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Source: Journal of Wildlife Diseases, 43(1) : 75-81

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-43.1.75

Journal of Wildlife Diseases, 43(1), 2007, pp. 75–81

MANNHEIMIA (PASTEURELLA) HAEMOLYTICA LEUKOTOXIN UTILIZES CD18 AS ITS RECEPTOR ON BIGHORN SHEEP LEUKOCYTES

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ABSTRACT: Pneumonia caused by *Mannheimia (Pasteurella) haemolytica* is a highly fatal disease of bighorn sheep (*Ovis canadensis*). Leukotoxin (Lkt), secreted by *M. haemolytica*, is an important virulence factor of this organism, and is cytolytic to bighorn sheep leukocytes. Previously, we have shown that CD18, the β subunit of β2 integrins, serves as the receptor for Lkt on bovine leukocytes. Furthermore, anti-CD18 antibodies inhibit Lkt-induced cytotoxicity of bighorn sheep leukocytes. Therefore, we hypothesized that Lkt utilizes CD18 as its receptor on bighorn sheep leukocytes. Confirmation of bighorn sheep CD18 as a receptor for Lkt requires the demonstration that the recombinant expression of bighorn sheep CD18 in Lkt-nonsusceptible cells renders them susceptible to Lkt. Therefore, we transfected cDNA encoding CD18 of bighorn sheep into a Lkt-nonsusceptible murine cell line. Cell surface expression of bighorn sheep CD18 on the transfectants was tested by flow cytometry with anti-CD18 antibodies. Transfectants stably expressing bighorn sheep CD18 on their surface were subjected to flow cytometric analysis for detection of Lkt binding, and cytotoxicity assays for detection of Lkt-induced cytotoxicity. Leukotoxin bound to the transfectants. More importantly, the transfectants were effectively lysed by Lkt in a concentration-dependent manner, whereas the parent cells were not. These results clearly indicate that *M. haemolytica* Lkt utilizes CD18 as a receptor on bighorn sheep leukocytes. Identification of CD18 as a receptor for Lkt on bighorn sheep leukocytes should enhance our understanding of the pathogenesis of pneumonia, which in turn should help in the development of control measures against this fatal disease of bighorn sheep.

Key words: Bighorn sheep, CD18, leukotoxin, *Mannheimia (Pasteurella) haemolytica*, *Ovis canadensis*, receptor.

INTRODUCTION

Mannheimia (Pasteurella) haemolytica causes pneumonia in bighorn sheep, domestic sheep, goats, and cattle (Ackermann and Brogden, 2000); however, bighorn sheep are particularly susceptible to pneumonia caused by this organism (Foreyt and Jessup, 1982). *Mannheimia haemolytica* produces a number of virulence factors which include leukotoxin (Lkt), lipopolysaccharide, a polysaccharide capsule, and outer membrane proteins (Confer et al., 1990). Of these, Lkt is widely accepted as the most important virulence factor (Jeyaseelan et al., 2002). Leukotoxin is a 102-kDa glycoprotein that is produced during the logarithmic-phase growth of the organism in vitro. This toxin is specific for ruminant leukocytes, and is responsible for leukocyte damage in the lungs of infected animals (Berggren et al., 1981). Leukotoxin is homologous with the exotoxins produced by several other gram-negative bacteria including *Escherichia coli* (Strathdee and Lo, 1989), *Actinobacillus pleuropneumoniae* (Devenish et al., 1989), and *Actinobacillus actinomycetemcomitans* (Kolodrubetz et al., 1989). The N-terminal half of the toxin consists of four hydrophobic domains, which are believed to be involved in pore formation in target cell membranes. The C-terminal third of the toxin contains a number of glycine-rich nonapeptide repeats which give rise to the term 'RTX' (repeats in toxin) toxins used to identify this family of toxins. This domain is involved in the binding of Ca^{++} which is necessary for the biological activity of the toxin (Jeyaseelan et al., 2002). A domain composed of about 50–60 amino acids at the extreme C-

terminal end is involved in the transport of the toxin across the bacterial envelope.

Leukotoxin is cytotoxic to all the subsets of leukocytes (Jeyaseelan et al., 2002). Of the leukocytes, polymorphonuclear leukocytes (PMNs) are most susceptible to the cytotoxic effects of Lkt. Leukotoxin-induced PMN lysis and degranulation have been implicated as the primary cause of acute inflammation and lung injury characteristic of pneumonia caused by *M. haemolytica* (Slocombe et al., 1985). Identification and characterization of the receptor for Lkt on bighorn sheep leukocytes is an important prerequisite for understanding the pathogenesis of this disease.

Previous studies in our laboratory have shown that CD18, the β subunit of β2 integrins, is necessary and sufficient to mediate Lkt-induced cytotoxicity of leukocytes of cattle (Deshpande et al., 2002). The β2 integrins are leukocyte-specific integrins that are expressed on the cell surface as a heterodimer composed of the α subunit CD11 and the β subunit CD18 (reviewed in Gahmberg et al., 1998). CD18, the common β subunit of β2 integrins, associates with four distinct α chains to give rise to four different β2 integrins: CD11a/CD18 (LFA-1), CD11b/CD18 (Mac-1), CD11c/CD18 (CR4), and the recently identified CD11d/CD18, which has not been well characterized yet.

In this study, anti-CD18 antibodies inhibited the cytotoxic effects of Lkt on the leukocytes of bighorn sheep, prompting us to hypothesize that Lkt utilizes CD18 as its receptor on bighorn sheep leukocytes. One of the methods of unequivocal demonstration of the involvement of CD18 of bighorn sheep in mediating Lkt-induced cytotoxicity is to render Lkt-nonsusceptible cells susceptible to Lkt-induced cytotoxicity, by recombinant expression of CD18. The objective of this study was to transfect a Lkt-resistant murine cell line with cDNA encoding CD18 of bighorn sheep, and to determine the susceptibility of the transfectant cell line to Lkt-induced cytotoxicity.

MATERIALS AND METHODS

Cell lines and antibodies

The murine mastocytoma cell line P815 was propagated in Roswell Park Memorial Institute Medium (RPMI) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 20 μg/ml gentamicin. The transfectants were selected and propagated in the above medium containing 500 μg/ml Geneticin (Life Technologies, Rockville, Maryland, USA). The anti-bovine CD18 monoclonal antibody (MAb) BAQ30A (IgG1), and the anti-human CD18 MAb HUH82A (IgG2a) were obtained from Washington State University Monoclonal Antibody Center (Saalmuller et al., 2005). Both BAQ30A and HUH82A bind to bighorn sheep CD18, but only HUH82A inhibits Lkt-induced cytotoxicity of bighorn sheep leukocytes. The MAb 8G12 (IgG1) specific for bovine respiratory syncytial virus (Klucas and Anderson, 1988), and MM113 (IgG2a) specific for bovine herpesvirus 1 (Srikumaran et al., 1990), which were used as isotype-matched control MAbs, were obtained from University of Nebraska-Lincoln Department of Veterinary and Biomedical Sciences.

Isolation of polymorphonuclear leukocytes (PMNs) and peripheral blood mononuclear cells (PBMCs) from bighorn sheep

Peripheral blood was collected by venipuncture from healthy bighorn sheep and subjected to Ficoll-Paque (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) density gradient centrifugation. Peripheral blood mononuclear cells were collected from the band at the plasma-Ficoll interface, and washed twice with ice-cold sterile phosphate-buffered saline (PBS; 130 mM NaCl, 1.55 mM KH_2PO_4, 5.1 mM Na_2HPO_4, pH 7). Polymorphonuclear leukocytes were isolated from the erythrocyte pellet by hypotonic lysis and washed three times in sterile ice-cold PBS.

Production of *M. haemolytica* Lkt

Production of Lkt from *M. haemolytica* strain A1 has been described previously (Gentry and Srikumaran, 1992). Briefly, bacteria grown to logarithmic phase (approximately 4.5 hr) in brain heart infusion broth (Difco, Detroit, Michigan, USA) were collected by centrifugation (13,500 × G for 20 min)

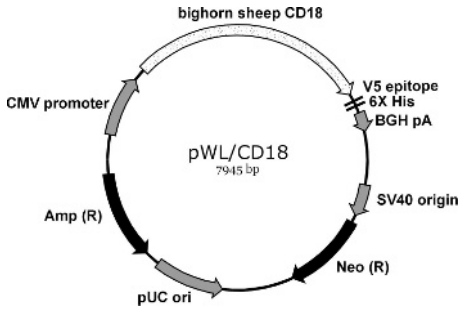

FIGURE 1. The pWL/CD18 mammalian expression vector carrying bighorn sheep CD18. This vector was obtained by cloning the bighorn sheep CD18 cDNA (Liu et al., 2006) into pcDNA3.1D/V5-His-TOPO (Invitrogen).

and resuspended in twice the original culture volume of RPMI 1640 medium supplemented with 4 mM L-glutamine (Sigma, St. Louis, Missouri, USA). After an additional 1–1.5 hr of growth at 37 C in the RPMI medium, the bacteria were removed from the culture by centrifugation (13,500 × G for 30 min) followed by filter-sterilization. The crude toxin in the form of culture supernatant was aliquoted and stored at −20 C. All experiments were performed with the same batch of toxin. For flow cytometry, biotinylated Lkt (Brown et al., 1997) kindly provided by Dr. Charles Czuprynski, University of Wisconsin, Madison, was used.

Transfection of bighorn sheep CD18 into P815 cells

The cloning, sequencing, and characterization of cDNA for bighorn sheep CD18 has been described previously (Liu et al., 2006). The mammalian expression vector carrying bighorn sheep CD18, pWL/CD18 (Fig. 1), was obtained by cloning the bighorn sheep CD18 cDNA into pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, California). P815 cells were transfected, for stable expression of bighorn sheep CD18, with pWL/CD18 using SuperFect transfection reagent (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. Briefly, 5×10^5 cells were incubated with 0.5 μg of pWL/CD18 and 2.5 μl of the transfection reagent. Forty-eight hours later, the cells were resuspended in selection medium supplemented with Geneticin (500 μg/ml), and plated into 96-well plates. Transfectants that continued to grow in the selection medium containing Geneticin were subjected to flow cytometric analysis.

Flow cytometric analysis for the cell surface expression of bovine CD18

The transfectants and parent cells (P815) were tested for cell-surface expression of bighorn sheep CD18 using anti-CD18 MAb BAQ30A by flow cytometric analysis according to the procedures described earlier (Deshpande et al., 2002). Briefly, 2.5×10^5 cells in 50 μl of FACS buffer (3% horse serum and 0.01% sodium azide in PBS), were incubated with 50 μl of MAb BAQ30A (15 μg/ml) at 4 C for 1 hr. Following three washes in FACS buffer, the cells were incubated with 50 μl of fluorescein isothiocyanate (FITC)-conjugated goat antibodies specific for murine Ig (KPL, Gaithersberg, Maryland, USA) at 4 C for 30 min. The cells were washed three times with FACS buffer, resuspended, and analyzed by a flow cytometer (FACSort, Becton-Dickinson Immunocytometry Systems, San Jose, California, USA). To enrich the number of transfectants expressing CD18 on their cell surface, anti-CD18 MAb-stained transfectants were sorted with a fluorescence-activated cell sorter (FACSVantage SE, Becton-Dickinson Immunocytometry Systems).

Flow cytometric analysis of Lkt-binding to transfectant cell lines

The transfectant cell lines and the parent cells were tested for Lkt binding by flow cytometry according to procedures described previously (Brown et al., 1997). Briefly, 5×10^5 cells in 50 μl of medium were incubated with 10 μg of biotinylated Lkt in 50 μl of medium at 4 C for 15 min. Following three washes in FACS buffer, the cells were incubated with 50 μl of alexafluor 488-conjugated streptavidin at 4 C for 20 min. The cells were washed three times with FACS buffer, resuspended, and analyzed with a flow cytometer (FACSort, Becton-Dickinson Immunocytometry Systems).

3-[4,5-dimethylthiazoyl-2-Yl]-2,5-diphenyl tetrazolium bromide (MTT) dye reduction assay for Lkt-induced cytotoxicity and Lkt neutralization

The susceptibility of bighorn sheep PMNs, PBMCs, transfectants, and parent cells (P815) to *M. haemolytica* Lkt-mediated cytotoxicity was determined by a previously described cytotoxicity assay (MTT dye reduction assay; Gentry and Srikumaran, 1991). This assay measures the ability of the endoplasmic reticulum–resident enzymes in viable cells to convert a tetrazolium dye (MTT; Sigma) into a purple formazan precipitate, which is later dissolved in acid isopropanol. The optical density (OD) of the end product, representing

the intensity of color developed, is directly proportional to the viability of the cells. Briefly, the cells were resuspended in colorless RPMI 1640 (RPMI 1640 without neutral red) at a concentration of 5×0^6/ml and seeded into 96-well round-bottom microtiter plates in duplicate (50 µl per well). Fifty microliters of serially diluted Lkt in colorless RPMI 1640 were added into each well and the plates were incubated at 37 C for 1 hr. The cells were centrifuged at 600 × G for 5 min following incubation, and the supernatant fluid was discarded. The cells were resuspended in 100 µl colorless RPMI 1640 and 20 µl of 0.5% MTT were added to each well. Following incubation at 37 C for 2 hr, the cells were centrifuged at 600 × G for 5 min, and the supernatant fluid was discarded. The remaining formazan precipitate was thoroughly dissolved in 100 µl acid isopropanol and the OD of the samples measured using an enzyme-linked immunosorbent assay reader. The percent cytotoxicity was calculated as follows: % cytotoxicity=(1−[OD of toxin-treated cells/OD of toxin-untreated cells])×100.

The ability of anti-CD18 MAb to inhibit Lkt-induced cytotoxicity of bighorn sheep PMNs and PBMCs was measured using this cytotoxicity assay. The assay was performed as described above, except that the cells were preincubated with anti-CD18 MAb HUH82A (undiluted culture supernatant) for 1 hr before the addition of Lkt.

RESULTS

Anti-CD18 MAb inhibited Lkt-induced cytotoxicity of bighorn sheep PMNs and PBMCs. Bighorn sheep PMNs and PBMCs were preincubated with the anti-CD18 MAb HUH82A or the isotype-matched control MAb MM113 before subjecting them to the cytotoxicity assay with Lkt. HUH82A inhibited the cytotoxicity of both PMNs and PBMCs, whereas MM113 did not (Fig. 2), suggesting that CD18 may serve as the receptor for Lkt on bighorn sheep leukocytes.

P815 cells transfected with cDNA for bighorn sheep CD18 expressed bighorn sheep CD18 on the cell surface. Flow cytometric analysis with the anti-CD18 MAb BAQ30A revealed cell-surface expression of CD18 by the transfectants. Fluorescence-activated cell sorting of the transfectants stained with anti-CD18 MAb

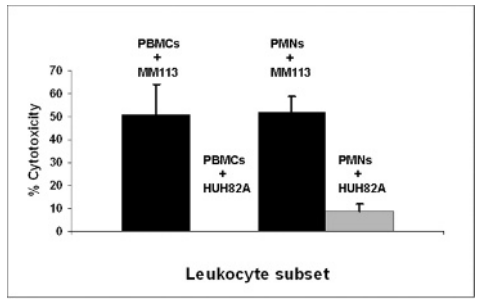

FIGURE 2. Anti-CD18 monoclonal antibody (MAb) inhibits leukotoxin (Lkt)-induced cytotoxicity of bighorn sheep peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs). The ability of anti-CD18 MAb to inhibit Lkt-induced cytotoxicity of bighorn sheep PBMCs and PMNs was determined by a cytotoxicity assay. The cells were preincubated with the anti-CD18 MAb HUH82A (PBMCs+HUH82A and PMNs+HUH82A) or the isotype-matched control MAb MM113 (PBMCs+MM113 and PMNs+MM113) before subjecting them to the cytotoxicity assay described under Materials and Methods. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

enhanced the number of cells expressing bighorn sheep CD18 in the population (Fig. 3A). Several clones expressing bighorn sheep CD18 were obtained from the sorted cell population. The clone D11 continued to express bighorn sheep CD18 in a stable manner (Fig. 3B), and hence this transfectant cell line was selected for further studies.

Leukotoxin bound to the transfectant cell line (clone D11) expressing bighorn sheep CD18. No binding was observed on the parent cell line P815 (Fig. 4), indicating that bighorn sheep CD18 serves as a receptor for Lkt.

Leukotoxin lysed the transfectant cell line D11 expressing bighorn sheep CD18 in a concentration-dependent manner (Fig. 5). Lysis was not observed with the parent cell line.

DISCUSSION

Previous studies (Li et al., 1999; Deshpande et al., 2002) revealed that CD18 serves as a receptor for Lkt of *M. haemolytica* on bovine leukocytes. Be-

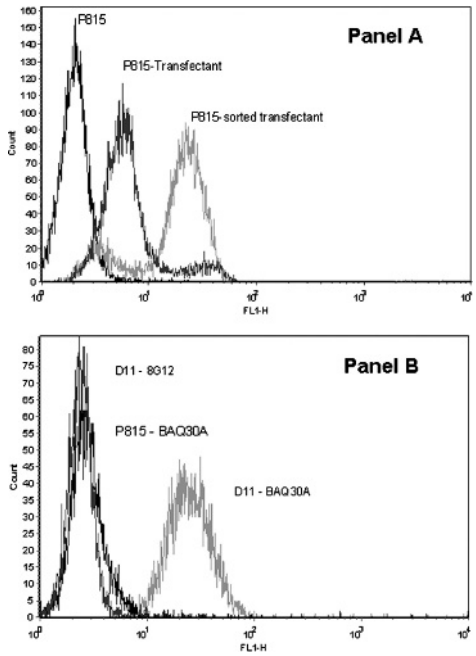

FIGURE 3. Cell surface expression of bighorn sheep CD18 on P815 cells transfected with cDNA for bighorn sheep CD18. P815 cells were transfected with the mammalian expression vector pWL/CD18 carrying bighorn sheep CD18 cDNA. Transfectants that continued to grow in the selection medium containing Geneticin were subjected to flow cytometric analysis with the MAb, BAQ30A, specific for bighorn sheep CD18, followed by FITC-conjugated goat antibodies to murine immunoglobulin. Transfectants exhibiting strong expression of CD18 were sorted with a fluorescence-activated cell sorter. Panel A shows CD18 expression of an unsorted population (P815 transfectants) and the sorted population (P815-sorted transfectants). Parent cells, P815, were used as the negative control. Panel B shows the CD18 expression of a transfectant clone D11 obtained from the sorted population. An isotype-matched control MAb with an irrelevant specificity (8G12) was used to show the specificity of CD18 staining. For clarity, the histograms with 8G12 have been omitted in Panel A. Results of one representative experiment out of three are shown.

cause the Lkt is cytolytic for the leukocytes of cattle, sheep, and goats, we speculated that it might be the case with bighorn sheep leukocytes also. Inhibition of Lkt-induced cytotoxicity of bighorn sheep PBMCs and PMNs by an anti-CD18 MAb (Fig. 2) prompted us to hypothesize that CD18 is the receptor for *M. haemolytica* Lkt on bighorn sheep

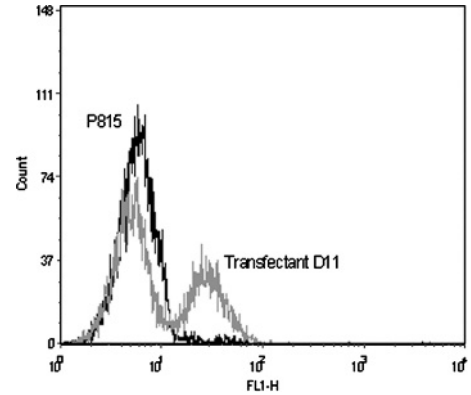

FIGURE 4. Leukotoxin binds to transfectants expressing bighorn sheep CD18, but not the parent cells. The transfectant clone expressing bighorn sheep CD18 (D11), or the parent cells (P815) were subjected to flow cytometric analysis with biotinylated Lkt, followed by Alexafluor 488-conjugated streptavidin. Results of one representative experiment out of three are shown.

leukocytes. We reasoned that recombinant expression of bighorn sheep CD18 on Lkt-nonsusceptible cells and examination of their susceptibility to Lkt-induced cytotoxicity should unequivocally demonstrate the role of CD18 in Lkt-induced cytotoxicity of bighorn sheep leukocytes. Trans-

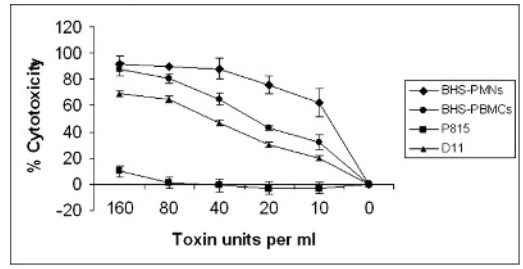

FIGURE 5. Leukotoxin lyses transfectants expressing bighorn sheep CD18, but not the parent cells, in a concentration-dependent manner. The transfectant clone expressing bighorn sheep CD18 (D11), the parent cells (P815), bighorn sheep PMNs (BHS-PMNs), or bighorn sheep PBMCs (BHS-PBMCs) were subjected to the MTT dye-reduction cytotoxicity assay with varying amounts of Lkt as described under Materials and Methods. The percentage of cytotoxicity was calculated as follows: %cytotoxicity=(1−[OD of toxin-treated cells/OD of toxin-untreated cells])×100. Results shown are the means of three independent experiments. The error bars indicate standard deviations of the means.

fection of Lkt-nonsusceptible cell line P815 and florescence-activated cell sorting resulted in the development of a clone, D11, that stably expressed bighorn sheep CD18 on the cell surface (Fig. 3). It has been well established that the α subunit (CD11) and the β subunit (CD18) of β2 integrins have to associate with each other in order to be transported to the plasma membrane, and expressed on the cell surface (Marlin et al., 1986). Therefore it is very likely that the transfected bighorn sheep CD18 molecule is expressed on the surface of the transfected cell line D11 as a heterodimer with the murine CD11a molecules of the parent cells, P815 (CD11b and CD11c molecules are not expressed on P815 cells; Deshpande et al., 2002). Flow cytometric analysis of Lkt binding to the transfectant cell line D11 strongly suggested that CD18 serves as a receptor for Lkt on bighorn sheep leukocytes. However, binding of Lkt to leukocytes is not considered specific because it has been demonstrated that Lkt also binds to nonruminant leukocytes (Jeyaseelan et al., 2000) without eliciting any cytolytic effects. Hence we tested the susceptibility of the transfectant cell line D11 to Lkt-mediated cytotoxicity in a cytotoxicity assay. In this assay, the transfectant cell line D11 was lysed by Lkt in a concentration-dependent manner, whereas the parent cell line P815 was not (Fig. 5). We also compared the Lkt-induced cytotoxicity of the transfectant cell line D11 with that of bighorn sheep PMNs and PBMCs. The percentage of cytotoxicity of the transfectant cell line was less than that of PMNs and PBMCs, which is to be expected because the transfectants express only the chimeric LFA-1 (bighorn sheep CD18 and mouse CD11a). In contrast, PMNs express all three integrins: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and CR-4 (CD11c/CD18) (Gahmberg et al., 1998). The PBMCs mostly comprise lymphocytes and monocytes. All lymphocytes express LFA-1, and a subpopulation of lymphocytes express Mac-1 and CR-4 as well. Monocytes express all three integrins, as do the PMNs, albeit at different levels.

In summary, we have shown the following: 1) inhibition of Lkt-induced cytotoxicity of bighorn sheep PBMCs and PMNs by anti-CD18 MAb, 2) binding of Lkt to the transfectant cell line expressing bighorn sheep CD18, and 3) Lkt–concentration-dependent lysis of the transfectant cell line. Taken together, these results clearly indicate that Lkt utilizes CD18 as its receptor on bighorn sheep leukocytes.

ACKNOWLEDGMENTS

This research was supported by funds from the Foundation for North American Wild Sheep and its Eastern, Idaho, Oregon, and Washington chapters.

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Received for publication 9 March 2006.