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Effect of Disodium Cromoglycate Treatment on Peripheral Blood Mononuclear Cell Adhesion to Cultured Endothelium in Allergic Asthmatics

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Summary

In this study we have compared the adhesion of peripheral blood mononuclear cells (PBMC) to human umbilical vein endothelial cells (HUVEC) in a healthy control group with two groups of allergic asthmatics, not treated or treated with disodium cromoglycate (DSCG). The adhesion and blocking experiments were performed by the flow cytometric adhesion assay. No differences in the adhesion of lymphocytes were observed in any of the groups. The monocytes obtained from DSCG non-treated patients have shown significant ($P<0.05$) enhancement of adhesion to HUVEC in comparison to healthy controls. The treatment of asthmatic patients with DSCG downregulated the monocyte adhesion to cultured endothelial cells (ECs) and this was comparable to the group of normal donors. The DSCG may have a therapeutic effect on the regulation of monocyte adhesion in inflammatory and allergic diseases. The binding ability of untreated asthmatic PBMC to cultured ECs was partially inhibited by monoclonal antibody anti-CD54, suggesting that the increased EC adhesiveness for monocytes from allergic asthmatics may be at least partially dependent on the ICAM-1 adhesion pathways. Our results also indicate that the blocking agent anti-CD18 was not essential for monocyte-endothelial interactions in allergic asthma.

Key words
Adhesion – Disodium cromoglycate – Allergic asthma

Introduction

In the past decade much progress has been made in the understanding of the emigration of leukocytes from the intravascular space. Leukocyte-endothelial cell interactions are thought to play an important role in a variety of inflammatory processes. Circulating leukocytes are directed to the site of infection by a complex of multi-step interactions of specific adhesion molecules (ADMs) located on the leukocytes and the vascular endothelium (Fan *et al.* 1993, Thiel *et al.* 1996).

In asthma and allergic disease, ADMs play an important and pivotal role in the infiltration of circulating cells to the target area of inflammation. Leukocytes are recruited initially by rolling and reversible binding to endothelial cells (ECs) through selectin ADMs and their ligands, followed by activation and stable binding to ECs through integrins and ICAM-1 (intercellular adhesion molecule 1, CD54) for neutrophils, and integrins, ICAM-1 and VCAM-1 (vascular cell adhesion molecule 1, CD106) for eosinophils and lymphocytes (Schroth 1996, Bloemen *et al.* 1997).

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Understanding the function of ADMs in asthma and allergic disease opens a possible therapeutic door for pharmacological agents directed at specific ADMs or at the antagonism of ADM expression. Agents that could modulate the interaction of leukocytes with the endothelium may, therefore, possess anti-inflammatory and immunosuppressive properties. Disodium cromoglycate (DSCG) was introduced into clinical practice in 1967 as a mast cell stabilizing drug for the treatment of patients with allergic asthma (McFadden 1987). This prophylactic agent inhibits degranulation of sensitized mast cells, prevents both the immediate and the late phase asthmatic response to a variety of immunologic challenges. The effects of DSCG may result in its ability to reduce the level of airway hyper-responsiveness in patients with bronchial asthma. The mechanisms may include reducing the influx and activation of inflammatory cells or directly affecting local nerve endings (Hoag and McFadden 1991). *In vitro* studies of DSCG have demonstrated the inhibition of ADM expression as an anti-inflammatory action and decrease of inflammatory cell number in the airways of asthmatic patients (Hoshino and Nakamura 1995, 1997).

ADM expression (CD11a - αL integrin chain of LFA-1 (lymphocyte function-associated antigen-1) complex, CD18 - subunit of β2 integrin, CD54, CD62L - L-selectin, CD49d - integrin α4, VLA (very late activation antigen-α4 chain) in peripheral blood of patients with mild asthma was evaluated by means of flow cytometry as a part of DSCG studies *in vitro*. The investigation, in our model situation, demonstrated a significant decrease of ICAM-1 on monocytes and CD49d expression on lymphocytes and monocytes after DSCG prophylactic medication (Jahnová *et al.* 1997). In the present study, we compare the adherence capacity of peripheral blood mononuclear cells (PBMC) from patients with allergic asthma, I) not treated and II) treated with DSCG, and III) healthy individuals. To assess the role of PBMC adhesion to human umbilical vein endothelial cells (HUVEC), we performed inhibition studies with blocking monoclonal antibodies (MoAbs) to CD54 and CD18.

Materials and Methods

Preparation and culture of HUVEC

Human umbilical cord veins were obtained from normal deliveries (kindly provided by the Second Department of Obstetrics, Faculty Hospital and Gynaecological Department of Dérer's Hospital, Bratislava). HUVEC were isolated and cultured by a modified method according to Jaffe *et al.* (1973). Briefly, primary ECs were harvested from human umbilical cord veins treated with 0.2 % collagenase (Sigma Chemical Co.) for 20 min at 37 °C. After incubation, the solution containing ECs was flushed out of the cord, and the cells were then centrifuged and resuspended in a culture medium that contained RPMI 1640 plus 15 % foetal calf serum (FCS), 1 % L-glutamine, antibiotics, heparin and 30 μg/ml EC growth supplement (Sigma Chemical Co.)

ECs were cultured in Petri dishes (Falcon) pretreated for 30 min with 1 % gelatine solution (Sigma Chemical Co.) in a humidified 5 % CO_2 incubator at 37 °C. The identity of the ECs was established by flow cytometry analysis with fluorescein isothiocyanate (FITC)-conjugated anti-ECs MoAb QBEND10 (Immunotech).

HUVEC from the first or second passage were used in all experiments. The confluent monolayered cells were detached with 0.1 % trypsin/ethylenediamine-tetraacetic acid (trypsin /EDTA) solution (Sigma Chemical Co.).

Study subjects

Allergic asthmatics (mean age 31 years), either not treated (n = 7) or treated with DSCG administered by inhalation of the powder by means of a spinhaler, one capsule (20 mg) 3–4 times daily for 6 weeks (n = 10), a clinical study evaluating the anti-inflammatory effect of DSCG on bronchial hyperreactivity. The subjects suffered from pollen rhinoconjunctivitis and mild bronchial asthma with clinical symptoms which ranged from chronic cough to a rare occurrence of asthmatic attacks (dyspnoic attacks). They had never been treated with any anti-inflammatory drugs, such as corticosteroids, or any oral antiallergic drugs and the investigation was not carried out during the pollen season. All individuals were tested before and after DSCG monotherapy by nonspecific inhalation challenge tests with acetylcholine followed by the method of Sterk *et al.* (1993). The statistical analysis confirmed the high significance of the bronchoprotective effect of DSCG in mild asthmatics (unpublished data).

Healthy control subjects were non-atopic adult volunteers (mean age 28 years; n = 13). All participating subjects were non-smokers.

Human PBMC

PBMC obtained from allergic asthmatics and healthy adult human volunteers were prepared by centrifugation on a Ficoll (Pharmacia, Uppsala, Sweden)-Tellebrix N 300 (Léčiva, Prague, Czech Republic) density gradient. After centrifugation, the PBMC were recovered at the gradient interface, washed and resuspended in RPMI 1640 supplemented with antibiotics and 10 % FCS. The

viability of isolated cells was determined by trypan blue exclusion. The purity of the preparation was assessed by flow cytometry analysis using lymphocyte and monocyte specific MoAb anti-CD45/anti-CD14 (LeucoGATE, Becton Dickinson).

Adhesion assay

A modification of the adhesion assay described previously by Benschop *et al.* (1992) was used. In brief, HUVEC monolayers were grown to confluence on gelatine-coated 24-well plates. After washing with phosphate-buffered saline (PBS), confluent EC monolayers were incubated with 25 U/ml IFN-γ (R & D Systems, UK) at 37 °C in 5 % CO_2 humidified air. After 24 h the freshly isolated PBMC ($1x10^5$ cells/well) were allowed to attach on the EC monolayer in the presence of 20 ng/ml lipopolysaccharide (LPS) (Sigma Chemical Co.). The plates were incubated for 1 h at 37 °C in a humidified incubator with 5 % CO_2, under static conditions. After removing non-adherent cells, the remaining cells were detached by trypsinisation and this mixture of cells was analysed using a flow cytometer. Each experiment was run in duplicate. The adherence index of PBMC, lymphocytes and monocytes was calculated by dividing the number of adhering leukocytes and the number of ECs.

Effect of MoAbs on cell adhesion

For the adhesion inhibition studies, 15 μl of MoAbs anti-CD54, anti-CD18 or the isotype control IgG1 (25 μg/ml) (Becton Dickinson) were used. The PBMC were tested for binding to HUVEC either in the presence of these antibodies or in the medium alone. The percentage inhibition was calculated as: (1 − adhesion in the presence of MoAb/ adhesion in the absence of MoAb) x 100 %.

Flow cytometry analysis

The mixture of cells obtained after adhesion of PBMC to ECs was washed in PBS and the cell populations were distinguished by flow cytometry (Coulter Epics XL, USA) based on scatter properties (forward and side scatter) according to Benschop *et al.* (1992).

Phenotypical analysis of the cells was performed using one-colour flow cytometry analysis. The cells were stained with 10 μl of appropriate FITC-conjugated MoAbs (Becton Dickinson) for 15 min at room temperature. Fluorescence data were collected on 5000 viable cells. The results are expressed in log units of fluorescence intensity.

Statistical analysis

The data were statistically analysed using unpaired Student s t-test. $P<0.05$ values were considered significant.

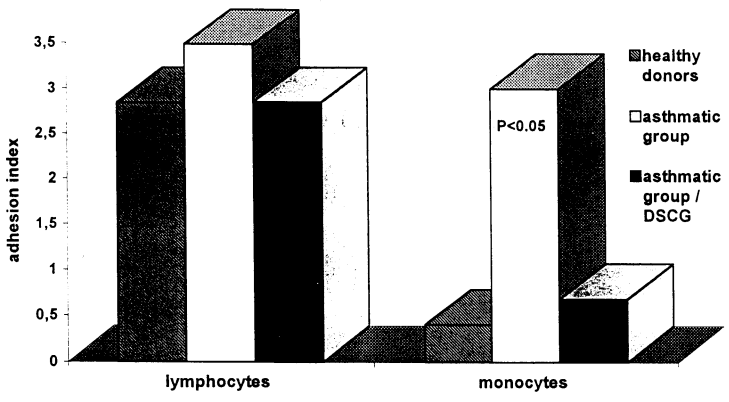


Fig. 1. *Adhesion of PBMC from healthy individuals (n = 13), patients with allergic asthma not treated (n = 7) and treated (n = 10) with DSCG to ECs. IFN-γ stimulated (25 U/ml) EC monolayers were co-incubated for one hour with freshly isolated PBMC in the presence of LPS (20 ng/ml). Adhesion of lymphocytes and monocytes to ECs was examined by the flow cytometric assay. $P<0.05$ compared with healthy control group and/or DSCG-treated asthmatic group.*

Results

PBMC adhesion to HUVEC monolayers

Because the adhesion of leukocytes to ECs is a crucial step for the subsequent extravasation of these cells into inflammatory sites, we investigated the difference in adherence capacity of freshly isolated PBMC from allergic asthmatic patients and healthy individuals to HUVEC. The asthmatics were divided into two groups: not treated or treated with DSCG.

In adhesion experiments under static conditions (Fig. 1), monocyte adhesion to HUVEC was significantly greater ($P<0.05$) in the asthmatic group not treated with DSCG (3.0±1.9) compared with healthy controls (0.6±0.4) and/or DSCG-

treated asthmatics (0.7±0.3). When we compared the adhesion of lymphocytes, no statistically significant differences could be observed in all groups (healthy donors 2.9±2.2, asthmatic groups not treated 3.5±1.7 and DSCG-treated 2.9±1.3). The adherence of lymphocytes when compared with monocytes significantly differed in both DSCG-treated asthmatics and the control group.

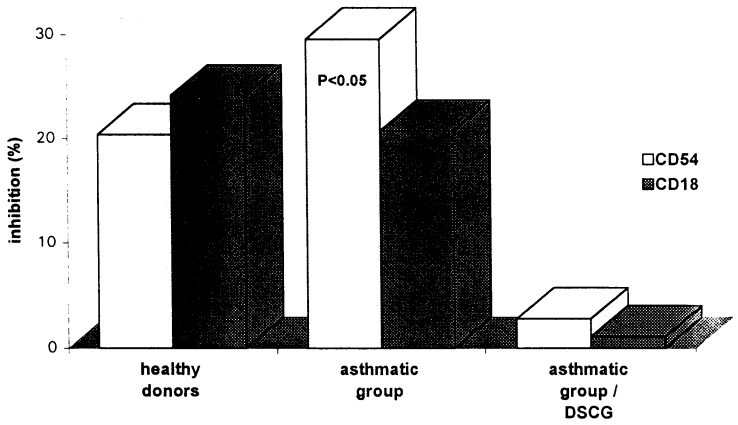
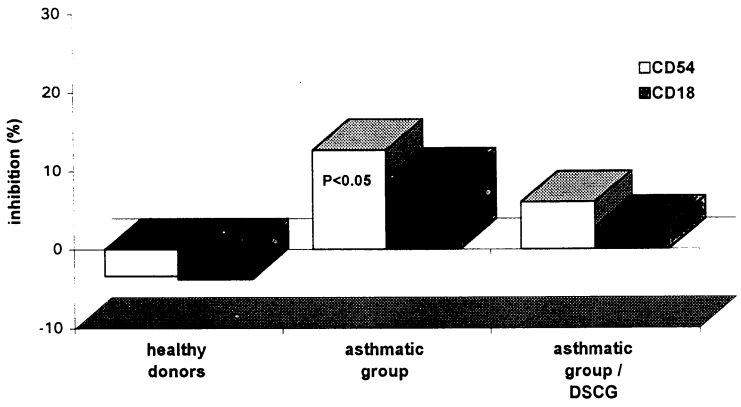


Fig. 2. *Inhibition of lymphocyte adhesion to ECs. Blocking activity of CD54, CD18 and isotype control IgG1 was tested in healthy donors (n = 5), DSCG-treated (n = 6) and not treated (n = 4) asthmatic groups. The MoAbs (25 μg/ml) were added to the stimulated HUVEC monolayers along with the PBMC ($1x10^5$ cells/well) and LPS (20 ng/ml). The zero level represents background inhibition in the presence of negative isotype control. P<0.05 compared with negative control inhibition.*

Fig. 3. *Inhibition of monocyte adhesion to ECs. PBMC were obtained from healthy adult volunteers (n = 5), from patients not treated (n = 4) or treated with DSCG (n = 6). EC monolayers were cultured with MoAbs (anti-CD54, anti-CD18) or isotype control (IgG1). The zero level represents background inhibition. P<0.05 compared with healthy control group.*



The effects of anti-CD54 and anti-CD18 MoAbs on PBMC adhesion to HUVEC

The blocking experiments were performed to ascertain the relative contributions of CD54 and CD18 to the adhesion of PBMC obtained from asthmatics and normal donors to HUVEC. The MoAbs anti-CD54 and anti-CD18 altered adhesion of lymphocytes from the not treated asthmatic group (30 % and 21 % inhibition, respectively) and healthy individuals (21 % and 25 % inhibition, respectively) was found, but significant values were obtained only in the first group with the blocking MoAb to CD54. Incubation of PBMC with either anti-CD54 or anti-CD18 MoAbs had no influence on lymphocyte adhesion in DSCG-treated asthmatics (Fig. 2).

The monocyte adhesion to HUVEC by PBMC from DSCG-untreated asthmatics was slightly inhibited by 13 % and 9 % with antibodies to CD54 or CD18, respectively, and the inhibition in the DSCG-treated group was 6 % for anti-CD54 and 3 % for anti-CD18 antibodies. Compared with healthy donors, the DSCG-untreated asthmatic group showed significant differences in CD54 blocking experiments, but there was no significant reduction of adhesion with CD18 blocking MoAb. Neither MoAb to CD54 nor to CD18 had inhibitory effects in the normal control group. There was no

significant distinction in monocyte binding between the normal control group and DSCG-treated asthmatics after inhibition with either anti-CD54 or anti-CD18 (Fig. 3).

Discussion

The adherence of lymphocytes and monocytes either to the neighbouring ECs or the extracellular matrix is a necessary initial step of the inflammatory, immunological and haemostatic responses (Coulombel *et al.* 1997). Leukocyte-EC interaction is a complex process that involves several specific and complementary molecules present on both cells, and different activation pathways (Galéa *et al.* 1993, Khalfoun *et al.* 1996).

In the present study, we have compared the adhesion of PBMC to HUVEC in a control healthy group with two groups of allergic asthmatics (treated and not treated with DSCG). The monocytes retrieved from DSCG-untreated allergic asthmatics showed significantly enhanced adhesion to HUVEC in comparison to healthy controls. This monocyte adhesion to ECs represents a part of a positive feedback system in which monocytes play a role in their own recruitment (Combe *et al.* 1995). It is well known that monocytes are a potentially large source of proinflammatory cytokines such as IL-1 and TNF. Co-cultures of monocytes and ECs induce secretion of IL-1 and TNF from these cells (Eierman *et al.* 1989, Takahashi *et al.* 1996). Some investigators studying human asthmatics have found that inflammatory mediators released by an anti-IgE-dependent challenge, promote or enhance the *in vitro* expression of ADMs (ICAM-1, VCAM-1, CD18) by cells involved in the pathogenesis of asthma. These subsequently mediate the migration of leukocytes to the target sites through initial rolling or reversible adhesion, leukocyte and vascular cell activation, and leukocyte activation-dependent adhesion (Caldero and Lockey 1992, Bentley *et al.* 1993, Schroth 1996).

It was recently demonstrated that DSCG treatment of asthmatic patients significantly decreased the adhesion of monocytes to ECs. Previous reports have shown that DSCG inhibits the ADM expression and may reduce the activation of neutrophils, eosinophils and monocytes *in vitro* (Hoag *et al.* 1991, Hoshino and Nakamura 1997, Jahnová *et al.* 1997). The precise mechanism by which DSCG affects these cells is unknown, but it may be related to an effect on cytoplasmic control of calcium influx into the cell (McFadden 1987). Based on our findings, the anti-inflammatory effect of DSCG treatment can, at least in part, be explained by the drug's suppressive effect on monocyte-EC interactions. The potency of DSCG as an exogenous regulator of monocyte adhesion might open up new therapeutic approaches aimed to protect against inflammatory tissue destruction, the first step in the pathogenesis of which is PBMC adhesion. Future therapeutic strategies on inflammatory and allergic diseases will undoubtedly include antiadhesive agents (Meng *et al.* 1991).

Our results showed that the PBMC binding ability of DSCG-untreated asthmatics to ECs could only be partially inhibited with the MoAb anti-CD54, but not with anti-CD18. We thus conclude that ICAM-1 may play a minor role in the increased EC adhesiveness for PBMC and that CD18 is not essential for the increased binding of monocytes from allergic asthmatics to cytokine-stimulated ECs. Several previous studies have indicated a partial inhibition effect of MoAbs to CD54 and/or CD18 on PBMC (Benschop *et al.* 1992, Jonjic *et al.* 1992, Beekhuizen *et al.* 1992, Calderon *et al.* 1992, Cartwright *et al.* 1995, Lukacs *et al.* 1994) and other blood cells (neutrophils, eosinophils) (Smith *et al.* 1991, Kyan-Aung *et al.* 1991, Malik and Lo 1996). Some other authors could not demonstrate an inhibitory activity of these MoAbs and have suggested that different molecules (E-selectin, VCAM-1) must have been involved in the increased binding of lymphocytes and/or monocytes to cytokine-stimulated ECs (Beekhuizen *et al.* 1991, 1992). Since a MoAb raised against ICAM-1 only caused partial inhibition of monocyte binding to ECs, we suggested that the presence of an alternative system other than ICAM-1 could be involved in mediating the monocyte adhesion to ECs according to a previous report (Maier *et al.* 1993).

In conclusion, this study provides evidence that monocytes of asthmatic patients become more activated than monocytes of healthy donors and that the DSCG treatment exerts antiadhesive effects and can modify monocyte adhesion. Our data have also demonstrated that the ICAM-1 was partially responsible for the binding of monocytes from allergic asthmatics to cultured ECs and that other adhesion pathways must thus also be involved. Therefore, the antiadhesive molecule therapy for asthma appears to show great promise.

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Reprint requests

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