Cobalamin (vitamin B_{12})-deficiency-induced changes in the proteome of rat cerebrospinal fluid

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We studied the changes in the proteome of CSF (cerebrospinal fluid) in two animal models of Cbl (cobalamin) deficiency: TGX (totally gastrectomized) rats and rats fed a Cbl-D (Cbl-deficient) diet. Two-dimensional PAGE was used to detect qualitative and quantitative variations in proteins in the CSF samples. The peak increase in total CSF protein concentration was observed 4 months after TG (total gastrectomy) and after 6 months of eating a Cbl-D diet. There is a specific increase 4 months after TG in the spots corresponding to α_1-antitrypsin and the *de novo* presence of thiostatin and haptoglobin β. Cbl-replacement treatment in 4-month-TGX rats corrected these alterations in the CSF proteome. However, most of the CSF proteome alterations attenuated in Cbl-untreated 8-month-TGX rats and in rats fed a Cbl-D diet for 16 months. Transthyretin concentration varied slightly in the CSF of both types of Cbl-D rat, whereas the relative abundance of prostaglandin D synthase rose sharply in the CSF of the rats fed a Cbl-D diet for 16 months. We have demonstrated previously that the histological and ultrastructural CNS (central nervous system) damage in both types of Cbl-D rat appears within 2–3 months of Cbl deficiency, and thus appears to precede the alterations in the CSF proteome. The CSF proteome patterns of rats in which phlogosis was induced in or outside the CNS are quite different from those of the CSF of Cbl-D rats. All these findings demonstrate that the alterations in the CSF proteome of Cbl-D rats are specifically linked to Cbl deficiency.

Key words: cerebrospinal fluid, cobalamin deficiency, prostaglandin D synthase, proteome, subacute combined degeneration, thiostatin.

INTRODUCTION

In recent years, we have identified a new pathogenetic mechanism by which chronic vitamin B_{12} [Cbl (cobalamin)] deficiency in adult human CNS (central nervous system) causes degenerative damage {classically known as SCD (subacute combined degeneration) [1]}, and thereby we have identified novel functions for Cbl [1,2]. In fact, we have demonstrated recently that the neuropathological morphological alterations caused by Cbl deficiency in the rat CNS are connected with the vitamin's fading ability to modulate in opposite directions the synthesis of a neurotoxic agent, TNF-α (tumour necrosis factor-α) [3], and a neurotrophic agent, EGF (epidermal growth factor) [4,5]. Chronic Cbl deficiency leads to an increased synthesis of TNF-α [3] together with a decreased synthesis of EGF in rat CNS [4], whereas Cbl-replacement treatment in Cbl-D (Cbl-deficient) rats substantially corrects this non-physiological imbalance between the two neuroactive factors [3,4]. Recently, we also demonstrated that Cbl positively regulates the levels of interleukin-6 (a well-known endogenous neuroprotectant [6]) in the CSF (cerebrospinal fluid) of the rat [6].

All of our findings demonstrate that Cbl plays a pivotal role in balancing the normal production of some cytokines and growth factors by CNS cells [1], which necessarily means that, regardless of its co-enzyme function, Cbl regulates in opposing ways the expression of some genes in mammalian CNS [1,4]. Some of the consequences of chronic Cbl deficiency for the mammalian genome have been emphasized previously by other authors, who have shown a significant negative correlation of Cbl deficiency with the increased *in vitro* and *in vivo* formation of micronuclei [7].

The term proteome refers to proteins expressed by a genome in a given tissue and/or organ at a given time [8]. One major advantage of the proteomic approach is that it allows the parallel evaluation of a large number of components instead of one or just a few selected markers, and it is free of the bias caused by the preselection of experimental targets. This is especially relevant when no definite pathological hypothesis has to be proved or disproved, but the aim is to attain a breakthrough in our understanding physiological or pathological mechanisms. The identification and characterization of CSF protein patterns for both human and experimental CNS-degenerative diseases is relevant to the development and identification of new marker(s) [the so-called DSP (disease-specific protein)] for studying the pathogenesis and neurochemistry of these diseases [9]. Furthermore, identification by proteomic methods of gene products in CSF also offers the possibility of quantifying changes in their levels in some experimental models of human neurodegenerative diseases, thus allowing the response to a given therapy to be evaluated. We have extensively studied the proteome of rat CSF, in which we have identified all of the major proteins present under normal conditions [10]. The reference map has been published recently [10]

Abbreviations used: APP, acute phase protein; Cbl, cobalamin; Cbl-D, Cbl-deficient; CNS, central nervous system; CSF, cerebrospinal fluid; 2-DE, two-dimensional electrophoresis; DSP, disease-specific protein; EGF, epidermal growth factor; Hp, haptoglobin; i.c.v., intracerebroventricular; i.m., intramuscular; LPS, lipopolysaccharide; α_1-MAP, α_1-major acute-phase protein; PGDS, prostaglandin D synthase; SCD, subacute combined degeneration; TG, total gastrectomy; TGX, totally gastrectomized; TNF-α, tumour necrosis factor α; TP, turpentine; TTR, transthyretin.

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and is available at http://linux.farma.unimi.it/RSPSG/otherfluids/CSF.html.

Given the apparent capacity of Cbl to influence the gene expression of mammalian cells, including those of the CNS, we hypothesized that Cbl deficiency could modify the proteome of rat CSF because some CSF proteins are derived from the CNS [8]. To this end, we determined the time course of changes in CSF protein patterns in rats made Cbl-D by means of TG (total gastrectomy) or chronic feeding with a Cbl-D diet. The specificity of the changes observed in the CSF proteome of Cbl-D rats was checked in two ways: first by investigating the effect of chronic Cbl-replacement treatment in TGX (totally gastrectomized) rats to assess the return of the CSF protein pattern to normality; secondly, by comparing the effects of Cbl deficiency on CSF proteome with those observed in the CSF proteome of rats in which a phlogistic reaction was induced primarily inside or outside the CNS. 2-DE (two-dimensional electrophoresis) on polyacrylamide gel was used to detect the qualitative and quantitative variations in the proteins in the CSF samples as it is the method of choice for the analytical study of the proteome (proteomics) [11–15].

MATERIALS AND METHODS

Animals and treatments

Non-inbred adult male albino Sprague–Dawley rats (Charles River Italia, Calco, Italy), weighing 250 g and all having the same age at the beginning of the experiments, were housed as described previously [16]. The procedures involving animals and their care were conducted in conformity with the institutional guidelines and in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1 Dec, 12, 1987, and [16a]). To induce Cbl-D neuropathy, the rat counterpart of human SCD [1], chronic Cbl deficiency was induced by means of TG or chronic feeding with a Cbl-D diet, as described elsewhere [16]. Laparotomized animals served as controls for TGX rats [16]. Body temperature was maintained at 37 °C during all of the surgical procedures and during recovery from anaesthesia (i.e. until normal locomotor activity was observed). All of the operated animals received short-term post-operative treatment with antibiotics [16]. To perform the i.c.v. (intracerebroventricular) microinjections, a polyethylene guide cannula was stereotactically placed in the left lateral ventricle under ketamine anaesthesia [3]; correct cannula placement was checked as reported previously [3].

Some TGX rats were given subcutaneous injections of Cbl once a week at a dose of 200 μg/100 g of body weight for the first 2 months after the operation, starting 7 days after TG (scheme A), and others were treated in the same way for the third and fourth post-operative months (scheme B), i.e. after CNS morphological abnormalities had already appeared [17]. An inflammatory reaction was primarily induced in the brain of normal rats by means of i.c.v. microinjections of LPS (lipopolysaccharide; Sigma, St. Louis, MO, U.S.A.). LPS was dissolved in sterile pyrogen-free saline and given to normal rats twice over 7 days subsequent to the attachment of the cannula, at a dose of 40 μg/100 g of body weight per microinjection [18]. Alternatively, an inflammatory reaction was induced primarily outside the CNS by means of a single i.m. (intramuscular) injection of TP (turpentine; Fluka Chemie, Buchs, Switzerland), at 300 μl/100 g of body weight, into normal rats. The controls for the LPS- or TP-treated rats had the same age and were given the corresponding number of i.c.v. microinjections or i.m. injections of an equal amount of sterile pyrogen-free saline.

Studies of CSF components

The rats were anaesthetized with chloral hydrate (350 mg/kg of body weight) given intraperitoneally, and CSF samples (100–150 μl each) were drawn by inserting a glass capillary (with a tip of $\approx$300 μm) connected to a flexible polyethylene tube into the cisterna magna under gentle suction [4]. Careful surgery prevented as much blood contamination as possible, and all of the CSF samples were centrifugated to determine the amount (if any) of erythrocyte contamination; those with more than 0.01 μl of visible erythrocytes were discarded [4]. Each experimental group consisted of three rats, from each of which one CSF sample was obtained. In the case of the LPS-treated rats, CSF was collected 24 h after the last microinjection; in the case of the TP-treated rats, the CSFs were collected 24 or 48 h after TP administration. The samples were stored at -20 °C until analysis by 2-DE and Cbl assay. CSF protein concentration was evaluated according to Bradford's method [19]. CSF does not require any processing for protein extraction, and so there is no need for normalization against variability in protein recovery. CSF volumes hardly change under normal conditions, and show limited variability even under extreme pathological conditions not affecting CSF circulation [20]. Therefore, the changes in the concentration of the individual CSF components (and hence the changes in the total amount of that component in a fixed-volume aliquot) directly reflect the changes in their total amounts in the bulk of the fluid. The Cbl concentrations were determined as described previously [5]. The post-operative administration of Cbl to TGX rats significantly increases CSF Cbl levels above those of Cbl-untreated TGX rats, regardless of whether Cbl administration begins 7 days (scheme A) or 2 months (scheme B) after TG [4].

2-DE and numerical analysis of the map spots

The 2-DE maps were obtained using the immobilized pH gradient–Dalt method [21]. The sample proteins were first resolved according to charge on a non-linear pH 4–10 immobilized pH gradient [22] in the presence of 8 M urea and 0.5 % carrier ampholytes. The anode-to-cathode distance was 8 cm. The identified proteins were then fractionated according to size using SDS/PAGE on 7.5–17.5 % polyacrylamide gradients. The sample loads consisted of 25 μl of CSF with 0.5 μl of 2-mercaptoethanol. Each CSF sample (i.e. one sample per rat) was run once. In a typical experiment, eight CSF samples from different experimental groups were processed in parallel. The sample application was at the cathode. After a ramp from 12.5 to 50 V/cm, the proteins were focused overnight at 50 V/cm, then for 2 h at 300 V/cm for a total of 10 000 V $\cdot$ h, at 15 °C in a Multiphor II horizontal chamber (Amersham Biosciences, Little Chalfont, Bucks., U.K.). Slab size was 14 cm $\times$ 16 cm $\times$ 0.15 cm; two immobilized-pH-gradient strips were run per SDS slab. The migration in SDS/PAGE was at 50 mA/slab, 450 V maximum, in a Protean II vertical chamber (Bio-Rad, Hercules, CA, U.S.A.). Silver staining was performed according to the method of Heukeshoven and Dernick [23] and developed for the same length of time (10 min) in all experiments. The protein patterns were scanned using a video camera under the control of NIH Image (release 1.62) and analysed by means of PDQUEST software (version 6.2.1; Bio-Rad) [24]. The spot volumes (integrated areas $\times$ integrated intensity) in the CSF samples from the treated rats are expressed as ratios to the corresponding volume in control animals. The spots were detected after background correction in the gel images, followed by the manual editing of all gels in one series. A match-set was created including all of the gels to be compared. A standard gel (master)

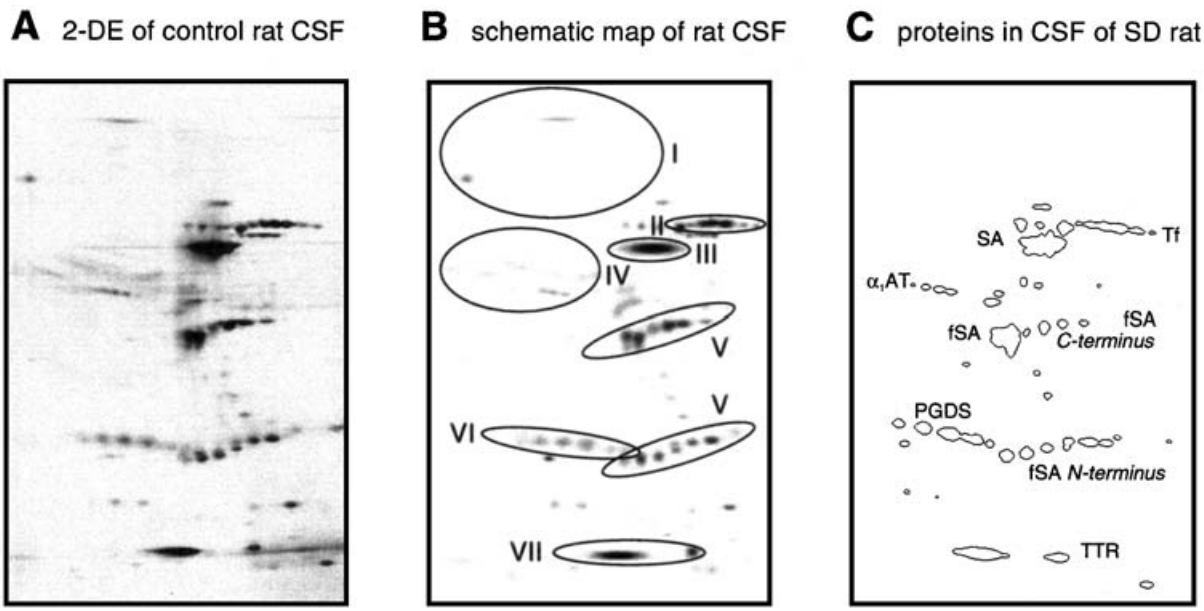

Figure 1 2-DE of CSF from Sprague–Dawley control rats

(**A**) Video-camera scan of a typical gel. (**B**) Schematic map of the CSF proteome produced by the PDQUEST image-analysis software. As detailed in the first paragraph of the Results section, the oval areas I–VII mark spot grouping for quantitative analysis. (**C**) Contour map (produced using Photoshop; Adobe Systems, San Francisco, CA, U.S.A.) on which identifications of the major protein spots are listed (for reference map, see [10] and http://linux.farma.unimi.it/RSPSG/otherfluids/CSF.html). For experimental details, see the Materials and methods section. Tf, transferrin; fSA, fragment of serum albumin; SA, serum albumin; α_1AT, α_1-antitrypsin.

was generated from the image with the highest spot number by including additional spots from the other gels.

Statistical analysis

As all of the gels in a given series were processed in one batch, and as all of the samples consisted of an invariable volume of CSF, the results were evaluated in terms of spot volumes (the integral of the pixel intensity over the spot area; i.e. the sum of the intensities of all of the pixels making up the object) without any further correction or normalization. The statistical significance of the differences between each experimental mean and the corresponding control mean was evaluated by Tukey's test. An α-level of 0.05 or less was fixed as the limit for statistical significance.

Studies of blood components

Red cell count, haematocrit, haemoglobin concentration and serum Cbl and folate levels were determined in blood collected from anesthetized rats by means of a cardiac puncture just before killing [16]. Anaemia and very low serum Cbl levels were observed in the TGX rats (results not shown), in accordance with our previous results [16]. The post-operative administration of Cbl to TGX rats for 2 months significantly increased serum Cbl levels above those of operated animals not given the vitamin, irrespective of whether it was begun 7 days (scheme A) or 2 months (scheme B) after TG [17]. The serum samples from the TP- or LPS-treated rats showed the typical pattern of APPs (acute phase proteins; not shown), in accordance with our previous results [25].

CNS histopathology

No histological check was made of the CNS lesions typical of SCD (i.e. the so-called spongy vacuolation of the white matter of CNS, especially of the spinal cord [16]), because we have previously studied them extensively and timed their onset and their sequence precisely [17]. Similarly, no histological check of the lesions induced by i.c.v. LPS microinjections was made because they have been widely shown to be consistent with a 'classical' inflammatory response [18].

RESULTS

Changes in the CSF proteome of Cbl-D rats

Figure 1 shows the reference protein pattern of rat CSF by means of 2-DE. Figure 1(A) contains the video camera scan of a silver-stained gel of a typical control CSF sample. The ovals (I–VII) in Figure 1(B) group the spots whose volumes were summed after quantification. Besides the albumin zone, four spot groups were defined to include all spots belonging to major identified proteins [transferrin, PGDS (prostaglandin D synthase; EC 5.3.99.2; also called β-trace protein), TTR (transthyretin) and albumin fragments]; two further groups were defined to include all spots inside specific gel areas (high-molecular-mass proteins and α-globulins), where little protein migrates in control samples (Figure 1C). Group I corresponds to high-molecular-mass proteins, group II to transferrin, group III to albumin, group IV to serum α-globulins, group V to albumin fragments, group VI to PGDS and group VII to TTR. The total CSF protein concentration in the control rats was 0.34 ± 0.11 mg/ml (means $\pm$ S.D.; $n = 3$) (in line with that reported by other authors [26]). In our control rats, we estimated a variation coefficient for protein concentration of as high as 33 %.

Figure 2 shows the 2-DE maps of CSF samples from the various groups of Cbl-D rats, and Figure 3 contains data from the quantitative evaluations of the same samples. Each panel in Figure 3 shows a histogram of the total protein concentration

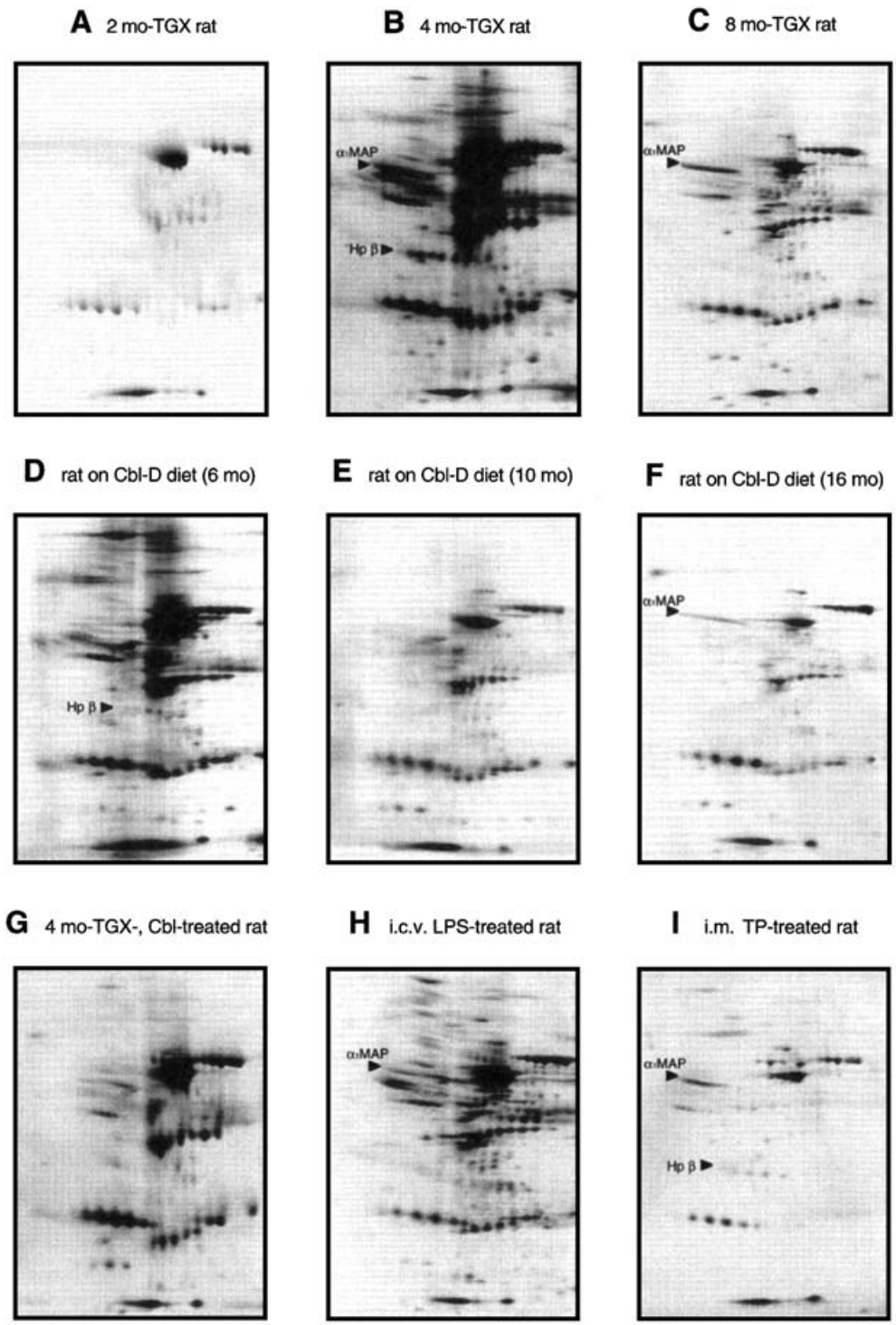

Figure 2 2-DE of CSF from rats in the various experimental groups

(**A**) Typical 2-month-TGX rat; (**B**) typical 4-month-TGX rat; (**C**) typical 8-month-TGX rat; (**D**) typical rat fed a Cbl-D diet for 6 months; (**E**) typical rat fed a Cbl-D diet for 10 months; (**F**) typical rat fed a Cbl-D diet for 16 months; (**G**) typical 4-month-TGX and Cbl-treated rat given Cbl subcutaneously during the third and fourth post-operative months (scheme B); (**H**) typical rat killed 7 days after the first LPS i.c.v. administration; (**I**) typical rat killed 48 h after TP i.m. administration. For further details, see the Materials and methods section. mo, month. α_1-MAP, α_1-major acute-phase protein; Hp, haptoglobin.

(C) and the abundance (volume) of proteins in groups I–VII, the data being expressed as ratios to the corresponding values in control rats. Figures 2(A)–2(C) and 3(A)–3(C) refer to the time-course effects of Cbl deficiency on the CSF proteome of TGX rats. Two months after TG (Figure 3A), total protein concentration was slightly (but not significantly) below control values; accordingly, the data for protein spot volumes normalized versus controls show a comparable reduction in the individual components, including

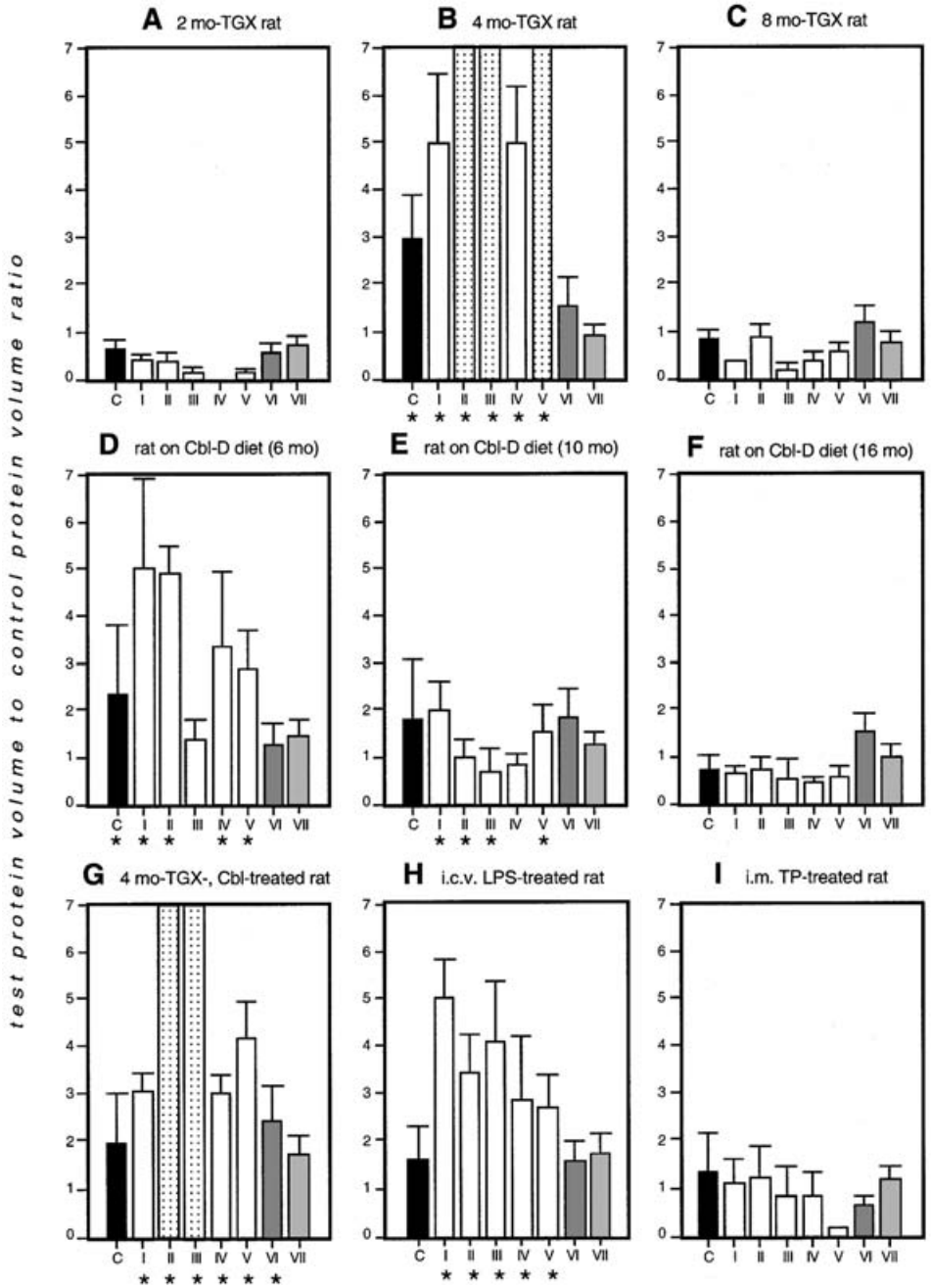

Figure 3 Quantitative data of CSF from rats in the various experimental groups

The data (means ± S.D.) are expressed as ratios between treated and controls CSF protein volumes. The sequence of the panels is as in Figure 2. In each panel, C (black) refers to total protein concentration; bars I–VII refer to protein groups defined in the first paragraph of the Results section and indicated in Figure 1(B). The bar for group VI (PGDS) is dark grey, for group VII (TTR) is light grey; the dotted black bars correspond to saturated spots. $^*P < 0.05$ versus corresponding control.

PGDS. Four months after TG (Figure 3B), total protein concentration is three times higher than in the controls. The albumin and transferrin spots are saturated, hence the increase of at least five times in the histogram is somewhat underestimated. The percentage increase is lower for TTR than for all of the other proteins. Of special relevance is the large increase in protein group IV; reference to Figure 2 shows that this is caused by a specific increase in the spots corresponding to α_1-antitrypsin, and even more to the *de novo* presence of thiostatin [α_1-MAP (α_1-major acute-phase protein)] [25] and Hp (haptoglobin) β chains (Figure 2B), both of which are serum proteins usually considered to be positive APPs [25]. The concentrations of both α_1-MAP and Hpβ were virtually zero in the control rat sera [25]. A sharp increase in the abundance of high-molecular-weight components (protein group I) was observed (Figure 3B), thus showing that even such a large protein as native Hp could diffuse freely from serum to CSF. The identification of these extra-CSF proteins (α_1-MAP and Hpβ) has been obtained by co-migration with a serum from rats with a

TP-induced inflammation (results not shown). Eight months after TG (Figure 3C), total protein concentration had nearly returned to control values. The Hpβ chains have disappeared from the CSF map, but α_1-MAP is still present (Figure 2C). This causes protein group IV to account for a larger percentage of total protein in these samples than in control CSF (Figure 3C); the same is true for PGDS, although its absolute concentration is only slightly higher than in the controls (Figure 3C).

Figures 2(D)–2(F) and 3(D)–3(F) show the time-course changes in the CSF pattern of the rats made Cbl-D by feeding a Cbl-D diet for different times. Total protein concentration was 2.3 times higher than in the controls after 6 months of feeding, 1.8 times higher after 10 months and back to near normal values after 16 months (Figures 3D–3F). After 6 months (Figure 3D) and, to a much lower extent, after 10 months (Figure 3E), the amount of high-molecular-mass proteins (group I) is higher than in the controls; no difference compared with the control was seen after 16 months (Figure 3F). The increase in PGDS protein was 1.3–1.8 times higher in the rats with diet-induced Cbl deficiency than in the control rats throughout the experimental time period (Figures 3D–3F); however, its relative abundance over total protein concentration sharply increased with time. There is no α_1-MAP in any of these samples, except a trace amount at the latest experimental time of Cbl-D diet feeding (Figure 2F).

Effects of post-TG Cbl replacement on CSF proteome

Post-TG Cbl-replacement treatment given during the first 2 months after TG (scheme A) did not significantly modify the CSF protein pattern (results not shown) compared with that of the Cbl-untreated 2-month-TGX rats (Figures 2A and 3A), which in turn was slightly different from the control (Figure 1A). Post-TG Cbl-replacement treatment during the third and fourth months after TG (scheme B; Figures 2G and 3G) drastically reduced the alterations seen in the CSF of the Cbl-untreated 4-month-TGX rats. Total protein concentration was reduced, but remained 2–3 times above that in the controls; the high-molecular-mass components (protein group I) and serum-derived proteins (protein group IV) were also drastically reduced. None of the APPs seen in the CSF of the Cbl-untreated 4-month-TGX rats was observed after Cbl-replacement treatment. The level of PGDS was only marginally reduced in the 4-month-TGX and Cbl-treated rats (Figures 2G and 3G).

The specificity of the CSF proteome changes in relation to Cbl deficiency

We verified the specific relationship between the CSF proteome alterations and Cbl deficiency by comparatively investigating the CSF proteome in rats in which acute phlogosis was induced by TP or LPS. In our previous study [17] we suspected that our TGX rats may develop an overgrowth of enteric flora probably leading to a phlogistic state, although chronic administration of antibiotics to TGX rats did not modify the severity of spongy vacuolation in the white matter of the spinal cord [17]. Furthermore, the presence of α_1-MAP and Hpβ in the CSF of the 4-month-TGX rats may have reflected an inflammatory condition occurring outside the CNS, because we never observed any histological or ultrastructural features of a phlogistic reaction in the CNS of our Cbl-D rats [16,27]. Figures 2(H) and 3(H) show the CSF pattern after i.c.v. microinjection of LPS. There was an increase in serum-derived high- and average-molecular-mass proteins (groups I and IV); α_1-MAP was detected but only at very low concentrations, together with some Hpβ. As expected, LPS treatment enhanced PGDS levels in the CSF proteome, since LPS is a well-known inducer of PGDS [28]. When an inflammatory reaction was induced by i.m. injection of TP, the main features of the CSF pattern involved a decrease in protein group V (albumin fragments) and an increase in PGDS, that very often became the major protein component. At 24 h after TP i.m. injection, no α_1-MAP was present in the CSF (results not shown), whereas, after an additional 24 h, it was clearly visible at relatively high concentrations (Figures 2I and 3I). After TP administration, there was no kallikrein-binding protein or α_2-HS (Heremans–Schmid)-glycoprotein.

DISCUSSION

Analysis of CSF abnormalities is still valuable and useful for understanding the pathogenesis of both CNS human and experimental diseases. Here we describe the first investigation into the regulation of the proteome pattern of rat CSF by Cbl.

Silver staining is highly sensitive when used to perform quantitative evaluations of biological fluids, but it suffers from a very narrow dynamic range that leads to easily saturated spots in the case of abundant sample components. This is a major drawback when the concentrations of major and minor proteins are very different. For instance, the complete lack of group IV proteins in the CSF of 2-month-TGX rats (Figures 2A and 3A) was probably due to their being a comparatively minor component in the control CSF; even a slight further reduction leads to their spots falling below the detection limit. In both Figures 3(B) and 3(G) several spots are saturated. As a result of these limitations, the recent upsurge in interest in proteomic investigations has led to a large number of reports dealing with the identification of individual proteins and the definition of reference maps (i.e. qualitative data), but much smaller amounts of quantitative data. A further specificity of biological fluid maps involves the extensive microheterogeneity of their glycoproteins [10,25]. For quantitative evaluation, all of the isoforms must be recognized and their contributions summed; this is sometimes a difficult task when different proteins migrate close to one another. Current investigations of biological fluids are very uncommon, except for our own work on rat sera and CSF [10,25]. In spite of the flaws discussed above and the limited number of our CSF samples, the quantitative data in the bar graphs of Figure 3 provide a graphic impression of the trends and make it possible to draw some important conclusions concerning the modulation of rat CSF proteome by Cbl.

A chronic lack of Cbl affects the pattern of rat CSF proteins in both a qualitative and quantitative way, as the abundance of common proteins is found to vary and/or proteins not present under reference conditions are detected in the CSF of Cbl-D animals. Furthermore, these changes are specifically linked to Cbl deficiency and vary in relation to its duration. However, the identification of a single Cbl-deficiency-linked marker (i.e. a DSP) in rat CSF is not straightforward.

We observed an increase in the total CSF protein concentration after prolonged Cbl deficiency, but the extent of this increase was higher in the TGX rats than in the rats fed a Cbl-D diet. The increase in total CSF protein concentration in Cbl-D rats is in keeping with the findings of Roos [29], who observed a significant increase in CSF proteins in most Cbl-D patients with impaired Cbl absorption after partial gastrectomy.

Taken together, our findings lead us to conclude that the overall changes in rat CSF proteome caused by prolonged Cbl deficiency do not seem to be causally connected with the occurrence of SCD-like lesions, but represent a specific epiphenomenon of vitamin deficiency, however induced. The changes in CSF proteome patterns of Cbl-D rats mainly affected the same group of CSF proteins at the experimental times at which the changes reached

their peak (i.e. 4 months after TG and after 6 months of a Cbl-D diet), and also when they seemed to be partially reduced in the long-term experiments of Cbl deficiency (i.e. 8 months after TG and after 16 months of a Cbl-D diet). In all of our Cbl-D rats the increase in total CSF protein concentrations temporally followed morphological CNS abnormalities and peaked 4 months after TG and after 6 months of a Cbl-D diet. At these experimental times, the spongy vacuolation and intramyelinic and interstitial oedema (i.e. the histological [16] and ultrastructural [27] hallmarks of CNS lesions of Cbl-D experimental neuropathy) are already present as they are induced after 2 months in TGX rats and 3 months in rats on a Cbl-D diet [16,27]. When considering the partial Cbl-unrelated reduction in CSF proteome abnormalities observed in the long-term experiments of Cbl deficiency (i.e. 8 months after TG and after 16 months of a Cbl-D diet), it should be remembered that a partial reduction in morphological CNS lesions has been observed in 4-month-TGX rats not receiving any Cbl-replacement therapy [17]. It therefore seems that this recovery of CSF proteome changes also temporally follows that of the morphological CNS lesions.

Among the CSF proteins, TTR and PGDS are of CNS origin [8]. TTR is most abundant in the choroid plexus but also present in the liver of mammals [30–32]. TTR concentrations varied slightly not only among the control samples but also across all test groups, except for the first tested experimental time (i.e. 6 months) for rats fed a Cbl-D diet. PGDS, also known as β-trace protein, is a lipocalin with enzymic activity that catalyses the conversion of prostaglandin H_2 to prostaglandin D_2 [33–36]. It is involved in a variety of CNS functions, such as sedation and sleep induction [37], and is likely to play important roles in maturation of the CNS and the maintenance of CNS physiology [35]. PGDS is selectively expressed in adult CNS on the rough-surfaced endoplasmatic reticulum and outer nuclear membrane of oligodendrocytes [34,38] and in leptomeninges [37,39]. Cultured astrocytes have been shown to release PGDS [40], and microglial cells synthesize larger amounts of prostanoids upon activation [28]. On the other hand, PGDS synthesis in neurons seems to be tiny [36]. In our TGX rats, we observed astroglial activation [27], but not microglial activation [41] or neuronal damage [16]; it is therefore tempting to speculate that the observed increase in PGDS in the CSF proteome of Cbl-D rats may be due to astrocytic activation. Both TTR and PGDS have been investigated as possible DSPs in human CSF for some human CNS disorders [35,42]. TTR has been proposed as a DSP for Alzheimer's disease [9]. Decreased PGDS levels have been associated with bacterial meningitis [43], and increased levels with subarachnoid haemorrhage [44]. In the CSF of rats made Cbl-D by either of the two experimental procedures we used, the absolute concentration of PGDS increased in connection with increased total protein concentration. On the other hand, a relative abundance of PGDS in CSF was observed after 16 months of feeding a Cbl-D diet, at which experimental time PGDS became one of the major protein components if not the most abundant CSF protein. Surprisingly, the increase in PGDS does not seem to be sensitive to Cbl-replacement treatment and is still present at the latest times of Cbl deficiency (i.e. 8 months after TG and 16 months on Cbl-D diet), when the other components of CSF proteome have returned to nearly normal levels. The fact that we found that TTR was hardly influenced by Cbl deficiency, and that PGDS was hardly affected by Cbl-replacement therapy, leads us to conclude that both CSF proteins can be considered to be relatively independent of Cbl status of the rat. For this reason, we considered these CSF proteins to be internal standards for our CSF samples, and the observed changes in CSF proteome patterns as real and not artifactual phenomena. This is further supported by the fact that Cbl-replacement treatment profoundly modifies the pattern of the CSF proteome both quantitatively and qualitatively, and only two spots were still saturated in the CSF proteome pattern of the 4-month-TGX and Cbl-treated rats (see Figure 3D).

Unlike what we have demonstrated for the regulation of EGF and TNF-α synthesis in rat CNS by Cbl [3,4], we certainly cannot say whether or not Cbl directly regulates the expression of some CSF proteins in CNS cells and elsewhere.

We thoroughly investigated the selectivity of the link between vitamin withdrawal and the overall changes observed in the CSF proteome of our TGX rats; we can say that they are specifically connected to a permanent Cbl-D status, since they are substantially corrected when the vitamin deficiency has been corrected. The CSF proteomes of TP- or LPS-treated rats differ from one another, and both differ from those observed after prolonged Cbl deficiency. Total CSF protein concentration increases in LPS-induced CNS inflammation, whereas it is normal in TP-induced inflammation outside CNS. At the present stage of our research, we do not have any explanation for the appearance of α_1-MAP and Hpβ in the CSF of Cbl-D rats at the late experimental times, because we did not observe any morphological signs of a phlogistic reaction in the CNS of Cbl-D rats [16,27] nor any lesions of the blood–brain barrier using NMR imaging (U. Guerrini, D. Veber and G. Scalabrino, unpublished work). The marginal changes in CSF TTR concentration caused by Cbl deficiency support the idea that chronic Cbl deficiency only slightly affects the function of choroid plexus epithelial cells, which are the most abundant in TTR mRNA and the cells forming the blood–CSF barrier [45]. However, it is worth noting that: (i) both α_1-MAP and Hpβ proteins disappeared from the maps after Cbl-replacement treatment, and (ii) the CSF levels of interleukin-6, a well-known APP-inducing cytokine [46,47], are selectively decreased in our Cbl-D rats [6].

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Received 6 January 2003/6 May 2003; accepted 28 May 2003
Published as BJ Immediate Publication 28 May 2003, DOI 10.1042/BJ20030059