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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: Expediente No. 06-074.675
Solicitud de patente a nombre de **Action Medicines S.L.**

Yo, DILIA MARIA RODRIGUEZ D'ALEMAN, actuando como apoderada sustituta de acuerdo con la sustitución adjunta, identificada como aparece al pie de mi firma, domiciliada en la Carrera 11 No. 86-53, piso 6, obrando como apoderada de la sociedad **Action Medicines S.L.**, domiciliada en Madrid, España, solicitante de la patente de la referencia, en respuesta al oficio No. 215 notificado por fijación en lista el 08 de enero de 2010, me permito acompañar los siguientes documentos solicitados por ese despacho:

1. NOUR A.F. & SALAM S. Preliminary clinical study with calcium dobesilate in fibrocystic disease of the breast, a pilot study, Invasive Treatment, 1986, Volume 12, No. 3, pages 233-242.
2. RUIZ, E & TEJERINA, T. Calcium dobesilate increases endothelium-dependent relaxation in endothelium-injured Rabbit aorta, Pharmacological Research, 1998, Volume 38, No. 5, pages 361-366.
3. GRABER, R. y col. Calcium dobesilate protects human peripheral blood mononuclear cells from oxidation and apoptosis. Apoptosis, 1998, Volume 3, No. 1, pages 41-49.

Espero con esto haber dado cabal cumplimiento a lo solicitado por ese despacho y de conformidad con lo indicado en los artículos 3º, inciso 3, 35 inciso 2 y 59 inciso 2 del CCA, al analizar este memorial, atentamente solicito se traten todas las cuestiones planteadas con motivo del mismo.

Señor Superintendente,

DILIA MARIA RODRIGUEZ D'ALEMAN
CC. 41.654.882
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PRELIMINARY CLINICAL STUDY WITH CALCIUM DOBESILATE*
IN FIBROCYSTIC DISEASE OF THE BREAST
A PILOT STUDY

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(A.R.E.)*

Summary

Fibrocystic disease of the breast is a benign tumour which usually occurs during the last decade of reproductive life as a result of hormonal imbalance. Thirty women diagnosed with fibrocystic breast disease were treated with calcium dobesilate* for 3 months. Disappearance or marked improvement was noticed in pain, tenderness, nodularity and nipple discharge. Mammography showed much less density in the parenchymatous and connective tissues. No recurrences were observed during the 6 months after cessation of therapy. The drug was devoid of side effects in this study.

INTRODUCTION

The female breast, like the endometrium, is under the hormonal influence of the menstrual cycle and shows a wide range of structural variation in health. It is during pregnancy and lactation that the changes are most marked. These changes may be within physiological limits or they may constitute, when exaggerated, the beginning of pathological lesions such as fibrocystic disease of the breast or fibroadenosis which is a benign tumour that should not be confused with carcinoma [1].

The cause of fibrocystic disease due to hormonal imbalance may probably be related to corpus luteum deficiency combined with excessive oestrogen and prolactin production. This condition is chiefly seen in the last decade of reproductive life especially in multiparae, but the main symptoms may not appear till after the menopause. Clinically, the woman complains either of pain,

* DOXIUM®, OM Laboratories.

87/80

A. FOUAD NOUR, S. SALAM

engorgement and tenderness or of lumps in the breast; the pain is usually worse during the menstrual period. Both breasts are often involved and there may be several lumps in each breast. The breast may feel coarsely granular because of the presence of small cysts that are as hard as a tumour, never soft and fluctuant. The axillary lymph nodes may be enlarged and tender.

The gross appearance of a surgical specimen of fibrocystic disease is characteristic and it is usually easy to make a naked-eye diagnosis. The tissue shows diffuse induration with cysts of varying size and number but usually they are small. The dilated ducts may be filled with putty-like material which can be squeezed out [2].

Histologically [1, 2], one or more of the following features may be present:

- 1) Glandular hyperplasia primarily involving the ducts. This appearance is likely to be found in younger women.
- 2) Cyst formation is very common. The cysts are usually small, they may be microscopic in size or may be as large as a cherry. The cysts are formed from dilated and hyperplastic ducts that have not undergone complete involution to a normal size.
- 3) Papillary formation may be present as a result of intraductal epithelial proliferation into the cyst spaces.
- 4) Apocrine epithelium appearing as large eosinophilic cells (stained pink with eosin) lining the whole, or part of the duct lumen. The apocrine epithelium is known to arise from primitive sweat gland epithelium.
- 5) Connective tissue hyperplasia is always found but varies greatly in amount. It is the specialized connective tissue of the gland fields which is involved, not the stroma of the breast. This disease may be related to connective tissue abnormalities.
- 6) Lymphatic infiltration is a very common feature.
- 7) Numerous thin-walled and fenestrated capillary blood vessels and initial lymphatics are present in the connective tissue which are the cause of oedema and lymph node swelling. Haemorrhage is common and blood stained discharge from the nipples is one of the characteristic symptoms.

The condition is regarded by some as pre-cancerous, but there is no unanimity on this point.

Hormonal imbalance is liable to alter the normal mechanisms of fluid and electrolyte equilibrium resulting in oedema into the interstitial spaces. This leads to the collapse of small lymphatic vessels thereby decreasing lymph

transport and increasing the amount of fluid and macromolecules present extravascularly. Tissue hyperplasia is enhanced and the reparative process diminished.

For the management of benign fibrocystic disease of the breast, salt and methylxanthines (present in coffee and tea) restriction, diuretics, tocopherol and hormone therapies (mainly testosterone derivatives) have been recommended [3].

The fact that calcium dobesilate is known to improve the structure of the small blood vessels, particularly collagen biosynthesis of their basement membranes and to enhance lymphatic flow [4-6] has encouraged us to evaluate it in the treatment of benign fibrocystic breast disease.

MATERIAL AND METHODS

Thirty women aged between 20 and 58 years (average: 39.3) with pain and tender masses in the breast diagnosed clinically, radiologically and by needle biopsy as fibrocystic disease were included in the trial. Patients with malignancy were excluded.

Calcium dobesilate was given orally in a dose of 500 mg 3 times daily continuously for 3 months. Patients were examined regularly every month. At the last examination mammograms were performed and needle biopsies were taken in some instances.

RESULTS

Clinically the breast masses disappeared gradually and were almost absent in most patients by the third month of treatment. Follow-up examinations 6 months after cessation of calcium dobesilate showed no recurrence.

Nodularity was markedly decreased and the associated pain and tenderness completely disappeared during treatment.

The nipple discharge (milky yellowish or reddish in colour) stopped during the first month of therapy and did not recur. In one case, the nipple discharge did not recur during 2 years follow-up.

Mammography performed after 3 months of therapy with calcium dobesilate showed a marked decrease in the generalised density of parenchymatous lobules and connective tissue stroma and this change was particularly obvious in 10 cases when compared to findings on entry to the trial.

The result of therapy was classified into 4 categories:

Very good: clinically, masses in the breast, nodularity, nipple discharge and pain completely disappeared during therapy. Mammography showed a remarkable decrease in generalised density when compared to findings before therapy.

Good: clinically, masses in the breast, nodularity, nipple discharge and pain completely disappeared during therapy. Mammography showed a less marked decrease in generalised density compared to findings before therapy.

Moderate: clinically, masses in the breast, nodularity, nipple discharge and pain were reduced but not absent during therapy. Mammography showed slight decrease in generalised density.

Nil: The condition remained clinically and radiologically unchanged.

The clinical data and overall results in these patients are shown in Table I.

The result of treatment with calcium dobesilate, both clinical and radiological, in 30 patients with fibrocystic disease of the breast is shown in table II. Figures 1 and 2 represent mammograms taken before and after therapy respectively showing a marked reduction in breast density due to diminution of coarseness and of proliferation of the parenchymatous tissue.

No side effects were observed during therapy.

DISCUSSION

Fibrocystic disease of the breast, or fibroadenosis, is a benign tumour which usually occurs when ovarian function is irregular and declining, chiefly in the last years of reproductive life, but it may also occur in young unmarried women who often suffer from ovarian hypoactivity. The masses, often in both breasts, are due to excessive hyperplasia of breast parenchyma, to increase in the connective tissue stroma surrounding the mammary gland and to microcirculatory and lymphatic disturbances. The latter are caused by focal discontinuity in the collagen of the basement membrane leading to extravasation of fluid from the capillary wall and inadequate drainage from the lymphatic system causing oedema which subsequently leads to an increase in tissue pressure and hyperplasia.

The consumption of methylxanthines in tea and coffee is known to increase circulating catecholamines and appears to be associated with the

84 83

CALCIUM DOBESILATE

TABLE I: Table indicating age, diagnosis and results of calcium dobesilate therapy in patients with fibrocystic disease of the breast.

Number of patients	Initials	Age (years)	Diagnosis	Results	
				Clinical	Radiological
1	H.S.	36	mass left breast radiolog. fibroadenosis	very good	good
2	K.S.	36	bilateral breast mass	very good	good
3	A.H.	40	left breast mass	very good	good
4	S.A.	33	right breast mass	very good	good
5	I.Y.	52	left breast mass	very good	good
6	M.F.	36	left breast mass	very good	good
7	M.S.	40	right breast mass	very good	good
8	K.F.	35	right breast mass	very good	good
9	A.M.	38	left breast mass	very good	good
10	N.A.	49	bilateral breast mass	very good	good
11	A.D.	28	left breast mass	very good	good
12	W.A.	34	right breast mass	very good	moderate
13	N.A.	22	left breast mass	very good	moderate
14	F.Z.	52	nipple discharge	very good	good
15	S.M.	45	right nipple discharge	very good	good
16	N.B.	53	nipple discharge	very good	good
17	E.R.	38	nipple discharge	very good	good
18	N.H.	38	right breast mass	very good	good
19	F.A.	39	right breast mass	good	nil
20	N.K.	35	right breast mass	very good	good
21	F.T.	50	left breast mass	very good	good
22	L.L.	52	left breast mass	good	good
23	K.A.S.	52	left breast mass	very good	good
24	S.Y.	35	painful left breast	very good	good
25	A.S.	58	left breast mass	very good	not done
26	A.A.	38	breast mass	very good	not followed
27	F.M.A.	38	nipple discharge	very good	not followed
28	R.B.	36	painful breast	very good	nil
29	A.M.	20	painful breast	good	nil
30	N.	22	painful breast mass	good	nil
		mean 39.3			

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A. FOUAD NOUR, S. SALAM

Fig. 1: Mammogram taken before treatment with calcium dobesilate. The breast shows areas of density due to coarseness and proliferation of parenchymatous tissue.

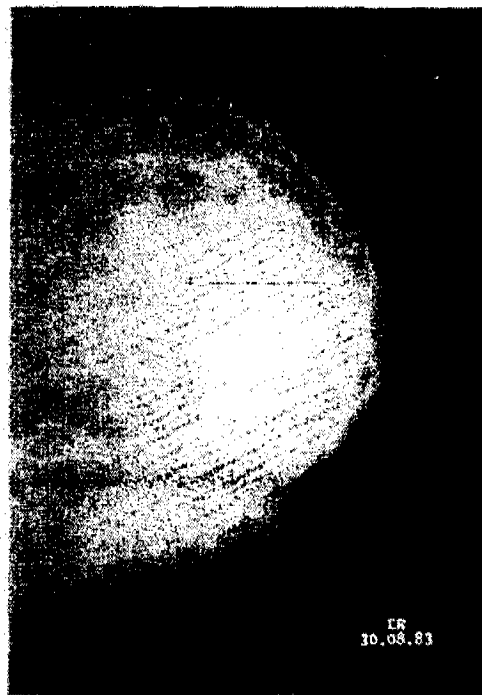
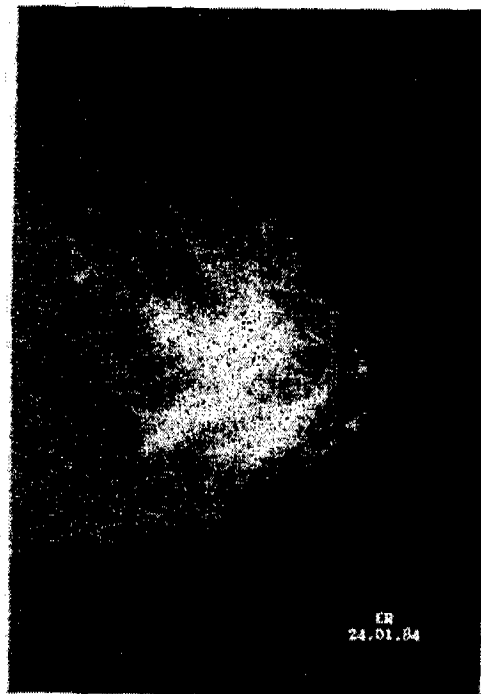


Fig. 2: Mammogram taken after calcium dobesilate therapy. The breast shows a marked diminution in density due to reduction in tissue proliferation.



86 93

TABLE II: *Clinical and radiological results of treatment with calcium dobesilate in 30 women with fibrocystic disease of the breast.*

Results	N	Very good	Good	Moderate	Nil
Clinical	30	26 (86.7%)	4 (13.3%)	—	—
Radiological	27*	—	21 (77.8%)	2 (7.4%)	4 (14.8%)

* 3 cases not evaluated.

development of benign fibrocystic disease in the American woman. Complete abstinence resulted in resolution of the disease in 82.5% of women and a significant improvement in another 15% [7]. In addition, a lower frequency of fibrocystic disease was found among users of oral contraceptives than among non-users [8].

On the other hand, high oestrogen and prolactin concentrations have been reported in patients with fibrocystic disease of the breast; this explains why a prolactin inhibitor like bromocriptine and an anti-oestrogen, tamoxifen, have recently been used [9] in this disease with somewhat encouraging results.

In the present study, 30 women with benign fibrocystic disease were treated continuously for three months with calcium dobesilate in a dose of 500 mg 3 times daily. During this period, the patients experienced marked relief from pain and mammary tension, the nipple discharge stopped from the first month, the nodules became smaller and disappeared completely in most patients by the third month of treatment. Mammograms taken after treatment showed much less density in the parenchymatous tissue and connective tissue stroma. There were no recurrences during the 6 months after the drug had been discontinued.

Our results do not allow a complete explanation of the mechanism of action of calcium dobesilate, but we may say that the drug's action seems to be mediated in part by its effects in strengthening capillary blood vessels, by its influence on collagen biosynthesis and by its anti-oedematous action through activation of the lymphatic system. Clinically, these results indicate the possible usefulness of calcium dobesilate in treating patients with benign fibrocystic disease of the breast. Moreover, the drug is safe and appears devoid of adverse reactions. For these reasons, we have now decided to conduct a further trial using the double-blind technique.

86
87
A. FOUAD NOUR, S. SALAM

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RÉSUMÉ

La maladie fibrokystique des seins est en général la conséquence bénigne d'un déséquilibre hormonal apparaissant dans la quarantaine. Les auteurs ont traité 30 patientes présentant cette affection par l'administration de dobésilate de calcium pendant 3 mois. Une disparition ou une amélioration marquée des signes objectifs, tels que douleurs, nodosités et écoulement mammaire, fut observée. Les mammographies montrent une diminution de la densité des tissus parenchymateux et conjonctifs. Aucune récurrence ne fut observée au cours des 6 mois qui suivirent l'interruption du traitement. Aucun effet indésirable ne fut signalé.

SAMENVATTING

Fibrokysteuze mastosis is gewoonlijk een goedaardig gevolg van een hormonaal onevenwicht bij de vrouw in de laatste tien jaren van haar vruchtbaarheidsperiode. De auteurs hebben 30 gevallen tijdens 3 maanden behandeld met calcium dobesilaat. De objectieve tekens, zoals pijn, knobbels en melkafschei-

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CALCIUM DOBESILATE INCREASES ENDOTHELIUM-DEPENDENT RELAXATION IN ENDOTHELIUM-INJURED RABBIT AORTA

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Calcium dobesilate (DOBE) is an orally administered angioprotective agent which is used in some vascular diseases such as diabetic retinopathy, although its mechanism of action is not yet fully understood. The aim of this work was to correlate previous '*in vitro*' findings carried out in our laboratory with an '*ex vivo*' model of endothelium-injury by overdose of vitamin D₂. Male New Zealand White rabbits were used. The study was divided into two protocols. Protocol 1: 10 days of treatment; and Protocol 2: 30 days of treatment. Rabbits in each group were treated with vitamin D₂ (200 000 IU day⁻¹) for the first 2 days and two groups were subsequently treated with DOBE at different doses (50 mg kg⁻¹ per day or 500 mg kg⁻¹ per day). The concentration-response curve induced by NA (10⁻⁸-10⁻⁴ M) in aorta arteries was shifted downwards in the groups treated with DOBE (in both Protocol 1 and 2), whereas only in Protocol 2 (30 days of treatment) was this curve affected in the hypervitaminic group. The endothelium-dependent relaxation induced by ACh (10⁻⁶-10⁻⁵ M) decreased in the hypervitaminic groups (in both Protocol 1 and 2) but only in Protocol 2 (30 days of treatment) was the endothelium-dependent relaxation restored to normal (control, untreated group) in both DOBE-treated groups. The endothelium-independent relaxation induced by sodium nitroprusside (SNP) (10⁻⁸-10⁻⁴ M) decreased in the hypervitaminic groups only in Protocol 1. We did not find differences in the DOBE-treated groups in any protocol compared with the control (untreated) group. These findings show evidence that DOBE restored endothelial functionality in endothelium-injured rabbit aorta only after 30 days of treatment.

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KEY WORDS: calcium dobesilate, endothelium-injured arteries, rabbit, hypervitaminosis.

INTRODUCTION

Calcium dobesilate (DOBE) (calcium 2,5-dihydroxybenzene sulphonate) is an orally administered angioprotective agent which, in addition to lowering blood, plasma and aqueous humor hyperviscosity [1, 2] reduces microvascular hyperpermeability [3], possesses platelet antiaggregatory activity [4], and has been successfully used in the treatment of chronic venous insufficiency [5] and diabetic retinopathy [6]. It has been described in previous reports [7, 8] that DOBE increased endothelium-dependent relaxation in isolated rabbit aorta, in an '*in vitro*' model and that this increase was due to an increase in synthesis/release of EDRF and to an additional mechanism such as a scavenging effect of DOBE on superoxide anion radicals. In order to correlate these actions of DOBE '*in vitro*' with those '*ex vivo*', we have tested the effects of DOBE on endothelium-

dependent relaxation in endothelium-injured rabbit aorta. Endothelium-injury was induced by adding an overdose of vitamin D₂ in the animal chow. It is well-known that hypervitaminosis with D₂ causes degeneration of both endothelial and vascular smooth muscle cells followed by smooth muscle cell proliferation [9].

MATERIALS AND METHODS

General procedure

Eighty male New Zealand white rabbits weighing 2.5-3.0 kg, obtained from Biocentre S.A. (Barcelona, Spain), were used in this study. The study was divided into two protocols as shown in Table I.

Administration of vitamin D₂ and calcium dobesilate

Animals were fed a standard diet 7 days before the start of the experiment.

*Corresponding author.

90 89

Table I
Protocols used in this study

	No. of rabbits	Treatment	Days of treatment
<i>Protocol 1</i>			
Group 1	10	Control (untreated group)	10
Group 2	10	Hypervitaminosis D ₂	10
Group 3	10	Hyper. + DOBE (50 mg kg ⁻¹ per day)	10
Group 4	10	Hyper. + DOBE (500 mg kg ⁻¹ per day)	10
<i>Protocol 2</i>			
Group 5	10	Control (untreated group)	30
Group 6	10	Hypervitaminosis D ₂	30
Group 7	10	Hyper. + DOBE (50 mg kg ⁻¹ per day)	30
Group 8	10	Hyper. + DOBE (500 mg kg ⁻¹ per day)	30

The rabbits were given 200 000 IU day⁻¹ of vitamin D₂ for the two first days of the experiments in order to damage the endothelium [9]. Vitamin D₂ was dissolved in 2 ml ethanol and the solution was vaporised onto the rabbit chow. Ethanol was evaporated at room temperature overnight in order to prevent the degradation of vitamin D₂. Hypervitaminosis was induced in all treated-groups (Groups 2, 3, 4, 6, 7 and 8) and rabbits followed the protocols shown in Table I. Calcium dobesilate was prepared everyday by dissolving it in drinking water. Animals were housing in individual cages.

At the end of each period, animals were anaesthetised with ethyl ether and killed by exsanguination from the common carotid.

Isolated aortic preparations

The thoracic aorta was rapidly removed and placed in Godfraind solution of the following composition (mM): NaCl, 121; KCl, 5.8; NaHCO₃, 14.9; MgCl₂, 1.22; glucose, 11; and Ca₂Cl, 1.25. Adherent fat and surrounding tissue were cleaned off and the arteries were cut into rings approximately 2–3 mm wide. The rings were then suspended between two stainless steel hooks in organ baths containing 10 ml of Godfraind solution. The solution was kept at 36 ± 0.5°C and gassed continuously with a 95% O₂–5% CO₂ gas mixture. The aorta rings were mounted under 2 g tension. Each preparation was allowed to equilibrate for 90–120 min. Contractile responses were measured isometrically by means of force-displacement transducers (Grass FT 03) and were recorded on a Grass polygraph as previously described [10]. After equilibration the following experiments were carried out in arteries from each protocol.

(1) A cumulative concentration–response curve to noradrenaline (NA) (10⁻⁸–10⁻⁴ M) was made by

adding it cumulatively in 1 log unit increments beginning with 10⁻⁸ M.

(2) In order to investigate whether DOBE was able to enhance endothelium-dependent relaxation in endothelium-injured rabbit aorta, an initial contraction with NA 10⁻⁶ M was induced and when the plateau was reached, a concentration–response curve to acetylcholine (ACh) (10⁻⁸–10⁻⁵ M) was made by adding aliquots of the relaxing agonist.

(3) We also studied the endothelium-independent relaxation induced by sodium nitroprusside (SNP). An initial contraction by NA 10⁻⁶ M was induced and when the plateau was reached, a concentration–response curve to SNP (10⁻⁸–10⁻⁴ M) was made by adding aliquots of the relaxing agonist.

Drugs

The following drugs were used: calcium dobesilate (a generous gift from Laboratories Esteve), noradrenaline bitartrate (Sigma), acetylcholine chloride (Sigma) and sodium nitroprusside (Sigma). All drugs were dissolved in deionized water; working solutions were made in Godfraind solution. The concentrations for each chemical or drug are expressed as final concentrations in the chamber in terms of the salt. Ascorbic acid was added to each daily prepared solution of noradrenaline.

Statistical analysis

The results are expressed throughout as mean ± SEM of 10 rabbits in each group. All protocols concerning animals were approved by the Complutense University of Madrid (EEC official registration 28079-15ABC). Comparisons between the different groups were performed using Student's t-test or two-way ANOVA when required. A level of probability *P* < 0.05 was accepted as statistically significant.

RESULTS

Results obtained in rabbits treated with DOBE for 10 (Protocol 1) or 30 days (Protocol 2)

Effect of treatments on concentration–response curves to NA In Protocol 1, in all groups of rabbits, NA (10⁻⁸–10⁻⁴ M) caused a contraction in a concentration-dependent manner. We did not find any significant differences between endothelium-injured rabbits (Group 2) and the control group (untreated group, Group 1). The maximal contractile response induced by NA 10⁻⁴ M was 96.1 ± 9.2% in Group 2 (with respect to 100% of control group, Group 1). DOBE-treatment shifted the concentration–response curve induced by NA (10⁻⁸–10⁻⁴ M) downwards. In arteries obtained from Group 3 (vitamin D₂ plus 50 mg DOBE kg⁻¹ per day) and Group 4

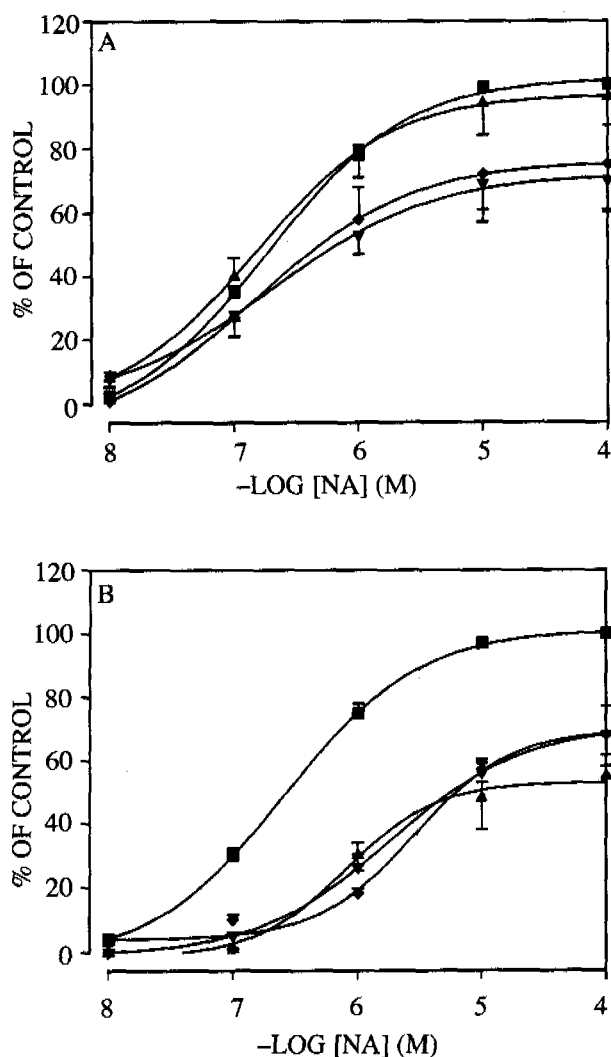


Fig. 1. Protocol 1 (panel A) and Protocol 2 (panel B). Effect of treatment on the concentration-response curve to NA (10^{-8} – 10^{-4} M). Each point shows the mean $\pm$ SEM of 10 experiments: ■, Groups 1 and 5 (control groups); ▲, Groups 2 and 6 (hypervitaminosis); ▼, Groups 3 and 7 (hypervitaminosis plus 50 mg DOBE kg^{-1}); ◆, Groups 4 and 8 (hypervitaminosis plus 500 mg DOBE kg^{-1}).

(vitamin D_2 plus 500 mg DOBE kg^{-1} per day), the maximal contractile effects, induced by NA 10^{-4} M, were, respectively, $79.1 \pm 9.2\%$ ($P < 0.05$, $n = 10$) and $70.2 \pm 8.0\%$ ($P < 0.01$, $n = 10$) of control (untreated rabbits, Group 1). As shown in Fig. 1 (panel A), we did not find any differences between the DOBE treated groups (Groups 3 and 4).

In Protocol 2, NA (10^{-8} – 10^{-4} M) induced a contraction in a concentration-dependent manner in aortic rings isolated from the different groups of rabbits. In all treated groups (with vitamin D_2 or vitamin D_2 plus DOBE, Groups 6, 7 and 8) the concentration-response curve to NA was shifted downwards with respect to the control (untreated) group (Group 5). The maximal effect was $55.2 \pm 11.0\%$ ($P < 0.01$, $n = 10$) in the hypervitaminic group (Group 6), $70.8 \pm 5.2\%$ ($P < 0.001$, $n = 10$) in hyper-

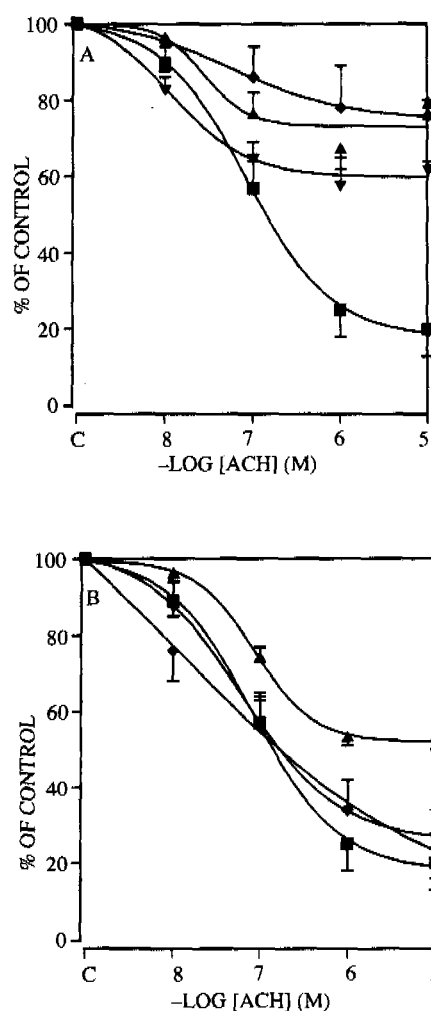


Fig. 2. Protocol 1 (panel A) and Protocol 2 (panel B). Effect of treatment on endothelium-dependent relaxation induced by ACh (10^{-8} – 10^{-5} M). Each point shows the mean $\pm$ SEM of 10 experiments: ■, Groups 1 and 5 (control groups); ▲, Groups 2 and 6 hypervitaminosis); ▼, Groups 3 and 7 (hypervitaminosis plus 50 mg DOBE kg^{-1}); ◆, Groups 4 and 8 (hypervitaminosis plus 500 mg DOBE kg^{-1}).

vitaminosis plus 50 mg DOBE kg^{-1} (Group 7) and $72.4 \pm 12.2\%$ ($P < 0.05$, $n = 10$) in hypervitaminosis plus 500 mg DOBE kg^{-1} (Group 8) as shown in Fig. 1 (pane B).

At the end of the treatment calcium dobesilate plasma-levels were analysed. We did not find any concentration of DOBE in controls (Group 5) whereas in DOBE-treated rabbits the plasma levels reached were 65.5 ± 3.2 ng ml^{-1} in Group 7 (hypervitaminic plus 50 mg DOBE kg^{-1} per day) and 119.3 ± 17.1 ng ml^{-1} in Group 8 (hypervitaminosis plus 500 mg DOBE kg^{-1} per day).

Effect of treatments on endothelium-dependent relaxation

Figure 2 (panel A) shows the endothelium-dependent relaxation induced by ACh 10^{-8} – 10^{-5} M in all groups studied in Protocol 1. The maximal relax-

92 91

ation obtained in the control group (Group 1) was $80.2 \pm 7.1\%$. In the hypervitaminic group (Group 2), the maximal relaxation induced by ACh 10^{-5} M decreased to $21.2 \pm 4.2\%$ ($P < 0.01$, $n = 10$).

Although the maximal relaxation induced by ACh 10^{-5} M increased significantly in DOBE-treated group (50 mg kg^{-1} per day, Group 3) from $21.2 \pm 4.2\%$ to $38.2 \pm 2.0\%$ ($P < 0.05$, $n = 10$) in hypervitaminic-treated and DOBE groups, respectively, the relaxation response curve to ACh was similar (using two-way ANOVA) in both, thus we cannot consider that DOBE treatment restored the relaxation induced by ACh in this protocol.

As shown in Fig. 2 (panel B), ACh (10^{-8} – 10^{-5} M) induced a relaxation in all groups tested Protocol 2, this effect being decreased in the hypervitaminic group (Group 6) with respect to control (untreated) group (Group 5) ($F_{1,36} = 4.5$, $P < 0.05$), thus the maximal relaxation induced by ACh 10^{-5} M was $80.2 \pm 7.1\%$ and $47.3 \pm 3.5\%$ ($P < 0.01$, $n = 10$) in Groups 5 and 6, respectively.

Group 7 (treated with vitamin D₂ plus the lowest dose of DOBE, 50 mg kg^{-1}) showed an increase in endothelium-dependent relaxation with respect to the hypervitaminic group (Group 6) ($73.2 \pm 7.3\%$ vs $47.3 \pm 3.5\%$, $P < 0.01$, $n = 10$). Likewise, in Group 8 (treated with vitamin D₂ plus the highest dose of DOBE, 500 mg kg^{-1}) we also found an increase in endothelium-dependent relaxation with respect to the hypervitaminic group (Group 6) (76.2 ± 8.4 vs 47.3 ± 3.5 , $P < 0.01$, $n = 10$).

In addition, we found no differences in terms of maximal relaxation between the DOBE-treated groups and the control (untreated) group, as shown in Fig. 2 (panel B).

Effect of treatments on endothelium-independent relaxation

The effects of DOBE on endothelium-independent relaxation induced by SNP (10^{-8} – 10^{-4} M) were also studied in all groups. We did not find any differences either in terms of maximal relaxant effect or sensitivity (EC_{50} values) in all the groups tested in Protocol 1, as shown in Fig. 3 (panel A).

The maximal relaxant responses in the different groups were: 92.4 ± 5.0 in Group 1 (control, untreated rabbit); $96.2 \pm 2.1\%$ in Group 2 (hypervitaminic group); $87.3 \pm 8.2\%$ in Group 3 (vitamin D₂ plus $50 \text{ mg DOBE kg}^{-1}$ per day); and $96.2 \pm 3.1\%$ in Group 4 (vitamin D₂ plus $500 \text{ mg DOBE kg}^{-1}$ per day).

As shown in Fig. 3 (panel B), in Protocol 2, the sensitivity to SNP decreased in the hypervitaminic group and this value was restored to normal in the DOBE-treated groups. On the other hand, the maximal effect of SNP was similar in all groups studied. The maximal relaxation was $92.4 \pm 5.2\%$ in the control (untreated) group and $81.2 \pm 7.3\%$ in the hypervitaminic group (Group 6); Fig. 3 (panel B).

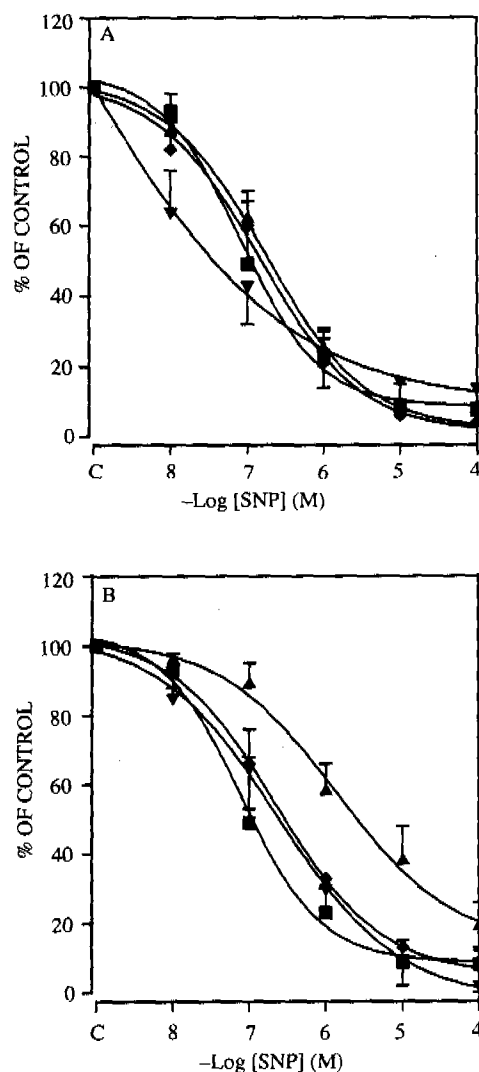


Fig. 3. Protocol 1 (panel A) and Protocol 2 (panel B). Effect of treatment on endothelium-independent relaxation induced by SNP (10^{-8} – 10^{-4} M). Each point shows the mean $\pm$ SEM of 10 experiments: ■, Groups 1 and 5 (control groups); ▲, Groups 2 and 6 hypervitaminosis; ▼, Groups 3 and 7 (hypervitaminosis plus $50 \text{ mg DOBE kg}^{-1}$); ◆, Groups 4 and 8 (hypervitaminosis plus $500 \text{ mg DOBE kg}^{-1}$).

DISCUSSION

A current hypothesis concerning several vascular pathologies, such as atherosclerosis or vascular disturbances which occur in diabetes, is that the initial lesion is damage to the endothelium [11, 12]. To initiate this chain of events experimentally, it is first necessary to denude the arteries of endothelium. Different techniques may produce opposite results, e.g. smooth muscle cell proliferation may occur after denudation caused by a balloon catheter [13, 14]. Some researchers have found that it is not the extent of endothelial injury but rather the trauma to the underlying media that is responsible for vascular lesion formation [15, 16]. This point was illustrated in experiments by Walker *et al.* [17] which showed

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that a superficial arterial injury made by a nylon catheter did not initiate any intimal lesion formation, whereas a wire filament of the same size, which caused a detectable injury to the media led to gross intimal thickening.

We tested the 'ex vivo' effects of calcium dobesilate on vascular smooth muscle and endothelium alterations which occur after treatment with an overdose of vitamin D₂, a state which causes endothelial injury as well as medial degeneration and calcification [9]. The aim was to correlate the 'in vitro' effect on rabbit aorta obtained with DOBE with an 'in vivo' model of vascular damage.

Figure 1 (panels A and B) shows that the concentration-response curve induced by NA in aorta arteries isolated from hypervitaminic-treated rabbits, is altered (shifted downwards) with respect to control (normal rabbits) in both protocols (after 10 or 30 days treatment). In both protocols (after 10 or 30 days of DOBE-treatment) the concentration-response curve to NA is shifted downwards, as occurs with DOBE in an 'in vitro' experiment [7]. We proposed in that report that DOBE might increase NA-induced NO release from endothelial cells and thus, decrease the concentration-response curve to NA. Another possible explanation is that DOBE could scavenge free radicals, avoiding the degradation of the NA-induced NO-release. In fact, we reported an 'in vitro' scavenger effect of DOBE on superoxide anions [8].

On the other hand, endothelium functionality measured as ACh-induced relaxation, is decreased in both protocols. This fact implies that the affecting of the endothelium is an early step in the mechanism of the damage by vitamin D₂. The decrease in endothelium-dependent relaxation induced by ACh with the vitamin D₂-treatment was restored to normal only after 30 days of treatment with DOBE but not after 10 days. A possible explanation of this different action could be that either the endothelium injury is partially recovered in the second protocol 'per se' or that DOBE needs a long time to act (more than 10 days, at least). In Protocol 2, the plasma levels of DOBE were 65.5 ± 3.2 ng ml⁻¹ (in Group 7) and 119.3 ± 17.1 ng ml⁻¹ (in Group 2). If we take into account the molecular weight of DOBE (MW = 418) these concentrations were approx. 2.7×10^{-5} M and 5×10^{-5} M. In previous studies [7, 8] we reported that DOBE 10^{-5} M enhanced the endothelium-dependent relaxation induced by ACh in rabbit aorta arteries [8] and that this effect was due to an increase in EDRF-synthesis since the effect was inhibited by L-NAME (an NO synthase inhibitor) and increased by L-Arg.

Endothelium-independent relaxation induced by SNP, in which the NO generated by SNP acts directly on vascular smooth muscle activating soluble guanylate cyclase, is altered only after 30 days of treatment (Protocol 2) but not after 10 days (Proto-

col 1). In addition, DOBE restored the endothelium-independent relaxation induced by sodium nitroprusside in Protocol 2 (after 30 days of treatment) suggesting that DOBE increases the affinity of sodium nitroprusside for vascular smooth muscle cells, although with only these experiments we cannot suggest the mechanism of this action.

Taken together, these results provide evidence that calcium dobesilate could be useful in the prevention of pathologies associated with vascular disorders such as diabetes mellitus, since it enhances both the endothelium-dependent relaxation and spontaneous release of NO in an 'ex vivo' model of endothelium injury.

ACKNOWLEDGEMENTS

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Calcium Dobesilate protects human peripheral blood mononuclear cells from oxidation and apoptosis

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The antioxidant effects of Calcium Dobesilate (CD, Doxium®) were investigated in relation to the oxidative status, apoptosis and *in vitro* proliferation of human peripheral blood mononuclear cells (PBMC) isolated from healthy donors. CD alone did not modify cell growth *in vitro* until 10 µM. This molecule counteracted oxidative damages generated by the high reducing sugar dR and was shown to reduce apoptosis by delaying both membrane permeability changes and DNA fragmentation. CD 10 µM affected in a time-dependent dynamics several parameters representative of the cellular oxidative status. In particular, CD significantly increased the activity of glutathione S-transferase (GST) after three days of treatment and also, but to a lower extent, the activity of γ-glutamyltransferase (γ-GT). Both enzymes are known to be involved in the glutathione (GSH) metabolic cycle. This enzymatic behaviour was reversed at seven days of treatment, with a significant GST decrease and a γ-GT activation. After seven days of CD exposure, the intracellular GSH content was enhanced and this resulted in a dramatic decrease in lipid peroxidation, underlining the powerful antioxidant properties of CD in human PBMC.

Key words: Acivicin; antioxidants; apoptosis; Calcium Dobesilate, Doxium®; deoxyribose; γ-glutamyltransferase; glutathione; glutathione S-transferase; human peripheral blood mononuclear cells; lipid peroxidation; membrane permeability.

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Introduction

Among the biochemical mechanisms responsible for apoptotic cell death of human T lymphocytes, particular attention has been devoted to those related to oxidative stress generated by oxygen free radicals and peroxides.^{1,2} The main features of oxidative stress are the loss of protective action of biological scavengers, such as glutathione (GSH), superoxide dismutase (SOD) and glutathione S-transferase (GST) enzyme activities. This results in an increased level of peroxides, hydroperoxides, aldehydes and free radicals. It has been demonstrated that oxygen radicals cause DNA damages in human lymphocytes.³ Bashir *et al.* observed that in apoptotic lymphocytes from patients with autoimmune diseases, there was increased genomic DNA damage and an enhanced susceptibility to cytotoxic killing by hydrogen peroxide. Peripheral blood lymphocytes from Type I diabetic subjects displayed increased breaks in single strand DNA when compared to control cells.⁴ Human quiescent peripheral blood mononuclear cells incubated with the reducing sugars D(-)-ribose and 2-deoxy-D-ribose (dR) showed characteristic patterns of apoptosis, such as DNA fragmentation and morphological changes, which can be prevented by exogenous antioxidants such as N-acetyl-L-cysteine (NAC)⁵ and/or other agents.³ Among molecules acting as antioxidants or scavengers of free radicals, there are the intracellular GSH, glutathione disulfide (GSSG), as well as antioxidant enzymes involved in the glutathione cycle, such as γ-glutamyltransferase (γ-GT) and glutathione-S-transferase (GST).^{6,7} Both enzymes play a crucial role in the protective mechanism against oxidative

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stress in lymphocytes by potentiating their immunological functions.^{8,9} In particular, the γ -GT ectoenzyme catalyzes the first step in the extracellular transpeptidation of the cellular anti-oxidant GSH via the γ -glutamyl cycle into amino-acid intermediates which are subsequently transported across the cell membrane and used in the *de novo* 'salvage' synthesis of intracellular GSH.¹⁰ Recent findings of our laboratory suggest that apoptosis induced in human lymphoblastoid CEM cells by the glucocorticoid dexamethasone involved a series of biochemical pathways, one of which might be the γ -GT upregulation.¹¹⁻¹³ GST exerts its protective functions using intracellular GSH through two main mechanisms. First, it catalyzes the transfer of sulfhydryl groups to alkylating molecules by forming GSH conjugated metabolites that are less toxic and easily excretable. Second, it catalyzes a peroxidase reaction with formation of oxidized glutathione GSSG.^{14,15} Therefore GST acts as an efficient detoxifier and repairer of oxidative damage to membrane lipids and DNA.

Among exogenous agents with pharmacologic effect, Calcium Dobesilate (calcium dihydroxy-2,5 benzenesulfonate, Doxium®) (CD) has been recently observed to express antioxidant properties *in vitro*. Doxium® appears to act as a scavenger for superoxide anions and as an inhibitor of membrane lipid peroxidation generated by oxygen free radicals in human erythrocytes and polymorphonuclear cells.¹⁶ On the other hand, recent studies have shown that Doxium® is an angioprotective agent widely used for the treatment of vascular diseases, in particular in diabetic retinopathy and in chronic venous insufficiency.¹⁷ In patients with diabetic retinopathy the drug has been shown to diminish both capillary permeability and fragility,^{18,19} to reduce blood viscosity²⁰ and also to inhibit platelet aggregation^{21,22} and thrombus formation.²³ Nevertheless, the precise cellular and biochemical mechanisms of action of CD *in vivo* and *in vitro* remain poorly understood. In particular, the effects of Doxium® on immunological functions, metabolic features and proliferative properties of human lymphocytes remain to be investigated.

Aims of this study were to examine the effects of Doxium® on *in vitro* proliferation, apoptosis and biochemical pathways involved in the control of oxidative status of human peripheral blood mononuclear cells (PBMC) isolated from healthy donors.

Materials and methods

CD was supplied by Laboratoires OM, CH-Geneva/Meyrin. Monoclonal antibodies (anti-CD3, anti-CD4 and anti-CD8) were purchased from Becton-Dickinson, CH-Basel. Glutathione assay kit was from Calbiochem AG, CH-Luzern. Ficoll-Hypaque was from Fakola AG, CH-Basel. All other reagents used for the various assays were purchased from SIGMA AG, CH-Buchs.

Lymphocyte isolation and treatment

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples from normal healthy individuals by Ficoll-Hypaque gradient, according to the conventional method.²⁴ Cells were washed twice with NaCl 0.9% pH 7.4, counted and characterized by staining with monoclonal antibodies anti-CD3, anti-CD4 and anti-CD8. PBMC population accounted for 84% of CD3 T lymphocytes, among which CD4 and CD8 lymphocytes amounted for 52% and 32% respectively, while 12% were B lymphocytes. Cell viability, determined by trypan blue exclusion, always exceeded 95%. Cells at the initial concentration of 10^6 cells/ml were cultured in RPMI 1640 supplemented by 5% FCS. Antioxidants and dR were dissolved in RPMI 1640 and added to the cultures as indicated in the Results section.

Enzyme activities

The enzyme assays were optimized for time, pH, protein and substrate concentration after homogenization of cell suspensions in Tris-HCl buffer (10 strokes at 4,000 rpm up and down). γ -glutamyl-transferase (γ -GT; EC 2.3.2.2) was assayed by incubating homogenates of PBMC (50 μ g protein) in 1 ml reaction mixture containing 20 mM Tris-HCl buffer pH 8.0, 60 mM glycylglycine, 300 mM NaCl and 2.5 mM γ -glutamyl-p-nitroanilide. The reaction was terminated by adding 2 ml 1.5 N acetic acid. The mixture was cleared by centrifugation ($800 \times g$, 20 min at 4°C) and the absorbance of the p-nitroaniline in the supernatant was measured at 410 nm with a Beckman DU-65 spectrophotometer.²⁵

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The glutathione S-transferase (GST; EC 2.5.1.18) activity was determined by the method of Anderson *et al.* with slight modifications. The GST activity was assayed in 1 ml of a reaction mixture containing 1 mM GSH, 1 mM CDNB (1-chloro-2,4-dinitrobenzene) dissolved in ethanol at a final concentration of 2.5% (v/v), 100 mM potassium phosphate pH 6.5 and 0.1% Triton X-100. The reaction was started by adding 50 µg of cell homogenate and incubating for 30 min at 25°C. The amount of dinitrobenzene (DNB) liberated from CDNB was measured with a Beckman DU-65 spectrophotometer at 340 nm.

Analysis of lipid peroxidation (MDA assay)

The binding of thiobarbituric acid to malondialdehyde (MDA) formed during lipid peroxidation results in a chromogenic complex. Briefly, the homogenate obtained by lysis of $1-5 \cdot 10^6$ cells with 10% ice-cold TCA was centrifuged at 800 g for 10 min. Aliquots of 1 ml of the supernatant were added to 1 ml of 0.67% thiobarbituric acid and heated in a boiling water bath for 10 min. After cooling, samples were read at 535 nm and the concentration of MDA determined by comparison with a standard reference curve.²⁶

Measurement of intracellular GSH content

Intracellular GSH content was measured on total cell homogenate by using the Glutathione Assay Kit from Calbiochem AG. The assay is based on a two steps chemical reaction: the formation of thioethers with a reagent and the mercaptans present in the sample and the successive alkaline β-elimination of the thioethers with the formation of a chromophoric thione detectable at 400 nm.

Characterization of apoptosis

Membrane permeability assay. The change in the permeability of the cell plasma membrane, measured by flow cytometry, was used to differentiate living from apoptotic and dead cells.¹¹ Cells which have an intact plasma membrane exclude dyes such as trypan blue and propidium iodide (PI), while dead

cells with damaged plasma membrane do not. Apoptotic cells have intermediate patterns of membrane permeability, which results in a variable dye uptake. Untreated or treated cells (10^5-10^6) were collected by centrifugation, resuspended in 0.5 ml NaCl 0.9%, pH 7.4 containing 5 µg/ml PI, and incubated for 7 min at room temperature and washed with NaCl. Histograms of cell subpopulations are obtained with a FACscan (Becton Dickinson) equipped with a Lysis II program, which distinguishes three main fluorescence peaks, corresponding to normal, apoptotic and dead cell populations.

Analysis of cell cycle and apoptosis. The cells ($3 \cdot 10^6$) were washed in NaCl 0.9% solution, pH 7.4 and nuclei were obtained with hypotonic choc by resuspending PBMC in 1 ml of solution of 0.1% sodium citrate containing 50 µg/ml of PI, 0.1% Triton X-100 and RNase-A (5 units/ml) for 1 h in the dark at 4°C. Thereafter nuclei were analyzed for cell cycle (G_0/G_1 , S and G_2+M) with a flow cytometry equipped with a Lysis II software. Fragmented DNA generated by apoptosis was visualized as a fluorescence subdiploid fluorescence peak occurring at the left of the G_0/G_1 diploid DNA peak.^{11,27}

Statistical analysis

Data were analyzed with Student's paired *t*-test (two tailed)

Results

Effects of CD alone or in combination with dR on PBMC growth

In order to investigate the effect of CD on human PBMC growth *in vitro*, cells (10^6 cells/ml) were incubated with various concentrations of CD (range 1 µM–10 mM) and the viability measured after 5 days of culture. Figure 1 shows that the percentage of living cells in presence of CD at the concentration from 1 µM to 10 µM was found to be similar to that of untreated PBMC or even slightly higher. In the presence of higher concentrations of CD (100 µM to 1 mM) there was a rapid decrease of living

cells accompanied by an enhancement of the percentage of apoptotic cells (about 30% at 1 mM CD). Finally, at 10 mM CD we observed an important increase in the number of dead cells (34%), probably due to the emergence of necrotic features at this very high concentration (Figure 1). On the basis of these results we decided to use 10 μ M CD for further experiments. The maximal effect of dR, known to be a potent inducer of apoptosis was reached at 10 mM with a 40% of PBMC growth inhibition (Figure 2). Since a pre-incubation of cells with 10 μ M of the antioxidant NAC completely abolished the cytotoxic effects of 10 mM dR (Figure 2, column NAC/dR), we could conclude that apoptosis induced by dR is probably due to the oxidative stress. In order to investigate the role of the CD as antioxidant agent, changes of permeability of cell membrane toward PI and the amplitude of the subdiploid DNA peak reflecting DNA fragmentation were investigated in PBMC treated with both 10 μ M CD and 10 mM dR. Both methods showed that 10 μ M CD exerted an antioxidative effect when it was added 24 h before dR (Figure 3, column CD/dR). In fact, the pre-treatment with CD resulted in a reduction of DNA fragmentation (restricted subdiploid peak) and permeability when compared to the values measured in PBMC with

dR alone. However, neither the addition of CD after the dR treatment (dR/CD) nor the contemporaneous administration of both CD and dR (CD + dR) could protect cells from dR-induced apoptosis (Figure 3).

Figure 2. Effect of deoxyribose (dR) and N-acetyl-L-cysteine (NAC) on PBMC viability (membrane permeability to propidium iodide) at 5 days of culture. NAC/dR indicates a preincubation of 24 h with 10 μ M NAC prior the addition of 10 mM dR. Values given are means $\pm$ SD of three separate experiments. ** Significantly different ($2 p < 0.01$) from the control.

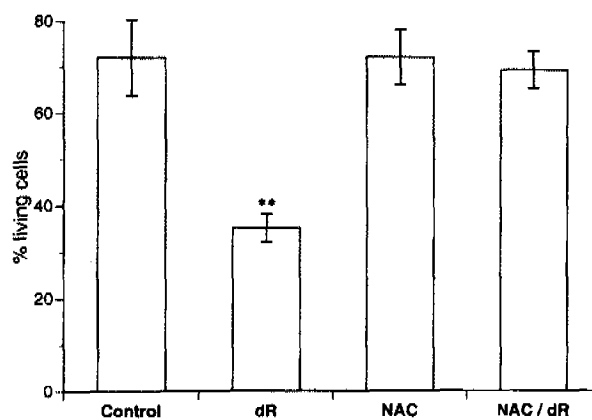
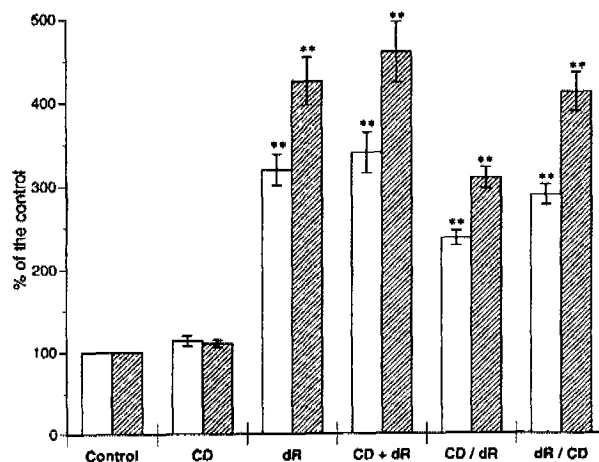
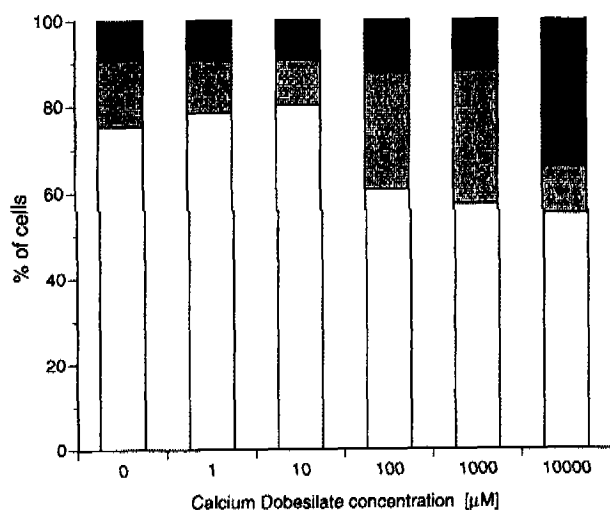


Figure 3. Effect of CD and dR on apoptosis in PBMC, expressed as % of controls. Cells have been incubated for 5 days with CD (10 μ M), dR (10 mM) or with combinations of both. CD + dR = treatment with both CD and dR; CD/dR = preincubation with CD 24 h before dR addition; dR/CD = preincubation with dR 24 h before CD addition. Apoptosis was determined by the permeability test (white bars) and by occurrence of the subdiploid DNA peak (grey bars). In control cells, the mean values for the permeability and the subdiploid DNA peak were $13.8 \pm 4.8\%$ and $2.5 \pm 1.2\%$, respectively. In treated cells, relative values are means $\pm$ SD of three separate experiments. ** Significantly different ($2 p < 0.01$) from the respective controls.

Figure 1. Effect of CD concentration on viability (membrane permeability to PI) of human PBMC at 5 days of culture. living, apoptotic and dead cells. Results are representative of four separate experiments. White bars = living; grey bars = apoptotic and black bars = dead cells. Results are representative of four separate experiments.



29 18

Effects of CD, NAC and dR on γ -GT and GST activities

The behaviour of both antioxidant enzymes γ -GT and GST was investigated in PBMC treated with the antioxidants NAC and CD and the reducing sugar dR in the culture conditions mentioned above. Two periods of culture have been considered,

day 3 (early phase) and day 7 (late phase). After three days of culture, 10 μ M of both antioxidant NAC or CD (a concentration which did not affect cell growth) induced a small activation of γ -GT (about 20%) whilst GST activity was highly enhanced (about 150%) when compared to the activity of control cells (Figure 4). At day 7 of culture γ -GT was highly stimulated in the presence

Figure 4. Effect of antioxidants (NAC and CD at 10 μ M) and oxidant (dR at 10 mM) on γ -GT (A) and GST (B) activities at 3 (white bars) and 7 days (grey bars) of culture. Enzyme activities in control cells represent 100%. Mean control values for γ -GT and GST activities at both times are similar and amounted to 8.6 ± 2.6 and 23.2 ± 2.8 nmol/min/mg respectively. Values given are means $\pm$ SD of three separate experiments. Significantly different (* $2 p < 0.05$ and ** $2 p < 0.01$) from the respective controls.

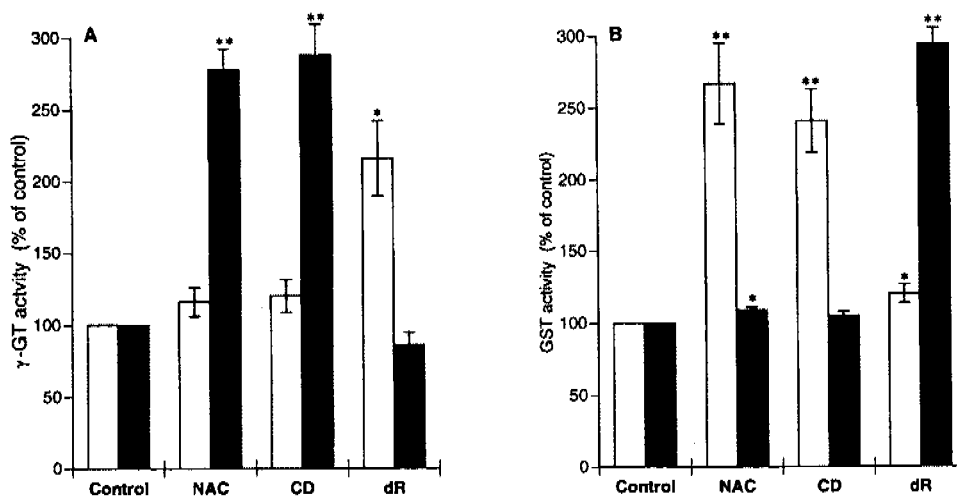
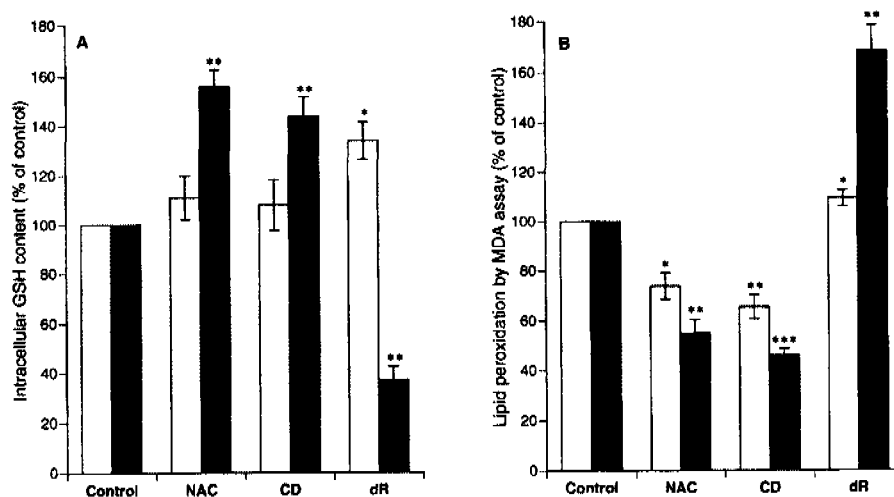


Figure 5. Effect of antioxidants (NAC and CD at 10 μ M) and oxidant (dR at 10 mM) on intracellular GSH (A) and on lipid peroxidation (B) at days 3 (white bars) and 7 (grey bars). Results are percentages of control values (100%). Mean control values at both times are similar and amounted to 12.2 ± 1.8 μ M GSH and 1.2 ± 0.5 nmol MDA/mg protein. MDA = malondialdehyde. Results are means $\pm$ SD of three distinct experiments. Significantly different (* $2 p < 0.05$; ** $2 p < 0.01$; *** $2 p < 0.001$) from the respective controls.



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of 10 μ M antioxidants (more than 150%), whereas GST underwent a significant reduction of activity with respect to that measured at day 3 (about 150%) (Figure 4). Interestingly, PBMC treated with 10 mM of the reducing sugar dR exhibited an inverse enzyme behaviour, namely the activation of γ -GT at day 3 (more than 100%) was followed by a strong inhibition at day 7 but accompanied by a strong stimulation of GST (about 200%). It should be noted that pre-treatment of PBMC with either NAC or CD before exposure to dR resulted in an intermediate enzymatic response with respect to that observed when antioxidants or dR were added alone (data not shown).

Effects of CD, NAC and dR on GSH content and lipid peroxidation

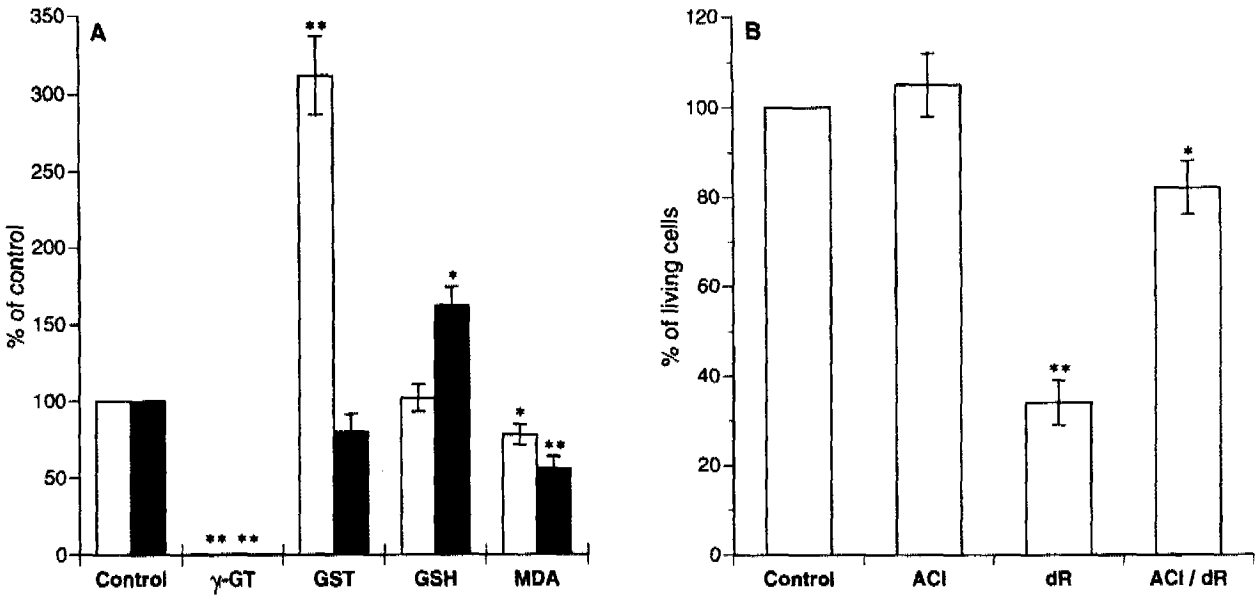
We measured a progressive accumulation of intracellular GSH at day 7 in the presence of 10 μ M of both antioxidant agents (CD, NAC), caused by γ -GT activation while GST remained unmodified (Figure 5). PBMC exposed to dR exhibited at day 7 an opposing enzyme behaviour leading to a

dramatic reduction in GSH content accompanied by a significant increase of MDA, an indicator of lipid peroxidation (Figure 5). In antioxidant-treated cells the GSH level was increased slightly at day 3 but markedly at day 7, in contrast to MDA level which strongly decreased below control values (Figure 5).

Effects of γ -GT inhibitor on GSH metabolism and cell growth

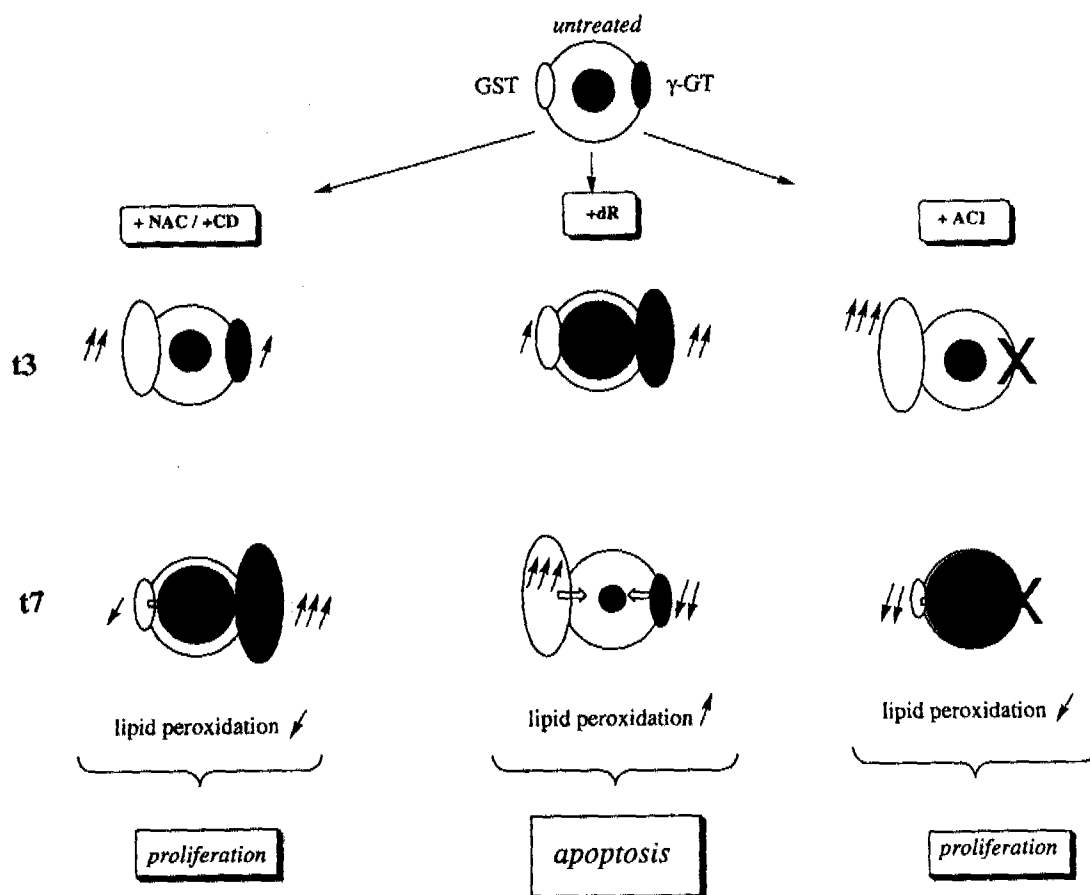
Acivicin 10 μ M added to PBMC induced a complete, irreversible and persistent inhibition of γ -GT activity but unexpectedly allowed a strong activation of GST (+200%) at day 3 followed by its reduction slightly below the control level at day 7 (Figure 6A). Such enzymatic response was quite different to that observed in cells treated with antioxidants NAC and/or CD at day 7. However, like the latter antioxidants, acivicin induced a rise in GSH content and a reduction of lipid peroxidation (Figure 6A) without affecting neither proliferation nor permeability of PBMC (Figure 6B). Extremely importantly, as with NAC and CD,

Figure 6. (A) Effect of acivicin (10 μ M) on γ -GT and GST activity, intracellular GSH and lipid peroxidation at days 3 (white bars) and 7 (grey bars) of treatment. Data are expressed as percentage of control cells. Absolute values in controls are the same as in Figures 4 and 5. (B) Effect of acivicin (ACI) and dR alone or in combination on per cent of living cells determined by P1 permeability. ACI/dR = preincubation with ACI 24 h before dR addition. Values given are means $\pm$ SD of three distinct experiments. Significantly different (* 2 p < 0.05 and ** 2 p < 0.01) from the respective controls.



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Figure 7. Hypothetical scheme of action of oxidant and antioxidant agents on enzyme activities, GSH content, lipid peroxidation and PBMC viability. *t3* = day 3 of treatment; *t7* = day 7 of treatment; X = complete inhibition of enzyme activity; upward arrow = increment; downward arrow = decrement.



a pre-treatment with acivicin gave protection against the oxidant effects of dR (Figure 6B, column ACI (acivicin)/dR).

Discussion

The results of the present study indicate that CD at micromolar concentration has antioxidant properties and prevents human PBMC from the oxidative damages induced by high reducing deoxyribose.^{28,29} In analogy to the antioxidant N-acetyl cysteine (NAC), CD exerted its effects by increasing the intracellular concentration of the reduced GSH. It is known that the cytoplasmic GSH is the result of two enzyme activities, namely the γ-GT responsible for its synthesis³⁰ and the GST which intervenes in its catabolism. Our findings

revealed that in human PBMC the behaviour of both enzymes was found to be dependent on the duration and the nature of the treatment (Figure 7). At three days of culture in the presence of the antioxidant NAC and/or CD alone, a significant increment of GST was accompanied by a slight change of γ-GT, whereas in the presence of the reducing aldose dR there was a significant increase of the γ-GT but a low activation of GST. At seven days of treatment with these antioxidants there was a drastic change in both enzyme activities. In fact, human PBMC in the presence of NAC and/or CD alone showed an activation of the γ-GT synthetic pathway and a concomitant reduction of GST activity. On the other hand, in the presence of dR the GSH-consumptive way mediated by GST was highly activated whilst the γ-GT was strongly inhibited, provoking an increased permeability of

the plasma membrane, a reinforced lipid peroxidation, and the occurrence of a subdiploid DNA peak, which finally led to apoptosis. The observed activation of γ -GT and to a much lesser extent of GST by dR at day 3 deserves some comments because the behaviour of both enzymes was unexpectedly in contrast with that observed in the late phase of apoptosis. Such an enzyme activation was accompanied by a significant increase of the intracellular GSH content and a stable level of the lipid peroxidation, which might suggest that PBMC could counteract injuries in the early phases of 10 mM dR treatment. In reality, these cells were in a pre-apoptotic stage, the rise of γ -GT-mediated GSH synthesis being insufficient to overcome the persistent effect of dR. In fact, this aldose provoked irreversible apoptotic features such as membrane damages, dramatic fall in GSH and rise in lipid peroxidation — findings which confirmed those previously reported.^{5,31} In addition, the apoptotic effect of dR could not be blocked or reversed by culturing dR-treated PBMC with mitogens or growth factors (data not shown). Preliminary results from our laboratory have revealed that a large proportion of PBMC isolated from HIV-positive drug abusers had also an increased γ -GT activity and a perturbed membrane permeability.³² Both altered membrane phenomena might suggest that most PBMC from infected drug abusers had a metabolic-oxidative status similar to that expressed by PBMC treated with dR for three days, considered to be pre-apoptotic. The pre-apoptotic condition was confirmed by the fact that HIV-infected PBMC submitted to mitogenic ConA instead of proliferating, swiftly underwent apoptosis and displayed a dramatic increase in the γ -GT activity³² in analogy to what happened with PBMC submitted to dR oxidative stress. Our observations in dR-treated PBMC and in HIV-infected PBMC from drug abusers were consistent with previous reports postulating a pre-apoptotic stage in HIV-infected cells which became apoptotic as soon as they were stimulated with various mitogens.^{33–35} Other authors have documented that oxidative stress might play a role in the initiation of apoptosis by PBMC from HIV-seropositive patients.^{36,37} Therefore, in another series of experiments we have attempted to better understand the effective role of CD on GSH metabolic enzymes and apoptosis by culturing peripheral blood lymphocytes in the presence of acivicin (10

μ M), known to be a competitive inhibitor of the γ -GT.¹² While acivicin initially mimicked and strengthened the primary effect of both antioxidants NAC and CD on GST, later on a dramatic reduction of this enzyme occurred in association with the irreversible inhibition of γ -GT. A high level of the intracellular GSH and reduced lipid peroxidation were measured indicating that PBMC had recovered their functional capacity (Figure 7). It is noteworthy that both types of molecules, either the antioxidants NAC/CD or acivicin, ensured a high level of intracellular GSH which partially prevented the apoptotic process in PBMC induced by dR. In light of these findings one may conclude that CD exerted an antioxidant effect in human PBMC *in vitro* by alternative modulating γ -GT and GST enzymes, which are an antagonist to GSH metabolism. This maximal effect of CD implying a maximal content of the intracellular GSH culminated at day 7 with the maximal reduction of GST and the concomitant maximal increase of γ -GT activity. The conclusion is that CD-modulated events reduced lipid peroxidation and hence contributed to the prevention of apoptosis. The antioxidant effect of CD observed in PBMC was consistent with that observed in other cell systems such as human polymorphonuclear cells and erythrocytes and in bovine endothelial cells. In these cells, CD was shown to decrease the concentration of superoxide anions, PAF-induced chemiluminescence and free radicals.¹⁶

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103 162

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