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The effect of budesonide on lymphoid and non-lymphoid cell profiles, and Ia-antigen expression in rats with experimental colitis

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ABSTRAK

Marsetyawan HNE Soesatyo & Mary Palmen – *Pengaruh budesonide terhadap profil sel limfoid dan non-limfoid serta ekspresi antigen-Ia pada tikus dengan colitis eksperimental.*

Kortikosteroid merupakan obat yang efektif terhadap peradangan, seperti penyakit peradangan usus kronis. Penelitian ini bertujuan untuk mengetahui pengaruh pemberian kortikosteroid lokal, yakni budesonide colitis experimental pada tikus, khususnya pengaruhnya terhadap populasi sel limfoid dan nonlimfoid, serta ekspresi antigen-Ia (molekul *major histocompatibility complex* (MHC) klas II). Di samping itu diteliti pula peran molekul-molekul adhesi, seperti *intercellular adhesion molecules* (ICAM-1) dan *lymphocytes function associated antigen* (LFA-1) setelah pemberian obat tersebut.

Model colitis dibuat dengan pemberian secara intracolon zat hapten: *2,4,6-trinitrobenzene sulphonic acid* (TNBS) dalam etanol, berdasarkan metode standar. Obat budesonide diberikan dalam dosis tunggal dan ganda sebanyak 0,25 ml larutan 10^{-5} M secara lokal ke dalam colon dengan menggunakan kateter. Populasi sel limfoid dan nonlimfoid di sepanjang mukosa dan submukosa, termasuk plak Peyer (PP) dan jaringan limfoid di daerah proksimal colon diperiksa dengan teknik imunositokimiawi. Ekspresi MHC klas ii dan molekul adhesi diteliti menggunakan panel antibodi monoklonal (AbMo).

Dosis ganda budesonide (3x) sangat efektif untuk mengobati colitis akut; ini dapat diamati dari gejala-gejala klinis yang menghilang. Pemeriksaan histologik menunjukkan penurunan nyata jumlah subpopulasi makrofag, seperti sel $ED1^+$, $ED2^+$ dan $ED3^+$. Intensitas ekspresi Ia dan ekspresi ICAM-1, LFA-1 mengurang, sedangkan migrasi sel neutrofil dan sel radang lainnya menghilang.

Key words : inflammatory bowel disease – TNBS colitis – budesonide-lymphoid and non-lymphoid cell populations – MHC class II expression – adhesion molecules.

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INTRODUCTION

Treatment of chronic colitis, such as inflammatory bowel disease (IBD), is hampered by the fact that little is known about the underlying causes of the condition. To acquire deeper insight of the pathogenesis of this disorder, animal models for IBD are primarily important. Morris *et al.*[1] have developed a model in rats by means of administration of a hapten: 2,4,6- trinitrobenzene sulfonic acid (TNBS), dissolved in ethanol, into the distal colon. The model for IBD is proved to be reproducible.

Glucocorticoids have been widely used as anti-inflammatory agents since 40 years. With respect to IBD, hydrocortisone enemas were introduced for the treatment of ulcerative colitis and other inflammatory processes of the distal colon.[2,3] This drug, however, has high systemic influences. So, a new generation of cortico-

steroid, characterized by a high first pass metabolism in the liver, was developed.[4] One of the new corticosteroid is budesonide, which has high topical potency but little systemic effect. Recently budesonide has been on trials against IBD, in particular ulcerative colitis and proctitis.[5,6]

IBD is a chronic gastrointestinal disorder in which it's etiology remains unclear. Several reports indicated the involvement of local macrophages and dendritic cells (DC) in the pathogenesis of such a disease.[7,8] For instance, differences in numbers and heterogeneity of macrophages and DC were found between tissues obtained from IBD patients and normal individuals.

This study was aimed at examining the effect of local corticosteroid, *i.e.* budesonide treatment on TNBS-induced colitis in rats, with special emphasis on the lymphoid and non-lymphoid cell populations by using immunocytochemistry. In addition, the expression of MHC class II antigens and the adhesion molecules was also investigated.

MATERIALS AND METHODS

Animals

Male wistar rats, weighing about 250 g (Harlan Sprague Dawley, Zeist, The Netherlands) were used in the study. The animals were maintained under standard laboratory conditions with pelleted food formula and tap water *ad libitum*.

Drugs

TNBS (2,4,6-trinitrobenzene sulfonic acid) was purchased from Sigma Chemical Co., St. Louis, MO., USA. Budesonide was kindly provided by Dr. S.E. Svensjo, Astra Draco, Lund, Sweden.

Experimental design

Induction of colitis

Colitis was induced by intracolonic administration of TNBS in ethanol, as previously described by Morris *et al.*[1] with slight modifications. Under Hypnorm@ (Janssen Pharmaceutica BV, Tilburg) anesthesia, each rat received 30 mg TNBS dissolved in 0,25 ml 40% ethanol, using a catheter inserted approximately 8 cm in the colon from the anus. Subsequently, the rats were checked daily to see their general conditions, body weight, and quality of stools. The histological sections of the colon were prepared and examined in the study on day 1,7,14 and 28 after the induction.

Budesonide treatment

The micronized-budesonide was diluted to 10^{-2}M in 100% ethanol, as stock solution. This was then adjusted to the final concentration at 10^{-5}M dissolved in 0.9% saline as working solution. Each dose contained 0.25 ml 10^{-5}M budesonide and was administered locally through a catheter into the colon. This drug was given either once a day at day 1, or 3 times at day 1, 4 and 8 after TNBS administration.

Fifty two animals were divided into 4 groups. TNBS-ethanol was given to group A, B and C. Group A was treated with multiple doses of budesonide 3 times a day on day 1, 4 and 8 after TNBS treatment; group B with a single dose of budesonide on day 1 after TNBS; and group C received a placebo. Group D was non-IBD control, which only received budesonide at day 1, 4 and 8. On day 9, 15 and 18 after the induction of colitis, the rats were sacrificed. The effects of budesonide 2 times daily administered 24 h prior to colitis induction were also examined.

Morphological changes of the colon were examined by at least 3 independent observers. Any visible damage was scored on 0-5 scale, as described by Morris *et al.*,[1] in which, score 0 means no damage; score 1 to 5 represent different severity of colonic lesions (see TABLE 3). The inflammed and non-inflammed colon (including proximal colonic lymphoid tissues, PCLT), a part of small intestine (including Peyer's patches, PP), were collected and snap frozen in liquid nitrogen for immunocytochemistry.

Immunocytochemistry

Cryostat sections of 8 µm were picked up on slides, fixed in aceton and air-dried. The slides

were incubated for 60 minutes at room temperature with a solution of the first step of monoclonal antibodies (MoAbs, *see* TABLE 1) in 0.01 M phosphate-buffered saline (PBS), pH 7.4, with 0.5% bovine serum albumin (BSA). Afterwards, the slides were washed 3 times in PBS and then incubated with peroxidase conjugated rabbit antimouse serum, dilution 1:200 (Miles, Elkhart, USA) in PBS with 0.5% BSA and 1% normal rat serum, for 30 minutes. After being rinsed with PBS, the sections were stained for peroxidase activity[9] with 3.3'-diaminobenzidine-tetra-hydrochloride (Sigma, St. Louis, Mo., USA) in 0.5 mg/ml TRIS-HCl pH 7.6, containing freshly added 0.01% H_2O_2. After washing again with PBS, the slides were lightly counterstained

TABLE 1. – Monoclonal antibodies (Mo Ab's) used in immunocytochemistry

MoAb	Detection	Antigen	Ref
ED1	monocytes, almost all macrophages	cytoplasmic	10
ED2	tissue macrophages, mature macrophages	differentiation membrane antigen	10,11
ED3	macrophage subpopulation mainly in lymphoid organs	membrane, sheep erythrocyte receptor	10,12
OX6	MHC class II gene products	Ia-antigens	13
OX8	suppressor T-cells	CD8	14
OX19	T-cells	CD5	15
W3/25	helper T-cells	CD4	16
WT.1	adhesion molecule LFA-1	CD11a	17
IA-29	adhesion molecule ICAM-1	CD54	18

TABLE 2. – The lymphoid and non-lymphoid cells in the colon after TNBS-administration.

time (days)	mϕ	PMN	DC	T-cells	B-cells
0	induction of colitis				
1	+++	++	=	=	=
7	++++	+++	+	+	=
14	++++	+++	++	++	+
28	+++	++	+	++	+

mϕ: macrophages; PMN: polymorphonuclear; DC: dendritic cells; =: number of positive cells is the same as controls; +: 1-5 positive cells per microscopic field more than in the controls; ++: 6-10 positive cells per microscopic field more than in the controls; +++: 11-15 positive cells per microscopic field more than in the controls; ++++: 15 positive cells per microscopic field more than in the controls.

TABLE 3. – Effect of single and multiple doses of budesonide (Bud) on the number of rats showing symptoms and signs related to colonic inflammation

group	IBD + saline	IBD + 1x Bud	IBD + 3x Bud	3x Bud
no. rats	15	15	15	7
diarrhoea	7	6	2	0
colon wall-thickening	7	5	0	0
damage score				
0 – 2	1	0	13	7
3 – 5	15	14	2	0

Damage score : 0 = no damage; 1 = localized hyperemia ≤ 1 cm, but no ulcer; 2 = one ulcer; 3 = one ulcer and area of inflammation; 4 = two or more sites of ulceration/inflammation; 5 = two or more sites of ulceration/inflammation and site > 1cm length.

with haematoxylin, then dehydrated and mounted in Entellan@ mounting medium (Merck, FRG). Slides that will be used for quantification of positive cells were counterstained with 10 times diluted nuclear fast red (Merck, FRG), and then were analysed with the image analysis system (IBAS, Kontron Electronic, GMBH).

Quantification

The positive cells per microscopic field could be separated from the homogenous, relatively colorless background (objective magnification 40x). This was done semi-automatically, in which the threshold of the critical grey values was set interactively for each measurement. Thus, the degree of coloration of a specimen did not affect the morphometric measurements.

The percentage of positive cell staining was determined as follow,

$$\% \text{ positive cell staining} = \frac{\text{field area of positive cells}}{\text{total field area}} \times 100\%$$

Statistical analysis

The statistical significance of the differences was evaluated using Student's t-test and non parametric analysis. A level of $p < 0.05$ was considered significant.

RESULTS

General findings on TNBS-model

All animals treated with TNBS-ethanol developed both clinical and histopathological symptoms, such as diarrhea and transmural inflammation with or without ulceration. Damage scores from day 1 to day 28 are shown in TABLE 2. Colon wall thickening occurred in about 70% of the total IBD group. The animals with the most severe bowel lesion (damage score 5) reached to 60%.

Histologically, there was an influx of granulocytes and $ED1^+$ and $ED2^+$ macrophages during the acute phase of inflammation. In addition, $ED2^+$ macrophages were found not only in the basal of the crypts, but also at the upper parts. After the induction with TNBS-ethanol, the $ED3^+$ cells, which are normally present in the spleen and lymph nodes, were also found at low numbers in the colon. MHC class II expressions on dendritic cells around the crypts, and the upper region of lamina propria was increased, judged by their intensive staining with the corresponding MoAb. Interestingly, no such an expression was found on colonic epithelial cells during active disease. From day 7 and 14, the number of T and B cells, respectively, was increased (TABLE 2).

Budesonide treatment

After a single dose of 0.25 ml 10^{-5}M budesonide, no apparent improvement of the inflammation occurred. The multiple doses of this drug, however, dampened signs of acute inflammation. The clinical symptoms disappeared, and the ulceration recovered. The damage scores declined significantly ($p < 0.05$) on day 15 and 18 compared to that which received a placebo (FIGURE 1). Local budesonide administration had no obvious effect on the colonic mucosa of normal control rats.

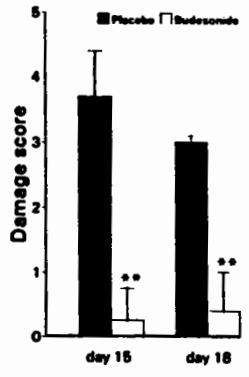

FIGURE 1. Damage scores of the colon at day 15 and 18 after induction of colitis. (■) colitis animals which received a placebo; (□) colitis animals which received budesonide; 3 times daily. (** p <0.001).

On day 15 (7 days after the last budesonide treatment) the percentages of $ED1^+$ and $ED2^+$ macrophages in the submucosa decreased (FIGURE 2A and 2B). The number of $ED3^+$ macrophages in the submucosa also decreased after

therapy. Among those three macrophage subpopulations, however, only the $ED1^+$ cells decreased enormously.

With respect to MHC class II expression, budesonide reduced the intensity of this Ia staining in the mucosa and submucosa. Hardly any MHC class II expression seen on dendritic cells around the crypts. The reduction of Ia staining was also observed in the colon of the controls treated with this drug.

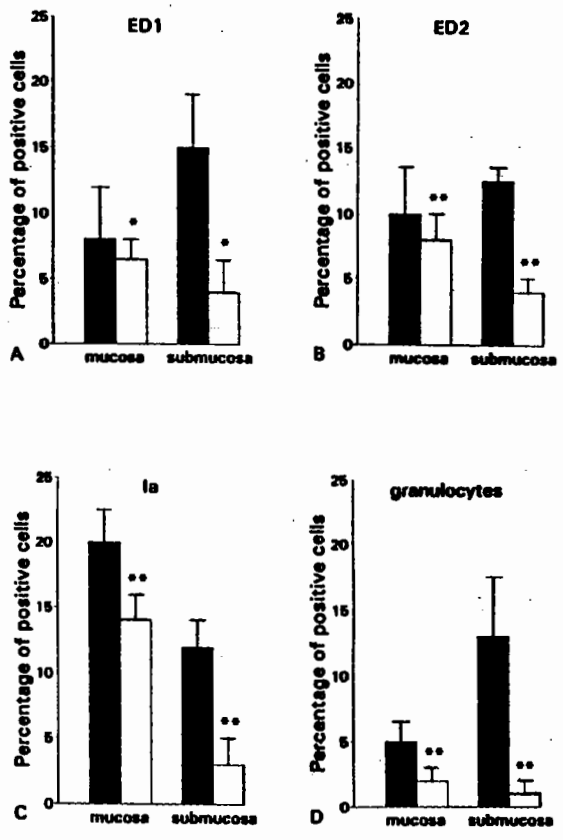

FIGURE 2. Percentage of different cell subpopulations at day 15 after induction of colitis. (■) colitis animals which received a placebo; (❑) colitis animals which received budesonide 3 times daily. (A) $ED1^+$ macrophages; (B) $ED2^+$ macrophages; (C) IA (MHC class II)$^+$ cells; (D) granulo cytes. (* $p < 0.05$; ** $p < 0.001$).

The percentage of granulocytes in both mucosal and submucosal sites was significantly decreased following the treatment with triple doses of budesonide (FIGURE 2D). A decrease was found in the number of positive B cells, as well as T cells in the mucosa of the colon. No apparent difference was observed in the number and distribution of both lymphoid and non-lymphoid cells in either PCLT or PP. Furthermore, the number of intercellular adhesion molecule-1 (ICAM-1)-bearing cells was also decreased in the colon. Similarly, there was a decrement of cells that express lymphocyte function-associated antigen-1 (LFA-1) molecules.

Administration of local budesonide prior to induction of IBD with TNBS, had no preventive effect. Most rats clearly developed megacolon, bowel wall thickening, and the damage score reached 5. This result was comparable to the group receiving a placebo.

DISCUSSION

This study describes the effect of budesonide on immunocompetent cells in TNBS-induced colitis. Although this drug has a potent topical influence, as previously demonstrated in the respiratory and skin diseases,[7] a single dose of 10^{-5}M budesonide presented locally in the colon did not have any effect on acute inflammation. In contrast to the multiple doses ($3x10^{-5}$M), which markedly produced a therapeutic effect. The latter doses seemed to affect only on mucosal lesions, whereas it did not influence the normal colonic tissue.

After budesonide treatment, a decrease was observed in $ED1^+$, $ED2^+$ and $ED3^+$ macrophage subpopulations. The reduction of $ED3^+$ macrophages is in contrast to the findings of Damoiseaux *et al.*,[19] who reported that the addition of corticosteroid to bone marrow cultures strongly induced the ED3 expression. The different result is possibly due to difference in test system, *i.e.* in vitro *vs* in vivo. A decrease in the number of macrophages at the sites of inflammation and a concomitant reduction of circulating monocytes after steroid treatment have been reported by several authors.[20-22] Guyre and Munck[23] described that monocytes and macrophages were among the most sensitive cells against anti-inflammatory effect of glucocorticoids. This drug could reduce the number of inflammatory cells in the inflammed airways to normal condition[24] by inducing apoptosis, inhibiting cell migration, and by decreasing the production of various

cytokines. It is likely that similar actions take place in the mucosa of colon during IBD.

After the induction of TNBS-mediated colitis, the intensity of MHC class II staining increased, and subsequently this expression was down-regulated by budesonide. Similar result were reported by Jevnikar[25] that the increment of expression of MHC molecules in autoimmune nephritis could be reduced by oral administration of corticosteroid.

Other interesting findings were accomplished, that budesonide modulated the expression of adhesion molecules, such as ICAM-1. This molecule was expressed on macrophages, dendritic cells and memory T cells, and is the ligand receptor for LFA-1. Our study demonstrated the reduction of ICAM-1^+ cells in the colon. This may be due to the reduction of ICAM-1 expression, which in turn causes a reduction of neutrophil migration into the lesion.

The mucosal T cells outnumbered B cells after TNBS induction and their number decreased gradually following the budesonide treatment. The changes of lymphoid cell profiles compared to normal situation were, however, not significant. In this study, the PP-and PCLT-B cells were not affected by this corticosteroid, though several reports[8,26] found a depletion of B cells in the dome area of PP and in the germinal center of PCLT. These conflicting results were possibly due to the use of different species and different route of drug administration.

CONCLUSION

Budesonide has a potential effect for the treatment of experimentally-induced colitis, particularly to non-lymphoid cells, like the $ED1^+$, $ED2^+$ and $ED3^+$ macrophage subpopulations and dendritic cells. These cells are suggested to play a role in the pathogenesis of IBD model through their MHC class II expression, and indirectly control the inflammatory cell migration by regulating the adhesion molecules for cellular interactions.

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