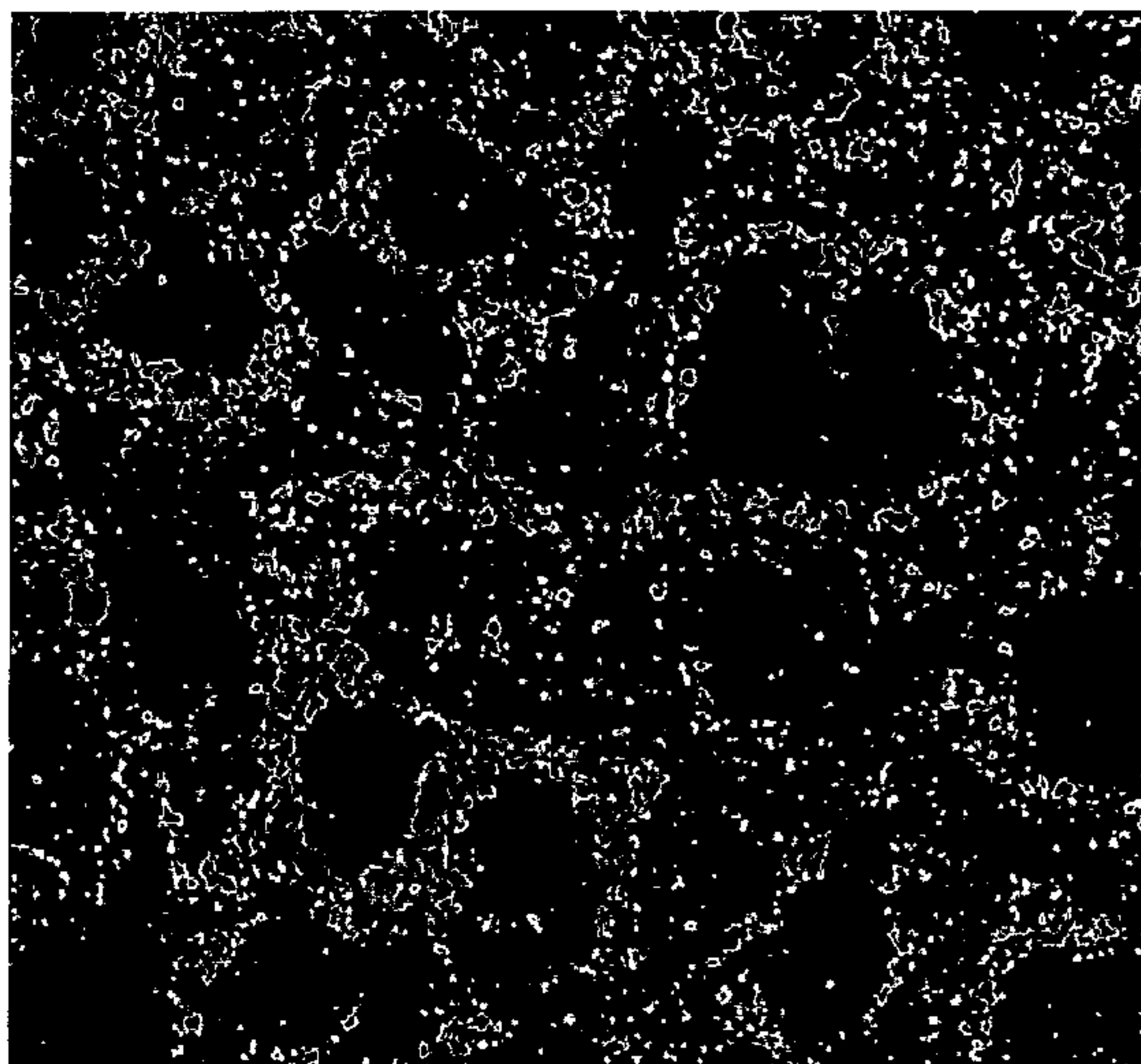
Note: 2 hour data illustrated in manuscript

Fig. 9E

Co-labelling of NF κ B p65 and PPAR γ in Caco2 cells following 2h exposure to *B. theta* and *S. enteritidis*

NF κ B p65

2nd antibody
conjugated to
Alexa-Fluor 488
(green)



PPAR γ

2nd antibody
conjugated to
Alexa-Fluor 568
(red)

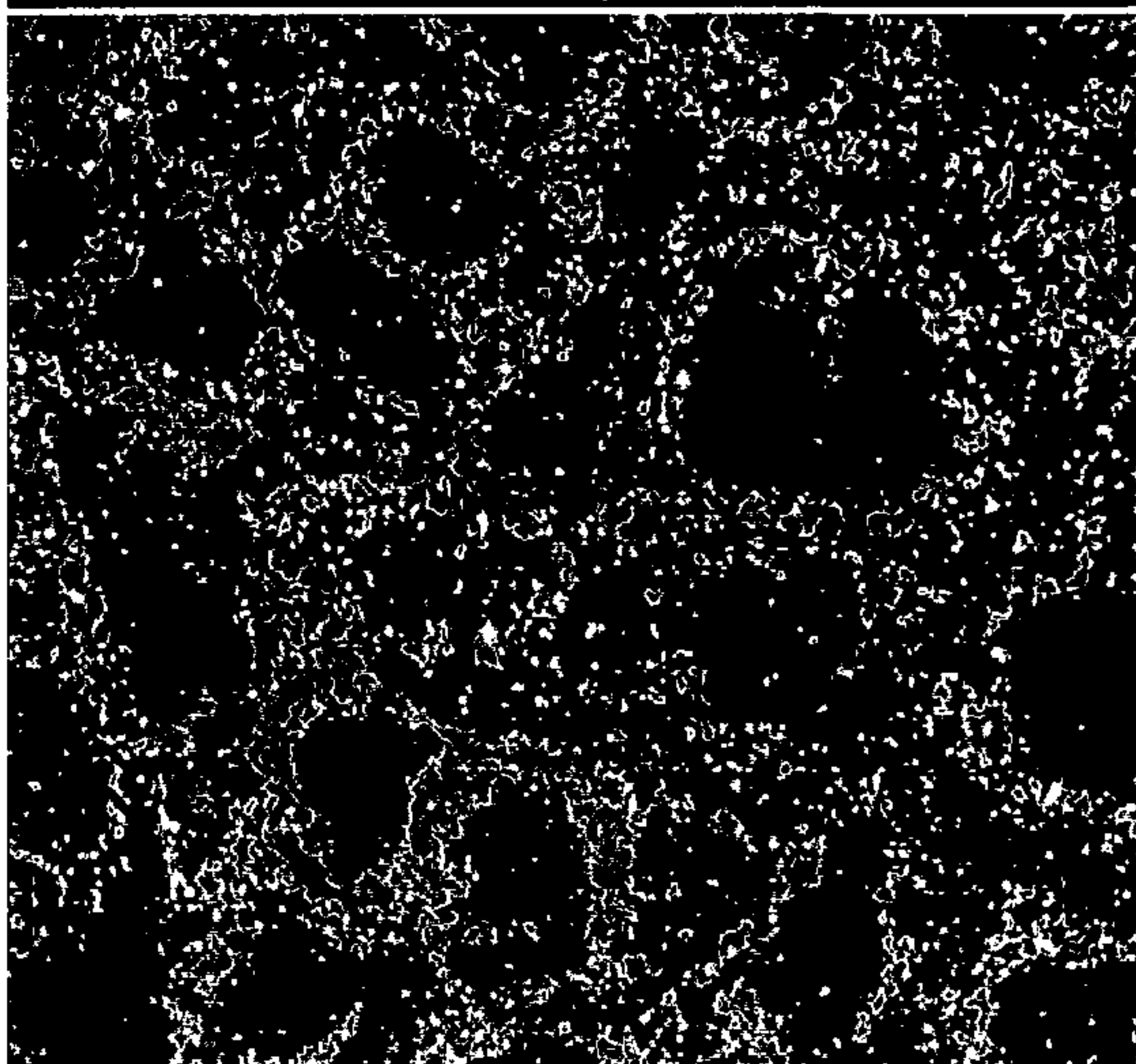


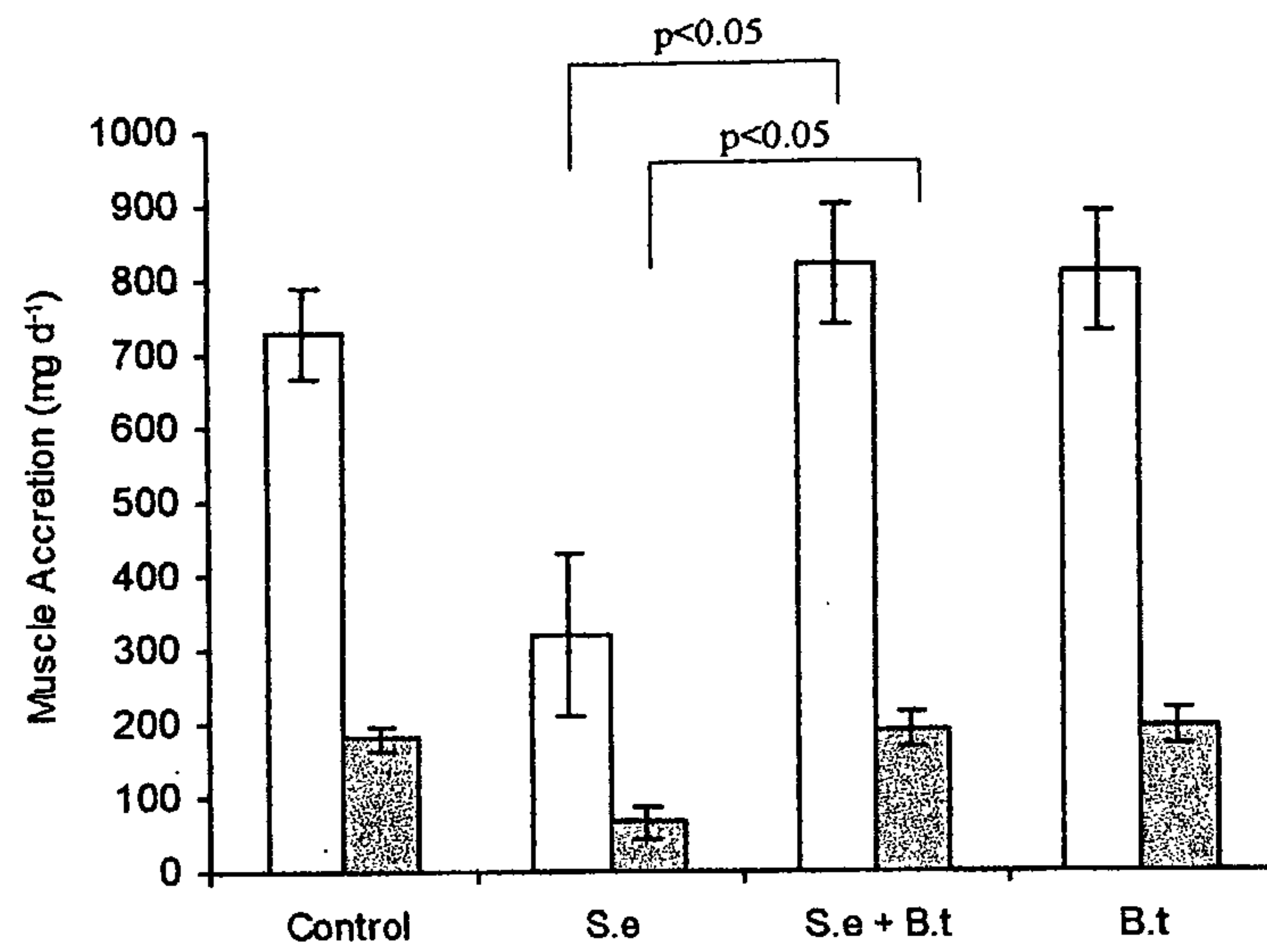
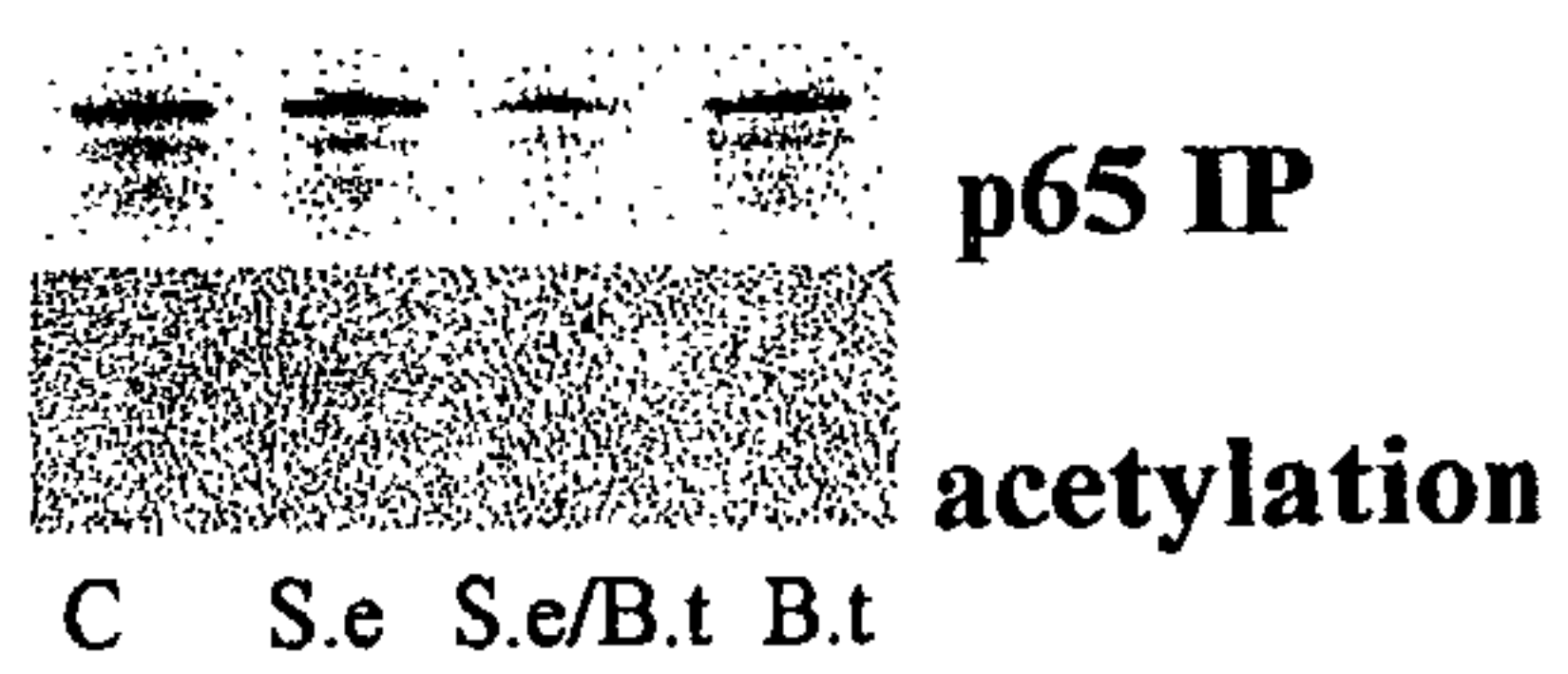
NF κ B p65

+

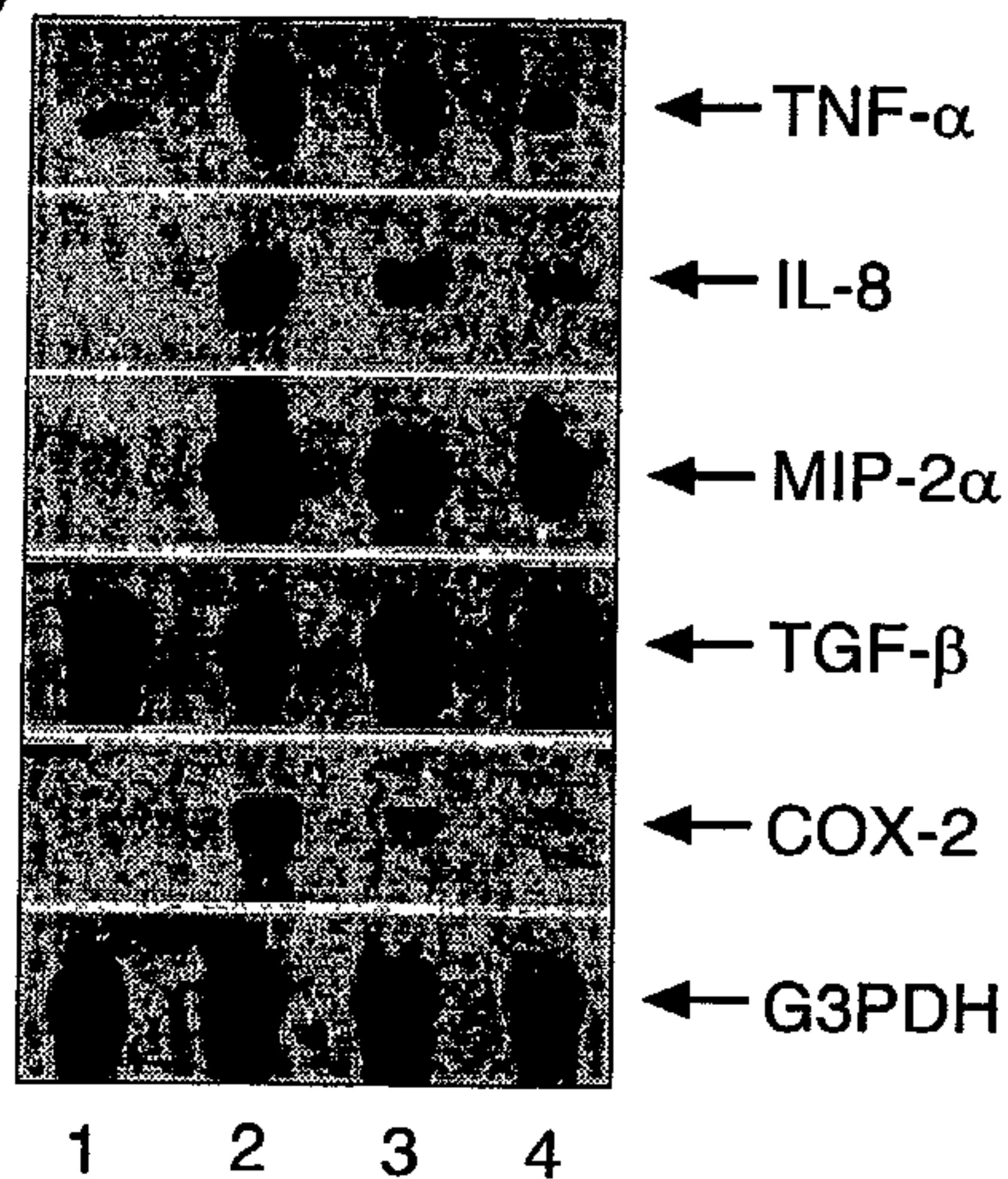
PPAR γ

Dual channel
image, regions
of co-localization
appear yellow.

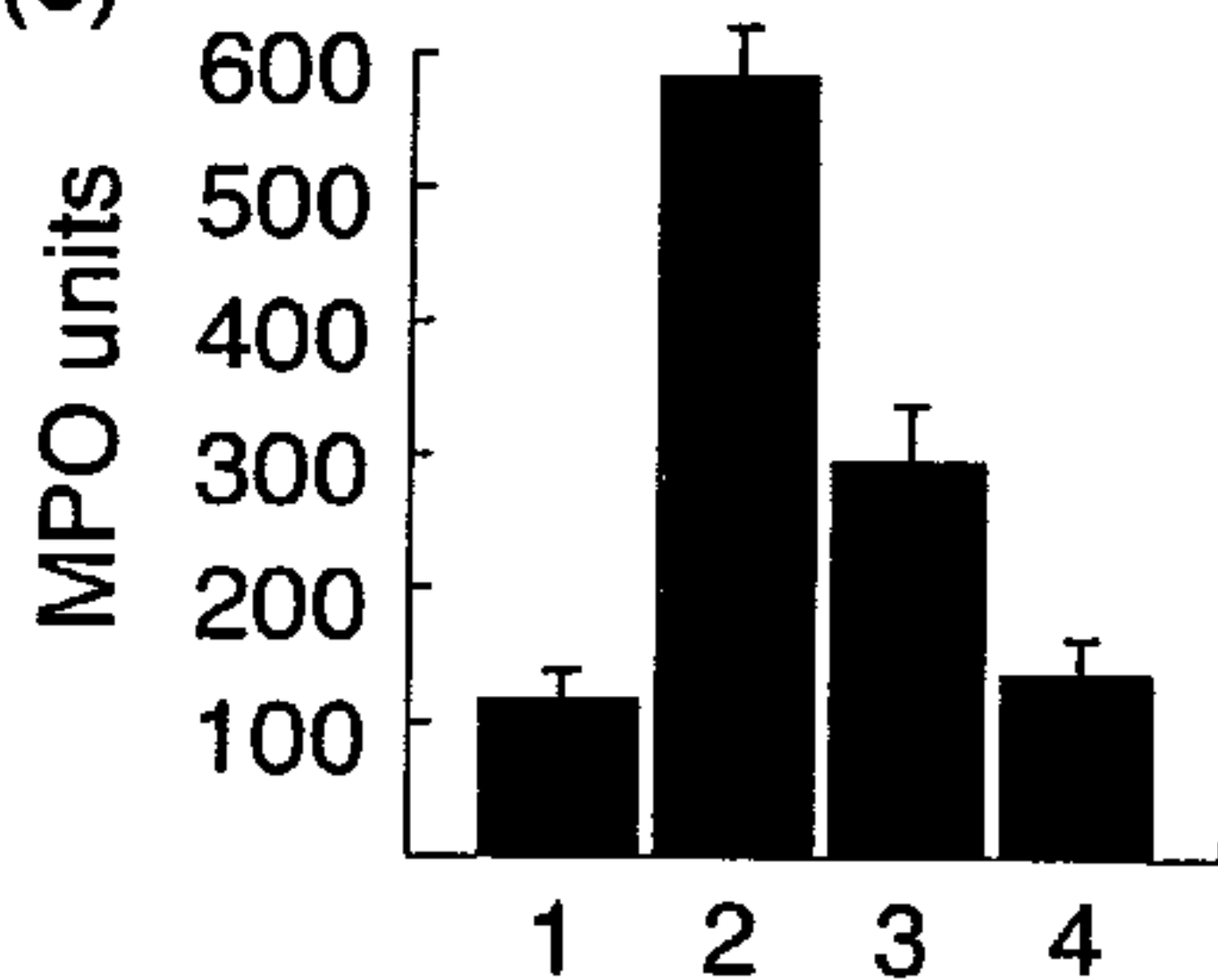


**Fig. 10****Fig. 11**

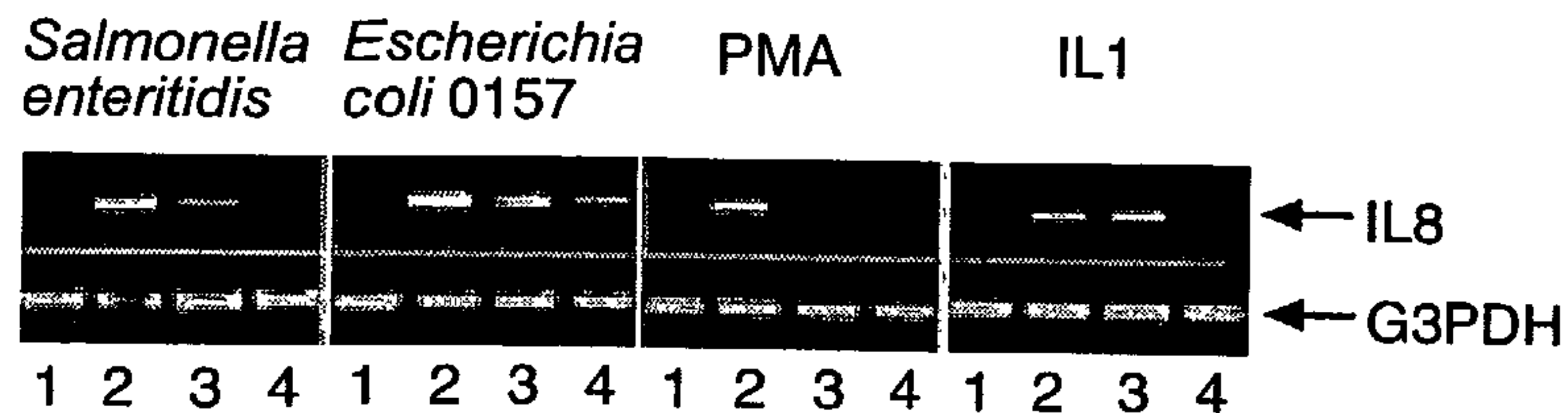
(a)



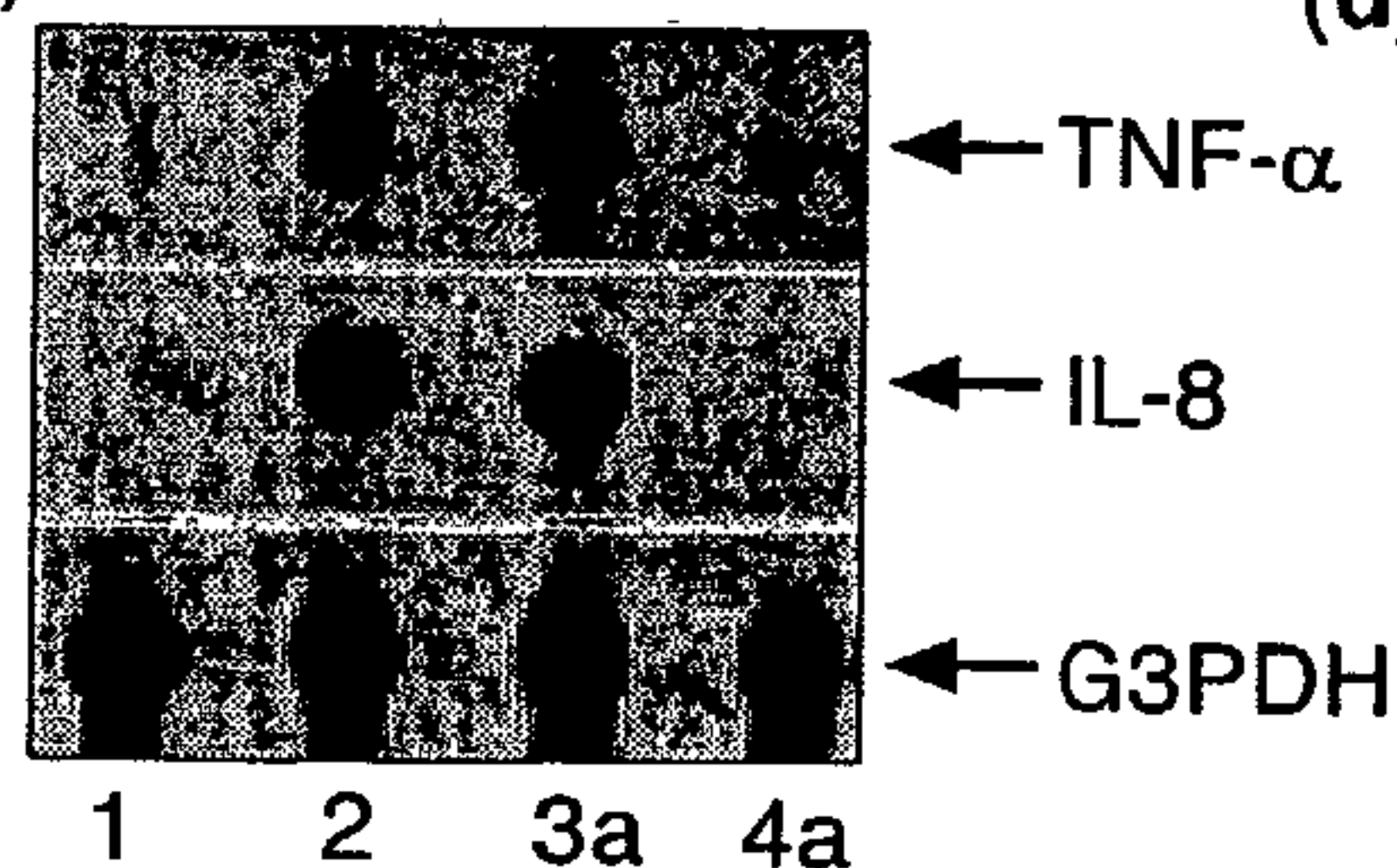
(e)



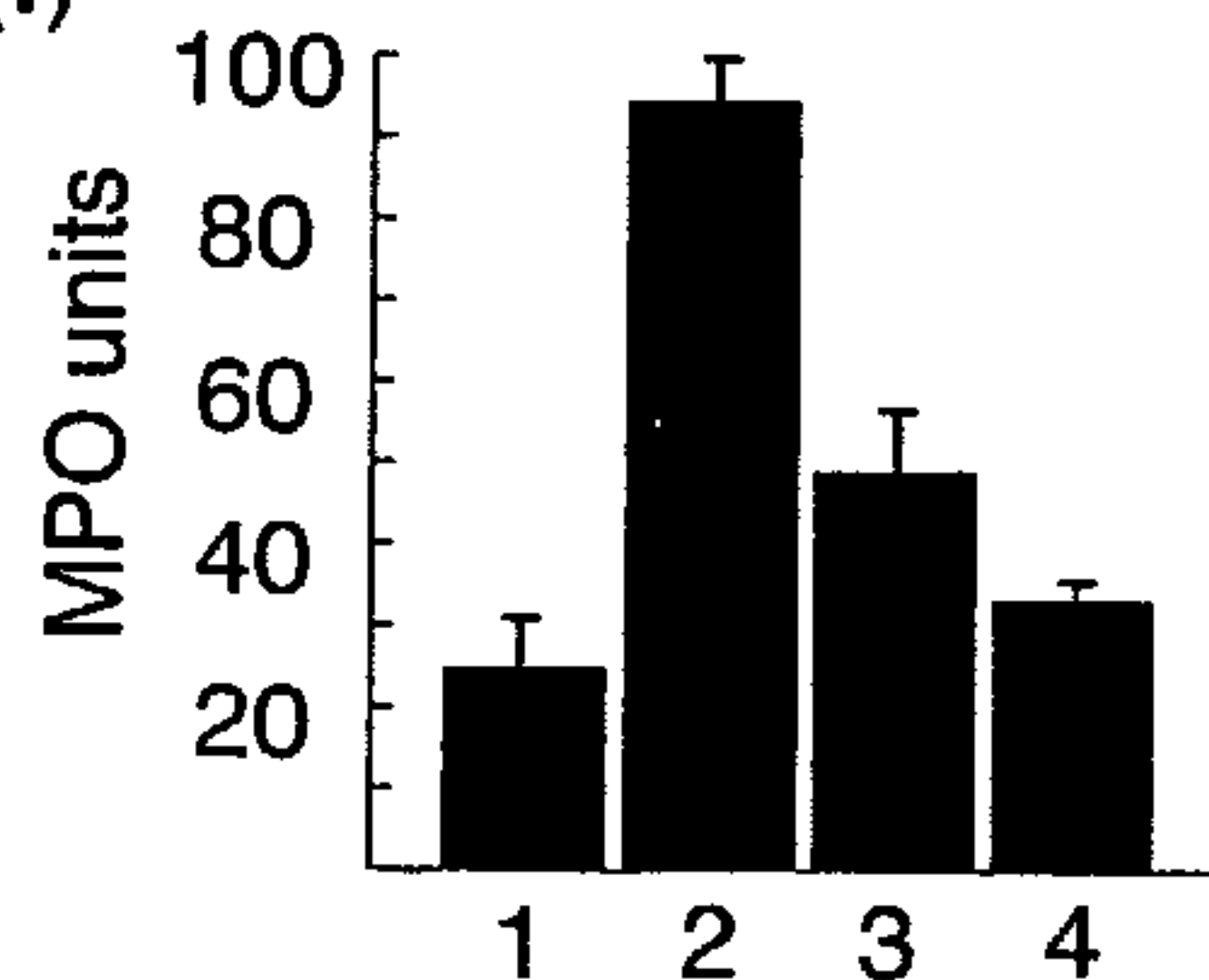
(b)



(c)



(f)



(d)

