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The compensatory 'rebound' of reactive astrogliosis: glial fibrillary acidic protein immunohistochemical analysis of reactive astrogliosis after a puncture wound to the brain of rats with portocaval anastomosis*

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Summary. This study was designed to compare the degree of reactive astrogliosis occurring around a puncture wound in the brain of normal rats and at different intervals after a similar puncture wound in rats with a portocaval anastomosis. The gliosis was evaluated by the number of astrocytes, the thickness of their processes and the intensity of the glial fibrillary acidic protein immunoreactivity. After the puncture wound in the brain of rats with a portocaval anastomosis, the gliosis varied at different intervals being: (1) decreased at 10 days, (2) markedly increased at 5 weeks and (3) significantly decreased at 8, 12, and 16 weeks. These findings suggest that 5 weeks after portocaval anastomosis, an active proliferation of the metabolically altered astrocytes occurs with heightened synthesis of glial fibrillary acidic protein in the period of adaptive compensation, the so-called compensatory 'rebound'. At 8 weeks or more after portocaval anastomosis, these altered astrocytes were considered to be in the phase of decompensation and incapable of maintaining the reactive response which occurred in normal rats. The compensatory rebound and decompensatory 'decline' illustrate the dynamic plasticity of the reactive astrogliosis.

Key words: Reactive astrogliosis – Portocaval anastomotic encephalopathy – Puncture wound – Compensatory and decompensatory phases of Alzheimer II gliosis

Reactive astrogliosis (RA) is the common response to injury of the central nervous system and a highlight of neuropathology and neurosurgery [8, 10]. Besides hypertrophy and hyperplasia of astrocytes, a characteristic feature is the increased synthesis of glial fibrillary acidic protein (GFAP) and a strongly positive immunohistochemical reaction [10].

It is well known that the pathological basis of hepatic encephalopathy, including Wilson's disease, is a generalized involvement of the astroglial defence in detoxifying the excess ammonia and related metabolites to safeguard the neurons. This defence coincides with the development of Alzheimer-type II (A-II) astrocytes which have large lobulated nuclei (15–20 μm) with prominent nucleoli. The failure or decompensation of these A-II astrocytes may be the pathogenetic mechanism of the functional disturbances which occur in the CNS in hepatic coma [1, 2, 18, 19, 20].

In the present study, we compared the glial response 4 days after a puncture wound to the brain of normal rats with the reactivity of the metabolically altered astrocytes after a similar wound in rats at various intervals after portocaval anastomosis (PCA). Significant differences were found in the reactivity of the altered astrocytes at the different intervals after PCA. Four days after the puncture wound in rats with a PCA of 10 days duration the RA was reduced, but markedly increased in rats with a PCA of 5 weeks duration while it was significantly reduced in rats with a PCA of 8, 12 and 16 weeks duration.

Materials and methods

Twenty-eight male Sprague-Dawley rats, weighing 250–430 g were divided into seven groups. Group I (N-N) was comprised of four normal rats without brain injury. Group II (N-P^4) of eight normal rats in which under ether anesthesia, a puncture wound was made in the right corpus striatum with a puncture needle 2.4 mm in external diameter by the method described by Cavanagh [4], and the reaction around it was examined 4 days later. In group III (PCA-10D-P^4), comprised of four rats, a PCA was made by the modified procedure of Lee and Fisher [14] and 10 days later a similar puncture wound was made in the brain and the reaction around it examined 4 days later. Groups IV, V, VI and VII

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(PCA-5W-P^4, PCA-8W-P^4, PCA-12W-P^4, PCA-16W-P^4, respectively) were each comprised of three rats in which a PCA made 5, 8, 12 and 16 weeks, respectively, before a puncture wound was made in the right corpus striatum with the reaction around being examined 4 days later.

Plasma ammonia was estimated by ion exchange, as described by Lu and Yu [15], once or several times in each animal.

The animals were killed 4 days after the puncture wound (P^4) had been made. They were perfused immediately via an aortic cannula with 10% formol-saline. The brains were bisected horizontally and sections of 5 μm were stained by hematoxylin and eosin, phosphotungstic acid hematoxylin (PTAH) and the peroxidase-antiperoxidase (PAP) technique for the GFAP immunohistochemical reaction as described by Ma et al. [16]. Rabbit anti-GFAP serum (Dako) in a dilution of 1:100, and sheep anti-rabbit IgG and rabbit PAP in dilutions of 1:200 were used. The control sera were diluted as follows: non-immune rabbit serum 1:100, and non-immune sheep serum 1:200.

Criteria for the RA and grading of astrocytes by GFAP immunohistochemical reaction

The following three criteria were used: (1) the number of proliferating astrocytes around the puncture wound; (2) the intensity of GFAP immunohistochemical reaction [3]: light yellow or yellow as grade 1 (normal astrocytes); light brown as grade 2 and dark brown as grade 3 (both of them represented reactive astrocytes); and (3) the width of the astrocyte processes at their conjunction with the perikaryon in GFAP-stained sections as measured by a micrometer (Shimadzu, Kyoto) at a magnification of 400X: <2.0 μm for grade 1 (normal astrocytes as in Fig. 3); 2.0–3.75 μm for grade 2 (reactive astrocytes as in Fig. 4); >3.75 μm for grade 3 (reactive astrocytes as in Fig. 5).

Enumeration of astrocytes and statistical evaluation

At a magnification of 400 X, in each section, the number and grading of GFAP-positive astrocytes around the wound in the corpus striatum were examined and recorded in each half of ten consecutive microscopic fields: each half field being 235 μm in radius and 86,747.43 μm^2 in area. The observations in group I (N-N) – normal controls – started from the center of the right corpus striatum perpendicularly to the lateral direction towards the cortex, and in groups II, III, IV,V,VI and VII (N-P^4, PCA-10D-P^4, PCA-5W-P^4, PCA-8W-P^4, PCA-12W-P^4, and PCA-16W-P^4) from the edge of the puncture hole in the corpus striatum laterally towards the cortex.

Statistical evaluation was performed using *t*-test, chi-square test, Dunnett's test, the rank-sum test of Krukal-Wallis and analysis of variance (ANOVA).

Results

Blood ammonia levels

In normal control rats the blood ammonia was 88.71 μg/100 ml. In rats with PCA, the blood ammonia was elevated 1 week after the PCA and remained high throughout the experimental period. The mean value was 264.0 μg/100 ml (Table 1).

Number of GFAP-positive astrocytes

The number of astrocytes in ten areas of 86,747.43 μm^2 was counted in every rat (Table 1). In non-punctured normal rats (N-N) the mean number was 186.75 (SE: 42.33). Four days after the brain puncture in normal rats (N-P^4), the mean number was 209.90 (SE: 25.43). In rats where PCA had been established 10 days, and 5, 8, 12, and 16 weeks previously, the mean numbers were 164.50 (SE: 14.67), 319.00 (SE: 21.38), 170.00 (SE: 50.74), 127.70 (SE: 28.15), and 154.30 (SE: 32.74), respectively. The mean numbers of astrocytes in the non-punctured normal (N-N) and punctured normal (N-P^4) rats was not statistically significant (Table 1[a]). In the five groups with PCA the mean numbers of astrocytes varied remarkably compared with the punctured normal rats (N-P^4), being reduced at 10 days (PCA-10D-P^4), markedly increase at

Table 1. An overall comparison of ammonia levels, mean numbers of astrocytes, and gradings of astrocytes by glial fibrillary acidic protein (GFAP) immunohistochemical reaction

Experimental groups					PCA – P^4				
			N-N (n = 4	N-P^4 (n = 8)	10D (n = 4)	5W (n = 3)	8W (n = 3)	12W (n = 3)	16W (n = 3)
Ammonia level (ug %)		Data mean	88.71	113.47	339.25	468.40	465.67	260.00	376.00
		SE	13.22	10.27	88.51	76.59	99.92	28.36	33.80
No. of astrocytes per 86,747.43 μm^2		$\overline{X}$i	186.75	209.90[a]	164.50	319.00[b]	170.00	127.70	154.30
		SE	42.33	25.43	14.67	21.38	50.74	28.15	32.74
Grading of astrocytes by GFAP immunohistochemical reaction[c] (% of total)	Grade 1	%	100.00	52.59	67.02	43.26	63.87	61.68	72.67
	Grade 2	%	0	35.14	26.60	32.60	24.61	25.46	21.12
	Grade 3	%	0	12.27	6.38	24.14	11.52	12.86	6.21
	Total no.		747	1679	658	957	512	381	483

[a] N-P^4 vs. N-N: t=0.50; P>0.05 (t-test)

[b] PCA-5W-P^4 vs. N-P^4: P<0.05 (Dunnett's test); result of ANOVA: F=3.73; P<0.05

[c] Result of rank-sum test and chi-square test: P<0.01. (The decrease in grade 1 & increase in grade 3 astrocytes were both significant in 5W vs. 10D, 8W, 12W & 16W, and most prominent in 5W vs N-P^4 & N-N.)

5 weeks (PCA-5W-P^4), and significantly reduced at 8, 12, and 16 weeks (PCA-8W-P^4, PCA-12W-P^4, and PCA-16W-P^4). A significant statistical difference was found by Dunnett's test ($P<0.05$) and ANOVA ($F = 3.73$; $P<0.05$) between groups I (N-N) and IV (PCA-5W-P^4) (Table 1^b), but a significant difference between other groups was not found.

Grading of astrocytes by GFAP immunohistochemical reaction

In group I (normal rats, N-N) the astrocytes showed grade 1 GFAP-positive staining while in group II (normal rats with a puncture wound, N-P^4) the astrocytes showed GFAP-positive staining from grades 1 to 3 (Table 1).

In all the experimental groups, a certain number of grade 1 astrocytes were undergoing transformation to astrocytes grades 2 and 3. Hence the percentages of grade 1 astrocytes were undergoing reduction as compared with the normal control group (N-N) (Table 1). This transformation represented an increase in the synthesis of GFAP (gliofibrillogenesis) and prominent hypertrophy of the astrocytes. Significant differences were found in the rats of groups II (N-P^4) to VII (PCA-16W-P^4) by the rank-sum test of Krukal-Wallis and chi-square test (Table 1^c). This reveals that grading of astrocytes by the GFAP immunohistochemical reaction is more meaningful than mere enumeration of the astrocytes in evaluating the degree of RA. The most striking effect was seen in group IV (PCA-5W-P^4) in which the percentage of grade 1 astrocytes fell to 43.26% while the percentage of grades 2 and 3 astrocytes increased from zero to 32.60% and from zero 24.14%, respectively, and particularly the latter (grade 3), being most remarkable as compared to other experimental groups (Table 1^c; Figs. 1, 3–6). This coincides with the finding of the significant difference in hyperplasia of astrocytes in groups II (N-P^4) and IV (PCA-5W-P^4) (Table 1^b).

Figure 1 illustrates the "compensatory 'rebound'" of RA in group IV (PCA-5W-P^4). However, in the 16 rats with PCA (PCA-P^4), the mean number of reactive astrocytes grade 2 in nine areas of 86,747.43 μm^2 at the edge of the puncture wound was less than the mean number of astrocytes in the same areas in 8 rats without PCA (group II, N-P^4). Hence, in Fig. 2, the curve of PCA-P^4 was lower than that of N-P^4.

Discussion

The intriguing phenomenon of compensatory 'rebound' of reactive gliosis has aroused our interest on several points. Firstly in our study of rats with a PCA and a puncture wound in the corpus striatum, we found that the Alzheimer-type II (A-II) astrocytes were completely GFAP negative but the Alzheimer-type I (A-I) astrocytes (giant multinucleated astrocytes with a large amount of eosinophilic cytoplasm) were strongly GFAP

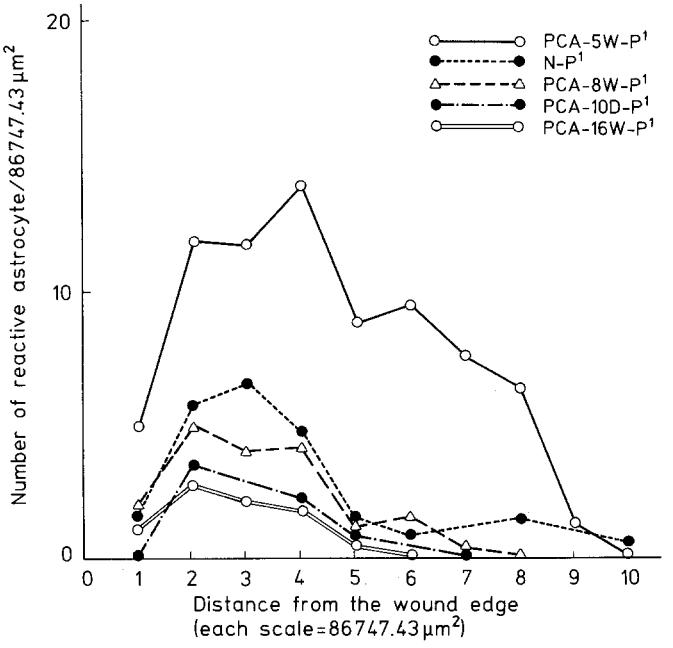

Fig. 1. Comparison of the mean numbers of grade 3 glial fibrillary acidic protein (GFAP) positive astrocytes of the 4 PCA-P^4 groups (PCA-10D-P^4, PCA-5W-P^4, PCA-8W-P^4, PCA-16W-P^4) with the control group (N-P4) in each scale lateral to the wound edge: markedly increased in PCA-5W-P^4, significantly reduced in PCA-10D-P^4, PCA-8W-P^4, and PCA-16W-P^4 as compared with N-P^4. Cytoplasmic staining: dark brown; width of process: >3.75 μm

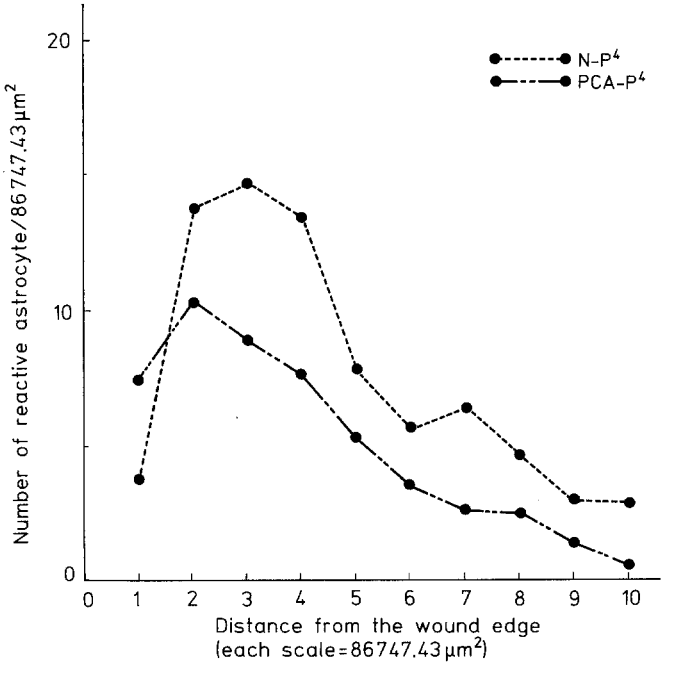

Fig. 2. Comparison of the mean numbers of grade 2 GFAP-positive astrocytes of the combined PCA-P^4 (the combination of five groups) with the control group (N-P^4) in each scale lateral to the wound edge: markedly decreased in PCA-P^4 as a whole, as compared with N-P^4. Cytoplasmic staining: light brown; width of process: 2.0–3.75 μm

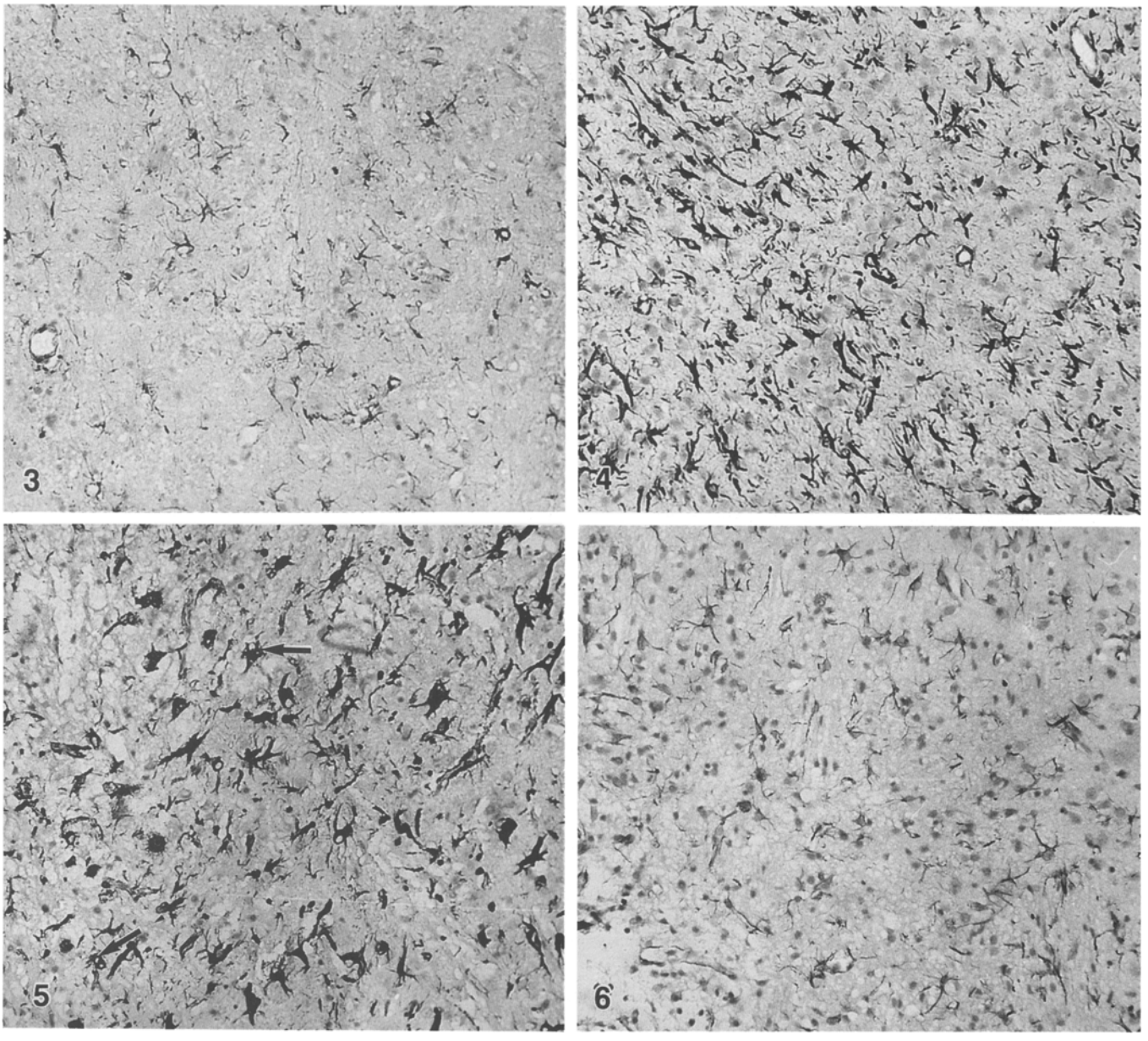

Fig. 3. Normal nonpunctured control (N-N). Right corpus striatum showing sparsely distributed grade 1 GFAP-positive astrocytes (light yellow or yellow in color with the width of cytoplasmic process less than 2.0 μm). GFAP-peroxidase-antiperoxidase (PAP) stain with hematoxylin, × 140

Fig. 4. Normal punctured group (N-P^4). About half of the proliferating reactive astrocytes (around the wound) are in grade 2 or 3 of GFAP immunohistochemical reaction with thicker processes. GFAP-PAP stain with hematoxylin, × 140

Fig. 5. PCA-5W-P^4 group. More than half of the proliferating reactive astrocytes (around the wound) are in grade 2 or 3 of GFAP immunohistochemical reaction, most prominent in grade 3 (dark brown in color with the width of cytoplasmic process greater than 3.75 μm), some of them are definitely Alzheimer-type I cells (*arrows*). GFAP-PAP stain with hematoxylin, × 140

Fig. 6. PCA-16W-P^4 group. These underdeveloped and faintly stained reactive astrocytes with thin and delicate processes (around the wound) are probably the characteristic of the failure of RA in an already decompensated or decompensating astroglial system. Similar picture was noted also in PGA-10D-P^4, PCA-8W-P^4, and PCA-12W-P^4. GFAP-PAP stain with hematoxylin, × 140

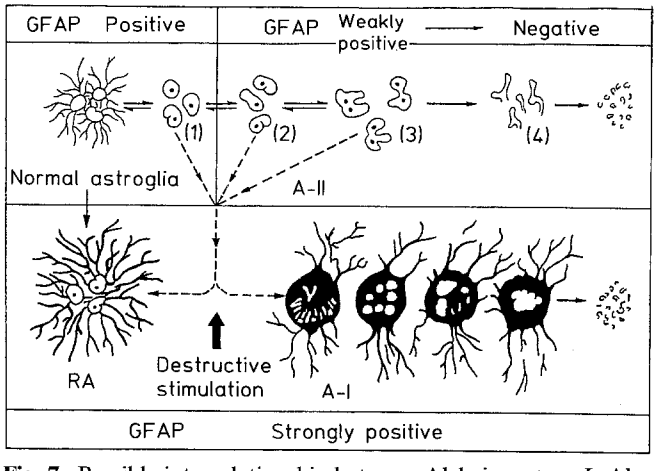

Fig. 7. Possible interrelationship between Alzheimer-type I, Alzheimer-type II astroglia and reactive astrogliosis and their expressions in GFAP immunoreactivity. (*1*) Intermediate between normal and Alzheimer-type II astroglia; (*2*), (*3*) well-developed nuclei of Alzheimer-type II cells; (*4*) shrunken nuclei of Alzheimer-type II cells. From Ma et al. [16] with revision

positive and had hypertrophic cytoplasmic processes [25]. Secondly, in our study of Wilson's disease [16], we saw that A-II astrocytes developed in two stages, compensatory and decompensatory. In the compensatory stage, the nuclei are large with prominent nucleoli while in the decompensatory stage, the nuclei are shrunken or ruptured. The development of strongly GFAP-positive A-I astrocytes was found to be inversely proportional to number of shrunken or decompensatory A-II cells and directly proportional to the capability of RA. When A-II astrocytes become decompensated their response to injury would be greatly diminished or lost and A-I astrocytes (a special variant of reactive astrocytes) would no longer appear [16]. If GFAP-negative A-II astrocytes were in their compensatory stage [Fig. 7 (1), (2), (3)], their ability to participate in reactive gliosis after injury would be intense with the appearance of numerous strongly GFAP-positive astrocytes including A-I astrocytes, which exhibit the so-called colchicine-like mitosis (Figs. 5, 7). Thirdly, Sobel and colleagues in one of their cases of hepatic encephalopathy in 1981 [22] had already noticed strongly GFAP-positive astrocytes within an old cortical infarct and numerous astrocytes surrounding the infarct were GFAP-negative A-II.

Other workers [1, 2, 5, 7, 18, 19, 21, 26] noted that in experimental hepatic encephalopathy there occurred a progressive and a regressive phase in the sequence of changes in the function, metabolism and morphology of A-II astrocytes. These phases correspond to the compensatory and decompensatory stages which we reported in Wilson's disease [16]. The present findings suggest that the reactive gliotic response to injury would be greatly heightened with increased synthesis of GFAP in the compensatory stage of A-II change seen in group IV (PCA-5W-P^4) [Figs. 1, 7 (1), (2), (3)]. Further, it would be much more active than the ordinary gliotic response to injury by normal astrocytes seen in group II (N-P^4) (Fig. 1). If the diseased glial cells are going to be or have already been in the decompensatory stage (groups V, VI and VII), the gliotic response would be significantly weakened (Figs. 1, 2, 4, 6 and Table 1). Prior to active 'rebound' there would be a "lag phase" as in group III (PCA-10D-P^4). This may reflect possibly, prematurity or incompetence of the astroglial compensatory process.

We consider that this active "rebound" phenomenon reflects the potential of the dynamic plasticity of reactive gliosis being manifested by an adaptive process which reaches its peak in the development of A-II astrocytes [5], and which is seen in the rats of group IV (PCA-5W-P^4), but not in the other groups.

Kitamura et al. [13] and Watanabe et al. [24] made in situ hybridization studies on the VIIth cranial nuclei of the rat damaged by transection of the facial nerve using GFAP cDNA probes derived from fetal calf cord GFAP mRNA. They found that astrocytes in the cranial nuclei of the normal facial nerve did not show GFAP immunoreactivity but that in damaged nuclei the astrocytes became GFAP positive. In the astrocyte cytoplasm, many silver grains occurred in the damaged nuclei but few in the undamaged facial nuclei. Their studies suggest that some chemical mediators liberated from the damaged axons or neurons may activate the GFAP gene of the surrounding astrocytes. The process of activation or induction of the GFAP gene in the compensated A-II astrocytes after injury may be more intense than in normal astrocytes following a similar injury. This will form the subject of a future investigation.

This new idea of the compensatory potential of RA may in turn add more weight in support of the possible interrelationship of the A-I and A-II astrocytes and the reactive gliosis as demonstrated in the study on Wilson's disease by Ma et al. [16] (Fig. 7), which has been controversial since its original description by von Hosslin and Alzheimer in 1912.

Neuropathologists have recognized new concepts of reactive gliosis in recent years, such as its multifunctional response, not just limited to healing, detoxification [1, 7, 19], phagocytosis [17] and ionic balance [19], but also encompassing immuno-protective and immuno-pathological mechanisms including the secretion of interleukins [6, 11, 12] and interferon [10, 23]. In addition, reactive astrocytes may be derived not only from normal, but also from abnormal astroglia (Fig. 7). Finally, the reserve potential of reactive gliosis is plastic and inducible [13, 24], e.g., diseased astrocytes prior to decompensation may exhibit a compensatory 'rebound' in gliosis following injury.

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