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(54) Titre : THERAPIE ANTIVIRUS POUR LE TRAITEMENT DE MALADIES RESPIRATOIRES
(54) Title: INTERFERON-BETA FOR ANTI-VIRUS THERAPY FOR RESPIRATORY DISEASES

(57) Abrégé/Abstract:
The present invention provides the use of IFN-beta, an agent that increases the expression of IFN-beta, or a polynucleotide which is capable of expressing IFN-beta or said agent for the manufacture of a medicament for the treatment of rhinovirus-induced exacerbation of a respiratory disease selected from asthma and chronic obstructive pulmonary disease, wherein said treatment is by airway delivery of said medicament, e.g. by use of an aerosol nebuliser.

ABSTRACT

The present invention provides the use of IFN-beta, an agent that increases the expression of IFN-beta, or a polynucleotide which is capable of expressing IFN-beta or said agent for the manufacture of a medicament for the treatment of rhinovirus-induced exacerbation of a respiratory disease selected from asthma and chronic obstructive pulmonary disease, wherein said treatment is by airway delivery of said medicament, e.g. by use of an aerosol nebuliser.

INTERFERON-BETA FOR ANTI-VIRUS THERAPY FOR RESPIRATORY DISEASES

Field of the Invention

The invention relates to anti-virus therapy for respiratory diseases. More specifically, the invention relates to the treatment of rhinovirus-induced exacerbations of asthma or chronic obstructive respiratory disease (COPD) by airway delivery of interferon-β (IFN-β) or an agent that increases IFN-β expression. Both asthma and COPD are examples of inflammatory airways disease in which the common cold virus (rhinovirus) is recognised to cause exacerbations associated with severe clinical problems.

Background of the invention

Viral respiratory tract infections lead to the exacerbation of a number of respiratory diseases. In fact, viral respiratory tract infections are responsible for 85% of asthma exacerbations (Johnston *et al.*, BMJ, 1995; 310: 1225-8; Nicholson *et al.*, BMJ, 1993; 307: 982-6), including the most severe requiring hospitalisation (Johnston *et al.*, Am. J. Respir. Crit. Care Med. 1996; 154: 654-660). It is of concern that viral infections can trigger severe asthma exacerbations even when there is good asthma control by compliant patients taking optimal doses of inhaled corticosteroids (Reddel *et al.*, Lancet, 1999; 353: 364-369). The most common pathogen associated with asthma exacerbations is rhinovirus. Infection with rhinovirus leads to the release of inflammatory mediators (Teran *et al.*, Am. J. Respir. Crit. Care Med. 1997; 155: 1362-1366) and increased bronchial responsiveness (Grunberg *et al.*, Am. J. Respir. Crit. Care Med. 1997; 156: 609-616).

Subjects with asthma do not appear to be more susceptible in acquiring viral respiratory tract infections but they do have more severe lower respiratory tract symptoms (Corne *et al.*, Lancet, 2002; 359: 831-834). Although rhinovirus is known to infect bronchial epithelial cells (Gern *et al.*, Am. J. Respir. Crit. Care Med. 1997; 155: 1159-1161) and has been isolated from the lower airway (Papadopoulos *et al.*, J. Infect. Dis., 2000; 1821: 1875-1884; Gern *et al.*, Am. J. Respir. Crit. Care Med. 1997; 155: 1159-1161), the reasons why the asthmatic lower respiratory tract is more prone to the effects of infection with rhinovirus are unclear. It is therefore necessary to determine why asthmatic bronchial epithelial cells have an abnormal response(s) to virus infection that causes increased viral replication and shedding leading to

prolonged and augmented pro-inflammatory responses and associated exacerbation of asthma symptoms. It is also necessary to provide treatments for virally-induced exacerbations of asthma.

Surprisingly, it has been found that asthmatic bronchial cells are abnormal in their response to viral infection leading to increased virion production compared to healthy normal controls. This is despite the fact that both asthmatic and healthy cells mount an early inflammatory response to infection. It has also been shown that asthmatic cells are more resistant to early apoptosis following infection and have a deficient type I interferon response. This early apoptotic response is a key protective mechanism since inhibition of apoptosis in healthy control cells leads to enhanced viral yield. Therefore the increased virion production by asthmatic bronchial epithelial cells is associated with the ability of the cells to bypass apoptosis. Furthermore, it has been found that induction of apoptosis in asthmatic bronchial epithelial cells using IFN-β causes a significant reduction in infectious virion production. The invention therefore relates to the treatment of virally-induced exacerbations of asthma using an apoptosis-inducing agent, preferably IFN-β or an analog thereof.

US Patent no. 6,030,609 has previously proposed a method for treating respiratory syncytial virus (RSV) infection in the airways by aerosol delivery of IFN-β. This proposal was made solely on the basis of experiments with cultured lung epithelial cells. There is no mention in US Patent no. 6,030,609 of asthma and more particularly rhinovirus–induced exacerbation of asthma, which as indicated above is a serious clinical problem. Indeed, it is not possible to extrapolate from the experiments reported in US Patent no. 6,030,609 that IFN-β would be effective in treating rhinovirus-induced exacerbation of asthma, as RSV is known to produce proteins that interfere with Type I interferon production (Bossert & Conzelmann, Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells. J Virol. (2002) 76, 4287-93; and Spann et al., Suppression of the induction of alpha, beta, and lambda interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages [corrected].J Virol. (2004) Apr;78(8):4363-9; Erratum in: J Virol. (2005) 78 (12):6705), whereas no similar activity is known to be produced by rhinovirus. Furthermore, although the first clinical trial in the general population using IFN-β-ser against experimental rhinovirus infection showed promising beneficial effects

(Higgins PG, Al-Nakib W, Willman J, Tyrrell DA. Interferon-beta ser as prophylaxis against experimental rhinovirus infection in volunteers. J. Interferon Res. (1986) 6:153-9), in a subsequent trial for prophylaxis of natural colds, IFN-β-ser was found to be ineffective (Sperber SJ, Levine PA, Sorrentino JV, Riker DK, Hayden FG. Ineffectiveness of recombinant interferon-beta serine nasal drops for prophylaxis of natural colds. J. Infect Dis. (1989) 160, 700-5), possibly because normal cells have an innate capacity to produce IFN-β in response to rhinovirus infection. As indicated above, the inventors in this instance have found that a key feature that distinguishes asthmatic epithelial cells is a deficient apoptotic response due to impaired production of IFN-β that enables viral replication to proceed unchecked, thereby contributing to prolonged symptoms and disease exacerbation. While treatment of such deficiency by use of IFN-β was first proposed by the inventors in relation to rhinovirus –induced exacerbation of asthma, it is now proposed to be equally applicable to rhinovirus-induced exacerbation of COPD, which encompasses a range of conditions including chronic bronchitis and emphysema.

Summary of the Invention

Accordingly, the invention provides the use of an agent selected from:

(a) interferon-β (IFN-β);

(b) an agent that increases IFN-β expression; or

(c) a polynucleotide capable of expressing (a) or (b);

for the manufacture of a medicament for the treatment of rhinovirus-induced exacerbation of a respiratory disease selected from asthma and COPD, wherein said treatment is by airway delivery of said medicament, e.g. by use of an aerosol nebuliser.

The invention further provides a method of treating in an individual rhinovirus-induced exacerbation of a respiratory disease selected from asthma and COPD comprising airway administration to the individual of an agent selected from the group consisting of :

(a) interferon-β (IFN-β);

(b) an agent that increases IFN-β expression; or

(c) a polynucleotide capable of expressing (a) or (b).

Such treatment may be prophylactic or therapeutic treatment. By "rhinovirus induced" will be understood induction solely by rhinovirus or virus comprising largely but not exclusively rhinovirus.

Brief description of the Figures

Figure 1 shows the proinflammatory responses of normal and asthmatic bronchial epithelial cells (BECs) following rhinovirus (RV) infection. Panels (a) and (c): Induction of IL-8 (a) and TNFα (c) mRNA 8h after RV-16 infection was measured by qPCR. Asthmatic cells had a median (IQR) fold induction of IL-8 of 33.2 (7.3, 208.6) compared to 101.4 (6.4, 802.9) in healthy controls with no significant difference between groups (p=0.8). Both groups demonstrated a significant increase in IL-8 mRNA compared to cells treated with medium alone (p=0.001) and UV inactivated RV (p=0.01). For TNFα mRNA, asthmatic cells had a median fold induction from baseline of 94.4 (5.8, 1001.4) compared to 272.9 (30, 676) in healthy control cells, with no significant difference between the groups (p=0.8). Both groups demonstrated a significant increase in TNF-α mRNA compared to cells treated with medium alone (p<0.01) and UV inactivated RV (p<0.01). Panels (b) and (d): IL-8 (b) and TNFα (d) protein production in the supernatant 48h after RV-16 infection was measured by ELISA. Median (IQR) levels of IL-8 were 922 pg/ml (868, 1065) in asthmatic cells compared to 705 pg/ml (414, 979) (p=0.6) in healthy controls. Both groups demonstrated a significant increase above cells treated with medium alone (61.4pg/ml, p<0.001) and UV inactivated RV-16 (43.8, P<0.01). Secretion of TNF-α was 10.4 pg/ml (6.9, 29.6) in RV-16 infected asthmatic cells and 24.6 pg/ml (9.2, 30.4) in RV-16 infected healthy control cells (p=0.7). Both groups demonstrated a significant increase above cells treated with medium alone (1.85pg/ml, p<0.01) and UV inactivated RV-16 (4.69, P<0.01). Panels (e) and (f): ICAM-1 expression was measured by flow cytometry immediately prior to RV-16 infection (e) or 24h after infection (f). Data are expressed as mean fluorescence intensity (MFI). Prior to infection, asthmatic cells had a tendency to a lower median MFI 31(12, 80) compared to healthy control cells 67 (34, 83) but this was not significant (p=0.3). After 24h, asthmatic cells had a significantly lower median MFI 54.6 (27.6, 145.2) compared to healthy control cells 110.4 (65, 195.3) (p=0.02). Graphs are box whisker plots, heavy line represents the median, upper box border represents 75th quartile, lower 25th

quartile, whiskers are 5th and 95th centiles. * = significantly different from cells treated with medium alone and UV inactivated RV-16

Figure 2 shows RV-16 replication and release from normal and asthmatic BECs. Panel (a): RV-16 release into the supernatant of infected cells was estimated by calculating the $TCID_{50}$ from the CPE in confluent monolayers of Ohio HeLa cells. Values have been log transformed; data points represent the geometric mean and the standard error of the mean. By 48h significantly more RV was detected from asthmatic cells with a mean $TCID_{50}$ of 3.99, compared to 0.54 in healthy control cells ($p<0.01$). Panel (b): RV-16 mRNA production was measured by qPCR after 8h of infection. Median (IQR) production from asthmatic cells was 21×10^5 (1.6×10^5, 97×10^5) compared to 0.4×10^5 (0.09×10^5, 0.6×10^5) from healthy controls ($p< 0.01$). Graphs are box whisker plots, heavy line represents the median, upper box border represents 75th quartile, lower 25th quartile, whiskers are 5th and 95th centile. Dots represent outliers. Panels (c) and (d): Cell lysis as a consequence of RV-16 infection was analysed based on LDH activity in culture supernatants. Values have been log transformed; data points represent the geometric mean and the standard error of the mean. Both groups demonstrated a progressive increase in LDH activity over time that was significantly increased from baseline by 24h ($p<0.01$) in asthmatic cells but not in healthy control cells even at 48h ($p=0.2$) (c). By 48h, the LDH activity from asthmatic cells showed a 3.4 mean fold increase from baseline compared to a 1.34 fold increase in the healthy control cells ($p<0.001$) (d). No significant change in LDH activity was seen in cells treated with medium alone or UV inactivated RV. * = results from asthmatic cells and healthy controls significantly different ($p<0.01$). Asthma = ●, Healthy controls = ○.

Figure 3 shows the changes in cell viability following RV-16 infection. Following RV-16 infection for 8h, cells were stained with Annexin-V conjugated to the flurochrome Phycoerythrin (PE) and the vital dye 7-Amino-actinomycin (7-AAD) and analysed by flow cytometry. Panel (a): Viable ($AxV^-/7AAD^-$) cell number was determined and expressed as % viability compared with cells treated with medium alone. Infection with RV-16 led to a significant reduction in median (IQR) cell viability in both asthmatic and control cells compared to medium alone ($p=0.03$). There was no significant reduction in viability in cells treated with inactivated RV-16 96 (91, 98)%. Asthmatic cells showed significantly better viability, median 80 (74, 86)%, compared to healthy controls 63 (51, 69)% ($p=0.002$). Panel (b): Apoptotic

($AxV^+/7AAD^-$) cells were also analysed 8h following RV-16 infection. While both groups demonstrated an increase in apoptosis with infection, asthmatic cells appeared more resistant with a fold increase of only 1.41 (1.35, 1.69), compared to 2.19 (1.98, 2.22) in healthy controls (p=0.02). Cells treated with medium alone did not show an increase in apoptosis. Cells treated with UV inactivated RV-16 did show a small increase above baseline, 1.2 (1.1, 1.4) (p=0.02). * = significantly different from cells treated with medium only (p<0.01). ** = significantly different from asthmatic cells (p<0.05).

Figure 4 shows caspase activity and its role following RV-16 infection. Panel (a): The time course for activation of Caspase 3/7 by RV-16 was determined using the Apo-One Homogenous Caspase 3/7 assay (Promega, Maddison, USA) with the readout adjusted for cell number. Values have been log transformed to enable them to be plotted over time; data points represent the geometric mean and the standard error of the mean. There was significant induction of active caspase 3/7 in response to infection reaching a plateau at 8h (p<0.01). Asthmatic cells showed a lower induction of active caspase 3/7 (mean (SEM) = 1.47 (0.1)) compared to healthy controls (mean (SEM) =2.16 (0.3); p=0.004). Panel (b): The effect of inhibition of caspase-3 using the inhibitor, ZVD-fmk, was measured by flow cytometry, as described in the legend to Figure 3. Cells were treated with RV-16 alone or with ZVD-fmk, before and after infection with RV-16. Results are expressed as the fold induction in apoptosis seen above control cells treated with medium alone. In asthmatic cells were there was a median (IQR) induction of apoptosis above baseline of 1.4 (1.35, 1.68) with RV-16 alone; pre-treatment of cells with the ZVD-fmk, had little effect on apoptosis (median (IQR) = 1.17 (0.96, 1.95); p>0.05). However, in healthy controls cells, RV-16 infection resulted in a median (IQR) induction of apoptosis above baseline of 2.19 ((1.98, 2.22) and this was abolished by pretreatment with ZVD-fmk (median (IQR) = 0.82 (0.78, 0.86); p=0.03). Panel (c): The effect of caspase-3 inhibition on RV-16 production was measured by HeLa titration assay on the BEC supernatant removed after 48h of infection. There was no difference seen in the $TCID_{50}$ in the supernatant removed from asthmatic cells infected with RV-16 (median (IQR) = 3.56 (3.50-3.62) compared to infected cells treated with ZVD-fmk (median (IQR) = 3.56 (3.5-3.62); p=0.94). However for healthy control BECs, the $TCID_{50}$ increased from a median (IQR) value of 0.6(0.4, 0.63) with infection alone to 2.78 (0.63, 6.32) (p=0.01) in the presence of RV-16 and ZVD-fmk. * = significantly different from asthmatic cells

((p<0.01). ** = significantly different from cells treated with RV-16 alone. Asthma = •, Healthy controls = o.

Figure 5 shows IFNβ production and its role in RV-16 infection. Panel (a): Induction of IFNβ mRNA was measured by qPCR after 8h of RV-16 infection. Asthmatic cells demonstrated a median (IQR) fold induction from baseline control of 0.3 (0.3, 0.8) which was not significantly different from cells treated with medium alone or UV inactivated RV-16 but was significantly less when compared to healthy controls 3.6 (3.4, 3.6) (p=0.004). Panel (b): Release of IFNβ into culture supernatants 48h post infection was measured by ELISA. For asthmatic BECs, median (IQR) IFNβ levels were 721 (464, 1290)pg/ml, compared to 1854 pg/ml (758, 3766) (p=0.03) in healthy controls. Both groups demonstrated a significant increase above cells treated with medium alone (56.4pg/ml, p<0.001) and UV inactivated RV-16 (113.8pg/ml, P<0.01). Panel (c): The effect of IFNβ on induction of apoptosis in RV-16 infected asthmatic cells was measured by FACS analysis as described in the legend to Figure 3. Asthmatic cells were either pre-treated with IFNβ (100IU) for 12h or exposed to RV-16 and then treated with IFN-β. To mimic the presence of viral RNA, cells were also exposed to poly(I):poly(C) a synthetic double stranded RNA oligonucleotide, instead of RV-16. Results are expressed as the fold induction in apoptosis seen above control cells treated with medium alone. There was significant increase in apoptosis in cells exposed to either IFN-β or RV-16 alone (median (IQR) induction of apoptosis = 1.11 (0.99, 1.94) or 1.57 (0.98, 1.98), respectively. Cells treated with RV-16 and IFNβ together showed a tendency to increased apoptosis (median (IQR) =3.75 (1.12, 5.25); p=0.11) while those pre-treated with IFN-β and then infected had a significant increase in induction of apoptosis (median (IQR) = 5.69 (2.19, 5.69)). Cells exposed to poly(I):poly(C) alone showed a small increase in apoptosis (median (IQR) = 1.92 (1.34, 4)) which was enhanced by treatment with IFN-β (median (IQR) = 5.56 (3.15, 5.56)) or pre-treatment with IFN-β (median (IQR) = 9.25 (3.46, 9.25); p< 0.05). Panel (d): The effect of IFNβ on viral yield from asthmatic cells was measured by HeLa titration assay using asthmatic BEC culture supernatants removed after 48h of infection. Cells were either pre-treated with IFNβ (100IU) for 12h and then exposed to RV-15 or were treated with IFNβ immediately following infection. There was a significant reduction in viral yield seen in cells treated with IFNβ following infection median log $TCID_{50}$ 2.78 (2, 3.56) and a further reduction in cells pre-treated with

IFNβ 1.12 (0.28, 1.34) compared to cells infected with RV-16 alone 3.56 (3.5-3.62) ($p<0.05$). * = significantly different from medium alone and asthmatic cells treated with RV-16. ** = significantly different from medium alone. # = significantly different from RV-16 infection alone. ## = significantly different from poly(I):poly(c) alone.

Figure 6 shows induction of IFN-β mRNA 8 hours after infection of primary BEC cultures from a non-COPD volunteer and a COPD patient with RV-16 (2moi). IFN-β mRNA was measured by reverse transcription quantitative PCR and normalised to IFN-β levels in untreated (SFM) controls.

Figure 7 shows a comparison of viral replication 24 hours after RV-16 infection (2moi) of BEC cultures from a non-COPD and a COPD patient. Virion production was measured as $TCID_{50}$/ml as determined by HeLa cell titration assay.

Figure 8 shows induction of IFN-β mRNA 8 hours after infection of primary BECs from a COPD patient with RV-16 (2moi) in the absence or presence of exogeneous IFN-β. IFN-β mRNA was measured by reverse transcription quantitative PCR and normalised to IFN-β levels in untreated (SFM) controls.

Figure 9 shows that IFN-β reduced RV-16 replication in BECs from a COPD patient. Cells were infected with RV-16 (2moi) in the absence or presence of exogeneous IFN-β (100 IU/ml). Virion production was measured as $TCID_{50}$/ml by HeLA cell titration assay.

Brief description of the Sequence Listing

SEQ ID NO: 1 shows the nucleotide sequence of human IFNβ-1a.

SEQ ID NO: 2 shows the amino acid sequence of human IFNβ-1a.

SEQ ID NO: 3 shows the nucleotide sequence of human IFNβ-1b.

SEQ ID NO: 4 shows the amino acid sequence of human IFNβ-1b. IFNβ-1b is identical to human IFNβ-1a except for replacement of the cysteine at residue 17 with serine.

Detailed description of the invention

As hereinbefore indicated, the present invention relates to new therapeutic uses for IFN-β. In particular, it relates, for example, to therapeutic use of IFN-β by airway delivery to promote apoptosis in bronchial epithelial cells of asthmatic patients

infected with rhinovirus. The invention as presented also extends to airway delivery of IFN-β to treat rhinovirus–induced exacerbation of COPD.

Definition of IFN-β

The term IFN-β as used herein will be understood to refer to any form or analog of IFN-β that retains the biological activity of native IFN-β and preferably retains the activity of IFN-β that is present in the lung and, in particular, the bronchial epithelium.

The IFN-β may be identical to or comprise the sequence of human IFNβ-1a (SEQ ID NO: 2) or human IFNβ-1b (SEQ ID NO: 4). IFN-β also refers to a variant polypeptide having an amino acid sequence which varies from that of SEQ ID NO: 2 or 4. Alternatively, IFN-β may be chemically-modified.

A variant of IFN-β may be a naturally occurring variant, for example a variant which is expressed by a non-human species. Also, variants of IFN-β include sequences which vary from SEQ ID NO: 2 or 4 but are not necessarily naturally occurring. Over the entire length of the amino acid sequence of SEQ ID NO: 2 or 4, a variant will preferably be at least 80% homologous to that sequence based on amino acid identity. More preferably, the polypeptide is at least 85% or 90% and more preferably at least 95%, 97% or 99% homologous based on amino acid identity to the amino acid sequence of SEQ ID NO: 2 or 4 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, amino acid identity over a stretch of 40 or more, for example 60, 80, 100, 120, 140 or 160 or more, contiguous amino acids ("hard homology").

Homology may be determined using any method known in the art. For example the UWGCG Package provides the BESTFIT program which can be used to calculate homology, for example used on its default settings (Devereux *et al* (1984) *Nucleic Acids Research* 12, p387-395). The PILEUP and BLAST algorithms can be used to calculate homology or line up sequences (such as identifying equivalent residues or corresponding sequences (typically on their default settings)), for example as described in Altschul S. F. (1993) J Mol Evol 36:290-300; Altschul, S.F *et al* (1990) J Mol Biol 215:403-10.

Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pair (HSPs) by identifying

short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighbourhood word score threshold (Altschul *et al*, supra). These initial neighbourhood word hits act as seeds for initiating searches to find HSP's containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extensions for the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1992) *Proc. Natl. Acad. Sci.* USA 89: 10915-10919) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

The BLAST algorithm performs a statistical analysis of the similarity between two sequences; see e.g., Karlin and Altschul (1993) *Proc. Natl. Acad. Sci.* USA 90: 5873-5787. One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P (N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a sequence is considered similar to another sequence if the smallest sum probability in comparison of the first sequence to the second sequence is less than about 1, preferably less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001.

Amino acid substitutions may be made to the amino acid sequence of SEQ ID NO: 1 or 2, for example from 1, 2, 3, 4 or 5 to 10, 20 or 30 substitutions. Conservative substitutions may be made, for example, according to Table 1. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

Table 1 – Conservative amino acid substitutions

NON-AROMATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar - charged	D E
		H K R
AROMATIC		H F W Y

One or more amino acid residues of the amino acid sequence of SEQ ID NO: 1 or 2 may alternatively or additionally be deleted. From 1, 2, 3, 4 or 5 to 10, 20 or 30 residues may be deleted, or more.

IFN-β also includes fragments of the above-mentioned sequences. Such fragments retain IFN-β activity. Fragments may be at least from 120 or 140 amino acids in length. Such fragments may be used to produce chimeric agents as described in more detail below.

IFN-β includes chimeric proteins comprising fragments or portions of SEQ ID NO: 2 or 4. One or more amino acids may be alternatively or additionally added to the polypeptides described above. An extension may be provided at the N-terminus or C-terminus of the amino acid sequence of SEQ ID NO: 2 or 4 or polypeptide variant or fragment thereof. The extension may be quite short, for example from 1 to 10 amino acids in length. Alternatively, the extension may be longer. A carrier protein may be fused to an amino acid sequence described above. A fusion protein incorporating one of the polypeptides described above can thus be used in the invention.

IFN-β also includes SEQ ID NO: 2 or 4 or variants thereof that have been chemically-modified. A number of side chain modifications are known in the art and may be made to the side chains of the proteins or peptides discussed above. Such modifications include, for example, glycosylation, phosphorylation, modifications of amino acids by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$, amidination with methylacetimidate or acylation with acetic anhydride. The modification is preferably glycosylation.

The IFN-β may be made synthetically or by recombinant means using methods known in the art. The amino acid sequence of proteins and polypeptides may be modified to include non-naturally occurring amino acids or to increase the stability of

the compound. When the proteins or peptides are produced by synthetic means, such amino acids may be introduced during production. The proteins or peptides may also be modified following either synthetic or recombinant production.

The IFN-β may also be produced using D-amino acids. In such cases the amino acids will be linked in reverse sequence in the C to N orientation. This is conventional in the art for producing such proteins or peptides.

The IFN-β may be produced in a cell by *in situ* expression of the polypeptide from a recombinant expression vector. The expression vector optionally carries an inducible promoter to control the expression of the polypeptide. The IFN-β or analog thereof may be produced in large scale following purification by any protein liquid chromatography system after recombinant expression. Preferred protein liquid chromatography systems include FPLC, AKTA systems, the Bio-Cad system, the Bio-Rad BioLogic system and the Gilson HPLC system.

Commercially available forms of IFN-β or analogs thereof may be used in the invention. Examples include Betaseron® and Avonex®.

Agents that increase IFN-β expression

The invention may also involve using an agent that increases endogenous expression of IFN-β in the lung or preferably the bronchial epithelium. The agents may act directly on the promoter or other regulatory sequences of the IFN-β gene. Such agents may act to reduce the constitutive silencing of the IFN-β promoter. Alternatively, the agent may stimulate cells to produce endogenous IFN-β by acting at receptors at the cell surface. Agents that increases endogenous expression of IFN-β of interest in relation to the present invention include, but are not limited to, poly(inosinic acid)-poly(cytidylic acid) (poly(IC)) and the ACE inhibitor perindopril.

Polynucleotides

The invention may also involve using a polynucleotide which is capable of expressing IFN-β or an agent that increases endogenous expression of IFN-β in lung airways. Such a polynucleotide may preferably be in the form of a vector capable of directing expression of IFN-β or an agent that induces IFN-β in the bronchial epithelium. The resulting IFN-β or agent may then have a therapeutic effect ("gene therapy"). The polynucleotide may encode any of the forms of IFN-β discussed above including the variants, fragments and chimeric proteins thereof.

The polynucleotide encoding IFN-β may comprise the human sequence (SEQ ID NO: 1 or 3) or a naturally occurring sequence variant, for example a variant which is expressed by a non-human species. Also, a polynucleotide encoding IFN-β include sequences which vary from SEQ ID NO: 1 or 3 but are not necessarily naturally occurring. Over the entire length of the amino acid sequence of SEQ ID NO: 1 or 3, a variant will preferably be at least 80% homologous to that sequence based on nucleotide identity. More preferably, the polynucleotide is at least 85% or 90% and more preferably at least 95%, 97% or 99% homologous based on nucleotide identity to the nucleotide of SEQ ID NO: 1 or 3 over the entire sequence. There may be at least 80%, for example at least 85%, 90% or 95%, nucleotide identity over a stretch of 40 or more, for example 60, 80, 100, 120, 140 or 160 or more, contiguous nucleotides ("hard homology"). Homology may be determined as discussed above.

The polynucleotides may comprise DNA or RNA but preferably comprise DNA. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to polynucleotides are known in the art. These include methylphosphate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art.

Polynucleotides such as a DNA polynucleotide may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques. The polynucleotides are typically provided in isolated and/or purified form.

Polynucleotides will generally be produced using recombinant means, for example using PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15-30 nucleotides) to a region of the required gene which it is desired to clone, bringing the primers into contact with DNA obtained from a suitable cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al*, 1989.

As hereinbefore indicated, preferably the polynucleotide is used in an expression vector wherein it is operably linked to a control sequence which is capable of providing for the expression of the coding sequence in the airways of human lung.

Expression vectors for use in accordance with the invention may be any type of vector conventionally employed for gene therapy. It may be a plasmid expression vector administered as naked DNA or complexed with one or more cationic amphiphiles, e.g one or more cationic lipids, e.g. in the form of DNA/liposomes. A viral vector may alternatively be employed. Vectors for expression of therapeutic proteins in the airways of human lung have previously been described. For example, Published International Application WO 01/91800 (Isis Innovation Limited) describes for such purpose expression vectors including the human ubiquitin C promoter or functional analogues thereof. The human ubiquitin C promoter has been shown to be capable of producing high level protein expression in the airways of mice over many weeks and hence has been proposed as a favoured promoter for use in airway gene therapy for a variety of respiratory diseases. Examples of expression vectors for use in directing transgene expression in airway epithelia have also been described in Chow et al. Proc. Natl. Acad. Sci. USA 1997; 94: 14695-14700. Such expression vectors can be administered via the airways, e.g into the nasal cavity or trachea.

Virally-induced exacerbations of respiratory disease

In the present invention, an apoptosis-inducing agent is used to treat virally-induced exacerbations of respiratory disease. A virally-induced exacerbation of a respiratory disease is an increase in the severity of a respiratory disease that results from the presence of a virus, such as rhinovirus. The virus typically leads to a worsening of the symptoms associated with the respiratory disease, a reduced response to therapy and in some cases hospitalisation. The virus typically infects the lung tissue, including or especially the bronchial epithelium. Generally, the virus results in the release of inflammatory mediators and increased bronchial responsiveness. As hereinbefore indicated, rhinovirus is recognised as a common pathogen trigger of asthma exacerbation. Similarly, rhinovirus may promote undesirable exacerbation of other respiratory diseases. Thus, respiratory diseases of interest in relation to the present invention also include conditions which may be labelled COPD

Therapy

Administration of IFN-β, an agent that increases IFN-β expression or a polynucleotide as discussed above may be either for prophylactic or therapeutic purpose. When provided prophylactically, the IFN-β, agent or polynucleotide is provided in advance of any exacerbation. The prophylactic administration of the IFN-β, agent or polynucleotide serves to prevent or attenuate any subsequent exacerbation. When provided therapeutically the IFN-β, agent or polynucleotide is provided at (or shortly after) the onset of a symptom of the exacerbation. The therapeutic administration of the IFN-β, agent or polynucleotide serves to attenuate any actual exacerbation. The individual treated may be any animal, but preferably the individual treated will be a human, most preferably an asthmatic human.

The IFN-β, agent or polynucleotide may be administered in a medicament or pharmaceutical composition suitable for airway delivery which will typically also include a pharmaceutically acceptable excipient. Such an "excipient" generally refers to a substantially inert material that is nontoxic and does not interact with other components of the composition in a deleterious manner.

Pharmaceutically acceptable excipients include, but are not limited to, liquids such as water, saline, polyethyleneglycol, hyaluronic acid, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulphates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

It is also preferred, although not required, that a composition or medicament comprising the therapeutic agent will contain a pharmaceutically acceptable carrier that serves as a stabilizer, particularly for peptide, protein, polynucleotide or other like agents. Examples of suitable carriers that also act as stabilizers for peptides include, without limitation, pharmaceutical grades of dextrose, sucrose, lactose, trehalose, mannitol, sorbitol, inositol, dextran, and the like. Other suitable carriers include, again without limitation, starch, cellulose, sodium or calcium phosphates, citric acid, tartaric acid, glycine, high molecular weight polyethylene glycols (PEGs), and combination thereof. It may also be useful to employ a charged lipid and/or detergent. Suitable charged lipids include, without limitation, phosphatidylcholines (lecithin), and the like. Detergents will typically be a nonionic, anionic, cationic or amphoteric surfactant. Examples of suitable surfactants include, for example, Tergitol® and

Triton® surfactants (Union Carbide Chemicals and Plastics, Danbury, CT), polyoxyethylenesorbitans, for example, TWEEN® surfactants (Atlas Chemical Industries, Wilmington, DE), polyoxyethylene ethers, for example Brij, pharmaceutically acceptable fatty acid esters, for example, lauryl sulfate and salts thereof (SDS), and like materials. A thorough discussion of pharmaceutically acceptable excipients, carriers, stabilizers and other auxiliary substances is available in Remingtons Pharmaceutical Sciences (Mack Pub. Co., N. J.1991).

A suitable composition for airway delivery of IFN-β may, for example, be formulated as described in US Patent no 6,030, 609 by dissolving lyophilised IFN-β in a pharmaceutically acceptable vehicle such as sterile distilled water or sterile physiological saline, optionally with addition of one or more carriers, stabilizers, surfactants or other agents in order to enhance effectiveness of the IFN-β active agent.

A composition comprising a prophylactically or therapeutically effective amount of the IFN-β, agent or polynucleotide described herein may conveniently be delivered to the lung airways by means of an aerosol nebuliser. An appropriate effective amount may be determined by appropriate clinical testing and will vary with for example the activity of the IFN-β administered or induced. The IFN-β, agent or polynucleotide may for example, be administered in microgram amounts. They are administered to the subject to be treated in a manner compatible with the dosage formulation, and in an amount that will be effective to bring about the desired effect. The amount to be delivered may be 1μg to 5 mg, for example 1 to 50μg, depending on the subject to be treated. The exact amount necessary will vary depending on the age and general condition of the individual being treated and agent selected, as well as other factors. For example, 250 μg of IFN-β may be administered every alternate day or 30μg of IFN-β may be administered weekly (Cook, J Neurol, 2003; 250 Suppl 4: 15-20; Durelli, J Neurol 2003; 250 Suppl 4: 9-14).

The IFN-β, agent or polynucleotide may be administered on its own or in combination with another therapeutic compound. In particular, the IFN-β, agent or polynucleotide may be administered in conjunction with a therapeutic compound used to treat the respiratory disease in the individual. The IFN-β, agent or polynucleotide and additional therapeutic compound may be formulated in the same or different compositions. In one embodiment, the IFN-β, agent or polynucleotide is administered to an individual with asthma in combination with an inhaled corticosteroid. The IFN-

β, agent or polynucleotide may be administered simultaneously, sequentially or separately with an inhaled corticosteroid.

Thus, in a further aspect of the present invention there is provided a product for treatment of asthma comprising for simultaneous, separate or sequential airway administration (i) a first agent selected from (a) IFN-β, (b) an agent that increases IFN-β expression and (c) a polynucleotide capable of expressing (a) or (b) and (ii) an inhaled corticosteriod. Preferably, such a product will provide for simultaneous, separate or sequential administration of IFN-β and an inhaled corticosteroid, for example, fluticasone, beclomethasone and budesonide.

A first agent as defined above and an inhaled corticosteriod may, for example, be provided in the form of a single pharmaceutical composition suitable for aerosol delivery to the airways.

The following examples are provided to illustrate the invention with respect to treatment of both rhinovirus-induced exacerbations of asthma and COPD.

Examples

Example 1: Study of bronchial epithelial cells from asthma patients

Materials and Methods

Subjects

All subjects were non-smokers, with no exacerbation of their lung disease or history of respiratory tract infection in the preceding 4 weeks. Allergy skin tests using a panel of common aero-allergens including house dust mite extract, grass pollen, tree pollen, cat dander, dog dander, *Candidia, Aspergillus* as well as negative (saline) and positive controls (histamine) controls. Tests were considered positive if there was a wheal response of 3mm or greater than the negative control. Lung function was assessed by spirometry, measuring forced expiratory volume in 1 second (FEV_1) and forced vital capacity (FVC). Bronchial hyper responsiveness was then assessed by histamine challenge, defined by a PC_{20} histamine less than 8mg/ml. Subjects with asthma were subdivided on a basis of clinical severity in accordance with the GINA guidelines (National, H., Lung and Blood Institute. Global strategy for asthma management and prevention 96-3659a, Bethseda, 1995).

Asthma was diagnosed on a consistent history with evidence of bronchial hyper responsiveness, defined by a PC_{20} histamine less than 8mg/ml. Asthmatic

subjects were classed as mild, with stable symptoms requiring treatment with salbutamol only as needed, less than 3 times per week and with moderate disease, with stable symptoms on inhaled beclomethasone of less than 1500µg per day. Healthy controls had no previous history of lung disease, normal lung function, no evidence of bronchial hyper responsiveness on histamine challenge and were non-atopic. The study was approved by the relevant ethics committees. All subjects gave written informed consent.

Table 2 outlines the characteristics of the subjects used in the studies. FEV_1 % predicted refers to the forced expiratory volume in 1 second expressed as a percentage of the predicted value. ICS refers to inhaled corticosteroids. Dose is expressed in dose of beclomethasone (BDP) in µg per day where 1µg BDP =1µg Budesonide or 0.5µg Fluticasone.

Table 2 – Subjects used in the studies.

	Asthma	**Healthy controls**	**P values**
Number	14	10	NA
Sex (% male)	69%	60%	P=0.6
Mean age (range)	32 (21-58)	29 (24-38)	P=0.4
Mean FEV_1 % predicted (sd)	77.3 (15.5)	110.3 (13.6)	P< 0.001
Mean dose of ICS, BDPµg/day (sd)	490 (260)	0	NA

Tissue culture

Epithelial cells were obtained by fibreoptic bronchoscopy in accordance with standard published guidelines, all subjects were premedicated with salbutamol (Hurd, J Allergy Clin Immunol, 1991; 88: 808-814) and cell culture was performed as previously described (Bucchieri, *et al.*, Am. J. Respir. Cell Mol. Biol., 2001; 27: 179-185). In brief cells were obtained using a sheathed nylon cytology brush by taking 5-10 brushings from second to third generation bronchi under direct vision. Primary cultures were established by seeding freshly brushed bronchial epithelial cells into culture dishes. Cells were cultured at 37°C and 5% carbon dioxide in hormonally supplemented bronchial epithelial growth medium (BEGM; Clonetics, San Diego, USA) containing 50U/ml penicillin and 50µg/ml streptomycin. Cells were cultured and passaged into tissue culture flasks using trypsin. At passage 2 cells were seeded

onto 12 well trays and cultured until 80% confluent (Bucchieri, *et al.*, Am J Respir Cell Mol Biol, 2001; 27: 179-185). Epithelial cell purity was checked by differential cell counts on cytospins of the harvested cells.

Cells were also treated alone or following infection with the major group RV-16. After infection cells were also treated with the caspase 3 inhibitor ZVD-fmk at 120μM (Calbiochem, La Jolla, CA, USA) and human IFNβ at 100IU (Sigma Chemical St Louis MO, USA).

Preparation and infection with RV

We generated RV-16 stocks by infecting cultures of Ohio HeLa cells as previously described (Papi and Johnston, J Biol Chem, 1999; 274: 9707-9720); cells and supernatants were harvested, cells were disrupted by freezing and thawing, cell debris was pelleted by low speed centrifugation and the clarified supernatant frozen at -70^0C.

RV titration was performed by exposing confluent monolayers of HeLa cells in 96-well plates to serial 10-fold dilutions of viral stock and cultured for 5 days at 37^0C in 5% CO_2. Cytopathic effect was assessed and the tissue culture infective dose of 50% ($TCID_{50}$/ml) was then determined and the multiplicity of infection (MOI) derived (Papi and Johnston, J Biol Chem, 1999; 274: 9707-9720). As a negative control for all experiments RV-16 was inactivated by exposure to UV irradiation at 1200μJ/cm^2 UV light for 30 minutes. Inactivation was confirmed by repeating viral titrations in HeLa cells.

The desired concentration of RV-16 was applied to cells that were gently shaken at 150rpm at room temperature for 1 hour. The medium was then removed and the wells washed twice with 1ml Hanks Balanced Salt Solution. Fresh medium was then applied and the cells cultured at 37.5^0C and 5% CO_2 for the desired time. As negative controls cells were treated with medium alone and UV inactivated RV-16.

Confirmation of infection of epithelial cells and quantification of viral production was assessed by HeLa titration assay (Papi and Johnston, J Biol Chem, 1999; 274: 9707-9720) and quantitative reverse transcription polymerase chain reaction (qPCR), as described below.

Analysis of cell viability

Viability and apoptosis were assessed by flow cytometry as previously described (Puddicombe *et al.*, Am J Respir Cell Mol Biol, 2003; 28: 61-68). Briefly 8h after RV infection, adherent cells were removed with trypsin and added to non-adherent cells. Cells were stained with Annexin-V conjugated to the flurochrome Phycoerythrin (PE) and the vital dye 7-Amino-actinomycin (7-AAD). Flow cytometric data were analysed using WinMDI 2.8. The active forms of caspase 3/7 were detected using the Apo-One Homogenous Caspase 3/7 assay (Promega, Maddison, USA). Cells were plated in quadruplicate for each condition. Two wells were stained with methylene blue and cell biomass estimated. The other two wells were lysed with lysis buffer and read on a fluorescent plate reader with an excitation wavelength of 485nm and emission of 530. Caspase activity was then corrected for cell biomass. Cell lysis was measured by determining the activity of lactate dehydrogenase (LDH) in the cell supernatant that had been removed and stored at room temperature for no longer than 48 hours. The LDH activity was measured at 37°C by an enzymatic rate method, using pyruvate as a substrate (Sigma, St Louis USA).

Reverse transcription quantitative PCR

Analysis of gene expression for IL-8, TNFα, ICAM-1, IFNβ and RV was carried out using RNA extracted from BECs using TRIzol reagent (Life Technologies, Paisley, UK); contaminating DNA was removed by deoxyribonuclease digestion on RNeasy Mini Kits (Qiagen, Crawley, West Sussex, UK) in accordance with manufacturer's instructions. Total RNA (1μg) was reverse transcribed using random hexamers or oligo $(dT)_{15}$ primers and avian myeloblastosis virus transcriptase from the Reverse Transcription System (Promega, Southampton, UK), following the manufacturer's protocol. Fluorogenic probes were labelled with the 5'-reporter dye 6-carboxy-fluorescein (FAM) and the 3'-quencher dye 6-carboxy-*N,N,N',N'*-tetramethyl-rhodamine (TAMRA).

Housekeeping gene primers and probe for 18S ribosomal RNA was obtained from Eurogentech (Eurogentech, Southampton, UK). No-template controls and reverse transcription-negative samples were also included as controls. The icycler PCR protocol was as follows: 95°C for 8min; followed by 42 cycles of denaturation at 95°C for 15 seconds followed by annealing at 60°C for 1 min and extension at 72°C

for 15 seconds. Quantitation and real-time detection of the PCR were followed on the on icycler sequence detection system, and after completion of the PCR, the thresholds for fluorescence emission baseline were set just above background levels on the FAM and VIC layers (~ 15 to 20 cycles). Standard curves were calculated from the delta CT and were constructed for target genes and the 18S rRNA endogenous control, and the amount of target and endogenous control were calculated. The data were normalized by using the ratio of the amount of target gene relative to endogenous control. Comparisons were made after 8 hours of infection, as this was the time of maximum mRNA induction for IL-8.

Quantification of RV-16 differed from above. The primers used to detect RV were 0.05 μM Picornavirus Forward Oligo (5'-GTG AAG AGC CCGC AGTG TGC T-3') and 0.30 μM Picornavirus Reverse Oligo (5'-GCT CGCA GGG TTA AGG TTA GCC-3'). A standard curve was constructed to quantify RV using the OL-26 - OL-27 amplicon (product of OL-26 and OL-27 primers cloned into PCR 2.1 TOPO (Invitrogen). The plasmid was grown in E. coli strain XL-1blue (Stratagene), purified by a maxiprep method using commercially available reagents (Qiagen), resuspended in Tris EDTA buffer pH 8.0 at 1ug/uL and stored at -80 C.

Expression of ICAM-1

ICAM-1 expression on cells were measured at baseline, immediately after infection and up to 24h after RV infection by flow cytometry as described above using a monoclonal antibody to ICAM-1 (eBioscience anti-human CD54) and a FITC labelled secondary (Dako, Denmark).

Measurement of inflammatory mediators by ELISA

Release of Interleukin (IL)-8 and Tumour Necrosis Factor-alpha (TNF-α) (R&D systems, Abingdon, UK) and Interferon-beta (IFN-β) (Biosource Nivelles Belgium) into culture supernatants was measured using enzyme-linked immunosorbent assays (ELISA) according to the manufacturer's instructions

Statistical analysis

Data was analysed using SPSS version 10.1 (SPSS Inc). As sample size was small and variables were not normally distributed the differences between the groups have been analysed using non-parametric tests; differences between two dependent

variables was analysed using the signed rank test, independent variables the Wilcoxon rank sum test and multiple comparisons the Kruskal Wallis test. A p value of <0.05 was considered significant.

Results

To compare responses of normal and asthmatic bronchial epithelial cells (BECs), primary cultures were grown from bronchial brushings obtained by fibreoptic bronchoscopy from clinically characterised volunteers. Dose and time courses for infection of BECs with RV-16 were optimised initially by measuring release of IL-8 in culture supernatants obtained from infected cells. From these experiments, a dose of RV-16 with an estimated MOI of 2 was selected for detailed study (data not shown).

Inflammatory response of normal and asthmatic BECs to RV-16 infection

To investigate differences between normal and asthmatic bronchial epithelial cells, we recruited 14 subjects with asthma and 10 normal healthy controls (see Table 2) to undertake fibreoptic bronchoscopy. The two subject groups were similar in terms of age and sex. All asthmatics had mild-moderate persistent symptoms and used inhaled corticosteroids regularly. The responses of the primary BEC cultures to RV-16 infection were compared first by measuring induction of IL-8 and TNFα mRNA expression and protein release (Figure 1a,c). BECs from either asthmatic or healthy controls showed a significant induction of IL-8 and TNFα mRNA 8h post RV infection and there was a significant increase in IL-8 and TNFα protein release 48h post infection (Figure 1b,d); there were no significant differences between the two groups. UV-inactivated RV did not trigger a proinflammatory response.

As cells were treated with a major group RV, susceptibility to infection would be expected to be dependent on expression of ICAM-1, the receptor for major group RV. To determine whether this differed between asthmatic and normal cells, ICAM levels were evaluated by flow cytometry. Prior to infection ICAM-1 expression was not significantly different in either group (Figure 1e). By 24h following infection, expression was similar in both groups (Figure 1f).

Infection, viral yields and cell lysis from primary bronchial epithelial cells

Following RV-16 infection of BEC cultures, recovery of viable RV was determined by transmission of infection and cytopathic effect (CPE) on Ohio HeLa

cells from the infected supernatant of BECs. CPE was not seen using supernatants obtained up to 8h after infection but after that virion yield following infection of the primary cultures, but thereafter rose steadily up to 48h. In contrast with the proinflammatory responses, asthmatic BECs had a significantly greater increase in RV-16 detected by 24h and 48h as measured by $TCID_{50}$ (Figure 2a). There was also a greater yield of RV-16 mRNA 8h post infection in asthma compared to healthy controls (Figure 2b). Given the equivalent levels of ICAM-1 expression this suggests that factors other than immediate susceptibility to infection were influencing viral yield from infected cells.

In parallel with the release of virus, there was a progressive increase in cell lysis, as measured by LDH activity, mirroring the increase in RV yield; by 48h, this was significantly greater in asthmatic cells (Figure 2c). Although there was no significant increase in LDH activity in cells treated with SFM alone at 48h, there was a small but significant increase in cells treated with UV inactivated RV-16 (data not shown), however this was small by comparison with that seen in active virus cultures. These results pointed to a link between viral yield and cell lysis and led to investigation of whether early changes in cell viability would predict viral yield.

BEC viability following RV-16 infection

As apoptosis is a natural defence that protects cells against virus replication, we characterised the nature of cell death in response to RV-16 using Annexin-V (AxV) and the nuclear stain, 7-aminoactinomycin D (7AAD), to discriminate phosphatidyl serine which has been externalised on the outer leaflet of apoptotic cells. Flow cytometric analysis revealed that there was a significant reduction in viable (ie. $AxV^-/7AAD^-$) cell number 8h following RV-16 infection of normal BECs. This was not seen in cells treated with medium alone or UV inactivated RV-16 suggesting a direct link between infection and cell death (Figure 3a). In contrast, infection of asthmatic BECs with RV-16 had a smaller effect on viability at 8h (Figure 3a). By comparing AxV+/7AAD- cells (ie. apoptotic cells) and AxV+/7AAD+ cells (ie. necrotic cells), the difference in overall viability between normal and asthmatic BECs was found to be due to a significant increase in apoptosis in the normal cultures (Figure 3b). The induction of apoptosis in infected cells was confirmed by demonstrating altered mitochondrial membrane permeability using the ApoAlert Mitochondrial Membrane sensor (Clontech, Palo Alto Ca, USA) (data not shown) and

by measuring activation of active caspase 3/7. In the latter case, there was significantly less active caspase in asthmatic BECs infected with RV-16 than normal BECs (Figure 4a).

Effects of inhibition of apoptosis and RV-16 production

As increased virion production by asthmatic BECs was associated with their ability to by-pass apoptosis, we investigated whether suppression of apoptosis in RV-16 infected normal BECs was sufficient to facilitate virion production. Thus, BECs were treated with the caspase 3 inhibitor (C3I), ZVD-fmk, before and following infection with RV-16. The inhibitor led to a marked reduction in apoptosis in the healthy control cells but had minimal effect on asthmatic cells compared to infection alone (Figure 4b). Treatment of cells from healthy controls with C3I also had a direct impact on RV-16 production, with a significant increase in transmissible infection at 48h, a similar increase was not seen in asthmatic cells treated with C3I (Figure 4c). These data provided a direct link between inhibition of early apoptosis and increased viral yield.

Evaluation of the innate anti-viral response of asthmatic epithelial cells.

To investigate the underlying mechanism linked to the abnormal anti-viral response by asthmatic BECs, we analysed expression of the type I interferon (IFN), IFN-β, which has been implicated as key regulator of apoptosis in response to virus infection (Samuel, Clin Microbiol Rev., 2001; 14: 778-809; Takaoka *et al.*, Nature., 2003; 424: 516-523). As observed with the proinflammatory cytokines, there was a significant increase in IFN-β mRNA expression by normal BECs 8h post RV-16 infection, however a similar increase was not seen in asthmatic cells (Figure 5a); there was also less IFN-β production by asthmatic cells 48h post RV-16 infection (Figure 5b). To confirm that this difference in IFN-β production was functionally relevant, we tested the ability of exogenous IFN-β to induce apoptosis in RV-16 infected asthmatic BECs. Figure 5c shows that pre-treatment of cells with IFN-β (100IU) with RV-16 caused a doubling in the number of apoptotic cells. IFN-β alone had no significant effect on the apoptotic index, but caused a marked induction of apoptosis in response to exposure to synthetic poly(I):poly(C), indicating a requirement for other signals involving recognition of double stranded RNA for commitment to apoptosis in response to IFN-β. In line with its ability to induce apoptosis of virally infected

asthmatic BEC, IFN-β caused a significant reduction in RV-16 infectious virion production (Figure 5d).

These results provide for the first time explanation for the tendency of asthmatic subjects to have lingering lower respiratory tract problems as a consequence of RV infection. Thus, regardless of asthmatic state, spread of RV from the upper to the lower respiratory tract can result in infection of bronchial epithelial cells and induction of an acute inflammatory response. While further infection is limited in non-asthmatic subjects by an innate antiviral response and induction of apoptosis in infected cells, a deficiency of IFN-β in asthma facilitates virion replication and cytolysis with adverse outcomes. These include increased risk of infection of neighbouring cells and an exaggerated inflammatory response in response to the cytolytic effects of the virus. Crucially, this defect can be restored *in vitro* by provision of exogeneous IFN-β, which can provide a brake on viral replication and minimise the self-perpetuating cycle of infection and inflammation. It follows that IFN-β, or agents that induce IFN-β, can be expected to have therapeutic utility during a virally-induced exacerbation of asthma.

Example 2: Study of bronchial epithelial cells from COPD patients

Chronic obstructive pulmonary disease is another example of an inflammatory airways disease in which the common cold virus causes exacerbations (Seemungal TA, Harper-Owen R, Bhowmik A, Jeffries DJ, Wedzicha JA.Detection of rhinovirus in induced sputum at exacerbation of chronic obstructive pulmonary disease. Eur Respir J. (2000) 16, 677-83) with those affected frequently requiring hospitalization (MacNee W. Acute exacerbations of COPD. Swiss Med Wkly. (2003) May 3; 133 (17-18):247-57). Based on the finding that bronchial epithelial cells from asthmatic subjects have a defective Type I interferon response, it was postulated that a similar deficiency in COPD could also explain the severity of lower respiratory tract symptoms in this group of patients. To investigate this possibility, archival samples of cultured bronchial epithelial cells were tested for their response to RV-16 infection. These cells were grown from bronchial brushings harvested from two subjects with

COPD (one male and one female, ages 61 and 57) and an age matched control without COPD (male, aged 64). The brushings were cultured as described for the asthma studies, except that at passage 0 the cells were cryopreserved at -170 to -180°C in BEGM medium containing 10% DMSO as a cryoprotective agent. Cryopreservation is routinely used for long-term storage of cell cultures.

When required for experimentation, the frozen cell cultures were rapidly thawed into 1ml of prewarmed BEGM and then reseeded into culture flasks containing fresh medium to allow expansion to passage 2, as for the cultures of bronchial epithelial cells from normal and asthmatic subjects described in example 1. At passage 2, the cells were seeded onto 12 well trays and cultured until 80% confluent. They were then exposed to RV-16 using the same protocols described above.

To compare the innate immune response of primary BEC cultures from a COPD and a non -COPD patient, induction of IFN-β mRNA was measured in response to infection with RV-16 (2 moi). As shown in Figure 6, the BECs from the non-COPD patient showed a 25-fold induction of IFNβ mRNA 8 hours after RV-16 infection whereas the response from the COPD BECs was less than one-third of this. Consistent with this poor innate immune response, virion production at 24 hours was an order of magnitude greater in the cells from the COPD subject (Figure 7).

It was next tested whether exogenous IFN-β could protect BECs from a COPD patient against virus replication. As shown in Figures 8 and 9, cells from a second COPD patient also showed poor induction of IFN-β in response to RV-16 infection. However, they were able to respond to exogenous IFN–β with a vigorous induction of IFN-β mRNA. This was accompanied by a marked suppression of RV-16 replication, with a one hundred fold reduction in $TCID_{50}$ which was less than that seen in the cells from the non -COPD volunteer.

These results suggest that, as found in the above-described studies of BECs from asthmatic subjects, BECs from COPD patients also have a poor innate immune response. This would help to explain why these patients have lingering lower respiratory tract problems as a consequence of RV infection. Based on the fact that IFN-β can induce its own expression and suppress RV- 16 replication, it follows that IFN-β, or agents that induce IFN-β, can be expected to have therapeutic utility during a virally-induced exacerbation of COPD, as well as asthma.

THE EMBODIMENTS OF THE INVENTION FOR WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An agent selected from:

(a) an agent that increases endogenous interferon beta (IFN-β) expression in the lung; or

(b) a polynucleotide which is capable of expressing such an agent

for use in the treatment of rhinovirus-induced exacerbation of a respiratory disease selected from asthma and chronic obstructive pulmonary disease (COPD), wherein said treatment is by airway delivery of said agent.

2. An agent for use as according to claim 1 wherein said respiratory disease is asthma.

3. An agent for use as according to claim 1 wherein said respiratory disease is COPD.

4. An agent for use according to any one of claims1 to 3 which increases endogenous IFN–β expression in the bronchial epithelium.

5. An agent for use according to claim 2 or 4 wherein said respiratory disease is asthma and wherein said agent is administered simultaneously, separately or sequentially with an inhaled corticosteroid.

Figure 1a

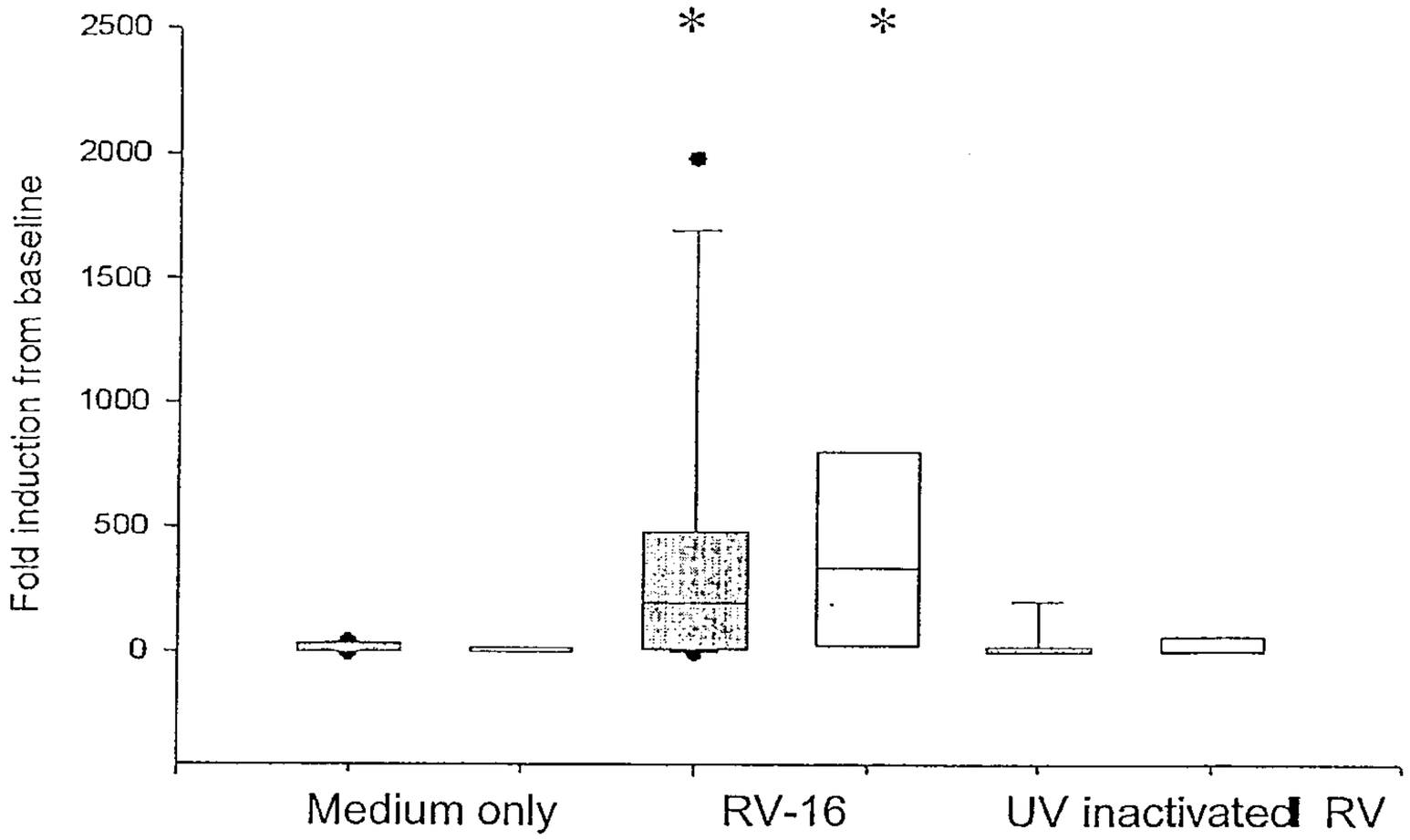


Figure 1b

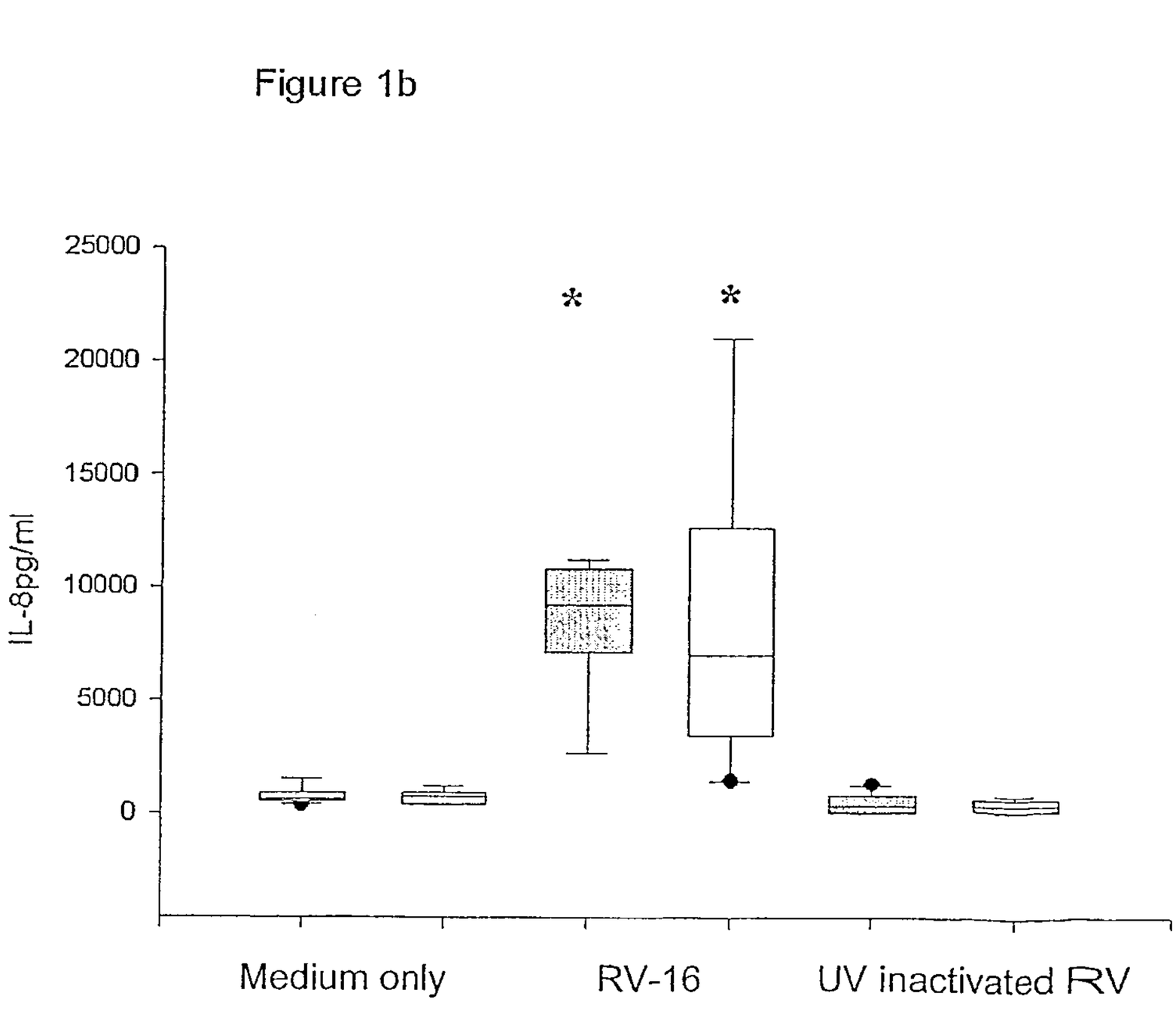


Figure 1c

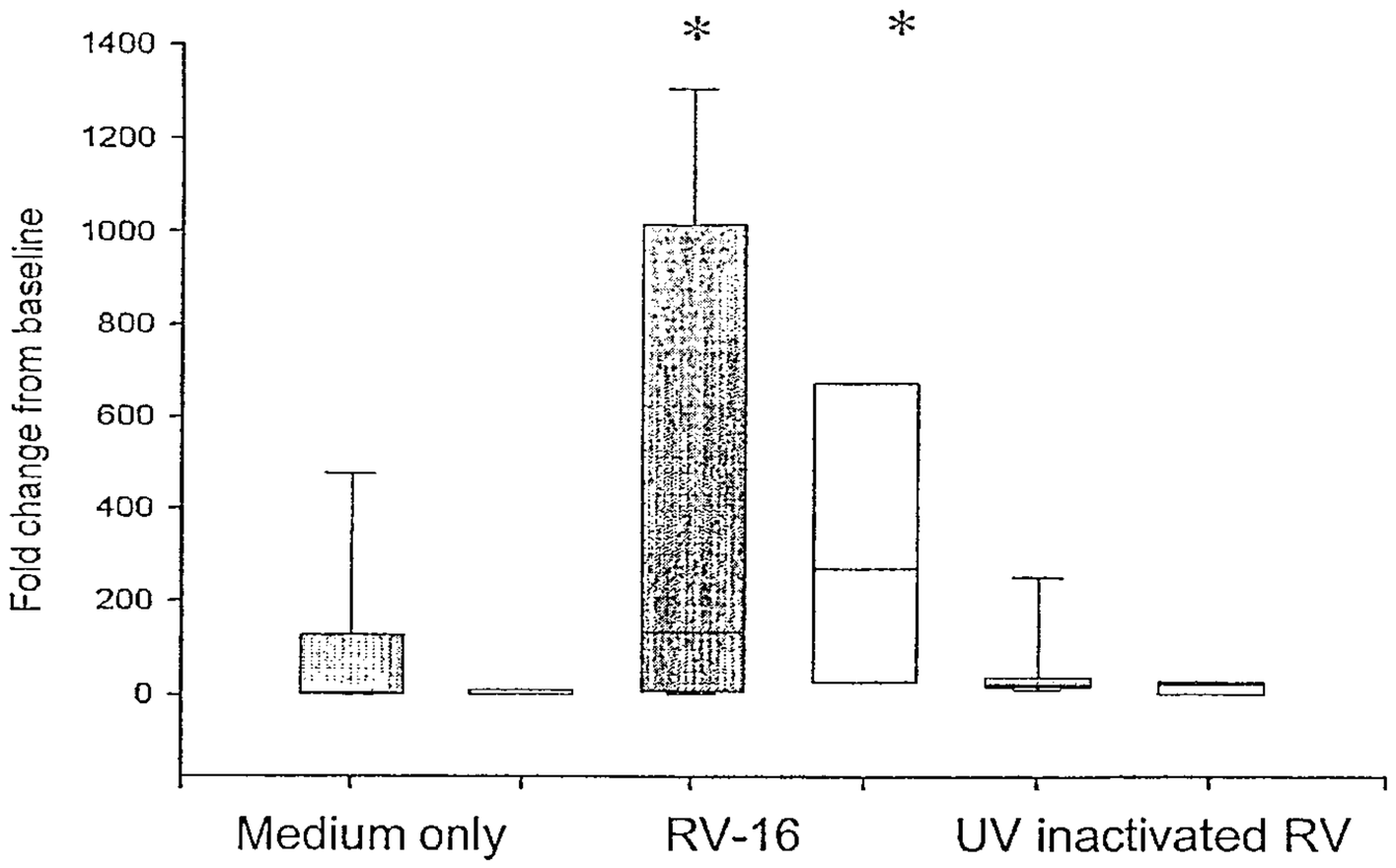


Figure 1d

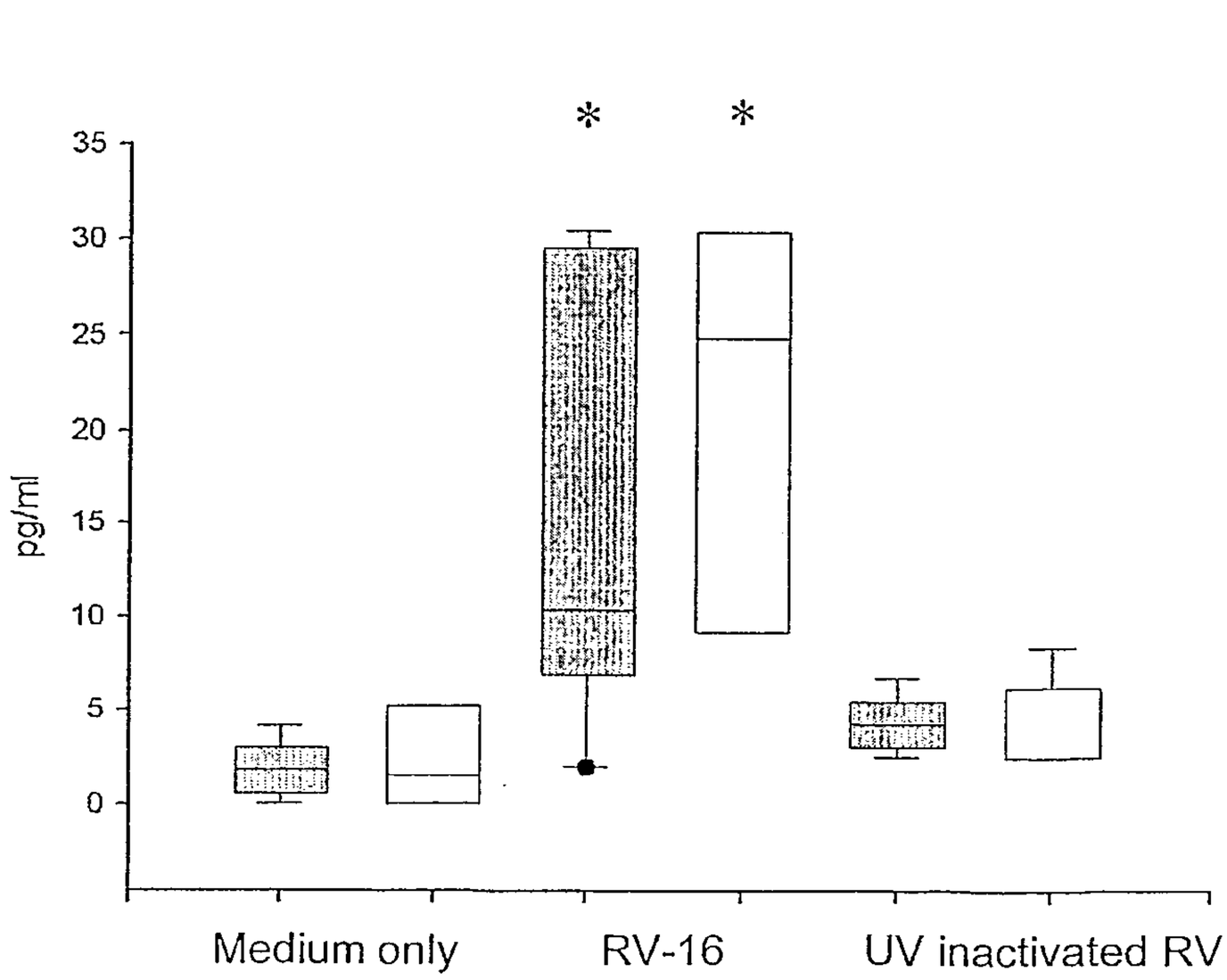


Figure 1e

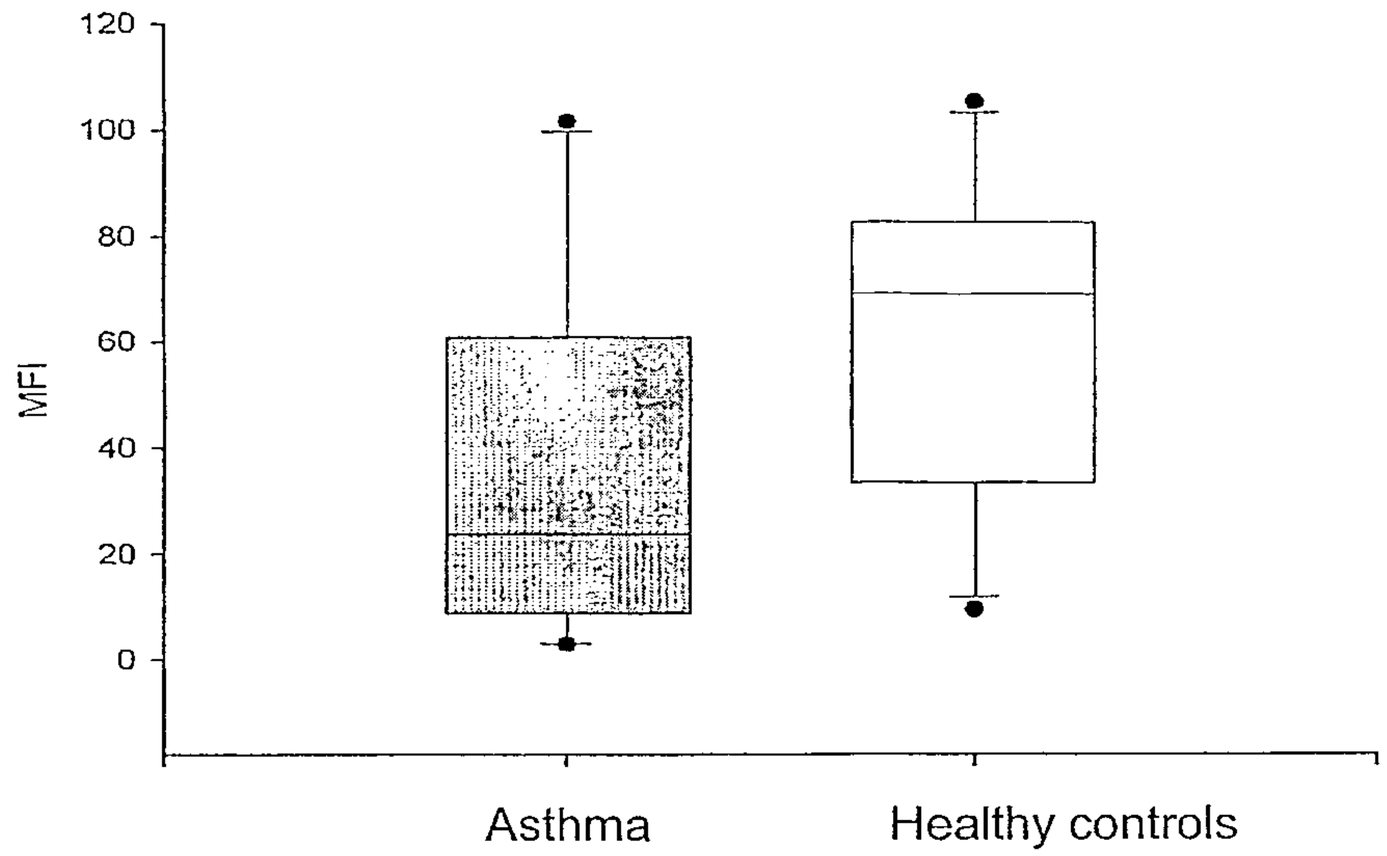


Figure 1f

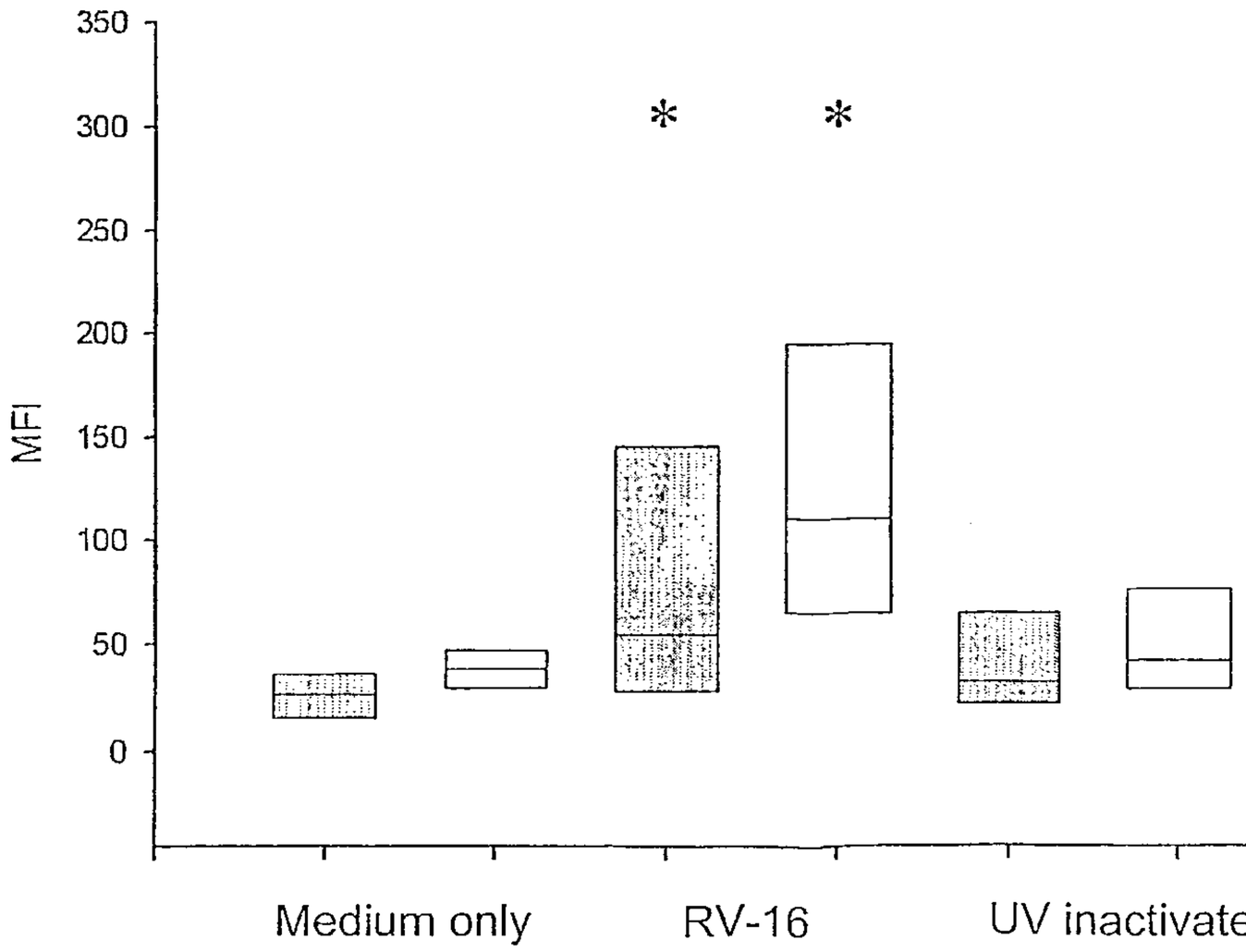


Figure 2a

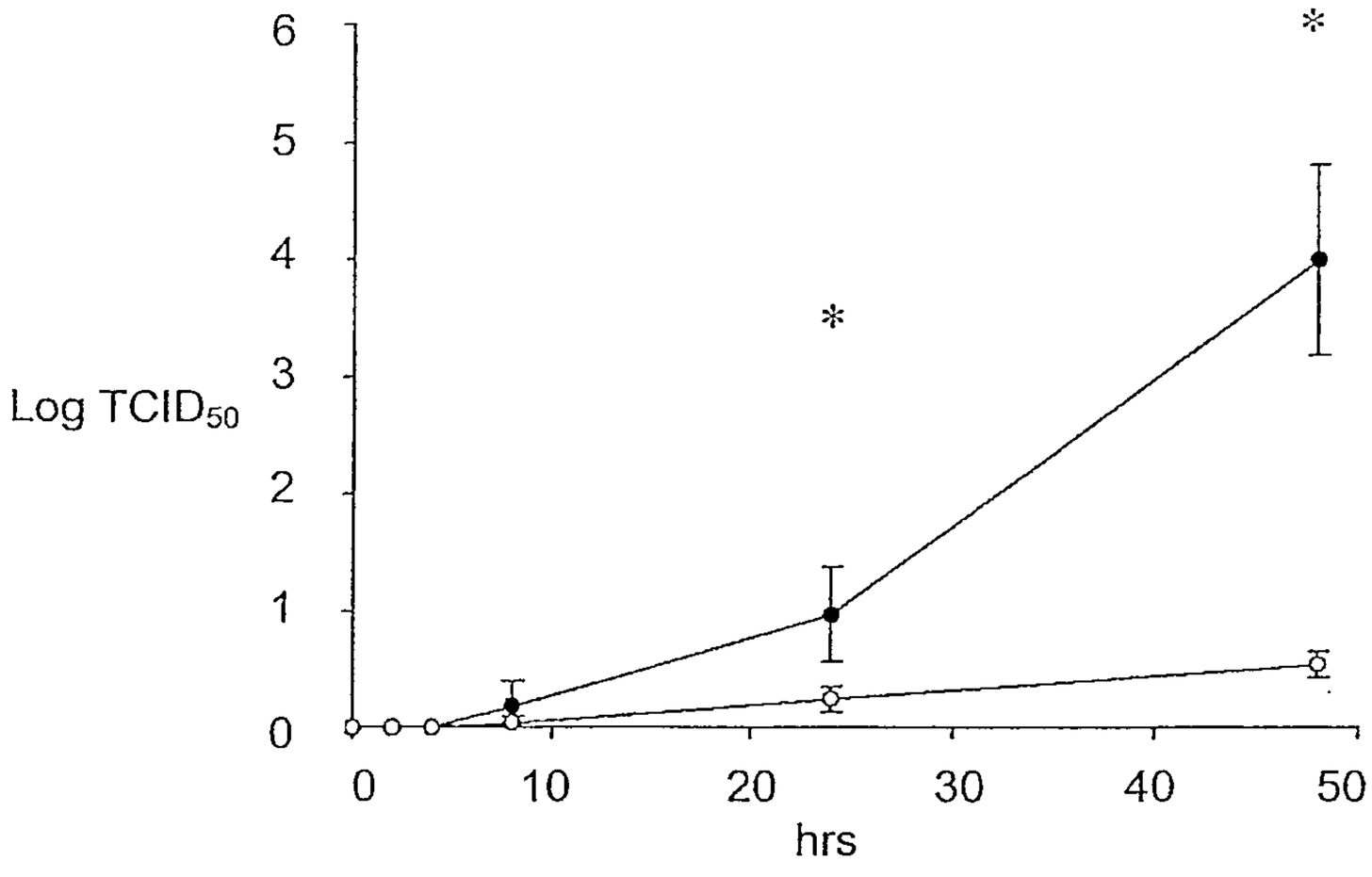


Figure 2b

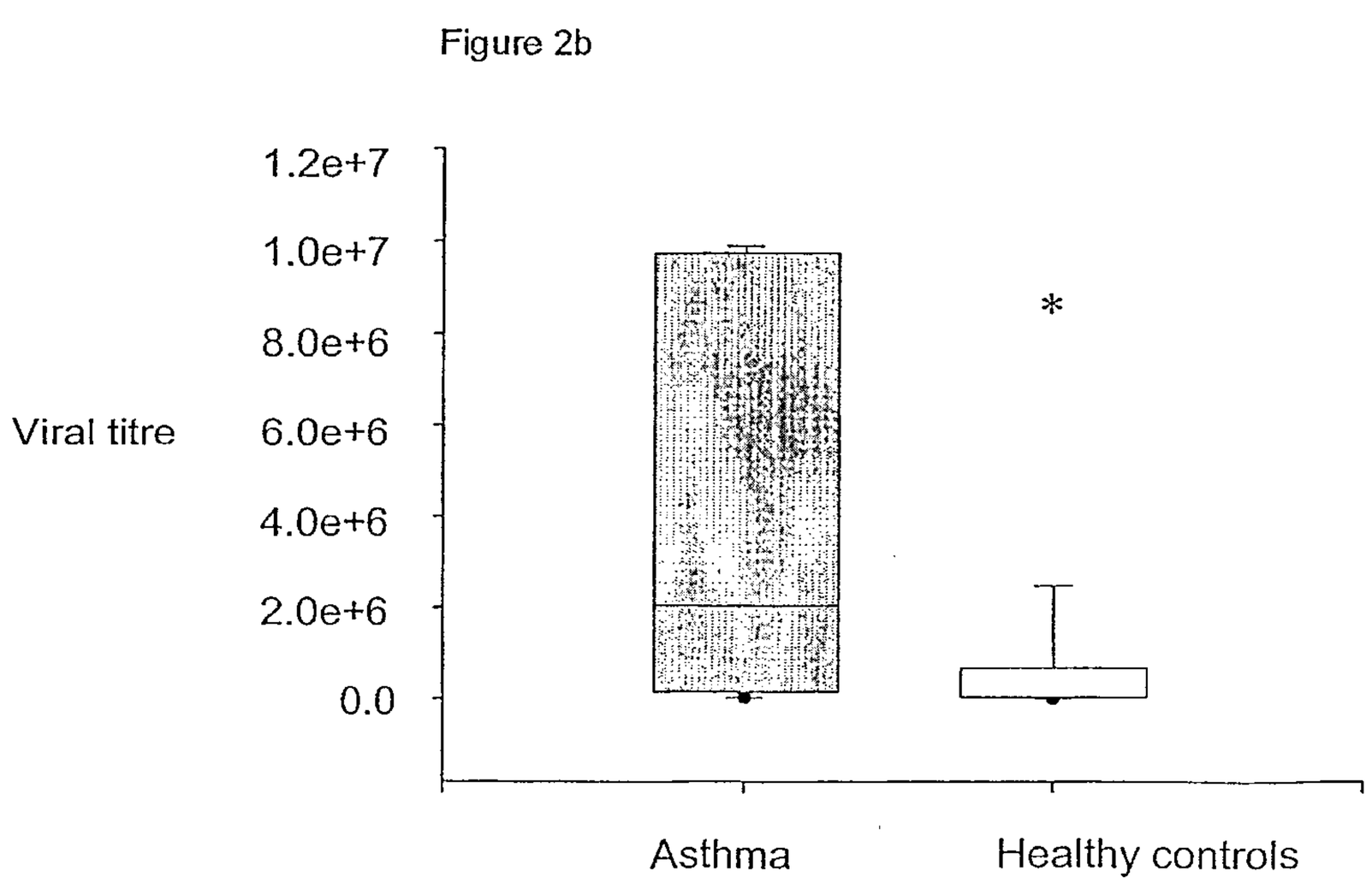


Figure 2c

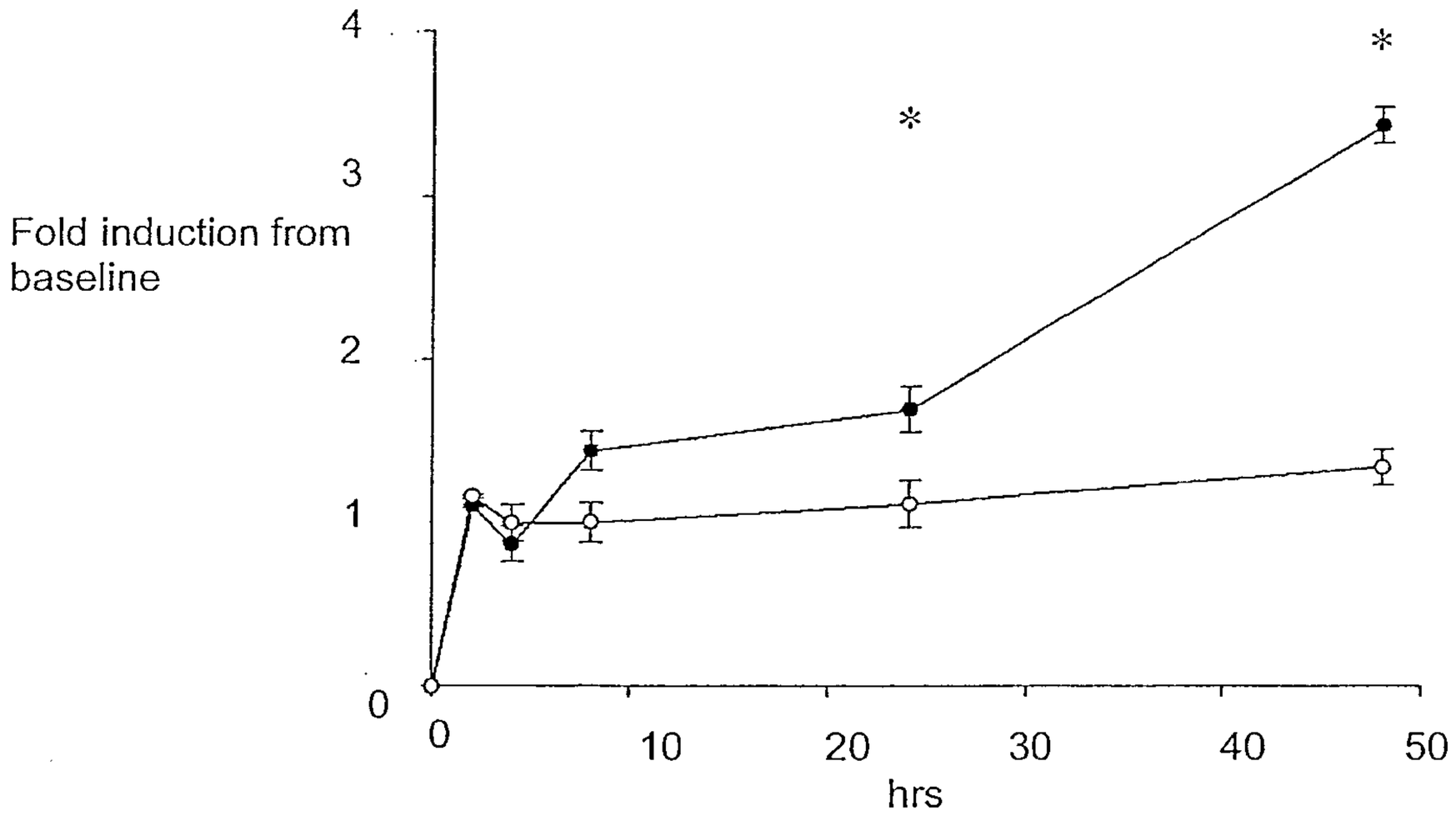


Figure 2d

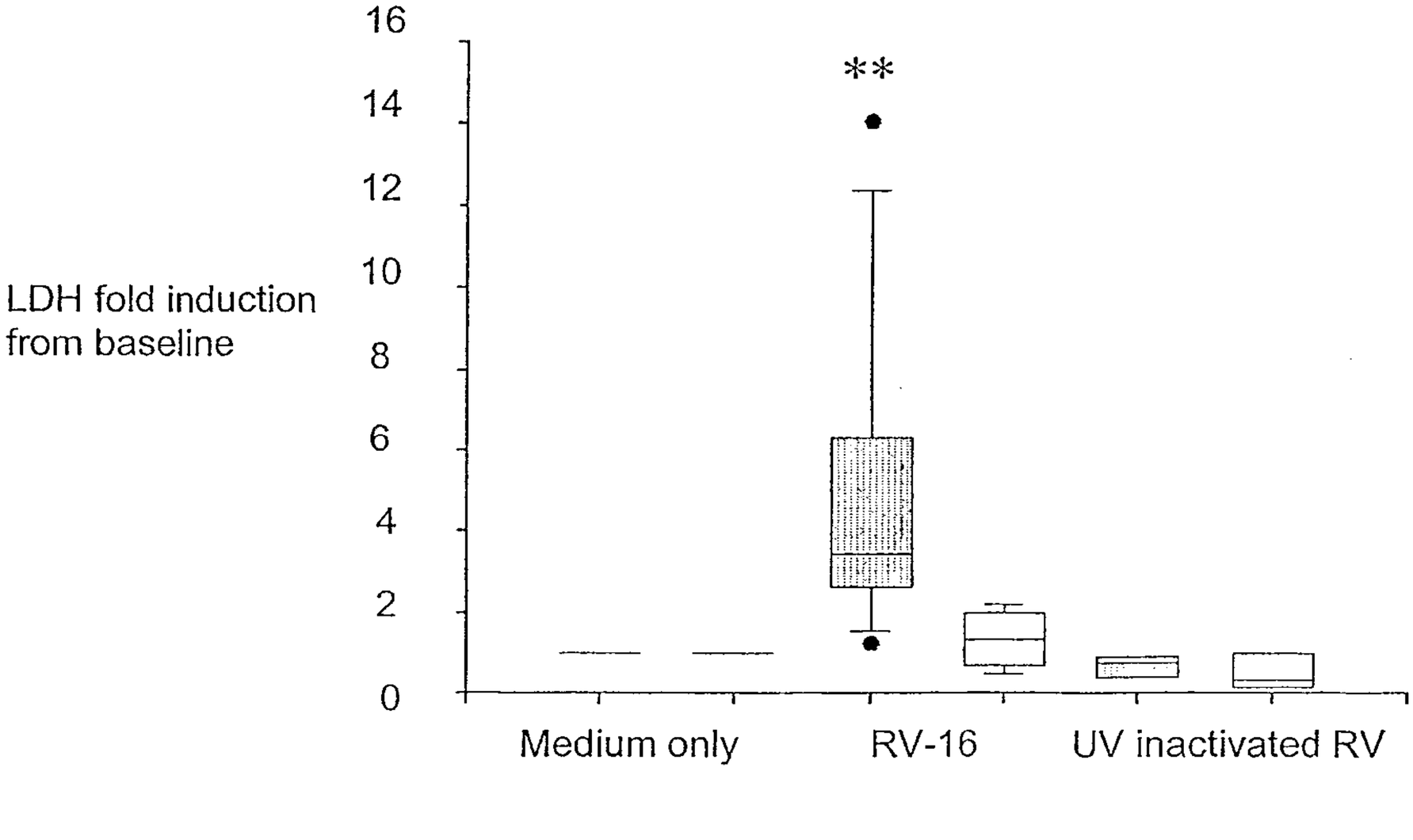


Figure 3a

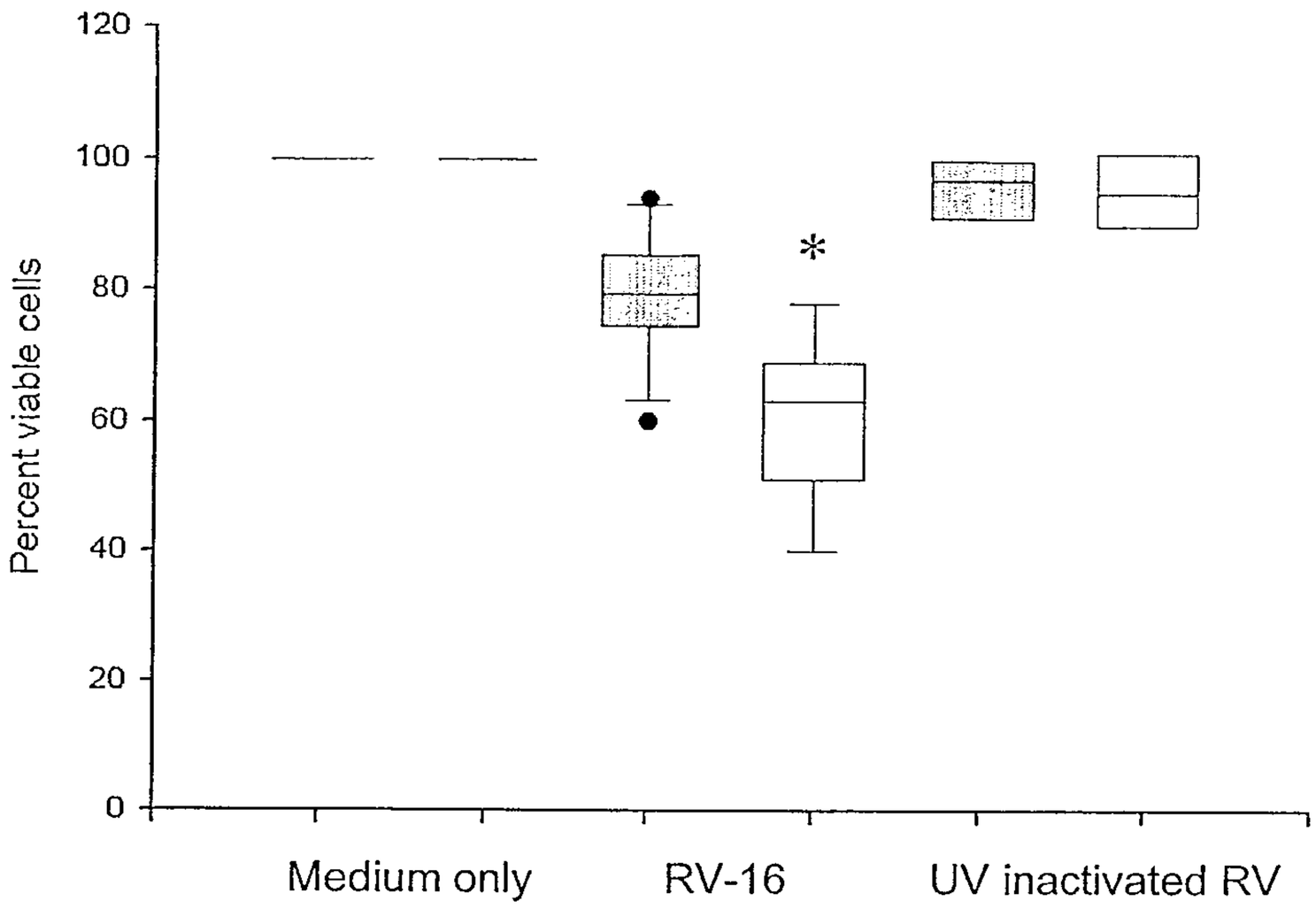


Figure 3b

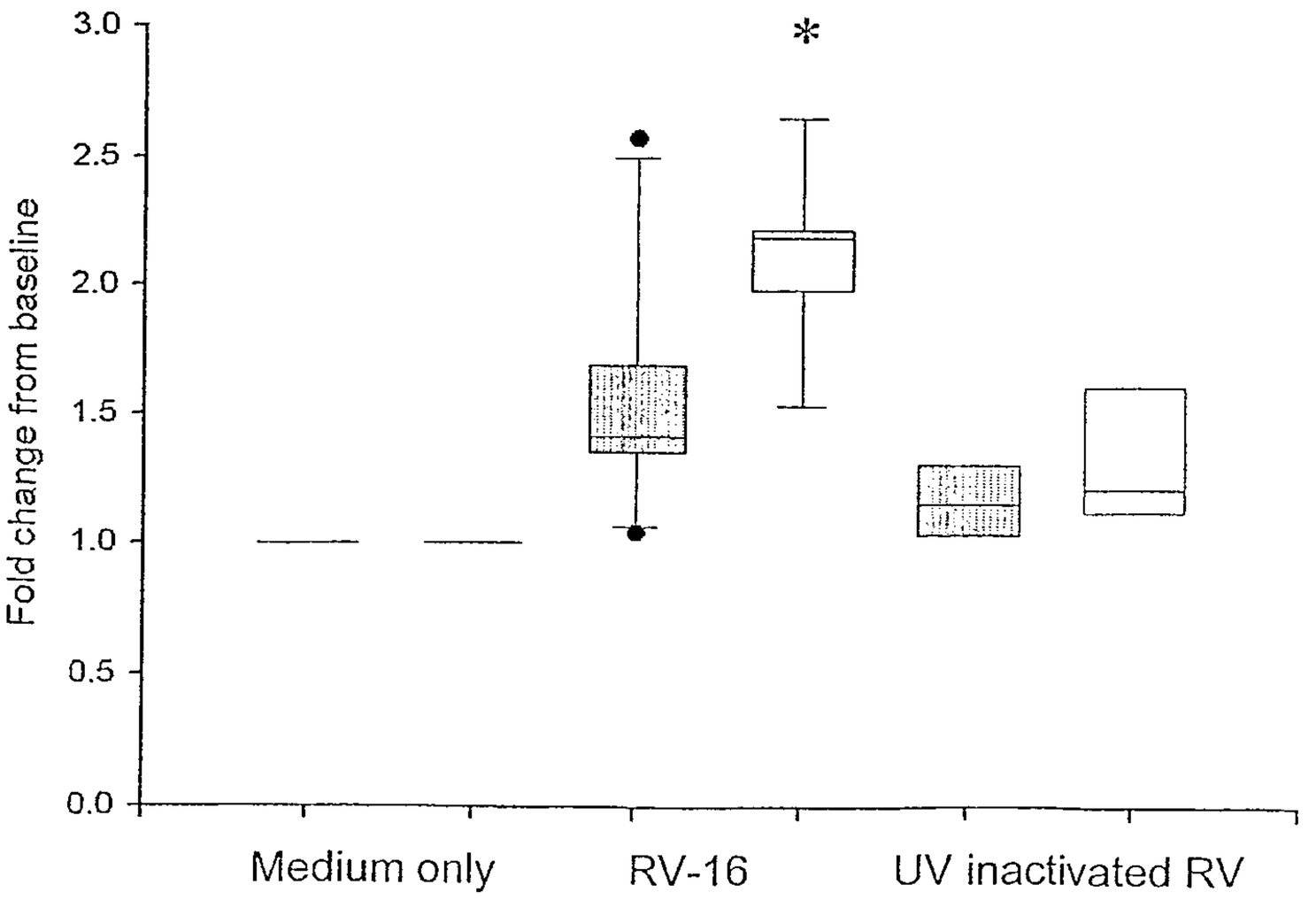


Figure 4a

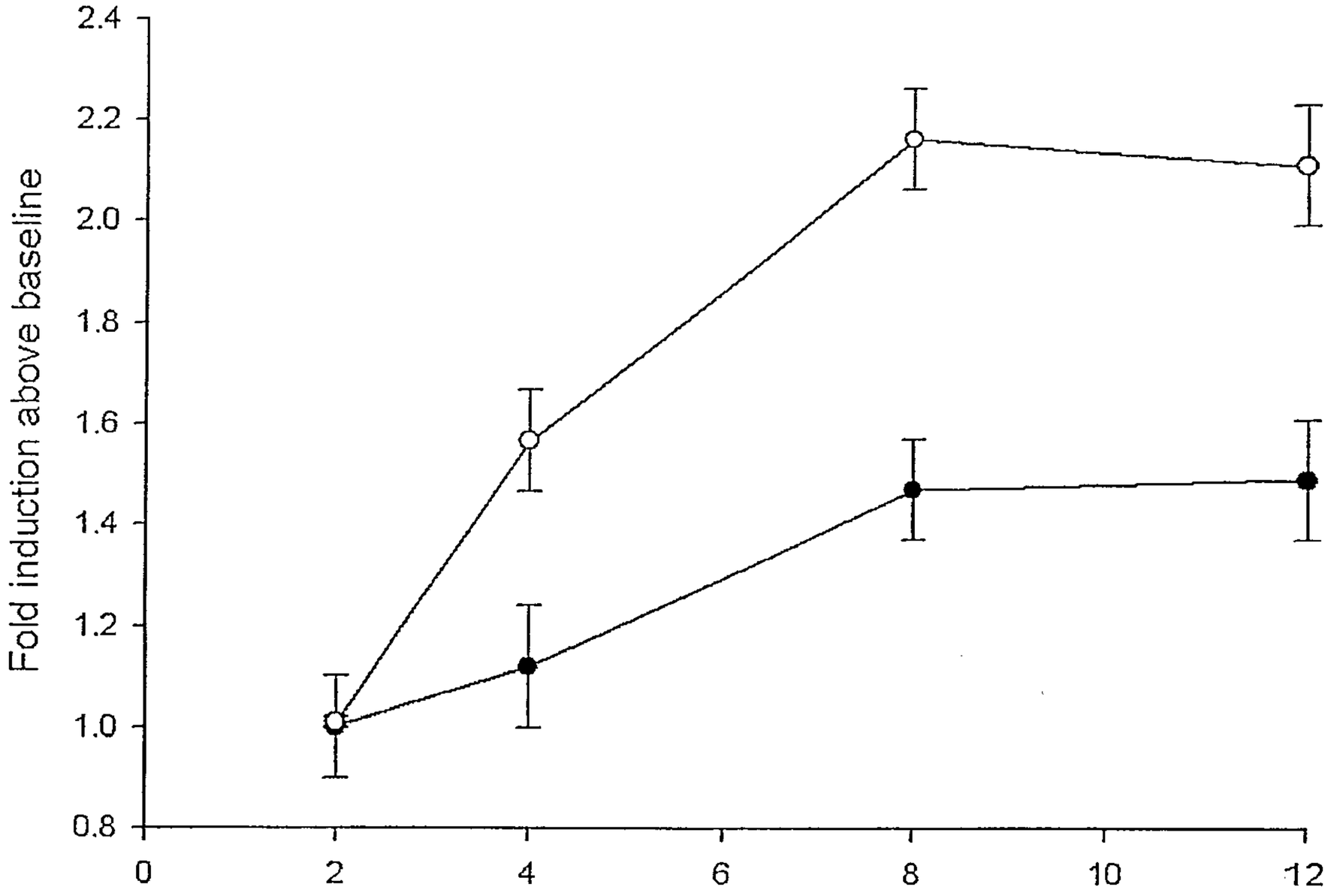

Fold induction above baseline
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0
2
4
6
8
10
12
hrs

Figure 4b

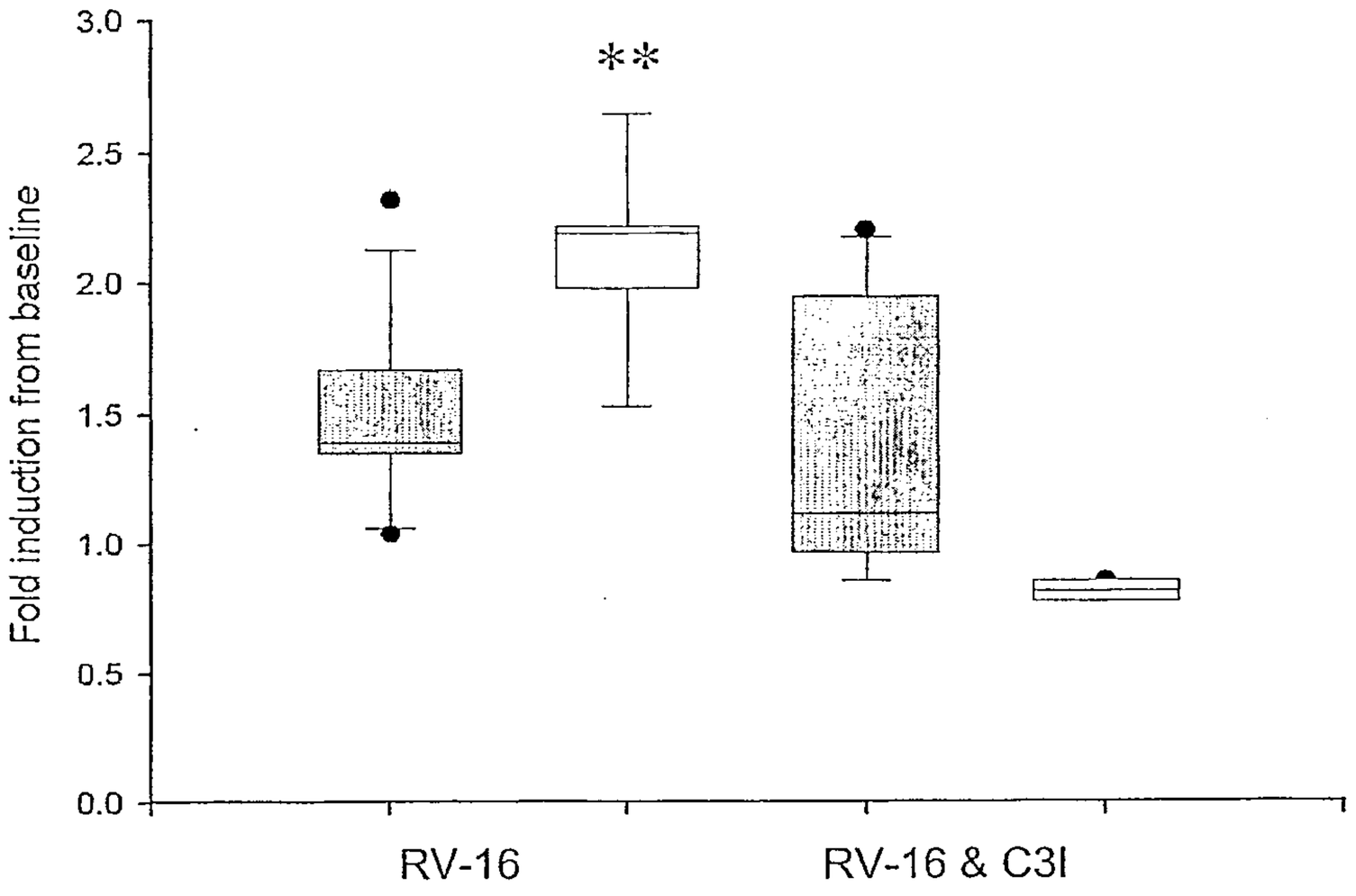


Figure 4c

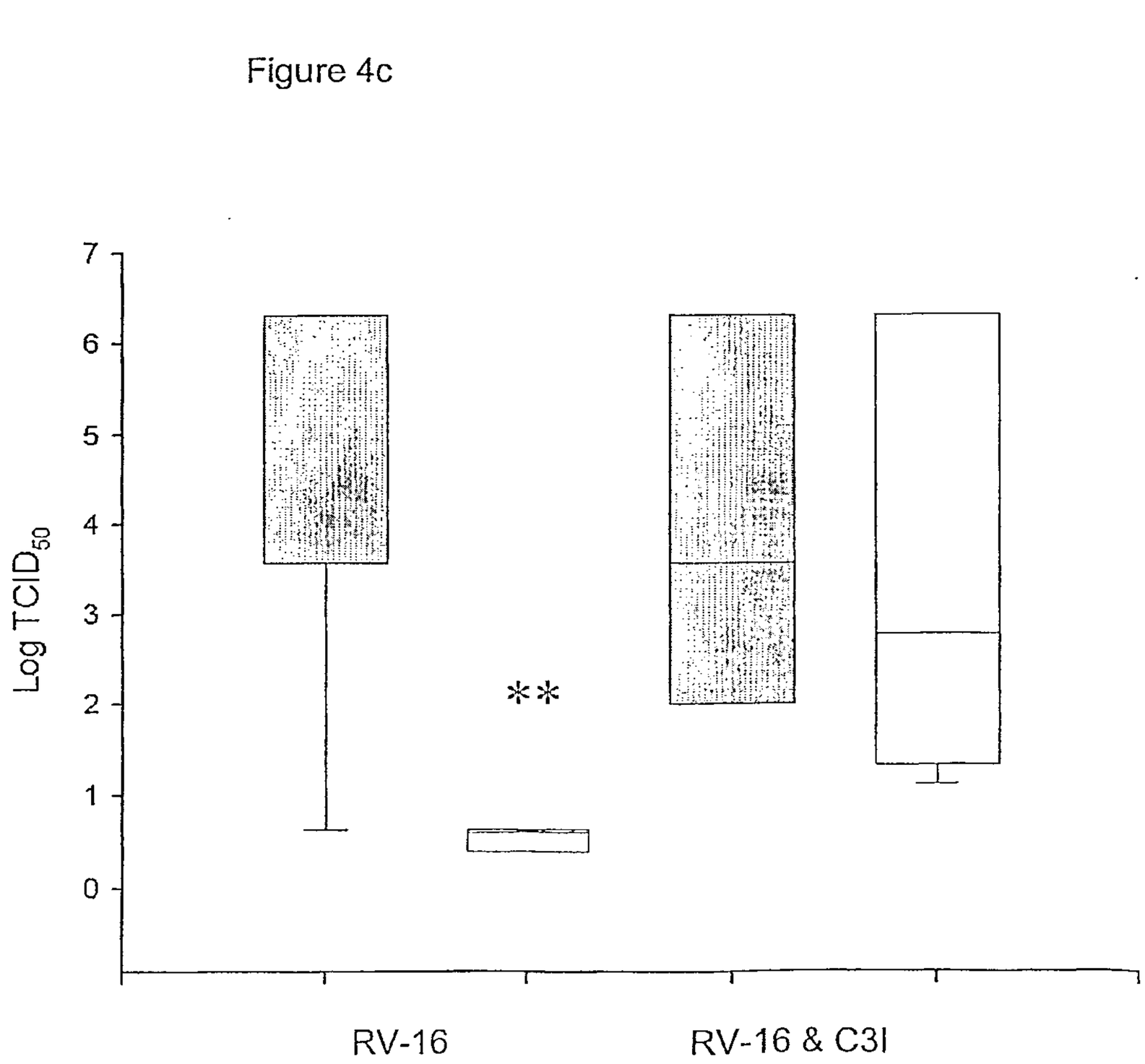


Figure 5a

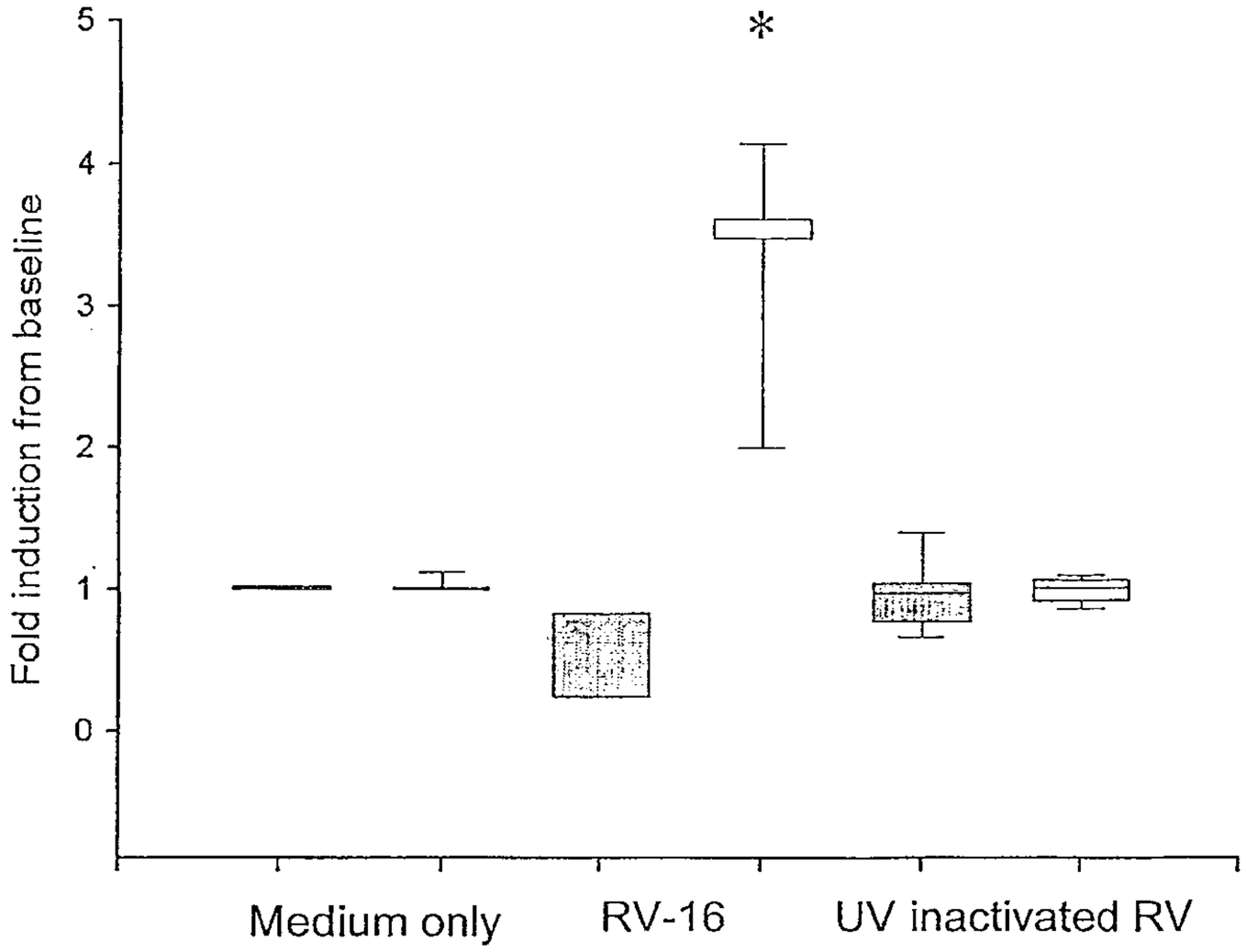


Figure 5b

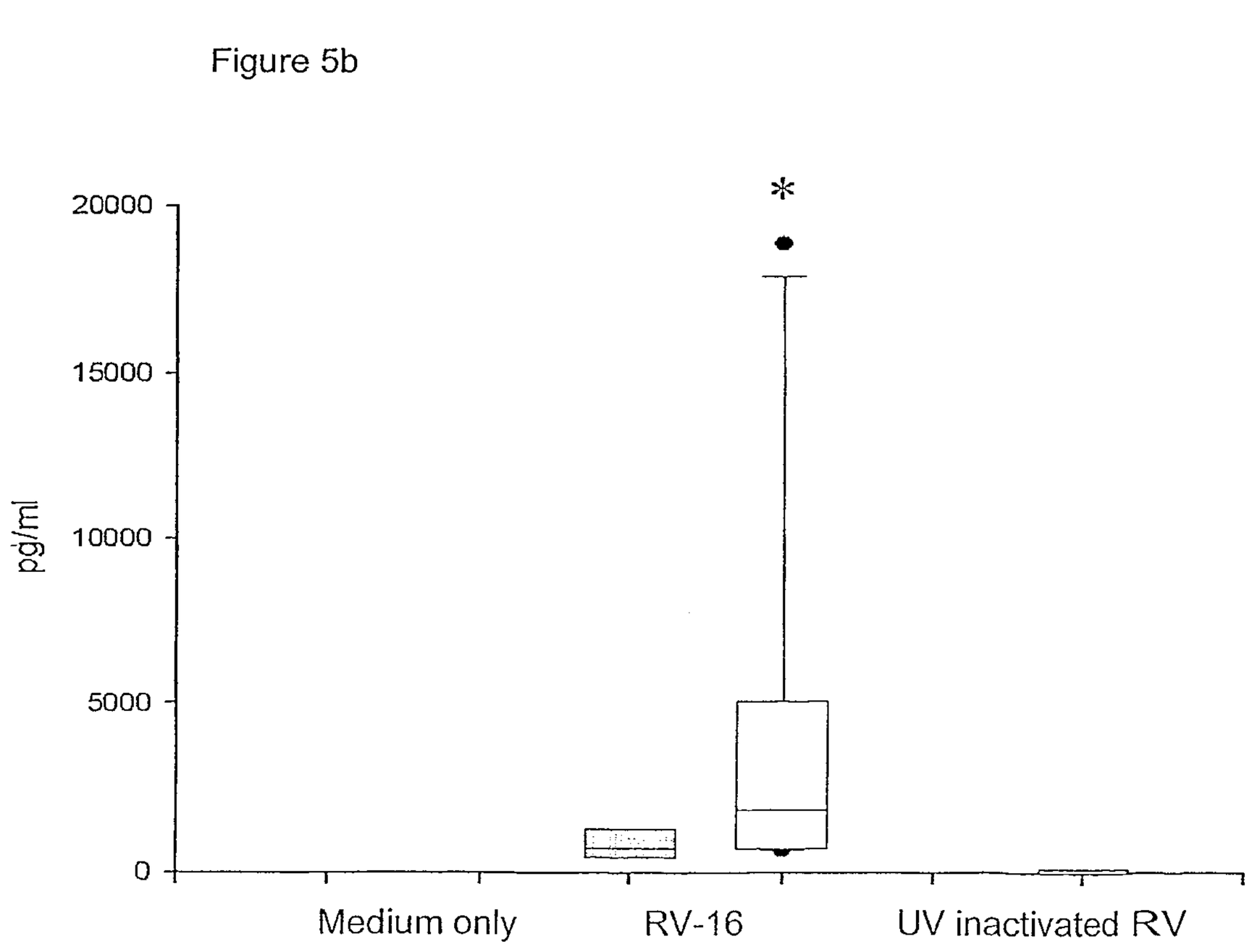


Figure 5c

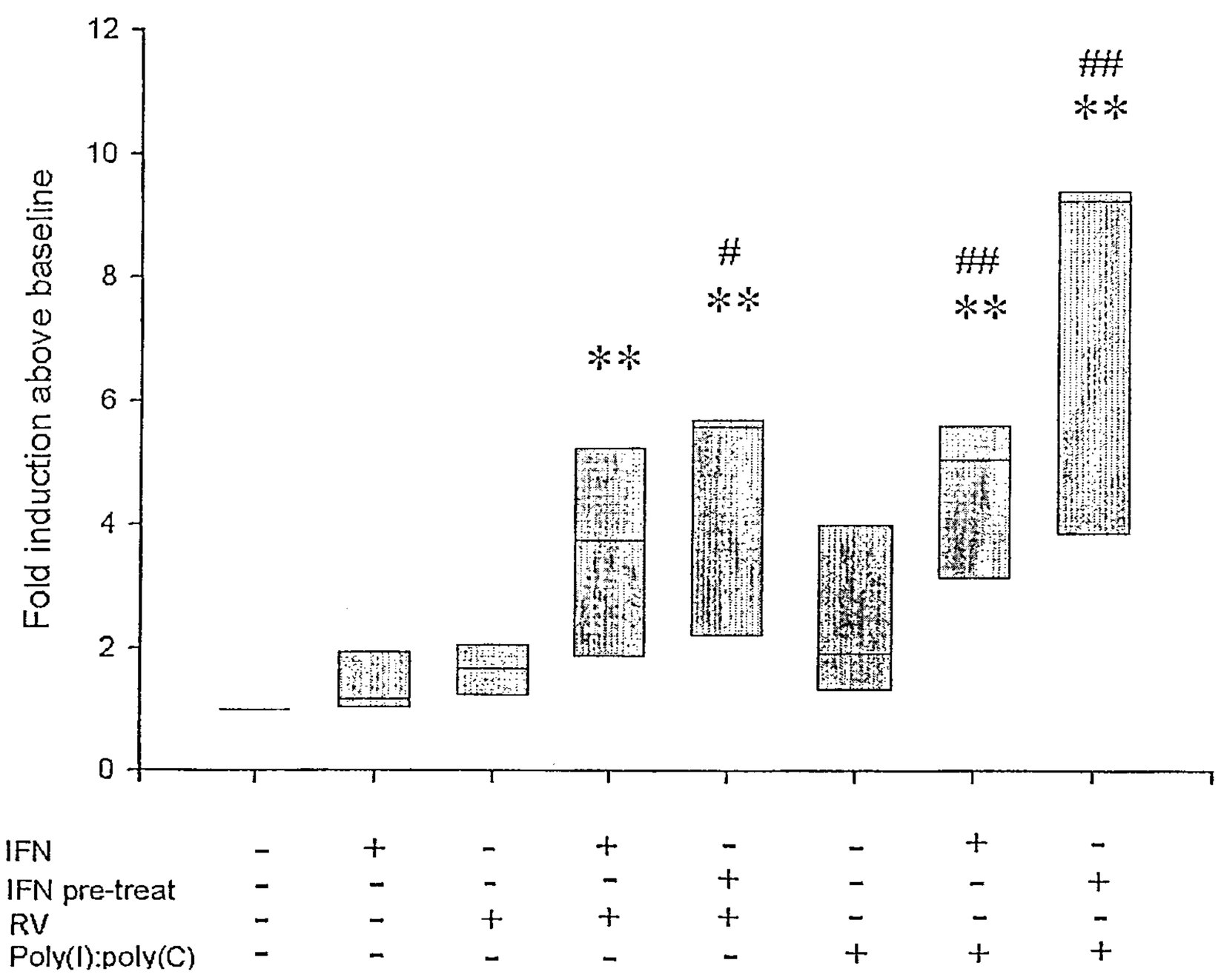
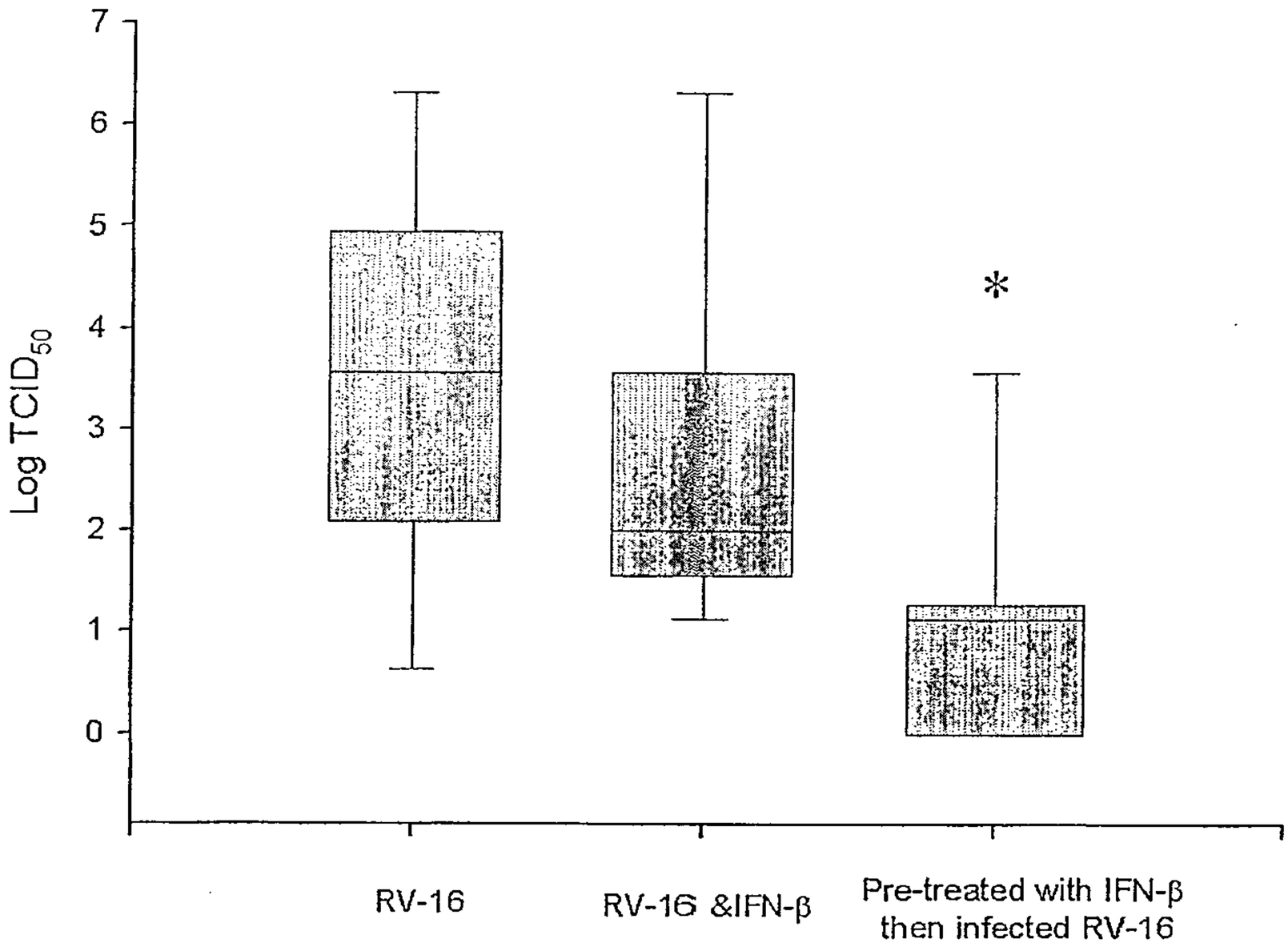

Fold induction above baseline
12
10
8
6
4
2
0
**
#
**
##
**
##
**
IFN - + - + - - + -
IFN pre-treat - - - - + - - +
RV - - + + + - - -
Poly(I):poly(C) - - - - - + + +

Figure 5d


Log TCID50
7
6
5
4
3
2
1
0
*
RV-16
RV-16 &IFN-β
Pre-treated with IFN-β
then infected RV-16

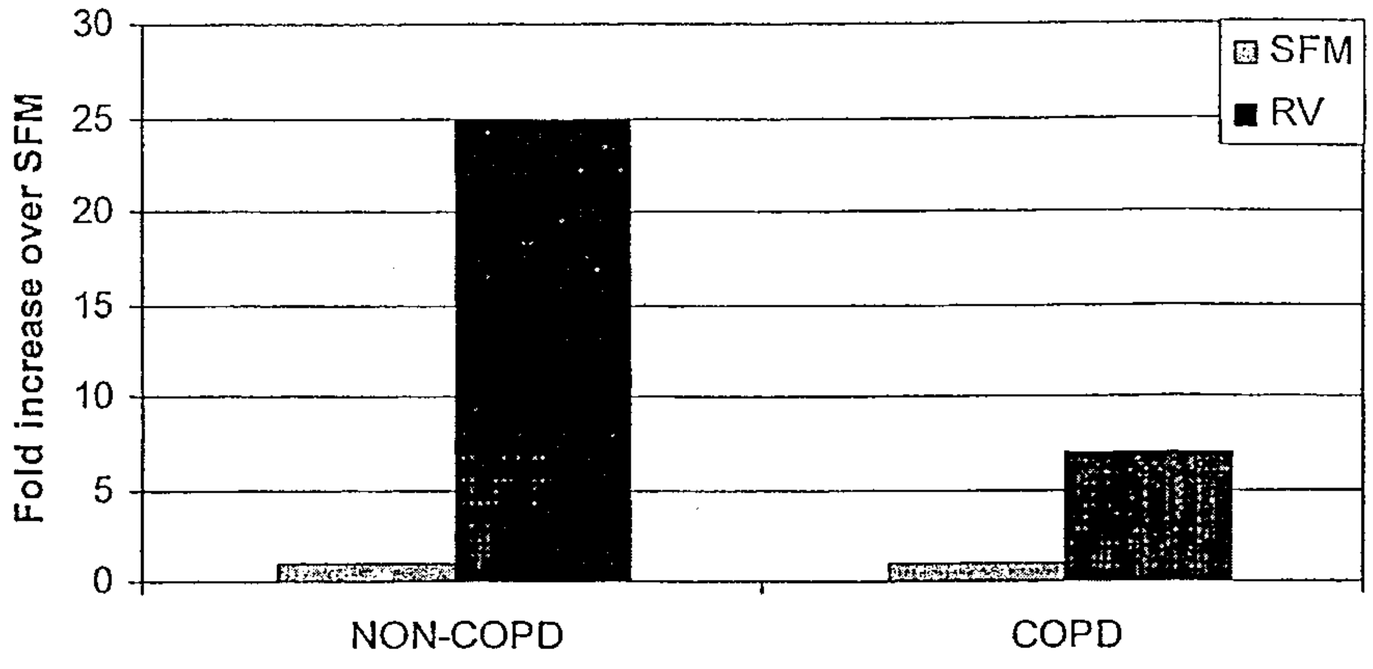


Figure 6.

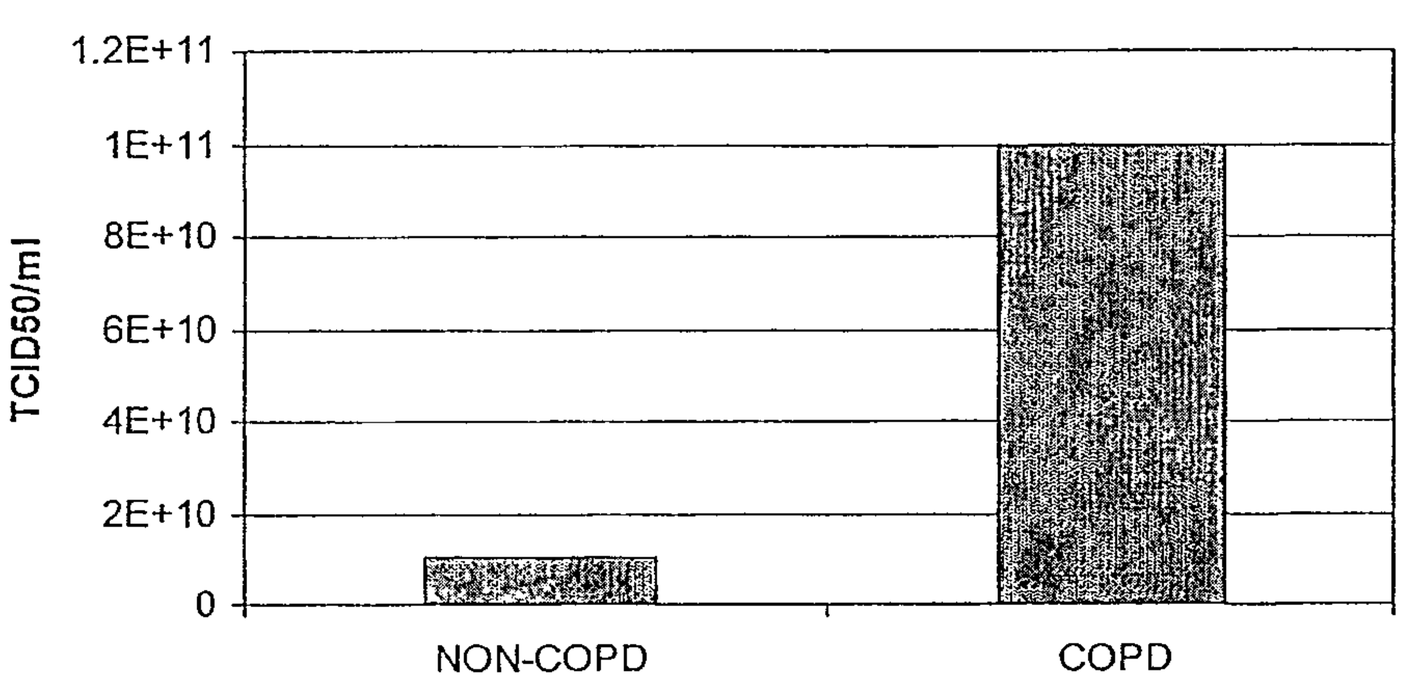


Figure 7.

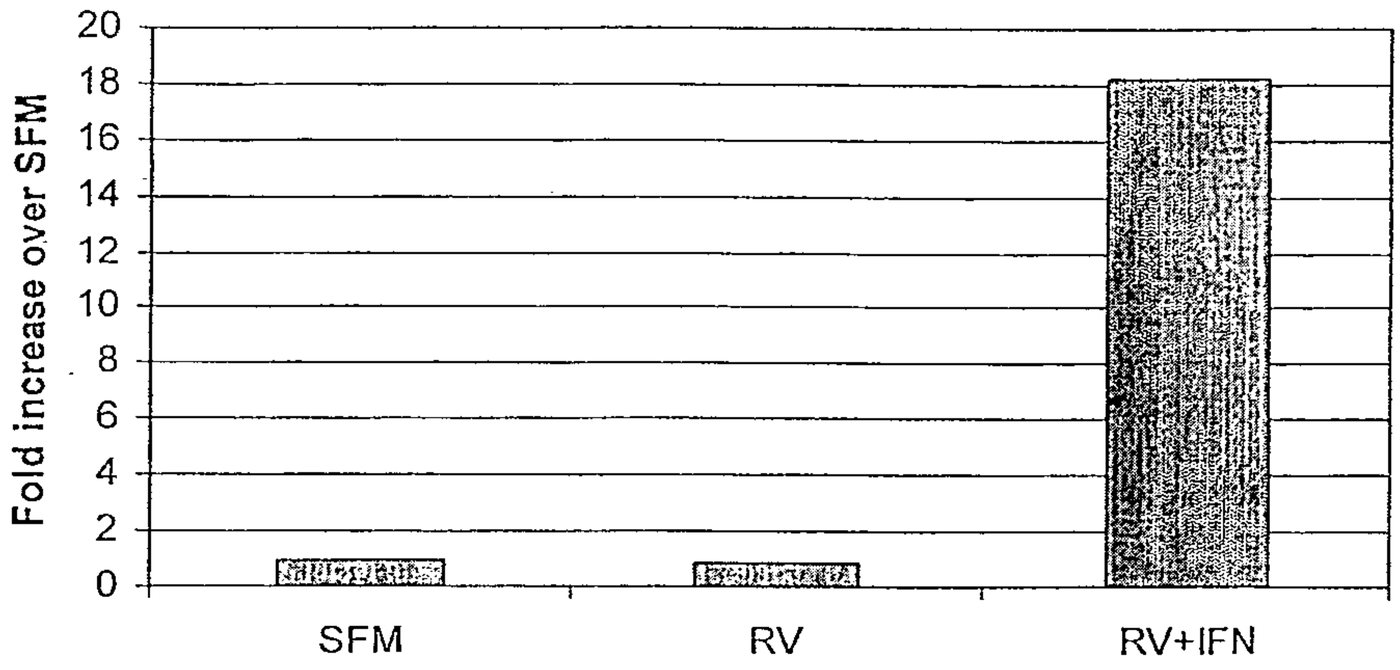


Figure 8.

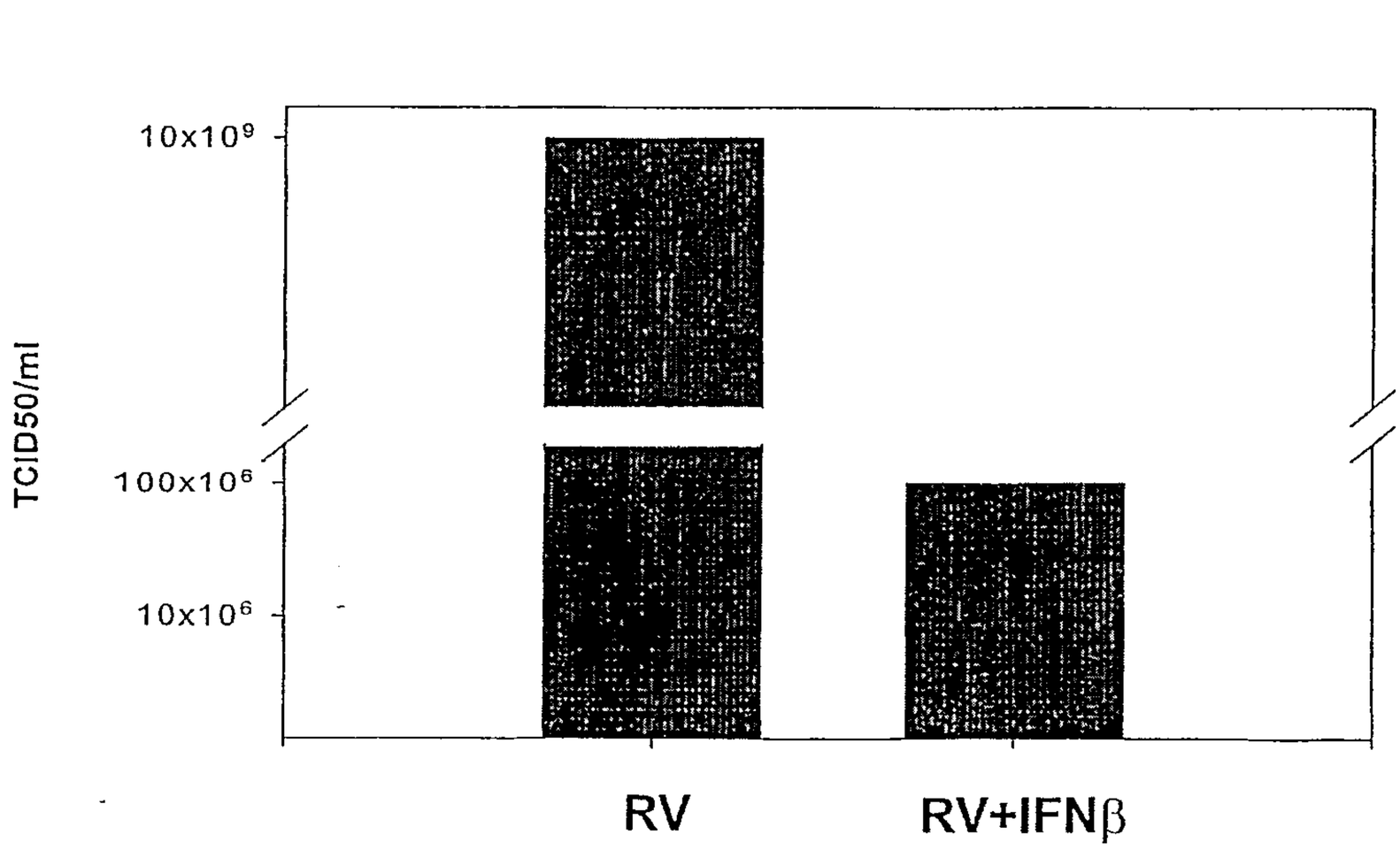


Figure 9.