British Journal of Pharmacology (1997) 120, 130–136

Inhibition by the immunosuppressive agent FK-506 of antigen-induced airways eosinophilia and bronchial hyperreactivity in mice

Seok-Yong Eum, Claudia Zuany-Amorim, Jean Lefort, Marina Pretolani & [1]B. Boris Vargaftig

Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur-INSERM 285, Institut Pasteur 25, Rue du Dr. Roux, 75015 Paris, France

1 The effect of the immunosuppressive agent, FK-506, on allergen-induced airways eosinophilia and bronchial hyperreactivity (BHR) in hyper IgE mice (BP2 selection) was investigated.

2 Administration of FK-506 at 2 mg kg^{-1} s.c., 1 h before and 5 h after the first of four ovalbumin challenges, reduced the recruitment of eosinophils into the bronchoalveolar lavage fluid (BALF) from $1.36 \pm 0.22 \times 10^5$ to $0.53 \pm 0.24 \times 10^5$ cells ml^{-1} ($n = 5-6$, $P < 0.05$; 60% inhibition), inhibited by 80% BHR in response to i.v. 5-HT and practically suppressed BHR in response to inhaled methacholine.

3 The antigen-induced interleukin (IL)-5 formation in the BALF and serum was inhibited by FK-506 by 75% in both instances.

4 FK-506 failed to modify the bronchoconstriction in BP2 mice, suggesting that different mechanisms are involved in acute bronchoconstriction and BHR.

5 The increased number of CD4+, CD8+, CD3+ T lymphocytes in the BALF of antigen-challenged mice was unaffected by FK-506.

6 These findings indicate that antigen-induced *in vivo* IL-5 release and eosinophil, but not T-cell, infiltration into the bronchial lumen of sensitized BP2 mice are targets for the anti-allergic activities of FK-506.

Keywords: Asthma; T lymphocytes; IL-5; eosinophils; bronchial hyperreactivity in mice; immunosuppression

Introduction

Asthma is characterized, amongst other features, by the infiltration of inflammatory cells into the airways and by bronchial hyperreactivity (BHR) (Boushey *et al*., 1980; Djukanovic *et al*., 1990; Arm & Lee, 1992). Evidence has accumulated that T lymphocytes play a central role in the pathogenesis of asthma by releasing a variety of interleukins (IL) which regulate and coordinate the immune and inflammatory responses (Azziwa *et al*., 1990; Walker *et al*, 1991; Busse *et al*., 1995). The observation that increased numbers of activated T cells are found during acute severe asthma (Corrigan *et al*., 1988; Robinson *et al*., 1993) and that this phenomenon correlates with the severity of the disease (Corrigan & Kay, 1990) further incriminates T cells in its aetiology. Similar observations have been made in animal models for allergen-induced airways inflammation and BHR (Frew *et al*., 1990; Lapa e Silva *et al*., 1993; Anderson & Coyle, 1994). The T cell-derived cytokines, IL-3, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are responsible for eosinophil tissue localization, prolongation of survival and activation (Silberstein & David, 1987; Owen *et al*., 1987; Lopez *et al*., 1988; Rothenberg *et al*., 1988). In particular, IL-5 is selective for eosinophil differentiation, priming and activation (Lopez *et al*., 1988). These observations suggest that, via the production of IL-5, T lymphocytes are deeply associated with the induction and the persistence of airway inflammatory responses.

We recently developed a murine model of antigen-induced airways eosinophilia and BHR using a selection of Biozzi mice, named BP2 (Biozzi *et al*., 1979), which produce high titers of IgE after immunization. We further demonstrated that the administration of an anti-IL-5 antibody prevents altogether antigen-induced airways eosinophilia and BHR (Eum *et al*., 1995). Since dexamethasone also reduces BHR and the *in vivo* production of IL-5 (Eum *et al*., 1996), we suggested that this cytokine plays an important role in the induction of BHR in mice.

In our previous study, we identified an increased number of CD4+ and CD8+ T lymphocytes in the bronchial wall of antigen-challenged mice (Eum *et al*., 1995), supporting a potentially important role for these cells in the development of airway inflammation.

FK-506, an immunosuppressive agent, inhibits T cell activation (Schreiber & Crabtree, 1992; Siekierka & Sigal, 1992; Widerrecht *et al*., 1993) and reduces the eosinophil infiltration into the airways (Lapa e Silva *et al*., 1995) and BHR in guinea-pigs (Akutsu *et al*., 1991) and in mice (Nagai *et al*, 1995). Accordingly, in this study, we investigated the effect of FK-506 on antigen-induced eosinophil and T cell accumulation in the BALF and on BHR in sensitized BP2 mice.

Methods

Mice

Male BP2 mice obtained from the Centre d'Elevage R. Janvier (BP5, 53940, Le Genest Saint-Isle, France) were maintained on standard laboratory chow and water *ad libitum* in the animal facilities of the Institut Pasteur. Mice ranging from 8–11 weeks (30–35 g) were used in the experiments.

Procedures for immunization and antigen provocation

Mice were immunized with 0.4 ml of a solution of 250 μg ml^{-1} ovalbumin mixed with 4 mg ml^{-1} $A1(OH)_3$, s.c., twice at a 7 day interval (100 μg per mouse). One week after the second injection, the immunized mice were challenged intranasally twice a day for two days (at 09 h 00 min and at 17 h 00 min) under light ether anaesthesia with 10 μg ovalbumin in 50 μl of

[1] Author for correspondence.

0.9% NaCl (saline) for about 10 s. Control mice were challenged with the same volume of saline. BHR was evaluated 24 h after the last antigen provocation.

BALF and serum preparations

BALF was recovered at the end of the evaluation of lung function, by injecting 8 volumes of 0.5 ml saline at room temperature. The total cell numbers were counted automatically (Coulter Counter ZM, Coultronics, Margency, France). The cell suspensions were cytocentrifuged (Hettich Universal, Tuttingen, Germany) and stained according to a May Grunwald Giemsa-derived method (Diff Quik, Baxter Dade AG, Duedingen, Switzerland). Differential cell counts were performed under a light microscope and the % of each cell population was obtained after counting at least 200 cells. Results are expressed as absolute numbers of total cells and of eosinophils ml^{-1} BALF.

In order to study the effect of FK-506 on the production of IL-5, the serum and BALF were recovered at 3 h after the last of the four antigen provocations. For serum collection, the abdominal cavity was opened and venous blood was collected from the post vena cava. Blood samples were allowed to clot at room temperature for 30 min and serum was collected after the centrifugation (500 *g* for 10 min) and stored at -20 °C until use. For the collection of BALF, the trachea was cannulated and a total volume of 1.5 ml of saline was instilled, as follows: a first sample of 0.5 ml was introduced and recovered and a second sample of 1 ml was introduced and recovered with a forward and backward movement for 3 cycles. This allowed a concentrated cell suspension to be obtained which was placed in plastic tubes on ice and centrifuged for 10 min, at 1850 *g*, 4°C (Jouan, Saint-Herblain, France). The supernatants were recovered and stored at -20°C until use.

Determination of IL-5 levels by enzyme immunometric assay

Immunometric assays developed by Dr C. Créminon (CEA, Gif sur Yvette, France) were performed in 96-well microtiters plates (MaxiSorps, Nuck, Roskilde, Denmark), coated with 10 μg ml^{-1} of the rat monoclonal antibody for murine IL-5 (TRFK-4), as described previously (Pradelles *et al*., 1985). The one-step procedure used for immunometric assays involved the simultaneous addition of 100 μl of the IL-5 standards (7.8–1,000 pg ml^{-1}) or experimental samples, and 100 μl of the second rat anti-IL-5 antibody, TRFK-5 conjugated to acetylcholinesterase (AChE) at a concentration of 10 Ellman units ml^{-1} (Grassi *et al*., 1989). After incubation for 18 h at 4°C, the plates were washed extensively and solid-phase bound AChE activity was determined colorimetrically by the addition of 200 μl of Ellman's medium (Ellman *et al*., 1961). Absorbance was read at 405 nm with an automatic microplate reader (Dinatech MR 5000; Dinatech Laboratories, Saint Cloud, France). The lower limit of detection of this assay is of approximately 5 pg IL-5 ml^{-1} sample.

TRFK-5 and TRFK-4 antibodies were purified from ascitic fluids of nude mice pre-injected i.v. with the appropriate hybridomas on a protein G column (HiTrap affinity columns, Pharmacia Biotechnology, Uppsala, Sweden) after precipitation by ammonium sulphate. The characteristics of these antibodies have been described in detail elsewhere (Schumacher *et al*., 1988).

Evaluation of lung function

Bronchoconstriction was measured by two methods. In the first, mice were anaesthetized with ethyl carbamate (15 mg 10 g^{-1}, i.p.), the trachea was cannulated and prepared for recording of dynamic compliance and airways resistance, by adapting the equipment of the computerized pulmonary analyser (Mumed PR800 system, UK) to mice airways at a tidal volume of 0.2 ml 10 g^{-1} and a frequency of 100 breaths min^{-1}. Mice were paralyzed with 10 μg of pancuronium bromide i.v., and airway resistance and dynamic lung compliance were calculated from the differential pressure between the airways and pleural cavity and the airflow. The effects of antigenic challenge on the airways responsiveness were evaluated using 5-hydroxytryptamine (5-HT) injected into the cannulated jugular vein at 10, 20, 40, 80, 160, 320 μg kg^{-1} in a volume of 100 μl 10 s^{-1} at 5 min intervals. Results are expressed as PD_{90} or PD_{270} (the amount of 5-HT needed to augment the bronchial resistance by 90% or 270% compared to the basal resistance, respectively). In the experiment on anaphylactic bronchoconstriction, ovalbumin at 50 mg kg^{-1} was injected i.v. to immunized mice and the results are presented as % increase of the bronchial resistance against the basal values.

In a second procedure, unrestrained conscious mice were placed in a whole body plethysmographic chamber (Buxco Electronics, Inc., Sharon, CT, USA), which analysed the respiratory waveforms. After stabilization for a few minutes, an aerosol of methacholine (3×10^{-2} M in the aerosolator) was delivered during 20 s. The airways resistance was expressed as Penh $=$[Te (expiratory time)/40% of Tr (relaxation time) $-1] \times$ Pef (peak expiratory flow)/Pif (peak inspiratory flow) $\times 0.67$. To calculate the ΔPenh (difference between the basal and maximal value), the average of 5 maximal values was used.

Treatment with FK-506

The solution for injection was prepared by dissolving FK-506 in a mixture of ethanol, Tween 80 and saline (1:0.2:8.8; v/v/v), according to the instructions of the manufacturer. Mice were injected with this solution at a dose of 2 mg kg^{-1}, s.c., 1 h before and 5 h after the first of four antigen challenges. Control mice received the same volume of vehicle.

Immunofluorescence analysis

The immunophenotyping of murine BAL cells from saline- or ovalbumin-challenged untreated or FK-506-treated mice was analysed by flow cytometry. Briefly, BAL cells collected 24 h after the last antigen challenge, were washed twice in PBS containing 3% FCS and 0.1% of sodium azide. Cells (2×10^5) were subsequently stained as described (Zuany-Amorim *et al*., 1994). The monoclonal antibodies used were rat IgG anti-CD4 FITC, rat IgG anti-CD8a FITC, hamster IgG anti-CD3ε phycoerytrin (PE), or the corresponding isotype matched controls. Stained cells were resuspended in PBS containing 3% FCS and 0.1% of sodium azide. Cell samples were analysed on a FACScan flow cytometer (Becton-Dickinson Immunocytometry, CA, U.S.A.). For each sample, 10,000 events were collected at 100 s and three-colour listmode data analysis was performed with Lysis II software (Becton-Dickinson). BAL cells were further analysed on the basis of light scatter properties in which the relative size (forward light scatter) and granularity (side angle scatter) of individual cell population was defined. Results are expressed as numbers of each T lymphocyte population ml^{-1} BALF.

Materials

Ovalbumin (5$\times$ crystallized) was from Immunobiological (Costa Mesa, U.S.A.). Aluminium hydroxide was from Merck (Darmstadt, Germany). 5-Hydroxytryptamine (5-HT), anti-CD4 FITC (clone H129.19), anti-CD8a FITC (clone 53-6.7), and ethyl carbamate were from Sigma (St. Louis, MO, U.S.A.). Methacholine (acetyl-β-methylcholine chloride) was from Aldrich-Chemie (Steinheim, Germany). Pancuronium bromide (Pavulon) was from Organon Teknika (France). FK-506 (17-allyl-1,14-dihydroxy-12-(2-(4-hydroxy-3-methoxy-cyclohexyl) 1-methylvinyl)-23,25-dimethoxy-13,19,21,27-tetramethyl-11,28-dioxa-4-azatricyclo (22,3,10) 4,9) octacos-18-ene-2,3,10,16-tetraone) was obtained from Fujisawa Pharmaceu-

ticals, Osaka, Japan. Tween-80 was from Fluka Chemika (Buchs, Switzerland). Anti-CD3ε phycoerytrin (clone 145-2C11) was from Pharmingen (San Diego, CA, U.S.A.), rat IgG-FITC and rat IgG-phycoerytrin were from Southern Biotechnology Associates (Birmingham, AL, U.S.A.). Buffer for flow cytometry was phosphate buffer (0.01 M, pH 7.5, Merck) containing NaCl (0.15 M, Carlo Erba Reagenti, Milano, Italy) and supplemented with 3% FCS and 0.1% of sodium azide (Merck).

Buffer for IL-5 determination by immunometric assay consisted of : 100 mM phosphate buffer pH 7.4, containing 150 mM NaCl, 0.1% bovine serum albumin and 0.01% sodium azide. Acetylcholinesterase (AChE) was purified from the electric eel *Electrophorus electricus* by affinity chromatography (Massoulie & Bon, 1976). The characteristics of the preparation of the tetrameric form of this enzyme (G4 form) which was used for antibodies labelling, have been described elsewhere (Grassi *et al*., 1988).

Statistical analysis

The results of each measurement are reported as mean ± standard error of the mean. The significance of differences between experimental and control group was examined by Student's unpaired t test and P values of less than 0.05 were considered significant.

Results

Effect of FK-506 on the eosinophil recruitment in the BALF

Total BALF cell and eosinophil numbers of ovalbumin-challenged mice were significantly increased, as compared to vehicle-treated saline-challenged mice (Figure 1). FK-506 administration failed to reduce total BALF cell numbers, but significantly reduced the eosinophil counts (from $1.36 \pm 0.22 \times 10^5$ to $0.53 \pm 0.24 \times 10^5$ cells ml^{-1}, $n = 5-6$, $P < 0.05$; Figure 1). There were no significant changes in the numbers of other leukocyte numbers in the BALF between FK-506-treated and control vehicle-treated mice (data not shown).

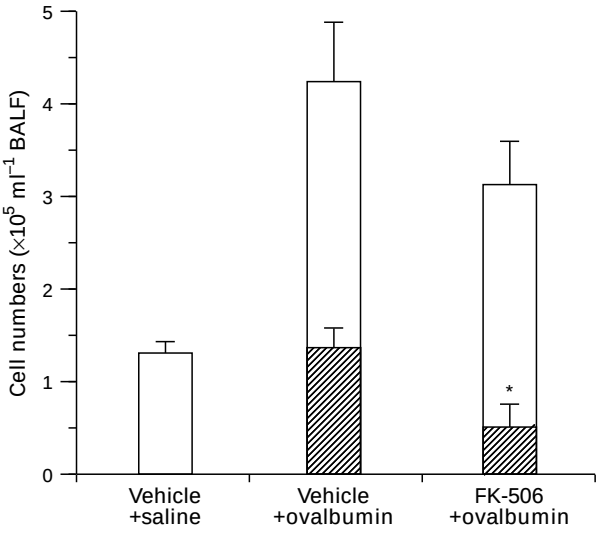

Figure 1 Effects of FK-506 (2 mg kg^{-1}, administered s.c. 1 h before and 5 h after the first of four antigen challenges) on the total BALF cell (open columns) and eosinophil numbers (hatched columns) after antigen challenges. The BALF was recovered 24 h after the last antigen provocation. Each value represents mean ± s.e.mean of 5–6 mice and significant differences are indicated: $*P < 0.05$, as compared to vehicle-treated ovalbumin-challenged animals.

Effect of FK-506 on bronchial responsiveness

Bronchoconstriction in response to i.v. 5-HT in vehicle-treated and ovalbumin-challenged mice was significantly augmented as compared to vehicle-treated and saline-challenged mice (from 52.0 ± 4.0 to 28.8 ± 1.8 μg kg^{-1} for PD_{270}, $n = 5-6$, $P < 0.01$, Figure 2). The administration of FK-506 augmented significantly the PD_{270} (from 28.8 ± 1.8 up to 47.9 ± 4.1 μg kg^{-1}, $n = 5-6$, $P < 0.01$). Similar results were obtained when the bronchial responsiveness was expressed as PD_{90} (Figure 2).

A significant increase in the intensity of bronchial resistance in response to aerolized methacholine was observed in vehicle-treated and ovalbumin-challenged mice, as compared to vehicle-treated and saline-challenged non-anaesthetized mice (from 2.7 ± 0.4 to 4.1 ± 0.2 for ΔPenh, $n = 5-6$, $P < 0.01$, Figure 3). The augmented responses to methacholine returned to the basal levels of saline-challenged mice in FK-506-treated animals (2.9 ± 0.2 for ΔPenh, $n = 5-6$, $P < 0.001$, Figure 3).

In order to study the effect of FK-506 on antigen-induced acute bronchoconstriction, immunized BP2 mice were treated with FK-506 twice a day for two days. Intravenous challenge with ovalbumin augmented markedly the bronchial resistance of immunized and vehicle-treated mice, and this was unaffected by FK-506 (Figure 4).

Effects of FK-506 on the production of IL-5 in the serum and in the BALF

The IL-5 levels were markedly increased 3–6 h after the last of the four antigen challenges (data not shown) in the BALF and serum. According, IL-5 release was evaluated 3 h after the last challenge. In vehicle-treated ovalbumin-challenged mice, the IL-5 levels in the BALF and in the serum were significantly augmented as compared to vehicle-treated saline-challenged mice. The production of IL-5 was significantly reduced in the BALF and in the serum of mice treated with FK-506 (Figure 5).

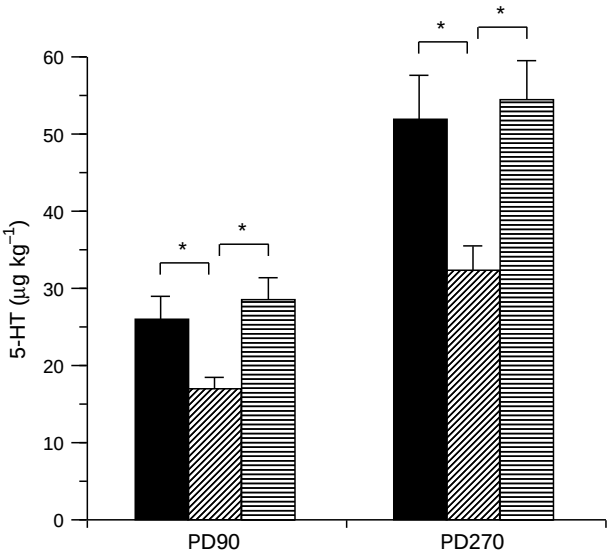

Figure 2 Effect of FK-506 (2 mg kg^{-1}, administered s.c. 1 h before and 5 h after the first of four antigen challenges) on bronchial responsiveness of anaesthetized BP2 mice in response to i.v. 5-HT, 24 h after the last antigen challenge; vehicle-treated saline-challenged (solid columns), vehicle-treated ovalbumin-challenged (hatched columns) and FK-506-treated ovalbumin-challenged (horizontally lined columns) mice. The bronchial resistance is expressed as PD_{90} and PD_{270} (see Methods). Each value represents mean ± s.e.mean of 5–6 mice and significant differences are indicated: $*P < 0.05$.

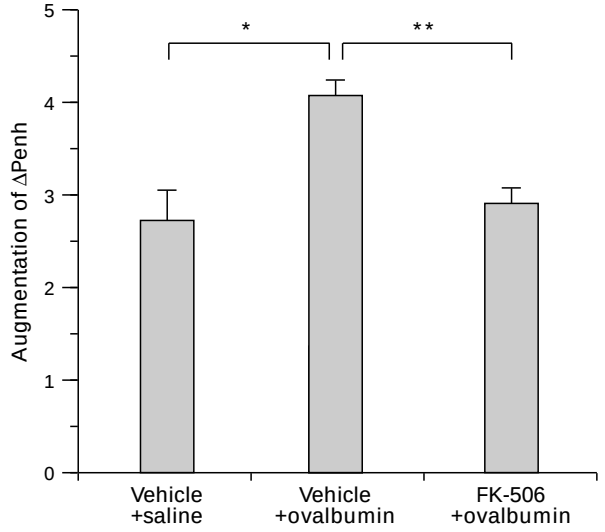

Figure 3 Effect of FK-506 on bronchial responsiveness to inhaled methacholine of non-anaesthetized BP2 mice after the last of the four antigen challenges. The bronchial resistance is expressed as Δ Penh (see Methods). Each value represents mean ± s.e.mean of 5–6 mice and significant differences are indicated: $*P<0.01$, $**P<0.001$.

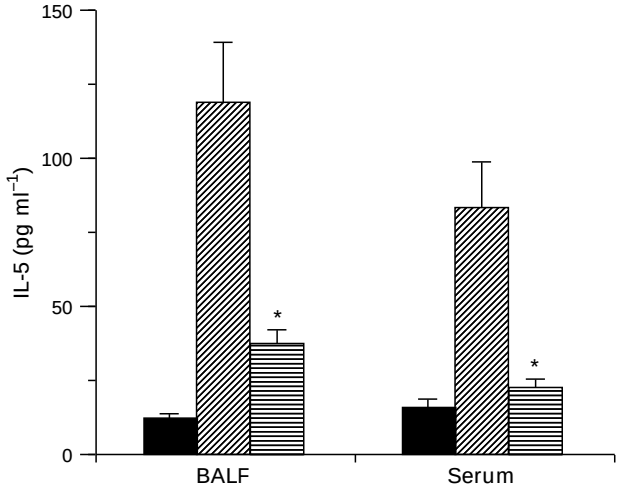

Figure 5 Effect of FK-506 on the IL-5 levels in the BALF and in the serum after four antigen provocations. The BALF and the sera were recovered 3 h after the last antigen challenge; vehicle-treated saline-challenged (solid columns), vehicle-treated ovalbumin-challenged (hatched columns) and FK-506-treated ovalbumin-challenged (horizontally lined columns) mice. Each value represents mean ± s.e.mean of 6–7 mice and significant differences are indicated: $*P<0.05$, as compared to vehicle-treated ovalbumin-challenged (hatched columns) animals.

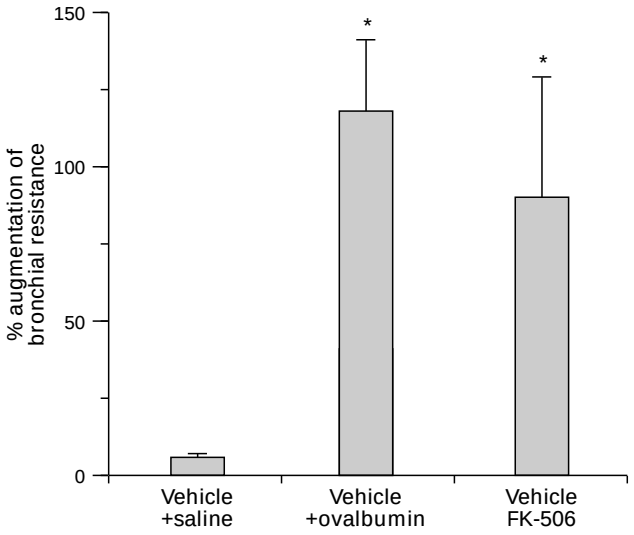

Figure 4 Failure of FK-506 to inhibit the antigen-induced bronchoconstriction in immunized BP2 mice. FK-506 was administered s.c. at 2 mg kg^{-1}, twice a day for two days, to the immunized mice and ovalbumin (50 mg kg^{-1}) was injected i.v., 24 h after the last treatment. Control immunized mice were injected with the same volume of saline. Each value represents mean ± s.e.mean of 5–7 mice and significant differences are indicated: $*P<0.01$, as compared to saline-injected animals.

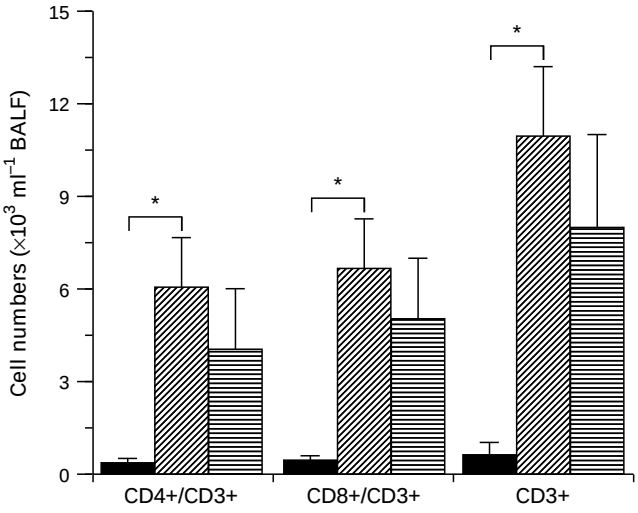

Figure 6 Effect of FK-506 on T lymphocyte infiltration in the BALF of immunized BP2 mice used 24 h after the last antigen challenge; vehicle-treated saline-challenged (solid columns), vehicle-treated ovalbumin-challenged (hatched columns) and FK-506-treated ovalbumin-challenged (horizontally lined columns) mice. BAL cells were recovered and double stained with either anti-CD3ε mAb, or with rat anti-murine CD4 or rat anti-murine CD8. Lymphocytes were identified as relative small, nongranular cells by their forward- and side-scatter characteristics. Results represent the mean ± s.e.mean of 3–6 experiments and are expressed as number of cells ml^{-1} of BALF: $*P<0.05$.

Effects of FK-506 on antigen-induced changes in the T-cell subsets in the BALF

Ovalbumin stimulation was followed by a marked rise in the numbers of $CD4^+/CD3^+$ and $CD8^+/CD3^+$ T-cells 24 h after the fourth antigen provocation. No significant inhibition in these numbers was observed after two treatments with FK-506, i.e., 2 mg kg^{-1}, 1 h before and 5 h after the first of four antigen challenges (Figure 6). Similar results were obtained, when FK-506 were administered four times, i.e., 1 h before each antigen challenge (data not shown).

Discussion

Eosinophils, the most prominent cell type infiltrating the airways of asthmatic subjects, are likely to play a role in the epithelial damage via the release of toxic granule proteins and the production of inflammatory mediators (Boushey *et al.*, 1980; Gleich, 1990). There is increasing evidence that the recruitment of eosinophils to the airways of asthmatics follows the activation of T lymphocytes (Walker *et al.*, 1992), and

indeed the presence of activated T cells has been demonstrated in blood, bronchial biopsies, and BALF of patients (Arm & Lee, 1992). These activated T cells are characterized by the expression of surface markers, including the IL-2 receptor (CD25), the human leukocyte antigen (HLA-DR), and the very late antigen (VLA-1) (Corrigan *et al*., 1988; Walker *et al*., 1991; Robinson *et al*., 1993). Lymphocyte-borne cytokines, which participate in the mobilization, accumulation and activation of eosinophils, include GM-CSF, IL-3 and IL-5 (Silberstein *et al*., 1987). The detection of mRNA encoding for IL-5 in bronchial biopsies from asthmatics strongly supports the concept that IL-5 released from the Th2 subset of T lymphocytes regulates eosinophil recruitment and function in asthma (Hamid *et al*., 1991). In the present study, we detected increased IL-5 production in the serum and in the BALF of antigen-challenged mice. Production of IL-5 may account for eosinophil recruitment into the airways and lung parenchyma following repeated antigen challenges, as supported by the effectiveness of a specific anti-IL-5 antibody (Eum *et al*., 1995). Different reports demonstrated a correlation between the recruitment of CD4+ T-lymphocytes and eosinophils into the airways. Indeed, CD4+ T-lymphocytes depletion inhibits antigen-induced eosinophil infiltration (Iwamoto *et al*., 1992; Nakajima *et al*., 1992; Hom & Estridge, 1994; Gavett *et al*., 1994) and reduces BHR (Gavett *et al*., 1994) in mice.

The immunosuppressive agent, FK-506, inhibits the *in vitro* production of T cell-derived cytokines such as IL-2, IL-3, and γ-interferon (INF-γ) (Kino *et al*., 1987; Sawada *et al*., 1987; Yoshimura *et al*., 1989). Furthermore, Mori *et al*. (1995) demonstrated that IL-5 production by blood mononuclear cells of asthmatic patients was suppressed *in vitro* by FK-506. The administration of this drug also inhibits the *in vitro* production of the mast cell-derived cytokines, IL-3, IL-4, and GM-CSF (Hatfield & Roehm, 1992). Furthermore, FK-506 prevented antigen-induced airway eosinophilia (Lapa e Silva *et al*., 1995) and BHR in guinea-pigs, but failed to reduce acute bronchoconstriction (Akutsu *et al*., 1995). Together, these findings indicate that FK-506 interferes with cytokine production triggered by allergic stimulation and with its consequences on airways inflammation and lung function. Nagai *et al*. (1995) demonstrated that FK-506 inhibited antigen-induced airway eosinophilia and BHR in BALB/c mice treated orally with 1 mg kg^{-1} for ten days before and during antigen challenges. In our present study, BP2 mice were treated with 2 mg kg^{-1} of FK-506 twice, i.e., before and during antigen challenge. Under these conditions, a 60% reduction of eosinophil numbers was noted, BHR being completely blocked, whether the bronchoconstrictor agent was administered i.v. or by aerosol. These results show that partial eosinophil recruitment to the airways can persist, BHR being suppressed. This partial recruitment may be due to insufficient suppression of IL-5 production or to other mediators, such as eotaxin, a recently described C-C chemokine (José *et al*., 1994), which may well operate in conjunction with IL-5. Eotaxin is expressed in allergic mice, and seems not to be produced exclusively by Th2 lymphocytes (Gonzalo *et al*., 1996). Its role in production/recruitment/activation of eosinophils had not as yet been defined. On the other hand, FK-506 inhibits immunoglobulin (Ig) E/FcεRI-triggered histamine release by human peripheral blood basophils (De Paulis *et al*., 1991) and 5-HT release by a rat basophilic leukaemic cell line (Hultsch *et al*., 1991). Nevertheless, in the present study, FK-506 failed to block the anaphylactic bronchoconstriction, in agreement with the results obtained in guinea-pigs (Akutsu *et al*., 1995). These results indicate that the effect of FK-506 may differ *in vitro* and *in vivo*, and suggest that its effectiveness against BHR results rather from an interference with the late eosinophils infiltration than with acute hypersensitivity reaction.

In agreement with the concept that IL-5 is central to eosinophil recruitment, FK-506 also reduced the production of IL-5 in the BALF and in the serum following antigenic provocation. T lymphocytes and mast cells are the major sources of IL-5, but since mast cell depletion fails to suppress eosinophil recruitment (Nogami *et al*., 1990; Okudairia *et al*., 1991), T lymphocytes seem to be more important for the development of airways eosinophilia via the production of lymphokines, in particular IL-5.

It has been demonstrated that FK-506 selectively and rapidly inhibits the expression of early T cell activation genes encoding for IL-2, IL-3, IL-4, GM-CSF, tumour-necrosis factor-α and INF-γ, but does not affect the expression of IL-1α or IL-1β (Tocci *et al*., 1989). Cytokine levels are elevated in lung tissues at 6 h and T cell numbers are elevated in the BALF 24 h after the antigenic challenge in mice (Garlisi *et al*., 1995). In the present study, we observed that lymphocyte numbers accumulating in the BALF after antigenic challenge were not reduced by KF-506 administration. This confirms the study of Yoshimura *et al*. (1989), who demonstrated that FK-506 does not inhibit the proliferation of cloned T cells driven by IL-2. Therefore, it seems likely that FK-506 affects the early stage of T cell activation rather than their proliferative stage. Nagai *et al*. (1995) failed to investigate the effect of FK-506 on infiltrated lymphocytes into the airways, even though they reported a reduction of IL-5. Together, these findings suggest that FK-506 down-regulates allergic airway eosinophilia by inhibiting T cell cytokine production.

In conclusion, the immunosuppressive agent, FK-506, inhibited the antigen-induced airways eosinophilia and suppressed BHR in response to i.v. 5-HT and aerosolized methacholine. FK-506 also reduced IL-5 production in the BALF and in the serum, but failed to modify lymphocyte numbers in the BALF. T lymphocytes residing in the lung are probably activated early upon antigen challenges and produce proinflammatory cytokines which may regulate the airways inflammatory reactions, resulting in BHR.

We thank Dr C. Créminon for developing the ELISA kits for IL-5 determination, Dr P. Minoprio for providing the hybridomas TRFK-5 and TRFK-4 and Dr K. Murato for providing FK-506.

References

AKUTSU, I., FUKUDA, T. & MAKINO, S. (1991). Inhibitory effect of FK-506 on the development of late asthmatic response and bronchial hyperresponsiveness. *J. Allergy Clin. Immunol*, **87,** 205, Abstract.

ANDERSON, G.P. & COYLE, A.J. (1994). TH2 and TH2-like cells in allergy and asthma: pharmacological perspectives. *Trends Pharmacol. Sci*., **15,** 324–332.

ARM, J.P. & LEE, T.H. (1992). The pathobiology of bronchial asthma. *Adv. Immunol*., **51,** 323–382.

AZZIWI, M., BRADLEY, B., JEFFERY, P.K., FREW, A.J., WARDLAW, A.J., KNOWLES, G., ASSOUFI, B., COLLINS, J.V., DURHAM, S. & KAY, A.B. (1990). Identification of activated T lymphocytes and eosinophils in bronchial biopsies in stable atopic asthma. *Am. Rev. Respir. Dis*., **142,** 1407–1413.

BIOZZI, G., MOUTON, D., SANT'ANNA, O.A., PASSOS, H.C., GENNARI, M., REIS, M.H., FERREIRA, V.C.A., HEUMANN, A.M., BOUTHILLIER, Y., IBANEZ, O.M., STIFFEL, C. & SIQUEIRA, M. (1979). Genetics of immunoresponsiveness to natural antigens in the mouse. *Curr. Top. Microbiol. Immunol*., **85,** 31–98.

BOUSHEY, H.A., HOLTZMAN, M.J., SHELLER, J.R. & NADEL, J.A. (1980). Bronchial hyperreactivity. *Am. Rev. Respir. Dis*., **121,** 389–413.

BUSSE, W.W., COFFMAN, R.L., GELFAND, E.W., KAY, A.B. & ROSENWASSER, L.J. (1995). Mechanisms of persistant airway inflammation in asthma: a role for T cells and T-cell products. *Am. J. Respir. Crit. Care Med*., **152,** 388–393.

CORRIGAN, C.J., HARTNELL, A. & KAY, A.B. (1988). T lymphocyte activation in acute severe asthma. *Lancet*, **1,** 1129 – 1132.

CORRIGAN, C.J. & KAY, A.B. (1990). CD4 T-lymphocyte activation in acute severe asthma. *Am. Rev. Respir. Dis.*, **141,** 970 – 977.

DE PAULIS, A., CIRILLO, R., CICCARELLI, A., DE CRESCENZO, G., ORIENTEE, A., MARONE, G., (1991). Characterization of the anti-inflammatory effect of FK-506 on human mast cells. *J. Immunol.*, **147**, 4278 – 4285.

DJUKANOVIC, R., ROCHE, W.K., WILSON, J.W., BEASLEY, C.R.W., TWENTYMAN, O.P., HOWARTH, P.H. & HOLGATE, S.T. (1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.*, **142,** 434 – 457.

ELLMAN, G., COURTNEY, K., ANDRES, V. & FEATHERSTONE, R. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.*, **7,** 88 – 95.

EUM, S.Y., HAILÉ, S., LEFORT, J., HUERRE, M. & VARGAFTIG, B.B. (1995). Eosinophil recruitment into the respiratory epithelium following antigenic challenge in hyper-IgE mice is accompanied by interleukin-5-dependent bronchial hyperresponsiveness. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 12290 – 12294.

EUM, S.-Y., CRÉMINON, C., HAILÉ, S., LEFORT, J., VARGAFTIG, B.B. (1996). Inhibition of airways inflammation by dexamethasone is followed by reduced bronchial hyperreactivity in BP2 mice. *Clin. Experiment. Allergy.*, **26**, 971 – 979.

FREW, A.J., MOQBEL, R., AZZAWI, M., HARTNELL, A., BARKANS, J., JEFFERY, P.K., KAY, B., SCHEPER, R.J., VARLEY, J., CHURCH, M.K. & HOLGATE, S.T. (1990). T lymphocytes and eosinophils in allergen-induced late-phase asthmatic reactions in the guinea pig. *Am. Rev. Respir. Dis.*, **141** 407 – 413.

GARLISI, C.G., FALCONE, A., KUNG, T.T., STELTS, D., PENNLINE, K.J., BEAVIS, A.J., SMITH, S.R., EGAN, R.W. & UMLAND, S.P. (1995). T cells are necessary for Th2 cytokine production and eosinophil accumulation in airways of antigen-challenged allergic mice. *Clin. Immunol. Immunopathol.*, **75,** 75 – 83.

GAVETT, S.H., CHEN, X., FINKELMAN, F. & WILLS-KARP, M. (1994). Depletion of murine CD4+ T lymphocytes prevents antigen-induced airway hyperreactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.*, **10,** 587 – 593.

GLEICH, G.J. (1990). The eosinophil and bronchial asthma : Current understanding. *J. Allergy Clin. Immunol.*, **85,** 422 – 436.

GONZALO, J.-A., JIA, G.-Q., AGUIRRE, V., FRIEND, D., COYLE, A.J., JENKINS, N.A., LIN, G.-S., KATZ, H., LICHTMAN, A., COPELAND, N., KOPF, M. & GUTIERREZ-RAMOS, J.-C. (1996). Mouse eotaxin expression parallels eosinophil accumulation during lung allergic inflammation but it is not restricted to a Th2-type response. *Immunity*, **4,** 1 – 14.

GRASSI, J., FROBERT, Y., LAMOURETTE, P. & LAGOUTTE, B. (1988). Screening of monoclonal antibodies using antigens labeled with acetylcholinesterase: applications to the peripheral proteins of photosystem 1. *Anal. Biochem.*, **168,** 436 – 450.

GRASSI, J., FROBERT, Y., PRADELLES, P., CHERCUITTE, D., GRUAZ, D., DAYER, J.M. & POUBELLE, P. (1989). Production of monoclonal antibodies against interleukins 1α and 1β: development of two enzyme immunometric assays (EIA) using acetylcholinesterase and application to biological media. *J. Immunol. Methods*, **123,** 193 – 210.

HAMID, Q., AZZAWI, M., YING, S., MOQBEL, R., WARDLAW, A.J., CORRIGAN, C.J., BRADLEY, B., DURHAM, S.R., COLLINS, J.V. & JEFFERY, P.K. (1991). Expression of mRNA for interleukin-5 in mucosal biopsies from asthma. *J. Clin. Invest.*, **87,** 1541 – 1546.

HATFIELD, S.M. & ROEHM, N.W. (1992). Cyclosporine and FK506 inhibition of murine mast cell cytokine production. *J. Pharmacol. Exp. Ther.*, **260,** 680 – 688.

HOM, J.T. & ESTRIDGE, T. (1994). Antigen-induced recruitment of eosinophils: Importance of CD4+ T cells, IL5, and mast cells. *Clin. Immunol. Immunopathol.*, **73,** 305 – 311.

HULTSCH, T., ALBERS, M.W., SCHREIBER, S.L., HOHMAN, R.J. (1991). Immunophilin ligands demonstrate common features of signal transduction leading to exocytosis or transcription. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 6229 – 6233.

IWAMOTO, I., TOMOE, S., TOMIOKA, H., TAKATSU, K. & YOSHIDA, S. (1992). Role of CD4+ T lymphocytes and interleukin-5 in antigen-induced eosinophil recruitment into the site of cutaneous late-phase reaction in mice. *J. Leukoc. Biol.*, **52,** 572 – 578.

JOSE, P.J., GRIFFITHS-JOHNSON, D.A., COLLINS, P.D., WALSH, D.T., MOQBEL, R., TOTTY, N.F., TRUONG, O., HSUAN, J.J. & WILLIAMS, T.J. (1994). Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea-pig model of allergic airways inflammation. *J. Exp. Med.*, **179,** 881 – 887.

KINO, T., HATANAKA, H., MIYATA, S., INAMURA, N., NISHIYAMA, M., YAJIMA, T., GOTO, T., OKUHARA, M., KOHSAKA, M., AOKI, H. & OCHIAI, T. (1987). FK-506, a novel immunosuppressant isolated from a Streptomyces; II. Immunosuppressive effect of FK-506 in vitro. *J. Antibiot.*, **XL**, 1256 – 1265.

LAPA E SILVA, T.R., BACHELET, C.M., PRETOLANI, M., BAKER, D., SCHEPER, R.J. & VARGAFTIG, B.B. (1993). Immunopathologic alterations in the bronchi of immunized guinea pigs. *Am. J. Respir. Cell Mol. Biol.*, **9,** 44 – 53.

LAPA E SILVA, T.R., RUFFIE, C., VARGAFTIG, B.B. & PRETOLANI, M. (1995). Modulation of bronchial inflammation in sensitized guinea pigs by FK-506, nedocromil sodium and dexamethasone. *Eur. Respir. J.*, **8,** 1321 – 1327.

LOPEZ, A.F., SANDERSON, C.J. GAMBLE, J.R., CAMPBELL, H.D., YOUNG, I.G. & VADAS, M.A. (1988). Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J. Exp. Med.*, **167,** 219 – 224.

MASSOULIÉ, J. & BON, C. (1976). Affinity chromatography of acetylcholinesterase: the importance of hydrophobic interactions. *Eur. J. Biochem.*, **68,** 531 – 539.

MORI, A., SUKO, M., NISHIZAKI, Y., KAMINUMA, O., KOBAYASHI, S., MATSUZAKI, G., YAMAMOTO, K., ITO, K., TSURUOKA, N. & OKUDAIRA, H. (1995). IL-5 production by CD4+ T cells of asthmatic patients is suppressed by glucocorticoids and the immunosuppressants FK506 and cyclosporin A. *Int. Immunol.*, **7,** 449 – 457.

NAGAI, H., YAMAGUCHI, S., TANAKA, H. & INAGAKI, N. (1995). Effect of some immunosuppressors on allergic bronchial inflammation and airway hyperresponsiveness in mice. *Int. Arch. Allergy Immunol.*, **108,** 189 – 195.

NAKAJIMA, H., IWAMOTO, I., TOMOE, S., MATSUMURA, R., TOMIOKA, H., TAKATSU, K. & YOSHIDA, S. (1992). CD4+ T-Lymphocytes and Interleukine-5 Mediate Antigen-induced Eosinophil Infiltration into the Mouse Trachea. *Am. Rev. Respir. Dis.*, **146,** 374 – 377.

NOGAMI, M., SUKO, M., OKUDAIRA, H., MIYAMOTO, T., SHIGA, J., ITO, M. & KASUYA, S. (1990). Experimental pulmonary eosinophilia in mice by *Ascaris suum* extract. *Am. Rev. Respir. Dis.*, **141,** 1289 – 1295.

OKUDAIRIA, H., NOGAMI, M., MATSUZAKI, G., DOHI, M., SUKO, M., KASUYA, S. & TAKATSU, K. (1991). T-cell-dependent accumulation of eosinophils in the lung and its inhibition by monoclonal anti-interleukin-5. *Int. Arch. Allergy Appl. Immunol.*, **94,** 171 – 173.

OWEN, W.F., ROTHENBERG, M.E., SILBERSTEIN, D.S., GASSON, J.C., STEVENS, R.L., AUSTEN, K.F. & SOBERMAN, R.J. (1987). Regulation of human eosinophil viability, density, and function by Granulocyte/Macrophage colony-stimulating factor in the presence of 3T3 fibroblasts. *J. Exp. Med.*, **166,** 129 – 141.

PRADELLES, P., GRASSI, J. & MACLOUF, J. (1985). Enzyme immunoassays of eicosanoids using acetylcholinesterase as label. *Anal. Chem.*, **57,** 1170 – 1173.

ROBINSON, D., HAMID, Q., BENTLY, A., YING, S., KAY, B. & DURHAM, S.R. (1993). Activation of CD4+ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J. Allergy Clin. Immunol.*, **92,** 313 – 324.

ROTHENBERG, M.E., OWEN, W.F., SILBERSTEIN, D.S., WOODS, J., SOBERMAN, R.J., AUSTEN, K.F. & STEVEN, R.L. (1988). Human eosinophils have prolonged survival, enhanced functional properties and become hypodense when exposed to human interleukin 3. *J. Clin. Invest.*, **81,** 1986 – 1992.

SAWADA, S., SUZUKI, G., KAWASE, Y. & TAKAKU, F. (1987). Novel immunosuppressive agent, FK506; In vitro effects on the cloned T cell activation. *J. Immunol.*, **139,** 1797 – 1803.

SCHREIBER, S.L. & CRABTREE, G.R. (1992). The mechanism of action of cyclosporin A and FK506. *Immunol. Today*, **13,** 136 – 142.

SCHUMACHER, J.H., O'GARRA, A., SHRADER, B., VAN KIMMENADE, A., BOND, M.W., MOSMANN, T.R. & COFFMAN, R.L. (1988). The characterization of four antibodies specific for mouse IL-5 and development of mouse and human IL-5 enzyme-linked immunosorbent assays. *J. Immunol.*, **141,** 1576 – 1581.

SIEKIERKA, J.J. & SIGAL, N.H. (1992). FK-506 and cyclosporin A: immunosuppressive mechanism of action and beyond. *Curr. Opin. Immunol.*, **4,** 548 – 552.

SILBERSTEIN, D.S. & DAVID, J.R. (1987). The regulation of human eosinophil function by cytokines. *Immunol. Today*, **8,** 380–385.

TOCCI, M.J., MATKOVICH, D.A., COLLIER, K.A., KWOK, P., DUMONT, F., LIN, S., DEGUDICIBUS, S., SIEKIERKA, J.J., CHIN, J. & HUTCHINSON, N.I. (1989). The immunosuppressant FK506 selectively inhibits expression of early T cell activation genes. *J. Immunol.*, **143,** 718–726.

WALKER, C., BODE, L.B.E., HANSEL, T.T., BLASER, K. & VIRCHOW, J.C. (1992). Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. *Am. Rev. Respir. Dis.*, **146,** 109–115.

WALKER, C., KAEGI, M.K., BRAUN, P. & BLASER, K. (1991). Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J. Allergy Clin. Immunol.*, **88,** 935–942.

WALKER, C., VIRCHOW, J.C., BRUIJNZEEL, P.L.B. & BLASER, K. (1991). T cell subsets and their soluble products regulate eosinophilia in allergic and non-allergic asthma. *J. Immunol.*, **146,** 1829–1835.

WIEDERRECHT, G., LAM, E., HUNG, S., MARTIN, M. & SIGAL, N. (1993). The mechanism of action of FK-506 and cyclosporin A. *Ann. N.Y. Acad. Sci.*, **609,** 9–19.

YOSHIMURA, N., MATSUI, S., HAMASHIMA, T. & OKA, T. (1989). Effect of a new immunosuppressive agent, FK506, on human lymphocyte responses in vitro; II. Inhibition of the production of IL-2 and IFN-γ, but not B cell-stimulating factor 2. *Transplantation*, **47,** 356–359.

ZUANY-AMORIM, C., LEDUC, D., VARGAFTIG, B.B. & PRETOLANI, M. (1994). Modulation by rm interferon-γ and CD4+ T-lymphocytes of allergic eosinophilic accumulation in the mice peritoneal cavity. *Ann. N.Y. Acad. Sci.*, **725,** 34–43.

(Received March 4, 1996
Revised June 4, 1996
Accepted September 19, 1996)