Free Rad. Res. Comms., Vol. 4, No. 1, pp. 51-60
Photocopying permitted by license only

Printed in Great Britain

EFFECT OF (+)CYANIDANOL-3 ON CELLULAR IMMUNE REACTIONS AND ON SUPEROXIDE DISMUTASE ACTIVITY *IN VITRO*

I. LÁNG, K. NÉKÁM, GY. DEÁK, G. MÜZES, C.R. GONZALEZ, J. KÁDÁR, A. PERL, P. GERGELY and J. FEHÉR

Division of Hepatology, Second Department of Medicine, Semmelweis University Medical School, Budapest, Hungary

(Received January 30th 1987)

Immunological and antioxidant effects of the widely used hepatoprotective agent (+)cyanidanol-3 were studied in a complex *in vitro* test system using isolated peripheral blood lymphocytes of patients with chronic alcoholic liver disease and of healthy donors. In both groups *in vitro* treatment with (+)cyanidanol-3 inhibited lectin-induced lymphocyte blast transformation and chemiluminescence in a dose-dependent fashion and increased the superoxide dismutase-expression of lymphocytes without influencing the percentage of T and active T cells and antibody-dependent and spontaneous lymphocytotoxicity. The originally lower Con A-induced T cell mediated lymphocytotoxicity of patients was completely abolished, while no significant effect was seen using healthy donors' lymphocytes. (+)cyanidanol-3 markedly decreased the Con A-induced suppressor activity of patients' lymphocytes, without influencing that of normal cells. These results suggest that both antioxidant and immunomodulatory effects play an important role in the mechanism of action of (+)cyanidanol-3.

KEY WORDS: (+)cyanidanol-3, cellular immunoreactivity, superoxide dismutase, alcoholic liver disease.

INTRODUCTION

(+)cyanidanol-3 (Catergen®, Col) is widely used in the treatment of chronic hepatic diseases. Its beneficial effects have been demonstrated in acute and chronic viral hepatitis, in ethanol-induced liver disease and in drug-induced hepatotoxicity.[1] Its main mechanism of action involves membrane protection[2,3] and antioxidant activity.[4,5] Recent studies, however, suggested an immunological effect, as well.[6-9] Under *in vitro* conditions it has been shown to increase the reduced T cell number of patients with chronic liver diseases,[9] to amplify LIF production,[6] and to enhance antigen – specific blast transformation.[9] This immunomodulatory effect of (+)cyanidanol-3 is no surprise since most of the potent hepatoprotective agents have immunological activity. Furthermore, scavengers of free radicals are known for their capability to alter immune functions in various ways.[11-13] They can inhibit the cytolysis mediated by human NK cells,[14] and LDCC effector cells.[15] Therefore we studied the effects of the free radical scavenger hepato-protective agent (+)cyanidanol-3 on a panel of cellular immune reactions while demonstrating its antioxidant activity

Address correspondence to: I. Láng, M.D., D.Sc., H-1088 Budapest, Szentkirályi utca 46, Pf. 277, Hungary

under similar *in vitro* conditions in patients with chronic alcoholic liver disease (CALD). Immunological mechanisms may be important in CALD,[16] and according to some data (+)cyanidanol-3 can be of benefit in the management of CALD[17,18] although contradictory results were also published.[19,20]

PATIENTS AND METHODS

Thirty-two patients with chronic alcoholic liver disease (CALD), 20 men and 12 women, mean age 46 ± 8 years and 28 age and sex matched healthy volunteers were involved in the study. Histological diagnosis was cirrhosis in 14 patients and fatty liver in 6 patients out of 20 patients with liver biopsy. All patients were heavy drinkers. Daily alcohol consumption exceeded 60 g in men and 30 g in women. Mean duration of alcoholism was 9 ± 3.6 years. Their general nutritional status was sufficient: no symptoms of hypovitaminosis or excessive weight loss were seen.

(+)cyanidanol-3, (+)-3′4′5,7-tetrahydroxy-flavan-3-ol: Catergen®, Zyma-Biogal, Nyon-Debrecen was used in various *in vitro* tests in a concentration range of 0.1–10.0 μg/ml (0.3448–34.48 μmol/l), including the concentration corresponding to the usual therapeutic dose, calculated according to the formula of Hirschaut.[21] (+)Cyanidanol-3 was dissolved in phosphate buffered saline.

Peripheral blood mononuclear cells (PBMC) were separated from heparinized venous blood on Ficoll(Pharmacia)-Uromiro (Bracco) gradient.[22] For rosette tests, cytotoxicity assays and flow cytofluorimetry phagocytic cells were removed by carbonyl-iron treatment or plastic adherence.

The percentage of total T cells have been determined according to the method of Jondal *et al.*:[22] 10^6 lymphocytes were mixed in the presence of 100 μl fetal salf serum (FCS) with 10^8 sheep red blood cells in TC 199 medium (final volume 400 μl) and centrifuged at 1200 rpm for 10 minutes. Rosetting cells (binding 3 or more erythrocytes) have been counted following overnight incubation on ice.

"Active" rosettes have been determined following the method of Yu[24] with slight modifications: 10^6 lymphocytes mixed with 10^8 sheep erythrocytes in 400 μl TC 199 medium were centrifuged as above and counted immediately for rosetting lymphocytes.

ADCC assay was performed according to the original method of Perlmann and Perlmann[25] with slight modifications: ^{51}Cr labelled chicken RBC (2.5×10^4) were added to plastic V-bottomed microplate (Greiner) and mixed with an appropriate amount of effector cells according to the desired effector/target cell ratio (10:1, 5:1 and 2.5:1). Anti-CRBC serum (final dilution 1:10.000) was added and the volume made up to 0.125 ml with medium. Appropriate controls for spontaneous release were included in each experiment. After 4 hrs incubation at 37°C radioactivity of the pellet and the supernatant was determined. Cell damage was expressed as per cent isotope release into the supernatant.

Natural killer (NK) assay was performed by the method of Jondal and Pross.[26] Briefly, 2×10^4 ^{51}Cr labelled K-562 targets were mixed with lymphocytes at an effector/target ratio of 50:1, 25:1 and 12.5:1, and incubated at 37°C for 4 hrs. Target cell damage was expressed as test tube isotope release minus spontaneous release.

LDCC activity was measured by the method of Perl et al.[27] Target cells derived from Cincinnati HEp-2 adherent human epipharynx carcinoma cell line (National Institute for Public Health, Budapest) were labelled with ^3H-thymidine. Effector to target

ratios were 50:1 and 25:1. LDCC cultures contained Con A at a final concentration of 25 μg/ml. Appropriate controls were included in each experiment. Culture plates were incubated for 24 hrs at 37 C. Detachment from the monolayer was used as indication of cell damage.

For the *blast transformation* assay separated PBMC were resuspended in TC-199 medium supplemented with 10% heat-inactivated fetal calf serum (Human, Budapest), antibiotics, and 25 mM HEPES (Serva). 2×10^5 cells in 5 replicates were placed into flat-bottomed microplates (Greiner). Con A (Calbiochem) or PHA (Pharmacia) was added to a final concentration of 25 μg/ml and 2 μg/ml, respectively. The cultures were kept in humidified atmosphere at 37°C for 72 hr. Eight hours before harvesting, 0.5 μCi ^{3}H-thymidine (UVVR, Czechoslovakia) was added to each well, the cultures were sucked off onto glass fibre filters (Whatman GF/c). Isotope determination was made in a Nuclear Chicago Isocap 300 counter and the results were expressed in cpm.

In the suppression test Con A stimulation was performed as described above. Details of the assay were published elsewhere[27]. Suppression was calculated according to the formula of Shou.[28]

Zymosan-induced luminol-amplified whole blood chemiluminescence was determined according to the method of Tono-oka *et al.*[29] Results are given as percentage of the control sample without (+)cyanidanol-3.

For the flow cytofluorimetric evaluation of superoxide dismutase, 10^7 lymphocytes were incubated at 4°C with 100 μl monoclonal mouse-anti-human CuZn-SOD antibody diluted to 1:200 in PBS for 90 mins. Following two washings in PBS cells were stained with 5 μl rabbit anti-mouse immunoglobulin conjugated with FITC (DAKO) for 30 mins at 4°C. After three washings in cold PBS lymphocyte counts were adjusted to 2–3 M cells/ml and fluorescence patterns of cell suspensions were evaluated using an FC 4800 A cytofluorograph (Bio Physics System, Inc.).

Statistical analyses were performed with Student's *t* test.

RESULTS

Effect of (+)cyanidanol-3 on lymphocyte viability and on the spontaneous release of target cells

In order to exclude direct toxicity of the drug on lymphocytes and on ADCC, NK and LDCC target cells, we studied the effect of the highest concentration used on lymphocyte viability (trypan blue exclusion test), on spontaneous chromium release (CRBC and K-562), and on spontaneous detachment of HEp-2 cells from the monolayer. No changes in these values, compared to those obtained in the absence of (+)cyanidanol-3, indicated that the drug exerted no direct toxic effect on these cells (data not shown).

Effect on T and "active" T lymphocytes

PBMC-s of healthy individuals and patients with CALD were incubated with (+)cyanidanol-3 for 6 hrs at 37°C *in vitro*. Its effects on total and early rosette formation are summarized in Table I. No significant effect on the percentage of T and Ta lymphocytes in a concentration range of 0.1–10.0 μg/ml were observed.

TABLE I
Effect of (+)cyanidanol-3 on T and "active" T lymphocyte subpopulations *in vitro*

(+)cyanidanol-3 μg/ml	healthy subjects		alcoholic liver disease	
	T(%)	T_a(%)	T(%)	T_a(%)
-	67 ± 2	33 ± 2	65 ± 2	31 ± 4
0.1	68 ± 3	35 ± 3	66 ± 3	33 ± 2
1.0	69 ± 4	32 ± 4	67 ± 3	34 ± 4
10.0	65 ± 3	31 ± 5	68 ± 4	30 ± 3

Mean ± SD

TABLE II
Effect of (+)cyanidanol-3 on ADCC and NK activity *in vitro*

(+)cyanidanol-3 μg/ml	healthy subjects		alcoholic liver disease	
	ADCC†	NK††	ADCC†	NK††
–	23.4 ± 6.2	34.7 ± 2.6	27.0 ± 4.5	33.6 ± 2.7
0.1	24.2 ± 6.7	38.6 ± 4.1	25.4 ± 4.9	34.0 ± 3.1
1.0	25.3 ± 7.1	35.9 ± 4.6	26.7 ± 5.2	33.4 ± 3.3
10.0	23.9 ± 5.8	36.4 ± 5.0	24.9 ± 4.4	31.2 ± 2.9

Mean ± SD
†: % cytotoxicity, effector:target cell = 5:1
††: % cytotoxicity, effector:target cell = 50:1
ADCC: antibody-dependent cell mediated cytotoxicity
NK: natural killer cell activity

Effect on antibody-dependent and spontaneous lymphocytotoxicity

The drug was present in both test systems during the whole incubation period. Results are shown in Table II. ADCC and NK activities of the patients were not significantly different from those of the healthy control group. Col had no significant influence on K and NK cytotoxicity in the concentration range used. Similar results were obtained using other effector:target ratios, as well (data not shown).

Effect on direct T cell mediated cytotoxicity

The effect of (+)cyanidanol-3 on the Con A – induced cytotoxicity of T lymphocytes of healthy subjects and of patients with CALD is demonstrated in Fig. 1. Ten μg/ml Col completely abolished the Con A – induced lymphocytotoxicity of patients with CALD (empty columns) without influencing the LDCC activity of healthy lymphocytes (spotted columns). "Negative" cytotoxicity i.e. an increased survival (= decreased detachment) of target cells can be explained with a feeder layer effect in the absence of cytotoxic potential. Mean LDCC activity of patients was significantly lower than that of the healthy control group.

Effect on lectin-induced blast transformation

(+)cyanidanol-3 decreased both PHA- and Con A- induced blastogenesis of healthy lymphocytes in a dose-dependent fashion (Fig. 2). Basically similar effect was seen on lymphocytes of patients with CALD (Fig. 3). Mean PHA-induced blastogenesis was

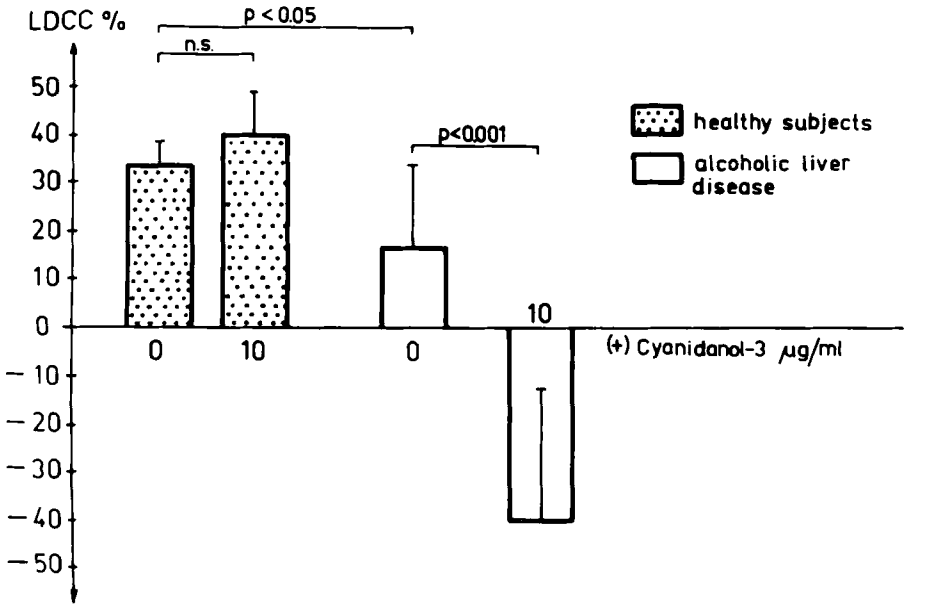

FIGURE 1 Effect of (+)cyanidanol-3 on LDCC activity *in vitro*. Mean ± SD.

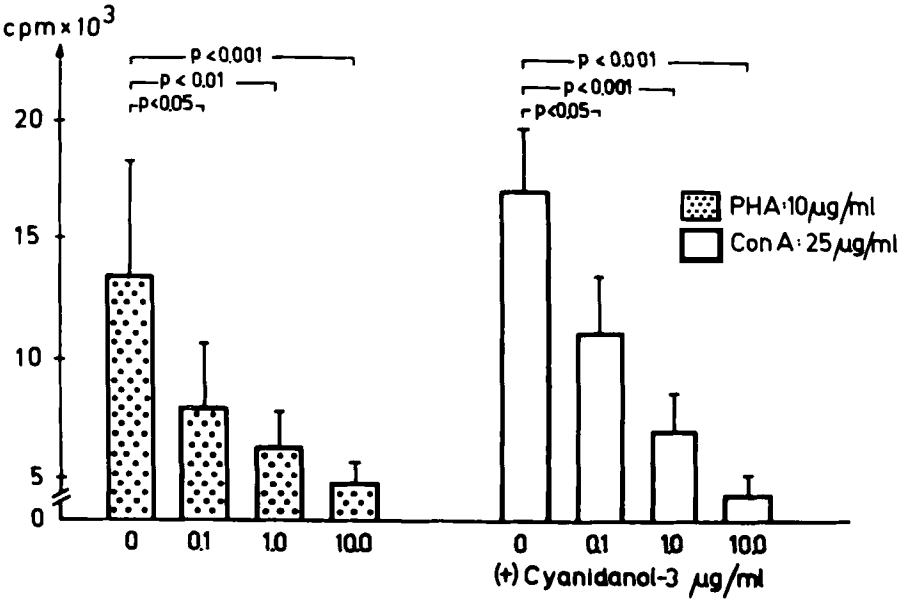

FIGURE 2 Effect of (+)cyanidanol-3 on lectin-induced blastogenesis of healthy lymphocytes *in vitro*. Mean ± SD.

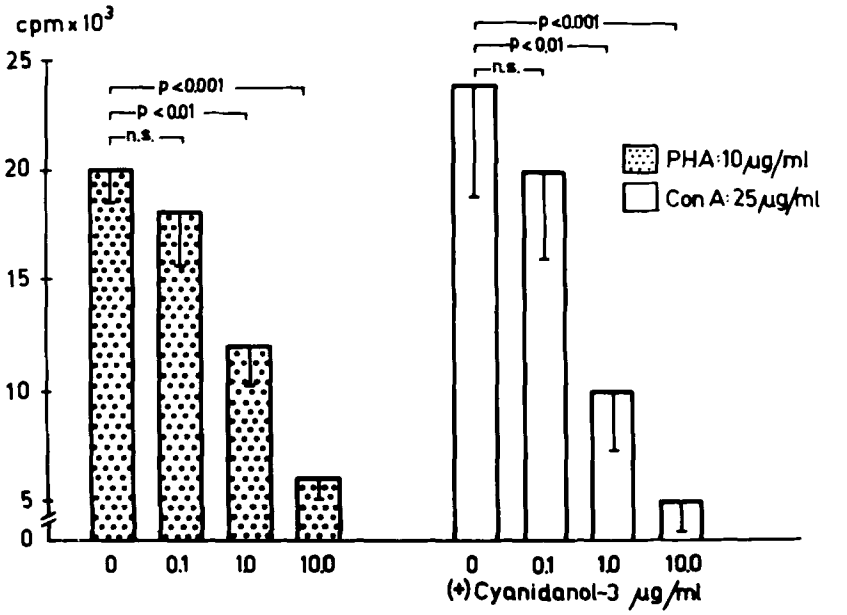

FIGURE 3 Effect of (+)cyanidanol-3 on lectin-induced blastogenesis of lymphocytes from patients with chronic alcoholic liver disease *in vitro*. Mean ± SD.

markedly ($p < 0.01$) lower, mean Con A-induced blast transformation was somewhat lower than the corresponding mean values of the healthy control group.

Effect on suppressor cell activity

In concentrations of 1 and 10 µg/ml (+)cyanidanol-3 exerted no significant influence on Con A-induced suppressor activity of healthy lymphocytes. On the other hand 10 µg/ml significantly decreased the Con A-induced suppressor activity of lymphocytes with CALD (Table III).

Effect on whole blood chemiluminescence

The effect of (+)cyanidanol-3 on Zymosan-induced, luminol-amplified whole blood chemiluminescence is shown in Fig. 4. The dose-dependent inhibition of chemiluminescence indirectly indicates the free radical scavenger capacity of drug *in vitro*.

TABLE III
Effect of (+)cyanidanol-3 on con A – induced suppressor cell activity

(+)cyanidanol-3 µg/ml	healthy subjects	alcoholic liver disease
–	32.2 ± 8.6	42.1 ± 3.9
1.0	31.4 ± 6.4	45.3 ± 4.4
10.0	33.7 ± 4.3	73.9 ± 3.2†

Mean ± SD, †: 73.9 ± 3.2 vs 42.1 ± 3.9: $p < 0.001$
Results given as percentage of the control value, i.e. cpm without suppression = 100%

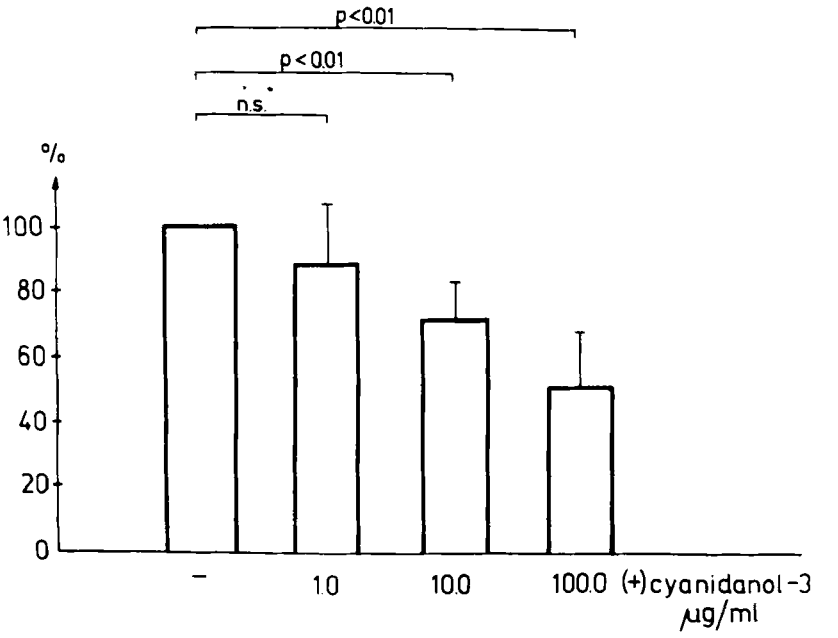

FIGURE 4 Effect of (+)cyanidanol-3 on Zymosan-induced chemiluminescence *in vitro*. Results are given as percentage of the control. Mean ± SD.

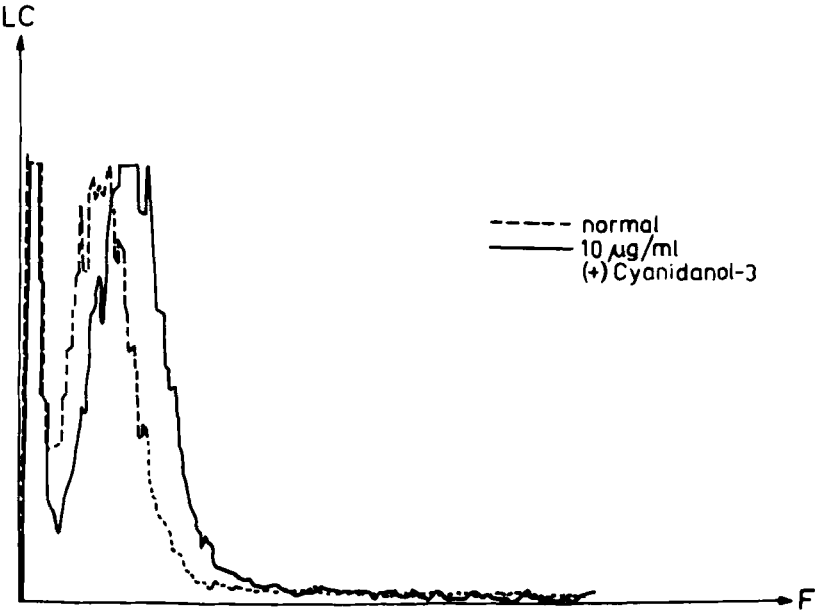

FIGURE 5 Effect of (+)cyanidanol-3 on the SOD-expression of healthy lymphocytes measured by flow-cytofluorimetry. Fluorescence histogram. LC: lymphocyte count. F: fluorescence intensity.

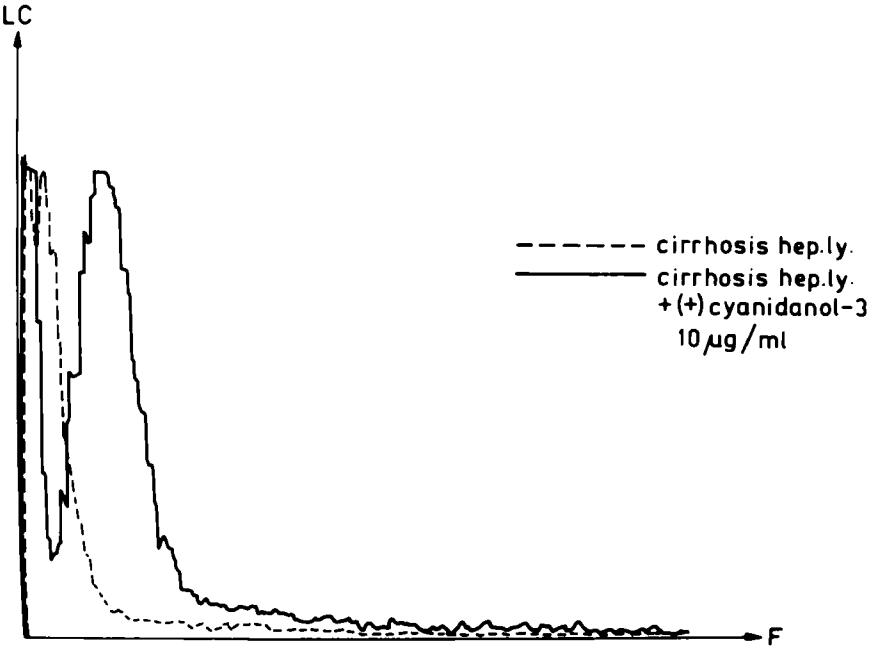

FIGURE 6 Effect of (+)cyanidanol-3 on the SOD-expression of lymphocytes from patients with chronic alcoholic liver disease measured by flow-cytofluorimetry. Fluorescence histogram. LC: lymphocyte count, F: fluorescence intensity.

Effect on the superoxide dismutase expression of lymphocytes

Ten μg/ml (+)cyanidanol-3 moderately increased te superoxide dismutase expression of healthy lymphocytes (Fig. 5): unbroken line as compared to dotted line. The superoxide dismutase expression of the patients' lymphocytes was originally decreased (dotted line of Fig. 6, as compared to dotten line of Fig. 5). The same concentration of (+)cyanidanol-3 restored this parameter (unbroken line of Fig. 6 as compared to dotted line of Fig. 6).

DISCUSSION

The flavonoid derivative (+)cyanidanol-3 has been widely used in the treatment of several hepatic disorders.[1] It was demonstrated to decrease the liver damaging effect of various toxic agents[31–33] mainly by its membrane stabilizing activity and strong radical scavenging ability.[2,5,32,34,25] It promoted the elimination of HBsAg from the blood,[36] and its beneficial effect was histologically proven in chronic active hepatitis, as well.[37] Considering the immunological response, it was demonstrated to inhibit Con A induced T suppressor cell activity,[10] and NK cytotoxicity,[14] to increase the reduced T cell number of patients with chronic liver diseases,[8] to amplify LIF production,[6] and to enhance antigen-specific blast transformation.[9] Lectin induced

immunoglobulin synthesis and blastogenesis was depressed in the presence of (+)cyanidanol-3.

These initial results prompted us to further investigate the immunological and antioxidant mechanisms of action of (+)cyanidanol-3 in a complex *in vitro* test system. In our hands both in healthy subjects and in patients, *in vitro* treatment in a concentration range including the concentration corresponding to the usual *in vivo* dosage inhibited lectin-induced lymphocyte blast transformation and whole blood chemiluminescence in a dose-dependent fashion, and increased superoxide dismutase expression of lymphocytes without influencing the percentage of T and active T cells and antibody-dependent and spontaneous lymphocytotoxicity. Con A-induced T cell mediated lymphocytotoxicity (LDCC) of patients was completely abolished by the drug, while no significant effect was seen using healthy donors' lymphocytes. 10 μg/ml (+)cyanidanol-3 markedly decreased the Con A-induced suppressor activity of patients' lymphocytes without influencing that of normal lymphocytes.

Summarizing these *in vitro* effects of (+)cyanidanol-3 on cellular immunoreactivity one can conclude that most of them are immunosuppressive with the exception of the decrease in suppressor activity. ADCC and NK activities were not influenced by (+)cyanidanol-3 in the *in vitro* concentration range used. On the other hand Duwe and Sutantiran reported an inhibitory effect on NK activity.[14,38] These contradictions might be explained by the different concentrations used. Concerning the inhibition of lectin-induced blast transformation and suppressor cell activity, our results are in agreement with those of Melinn and McLaughlin[15] and of Kakumu *et al.*[10] Our findings that (+)cyanidanol-3 inhibits mitogen induced blast transformation of healthy and patients' lymphocytes and also LDCC activity of patients' lymphocytes might be explained by the inhibition of the lipooxigenase pathway of arachidonic acid metabolism as suggested by Goodwin *et al.*[39] concerning the mechanism of action of glucocorticoids via leucotriene B_4 and by Melinn and McLaughlin discussing the action of other hydroxyl radical scavengers.[15]

Under *in vitro* conditions Sipos *et al.*[8] have demonstrated that (+)cyanidanol-3 could normalize the reduced T cell number in patients with chronic liver diseases. In our hands the originally normal percentage of T cells was not influenced by incubation with (+)cyanidanol-3. The inhibition of chemiluminescence can be explained either by a direct free radical scavenging activity or through the elevation of cAMP concentration.[39] The decreased superoxide dismutase expression of patients' lymphocytes might reflect the exhaustion of the enzyme caused by the prolonged excess of reactive oxygen intermediates in alcoholic patients. In this respect the fact that (+)cyanidanol-3 restored the low enzyme expression indirectly suggested it's free radical scavenging activity.

Further studies are under way in our laboratory to elucidate the possible *in vivo* significance of these *in vitro* observations.

Acknowledgements

This work was supported by grants from the Hungarian Academy of Sciences and Ministry of Health.

References

1. H.O. Conn, (Ed.), International Workshop on (+)Cyanidanol-3 in Diseases of the Liver, Academic Press, London-Toronto-Sydney, Grun and Stratton, New York-San Francisco, 1981.

2. D. Perrissoud, M.F. Maignan and G. Auderset, In (1), 21.
3. J. Fehér, H. Toncsev, L. Sréter, A. Cornides and A. Kiss. *Int. J. Tiss. React.*, **VI**, 75 (1984).
4. H. Kappus, In (1), 17.
5. T.F. Slater and R. Scott, In (1), 33.
6. J.J. Vallotton and P.C. Frei, In (1), 109.
7. J.P. Despont, and R. Cornille Brögger, In (1), 111.
8. J. Sipos, V. Gábor, Z. Tóth, K. Bartók and P. Ribiczey, In (1), 113.
9. S. Yamamoto, H. Suzuki and T. Oda, In (1), 143.
10. S. Kakumu, H. Murakami, and J. Kuriki, *Clin. Exp. Immunol.*, **52**, 430, (1983).
11. J.C. Fantone and P.A. Ward, *Amer. J. Pathol.*, **107**, 397, (1982).
12. A.P. Weetman, A.M. McGregor and R. Hall, *Clin. Immunol. Immunopathol.*, **28**, 39, (1983).
13. P. Kendall-Taylor, *Br. Med. J.*, **288**, 509, (1984).
14. A.K. Duwe, J. Werkmeister, J.C. Roder, R. Lauzon and U. Payne, *J. Immunol.*, **134**, 2637, (1985).
15. M. Melinn, and H. McLaughlin, *Immunology*, **58**, 197, (1986).
16. R.N.M. MacSween, *Acta Med. Scand.*, Suppl., **703**, 57, (1985).
17. S. Palmas, In (1), 167.
18. G. Ugarte, D. Bunout and Iturriaga, In (1), 181.
19. H. Henning, In (1), 177.
20. M.J. World, M.J. Aps, G.K. Shaw and A.D. Thomson, *Alcohol*, **19**, 23, (1984).
21. Y. Hirschaut, G.H. Weiss, and S. Perry, *Cancer Res.* **29**, 1732, (1969)
22. A. Boyum, *Scand. J. Clin. Lab. Invest.* Suppl., 97, **21**, 77, (1968).
23. M. Jondal, G. Holm and H. Wigzell, *J. Exp. Med.* **136**, 207, (1972).
24. D.T.Y. Yu, *J. Immunol.*, **115**, 91, (1975).
25. P. Perlmann and H. Perlmann *Cell. Immunol.*, **1**, 300, (1970).
26. M. Jondal and H. Pros, *Int. J. Cancer*, **15**, 956, (1975).
27. A. Perl, R. Gonzalez-Cabello, I. Láng, P. Somos and P. Gergely, *Cancer Immunol. Immunother.*, **15**, 155, (1983).
28. L. Shou, S.A. Schwartz and R.A. Good, *J. Exp. Med.*, **143**, 1100, (1976).
29. T. Tono-oka, N. Ueno, T. Matsumoto, M. Ohkawa and S. Matsumoto, *Clin. Immunol. Immunopathol.* **26**, 66, (1983).
30. P. Németh, L. Rácz, J. Varga, Á. Láng, A. Németh and Á. Németh, *Arch. Toxicol.*, Suppl. **8**, 288, (1985).
31. A. Gajdos, M. Gajdos-Török and R. Horn, *Biochem. Pharmacol.* **21**, 595, (1972).
32. P. Niebes and G. Ponard, *Biochem. Pharmacol.* **24**, 905, (1975).
33. A.E.M. McLean and L. Nutiall, *Biochem. Pharmacol.*, **27**, 425, (1978).
34. T.F. Slater, and M.N. Eakins, Interactions of (+)cyanidanol-3 with free radical generating systems. In: Bertelli, A. (Ed.), New Treands in the Therapy of Liver Diseases, Kargel, Basel, 1975, 84.
35. J. Fehér, A. Blázovics, A. Cornides, and A. Vereckei, *Br. J. Exp. Path.*, **66**, 161, (1985).
36. A.L. Blum, W. Doelle, K. Kortüm, P. Peter, G. Stohmeyer, P. Berthet, H. Goebell, S. Pelloni, H. Poulsen and N. Tygstrup, In (1), 81.
37. F. Demeulenaere, V.J. Desmet, E. Dupont, R. Fiasse, H. Gisselbrecht, F. Heully, R. Jeanpierre, J. Lecomte, G. Lennes, C. Macinot, P. Migeotte, J. Pirotte, G. Rauber, L. Ruyters and H. van Cauwenberge, In (1), 135.
38. M.S.D. Suthanthiran, P.S. Solomon, A.L. Williams, A. Rubin, A. Novogrodsky and K.H. Stenzel, *Nature*, **307**, 276, (1984).
39. J.S. Goodwin, D. Atluru, S. Sierakowski and E.A. Lianos, *J. Clin. Invest.*, **77**, 1244, (1986).

Accepted by Dr. J.V. Bannister