Endogenous benzodiazepine-like compounds and diazepam binding inhibitor in serum of patients with liver cirrhosis with and without overt encephalopathy

R Avallone, M L Zeneroli, I Venturini, L Corsi, P Schreier, M Kleinschnitz, C Ferrarese, F Farina, C Baraldi, N Pecora, M Frigo, M Baraldi

Abstract

Background/Aim—Despite some controversy, it has been suggested that endogenous benzodiazepine plays a role in the pathogenesis of hepatic encephalopathy. The aim of the present study was to evaluate the concentrations of endogenous benzodiazepines and the peptide, diazepam binding inhibitor, in the blood of patients with liver cirrhosis with and without overt encephalopathy, and to compare these levels with those of consumers of commercial benzodiazepines.

Subjects—Normal subjects (90), benzodiazepine consumers (14), and cirrhotic patients (113) were studied.

Methods—Endogenous benzodiazepines were measured by the radioligand binding technique after high performance liquid chromatography (HPLC) purification. The presence of diazepam and N-desmethyl-diazepam was assayed by HPLC-electrospray tandem mass spectrometry. Diazepam binding inhibitor was studied in serum by radioimmunoassay.

Results—Endogenous benzodiazepines were below the limit of detection in 7% of patients with encephalopathy. When detectable, their levels were at least comparable with those of benzodiazepine consumers and correlated with the liver dysfunction but not the stage of encephalopathy. Serum levels of diazepam binding inhibitor tended to decrease when endogenous benzodiazepines levels increased.

Conclusions—Endogenous benzodiazepines may accumulate in patients with liver cirrhosis during the course of the disease, and the phenomenon appears to be independent of the presence or absence of encephalopathy.

(Keywords: benzodiazepine consumers; diazepam binding inhibitor; endogenous benzodiazepines; liver cirrhosis; overt hepatic encephalopathy)

Hepatic encephalopathy is one of the major complications of liver cirrhosis, and it is a component of fulminating hepatic failure characterised by impairment of the central nervous system, which is believed to develop from increased tone of the inhibitory γ-aminobutyric acid (GABA<sub>γ</sub>) receptor system (for reviews, 1, 2). The involvement of this receptor system in overt hepatic encephalopathy (OHE), discovered in the 1980s during studies on GABA<sub>γ</sub> receptors in the brain of animals with OHE, was considered likely when specific benzodiazepine receptor antagonists were shown to revert the symptoms of encephalopathy in animal models and in patients. 3, 4 Later, the observation of an increased presence of endogenous benzodiazepine receptor ligands (BZDs) in animals and patients with OHE 5 suggested that this phenomenon may contribute to the enhancement of GABAergic neurotransmission. 5, 6 We cannot exclude, however, the possibility that compounds such as ammonia 7, 8 or neurosteroids 9 contribute to the above mentioned increased functional activity of the GABA<sub>γ</sub> receptor system. BZD-like compounds and ammonia may potentiate inhibitory GABAergic neurotransmission by acting synergistically. 10

The endogenous receptor ligands found in blood and brain during OHE 8 were called BZD-like substances since they are a mixture of the halogenated 1,4-benzodiazepines (such as diazepam) and non-halogenated BZDs (called “endozepines”). Although the chemical structure of the endozepines is not yet fully characterised, it is fair to surmise that they contribute to OHE.

Halogenated BZDs are naturally present in several plants and vegetables, 11 in brain tissues of different animal species and in man. 12 Their sources have not yet been clarified, but the observation that they are present in human brain samples stored since 1940 13 indicates that they do not derive from environmental pollution with synthetic BZDs, which have been produced commercially since 1959. These compounds and their precursors are components of our diet. 12 An exogenous biosynthetic pathway for the production of such compounds cannot, however, be excluded since we recently showed that a reduction in the intestinal bacterial flora caused by a non-absorbable antibiotic partially decreases the levels of these compounds in the blood. 11

Other endogenous BZDs such as the neuropeptide called diazepam binding inhibitor (DBI) and its metabolite, the octadecaneuropeptide, which decreases GABA<sub>γ</sub> neurotransmission, 12 have been found to be increased in the cerebrospinal fluid of patients with OHE 12 and in brain regions of rats with portacaval anastomosis. 22
Since few studies have been performed on endogenous circulating BZDs in patients with OHE due to fulminant hepatic failure or liver cirrhosis, the aim of the present study was to (a) evaluate the concentrations and nature of BZD-like compounds in the plasma of patients with liver cirrhosis with and without OHE, (b) compare the levels found in liver cirrhotic patients with those present in the plasma of consumers of commercial BZDs in order to estimate their pharmacological relevance, and (c) study the levels of DBI in both the patients and BZD consumers, bearing in mind that little is known about the mutual interaction of BZD compounds and DBI at the periphery.

Methods

Subjects (Tables 1 and 2)

We studied 113 patients with liver cirrhosis and 90 normal subjects, who appeared to be free of commercial BZD medication for at least three months as verified by patient, family, and medication records. Moreover 14 normal subjects who were habitual consumers of commercial BZDs were included in the study. The diagnosis of liver cirrhosis was based on biochemical tests and liver biopsy. Fifty nine patients showed no evidence of OHE while the other 54 showed different stages of impaired mental status. The stage of OHE was evaluated on the basis of electronencephalographic pattern. This test allowed the classification of the cirrhotic patients into the following categories: 59 with stage 0, 22 with stage I, 19 with stage II, eight with stage III, and five with stage IV. The functional status of the liver was clinically classified according to the Child–Pugh classification. Table 1 gives the characteristics of the patients included in the study, and table 2 contains laboratory data.

The 14 regular consumers of BZDs, who used diazepam 2 mg per day or lorazepam 2.5 mg per day as sedatives, had normal liver and kidney function.

### Table 1 Patient characteristics

| Child-Pugh class | Liver cirrhosis | BZD consumers |
|------------------|-----------------|---------------|
|                  | Controls        | A             | B              | C              | BZD consumers |
| No               | 90 (33)         | 41 (39)       | 14 (3)         |                |               |
| Women            | 45 (18)         | 28 (30)       | 3 (3)          |                |               |
| Age (y) (mean (SD)) | 63 (9)       | 59 (9)        | 57 (11)        | 50 (11)        | 65 (4)        |
| Aetiology of cirrhosis |         |               |                |                |                |
| Viral            | –               | 22 (27)       | 28 (3)         |                |               |
| Alcoholic        | –               | 6 (11)        | 6 (3)          |                |               |
| Overt encephalopathy (stage) |         | 5 (3)         | 5 (3)          |                |               |

A–C, functional status of the liver classified according to Child-Pugh.

### Table 2 Laboratory data on patients with liver cirrhosis

| Child-Pugh class | Bilirubin (µmol/l) | Prothrombin time (%) | Albumin (g/l) | Ammonia (µmol/l) |
|------------------|-------------------|----------------------|---------------|-----------------|
| A                | 15 (8)            | 75 (10)              | 37 (3)        | 33 (13)         |
| B                | 34 (29)           | 55 (9)               | 30 (5)        | 98 (44)         |
| C                | 97 (61)           | 40 (10)              | 28 (2)        | 84 (46)         |

Values are mean (SD). Normal values: bilirubin 2–18 µmol/l, prothrombin time 80–100%, albumin 3–5 g/l, venous ammonia 4–26 µmol/l.
DEGstation 5000/33 (Digital Equipment, Un-
terföhring, Germany) and ICIS 8.1 software
(Finnigan MAT). For HPLC, an Applied Bio-
system 140B Solvent Delivery System (Applied
Biosystems, Foster City, CA, USA) equipped
with two 10 ml syringes and a LiChrospher
60-RP select B column (100 × 2.0 mm internal
diameter; 5 µm; Knauer, Berlin, Germany)
was used. Aliquots of the lyophilised samples
were redissolved in 100 µl methanol/water/
acetonitrile (1:1:1, by vol.). Separations were
performed using a linear gradient. Solvent A
was 0.1% TFA in water, and solvent B was
acetonitrile. The gradient program was: 0–10
min, 20–80% solvent B. The loop injection was
5 µl and the solvent flow was set to 200 µl/min.
The mass spectrometer was operated in the
selected reaction monitoring mode, with argon
at a pressure of 0.27 Pa as collision gas.
Selected ion pairs for the simultaneous experi-
ment were [M+H]+ and the most abundant product
ions (m/z 217/140 (−30 eV) for diazepam
and m/z 285/257 (−30 eV) for diazepam). The
ion pairs represent the protonated molecular
ion [M+H]+ and the most abundant product
ion for each of the BZDs. The temperature of
the heating capillary was 250°C. The electro-
spray capillary was set to 5 kV and the electron
multiplier voltage was set to 2.3 kV. A sheath
gas pressure of 73 Pa and an auxiliary gas flow
of 5 litres/min were applied to support the
droplet formation. Nitrogen served both as
sheath and auxiliary gas. With this method a
signal-to-noise ratio of 2:1 was reached.

**QUANTIFICATION OF DBI**
The level of DBI-like immunoreactivity (DBI-
LI) in serum was assayed in 16 controls, in 19
patients with liver cirrhosis without OHE, in 12
patients with liver cirrhosis with OHE, and in
eight BZD consumers. To extract DBI and to
precipitate plasma proteins, 1 ml plasma was
diluted with 1 ml saline and 2 ml 2 M acetic
acid, heated at 90°C for 10 min followed by the
addition of 2 M NaOH (1 ml). After centrifuga-
tion at 20 000 g for 20 minutes, aliquots of
supernatants were lyophilised in triplicate and
used for DBI radioimmunoassay (DBI-RIA).
The characterisation of DBI immunoreactivity
detected in serum extracts by reverse phase
HPLC and the DBI-RIA, performed using antiser-
um produced in rabbits against human recombinant
DBI, were performed as previ-
ously described.

**STANDARDISATION ANALYSIS**
The Kruskal-Wallis test was used to determine
whether a given variable differed significantly
between groups. Comparisons between single
groups were performed by means of the
Mann-Whitney U test corrected as described
by Bonferroni.

**RESULTS**

**BZD-LIKE COMPOUNDS**
The extraction and purification of plasma sam-
10 les from normal subjects and from patients
with liver cirrhosis with and without OHE
showed the presence of at least 12 different
peaks, with a retention time ranging from 17 to
70 minutes under our chromatographic condi-
tions. The number of peaks found in each
patient ranged from one to four. The number of
fractions containing BZD ligands was consis-
tently lower in controls and in liver cirrhosis
without OHE (one or two peaks) than in
patients with OHE (three or four peaks).
Avallone, Zeneroli, Venturini, et al

Determination of 1,4-Benzodiazepines by HPLC-ESI-MS-MS (Fig 3)

Mass spectrometric studies utilising HPLC-ESI-MS-MS on the active fractions were performed on 12 controls, 37 liver cirrhosis cases without OHE, and 16 liver cirrhosis cases with stages I–IV of OHE. Diazepam and N-desmethyldiazepam were below the detection limit in normal subjects and in 34 of 37 of the patients without OHE. In the remaining three patients, trace amounts of both compounds were found in two, and in one there was only N-desmethyldiazepam. In liver cirrhosis with OHE the above two compounds were below the detection limit in two patients with stage I OHE and were represented only in trace amounts of N-desmethyldiazepam in two patients with stage III and IV OHE.
Endogenous benzodiazepines and liver cirrhosis

DIAZEPAM BINDING INHIBITOR (FIG 4)

The DBI-LI levels ranged between 0.31 and 2.37 nmol/l (mean value 1.04 nmol/l) in control subjects, between 0.28 and 1.01 nmol/l (mean value 0.55 nmol/l) in liver cirrhosis without OHE, and between 0.13 and 0.57 nmol/l (mean value 0.34 nmol/l) in liver cirrhosis with OHE. Interestingly, the levels of DBI-LI in BZD consumers ranged between 0.15 and 0.57 nmol/l (mean value 0.33 nmol/l). Kruskal-Wallis one way analysis of variance shows a significant difference between groups (p<0.0001). The Mann-Whitney U test adjusted using the Bonferroni correction shows that the levels found in liver cirrhotic patients with or without OHE were statistically different from controls (p<0.05 and p<0.001 respectively). The DBI-LI in BZD consumers was different from controls (p<0.005) and practically equal to those found in cirrhotic patients with or without OHE.

Discussion

We have shown in this study, which includes a large number of fully characterised liver cirrhotic patients, that: (1) endogenous BZD-like compounds are, under our experimental conditions, below the detection limit (2 nmol DE/l) in 51% of normal subjects, in 16% of liver cirrhotic patients without OHE, and in 7% of those with OHE; (2) when detectable, BZD levels rise in the serum of cirrhotic patients in correlation with worsening liver function, but not with the degree of OHE; (3) the measurable BZD-like compounds comprise both known BZDs such as diazepam and N-desmethyldiazepam and unknown BZD-like compounds, and these so called “endozepines” seem to represent most of the displacing ligands in plasma; (4) when detectable, the BZD levels found in patients with OHE were comparable with those present in BZD consumers with normal states of consciousness; (5) DBI-LI levels were found to be decreased in cirrhotic patients independently of the presence or absence of OHE. In BZD consumers, in whom BZD levels were constantly elevated, we found a significant reduction of DBI-LI. These data indicate an inverse correlation with the levels of circulating BZDs.

The finding that encephalopathy may occur in liver cirrhotic patients with very low levels of circulating BZD-like compounds, if not below the detection limit, is in line with the results of previous studies on patients with OHE due to fulminant hepatic failure. In these studies, only 60% of patients showed increased levels of BZDs in serum and only 55% had increased concentrations in the brain. These findings are in line with the concept that BZDs in serum diffuse passively into the brain and are in equilibrium with BZDs in the brain.

The finding that, when detectable, circulating BZD-like compounds reach concentrations comparable with those found in BZD consumers raises the question of what causes the difference in the response to BZDs by the brains of cirrhotic patients with OHE and those of BZD consumers.

Chronic exposure to commercial BZD produces tolerance represented by reduced GABA-BZD receptor function; this means that administration of increased doses of the drug is required to maintain the pharmacological effect. In contrast, in patients with liver cirrhosis, rather than tolerance, there is increased cerebral sensitivity to BZD administration. It has been shown that the reduction in BZD dose requirements in these patients is due to changes in the cerebral sensitivity more than to changes in drug disposition. Hence it seems fair to surmise that the enhanced GABAergic tone cannot be attributed to increased endogenous BZD-like compounds per se, but more to the presence of pre-existing brain dysfunction related, for example, to ammonia toxicity. In this situation, a concentration of circulating BZD-like compounds that would have no effect in a normal subject may facilitate sedation and worsen an episode of encephalopathy in a liver cirrhotic patient.

Finally, as regards the nature of the BZD-like compounds in serum, we found the presence of both diazepam and N-desmethyldiazepam by HPLC-ESI-MS-MS analysis. These halogenated compounds, however, represented less than 20% of the total BZD receptor ligands. This observation, which confirms the results of previous studies, indicates that most of these compounds are substances of unknown origin and nature called “endozepines”. Both
halogenated and non-halogenated BZDs were found inconsistently in patients with OHE and were sometimes not raised at all. It remains, however, unclear why BZDs accumulate in the blood of some liver cirrhotic patients and not in others with the same pathological condition. Retrospective control of the diet and therapy used in our patients as well as establishment of the aetiology of the liver cirrhosis did not show any substantial difference to explain this phenomenon.

As regards DBI, we found that the levels of this peptide are significantly decreased in those patients with liver cirrhosis and increased levels of BZDs independently of the presence or absence of OHE. The levels of DBI do not correlate with neuronal dysfunction or the severity of the liver disease. This finding would appear to exclude any direct effect of the liver dysfunction and the encephalopathy on the metabolism of this circulating peptide and suggests the presence in the periphery of a negative regulatory feedback mechanism exerted by BZDs on DBI. Accordingly, the same decrease is present in BZD consumers. The relation between DBI levels in plasma and those in the central nervous system is still poorly understood, as is also the regulation of its synthesis and metabolism in peripheral tissues. From these data we can surmise that the ratio between DBI and BZDs in the periphery is probably regulated by different mechanisms from those operating in the central nervous system. In liver cirrhotic patients with OHE, in fact, DBI was shown to be increased in cerebrospinal fluid in the presence of increased levels of BZDs, and the phenomenon was interpreted as an episode of compensatory reaction by DBI to an increased presence of BZDs.

Whatever the regulatory mechanism in the periphery may be, the described decrease in DBI in the blood of the liver cirrhotic patients may be of relevance from the metabolic point of view, since this peptide, through stimulation of peripheral BZD receptors, regulates the intermediate metabolism and steroid biosynthesis. In conclusion, endogenous compounds with sedative action may accumulate in patients with liver cirrhosis during the course of the disease, and the phenomenon appears to be independent of the presence or absence of encephalopathy. The observation that circulating BZD-like compounds reach levels comparable with those found in BZD consumers with a normal state of consciousness reinforces the concept that these compounds may be more effective in those patients with pre-existing altered brain function. This work was supported by a grant from MIRAAF (no 7240, 1991) and a grant from Modena University. We thank Professor H Alho, University of Tampere, Finland who kindly provided antisera raised in rabbits against human recombinant DBI. Preliminary data on the assay performed with the radioligand binding technique without previous HPLC purification were given as an oral presentation to the International Association for the Study of the Liver, Cancun, May 1994, and published in abstract form: Zeneroli ML, Venturini I, Avallone R, Ardizzone G, Demarini M, Porcella G, Baraldii M. Levels of endogenous benzodiazepine-like compounds in serum of liver cirrhotic patients with and without encephalopathy and in fulminant hepatic failure. Hepatology 1994:19:1431

1. Zeneroli ML. Hepatic encephalopathy. Experimental studies in a rat model of fulminant hepatic failure. J Hepatol. 1985;1:301–12.
2. Jones EA, Skolnick P, Gammal H, et al. The gamma-aminobutyric acid-A (GABA-A) receptor complex and hepatic encephalopathy. Some recent advances. Ann Intern Med 1989;110:532–46.
3. Baraldi M. Supersensitivity of GABA-A receptors in hepatic encephalopathy. Neurechim Res 1990;15:153–60.
4. Baraldi M, Zeneroli ML, Venturini I, et al. Supersensitivity of benzodiazepine receptors in hepatic encephalopathy due to fulminant hepatic failure in the rat: reversal by benzodiazepine antagonist. Clin Sci 1984;67:167–75.
5. Scollo Lazizzari G, Steinnmann E. Reversal of hepatic coma by benzodiazepine antagonist (Ro 15-1788). Lancet 1985;1:1324.
6. Barsky G, Meier PJ, Ziegler WH, et al. Reversal of hepatic coma by benzodiazepine antagonist (Ro 15-1788). Lancet 1985;1:1324–5.
7. Olomuza M, Guidotti A, Costa E, et al. Endogenous benzodiazepine in hepatic encephalopathy. Lancet 1989;1:491–2.
8. Basile AS, Gammal SH, Jones EA, et al. GABA-A receptor complex in an experimental model of hepatic encephalopathy: evidence for elevated levels of an endogenous benzodiazepine receptor complex ligand. J Neurochem 1989;53:1057–63.
9. Olomuza M, Rothstein JD, Guidotti A, et al. Endogenous benzodiazepine in human and animal hepatic encephalopathy. J Neurochem 1990;55:2015–23.
10. Mullen KD, Stauzer KM, Kaminsky-Russ K. “Endogenous” benzodiazepine activity in body fluids of patients with hepatic encephalopathy. Lancet 1990;336:81–3.
11. Baraldi M, Zeneroli ML, Rothstein JD, et al. Increased presence of benzodiazepine-like compounds in a rat model of hepatic encephalopathy. In: Bengtsson F, Jeppsson B, eds. Progress in hepatic encephalopathy. Miami: CRC Press, 1991: 155–60.
12. Basile AS, Hughes RD, Harrison PH, et al. Elevated brain concentrations of 1,4-benzodiazepines in fulminant hepatic failure. N Engl J Med 1991;325:475–8.
13. Basile AS, Harrison PMM, Hughes RD, et al. Relationship between plasma benzodiazepine receptor ligand concentrations and severity of hepatic encephalopathy. Hepatology 1994:19:112–21.
14. Mullen KD, Martin JV, Mendelson WB, et al. Could an endogenous benzodiazepine ligand contribute to hepatic encephalopathy? Lancet 1988;1:457–9.
15. Baraldi M, Pinelli G, Rucci P, et al. Toxins in hepatic encephalopathy: the role of the synergistic effect of ammonia, mercaptans and short chain fatty acids. Arch Toxicol 1984;1:103–5.
16. Inzata Y, Noreen MB. Ammonia-induced up-regulation of peripheral-type benzodiazepine receptors in cultured astrocytes labeled with [3H]PK11195. Neurosci Lett 1994; 175:35–8.
17. Basile AS, Jones EA. Ammonia and GABAergic neurotransmission: interrelated factors in the pathogenesis of hepatic encephalopathy. Hepatology 1997;25:1303–5.
18. Medina JH, Pena C, Levr D, Stein M, et al. Benzodiazepine-like molecules as well as other ligands for the brain benzodiazepine receptors, are relatively common constituents of plants. Biochem Biophys Acta Comm 1989;1507–51.
19. Sangameswaran L, De Blas AL. Demonstration of the benzodiazepine-like molecules in the mammalian brain with a monoclonal antibody to benzodiazepines. Pro Nat Acad US 1985;82:5560–4.
20. De Blas AL, Park D, Friedrich P. Endogenous benzodiazepine-like molecules in human, rat and bovine brains studied with a monoclonal antibody to benzodiazepines. Brain Res 1987;413:275–84.
21. Zeneroli ML, Venturini I, Stefaneli S, et al. Antibacterial activity of rifaximin reduces the levels of benzodiazepine-like compounds in patients with liver cirrhosis. Pharmacol Rev 1997;49:557–60.
22. Guidotti A, Forchetti CM, Corda MG, et al. Isolation, characterization and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. Pro Nat Acad US 1983;80:5331–5.
23. Rothstein JD, McPhann G, Guarneri P, et al. Cerebrospinal fluid content of diazepam binding inhibitor (DBI) in chronic hepatic encephalopathy. Ann Neurol 1989;26:57–62.
24. Butterworth RF, Tonon MC, Desy L, et al. Increased brain activity of the endogenous benzodiazepine receptor ligand, octadecaneuropeptide (ODN), following portacaval anastomosis in the rat. Peptides 1991;12:119–25.
25. Kennedy J, Parkhoo SP, Mcgilvray B, et al. Effect of extracorporeal liver perfusion on the electroencephalogram of patients in coma due to acute liver failure. Q J Med 1991;84:549–56.
26. Pugh RNH, Murray-Lyon IM, Dawson JL, et al. Transection of the oesophagus for bleeding oesophageal varices. Br Med J 1973;1:657–60.
27. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
28. Kleinschmidt M, Herdeich M, Schrieber P. Determination of 1,4-benzodiazepines by high-performance liquid chromatography-electrospray tandem mass spectrometry. J Chromatogr B Biomed Appl 1996;786:61–7.

for 1 July 1998. Downloaded from http://gut.bmj.com/ by guest. Protected by copyright.
29 Ferrarese C, Apollonio L, Frigo M, et al. Cerebrospinal fluid levels of diazepam-binding inhibitor in neurodegenerative disorders with dementia. *Neurology* 1990;40:632–5.
30 Ferrarese C, Appollonio I, Frigo M, et al. Distribution of a putative endogenous modulator of the GABAergic system in human brain. *Neurology* 1989;39:443–5.
31 Kolmer M, Pelto-Huikko M, Alho H. Germ-cell specific transcription of the diazepam binding inhibitor (DBI) gene in testis: evidence for translational control. *DNA Cell Biol* 1997;16:59–72.
32 Greenblatt DJ, Miller LG, Shader RI. Neurochemical and pharmacokinetic correlates of the clinical action of benzodiazepine hypnotic drugs. *Am J Med* 1990;88:194–236.
33 Branch RA, Morgan MH, James J, et al. Intravenous administration of diazepam in patients with chronic liver disease. *Gut* 1976;17:975–83.
34 Baktir GA, Fisch HU, Karluganis G, et al. Mechanism of the excessive sedative response of cirrhotics to benzodiazepine: model experiments with triazolam. *Hepatology* 1987;7:629–38.
35 Papadopoulos V, Berkovich A, Krueger KE, et al. Diazepam binding inhibitor and its processing products stimulate mitochondrial steroid biosynthesis via an interaction with mitochondrial benzodiazepine receptors. *Endocrinology* 1991;129:1481–7.