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IMMUNOMORPHOLOGY OF WILSONIAN AND HEPATIC GLIOPATHY *IN VITRO*

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Astrocytic abnormalities dominating the neuropathological picture of hepatic encephalopathy, underlie hypothesis of primary glial lesions as the background of the disease syndrome (Mossakowski 1966; Seitelberger 1970). Impairment of astrocytes in encephalopathies resulting from chronic and acute liver damage is similar, if not identical, to that observed in hepato-lenticular degeneration. In both pathological conditions it is characterized by concomitance of progressive and regressive astrocytic changes. The former, expressed as cellular proliferation and hypertrophy are considered to be the exponents of compensatory processes, while the latter, taking the form of non-specific degeneration, involving mostly cellular extensions, and leading to the appearance of cells deprived of processes are resultants of the direct action of the toxic factor or factors. Special types of glial abnormalities, considered as typical of hepato-cerebral pathology appear against the background of the above mentioned non-specific changes. These are Alzheimer cells, type I and II, and Opalski cells. Alzheimer cells, type II, known also under the names of naked nuclei or metabolic glia, are common for both Wilson's disease and hepatic encephalopathy, whereas those of type I are characteristic only of hepato-leniticular degeneration (Mossakowski 1978). Opalski cells, described originally as pathognomonic for Wilson's disease (Opalski 1930) appear in both processes, although they are less common in chronic hepatogenic encephalopathy and do not occur in the acute form (Mossakowski 1966; Mossakowski et al. 1974).

All the above mentioned pathological forms of astrocytes were obtained in tissue cultures, growing in nutrient media supplemented with either sera from patients with Wilson's disease and hepatic coma or exogenous compounds of copper and ammonia (Mossakowski et al. 1970). They were also found in cultures on medium with exogenous sodium

malonate, a chemical inhibitor of the succinic dehydrogenase system (Renkawek et al. 1973) and blood serum of rats, in which acute liver failure had been induced by thioacetamide intoxication (Kraśnicka et al. 1983).

Aiming at a further characteristics of astrocytic abnormalities in hepatogenic encephalopathy, a series of immunomorphological studies was undertaken. This seemed to be even more justified as Opalski cells are considered by some authors (Kryspin-Exner 1931; Greenfield 1963) as cells of mesodermal origin, contrary to the opinion of the author of their original description (Opalski 1930) and numerous further data concerning their morphology and metabolic properties (Mossakowski 1965). Evidence of specific glial markers could, therefore, settle the question of their histogenesis. Glial fibrillary acidic protein (GFAP), glutamine synthetase (GS) and glial antigen, described by Weinrauder and Lach (1975) were chosen as cellular markers.

MATERIAL AND METHODS

The studies were performed on organotypic cultures of newborn rat cerebella carried out either in Carrel flasks or Maximov's double coverslip chambers according to the method described by Kraśnicka and Mossakowski (1965). The nutrient medium, renewed twice weekly, contained 50% of normal human serum, 40% of buffered salt solution (Laboratory of Sera and Vaccines Production, Lublin, Poland) and 10% of chicken embryo extract. The medium was supplemented with glucose to the final concentration of 600 mg%, pH was adjusted to 7.0—7.3.

Selected cultures after 6—8, 10—12 and 14—15 days *in vitro* were divided into groups, which were exposed for 1—7 days to sera of untreated patients with Wilson's disease or the hypermmonemic form of hepatic coma and to exogenous copper acetate and ammonia chloride. The scheme of experiments, basically identical with that used in previous studies (Mossakowski et al. 1970) is presented in table 1.

The control cultures were grown for the whole time in routine nutrient media, without any of the above mentioned pathogenic factors. Cultures were controlled every day under a phase-contrast microscope and at chosen time intervals, depending on their general condition, were used for histological and immunomorphological studies.

For histological examination, cultures were fixed in Carnoy's solution and stained with toluidine blue.

Cultures for immunomorphological study were washed three times (15 min each time) in PBS (phosphate buffered physiological solution of sodium chloride), dried and then fixed in cold (— 20°C) acetone for 4 min. Fixed cultures were incubated in appropriate immune sera at room temperature for 30 min and washed in PBS (3 × 15 min). The second incubation was carried out in the $F(ab)_2$ -fragment of anti-rabbit

Table 1. Experimental scheme
Tabela 1. Schemat doświadczenia

Experimental group Grupa doświadczalna	Pathogenic factor Czynnik patogenny	Content in the nutrient medium Zawartość w środowisku odżywczym	Time of exposure Czas ekspozycji
I	serum from patients with hepatolenticular degeneration surowica chorych ze zwyrodnieniem wątrobowo-soczewkowym	50%	3–6 days dni
II	serum of patients with hepatic coma surowica chorych ze śpiączką wątrobową	50%	3–6 days dni
III	ammonia chloride chlorek amonu	100 mg%	1–3 days dni
IV	copper acetate octan miedzi	Cu^{++} 24.5 γ/ml	1–3 days dni

IgG, conjugated with fluorescein (Boehring A. G., FRG), diluted 1 : 20. Incubation lasted for 30 min and was carried out with sera from non-immunized rabbits (1 : 20) or PBS. The following anti-sera were used: 1) Anti-GFAP serum, obtained from Protein Laboratory, Copenhagen University (dr E. Bock). Dilution 1 in 100. 2) Anti-GS serum, obtained by subcutaneous immunization of albinotic rabbits with glutamine synthetase (Sigma) with complete Freund adjuvant and controlled in double immunodiffusion test (Weinrauder — in preparation). Dilution 1 in 20. 3) Anti-glial serum, obtained from a rabbit immunized by repeated intramuscular injections of rat brain extract emulgated with complete Freund adjuvant; to eliminate non-specific tissue antibodies, immune serum was additionally absorbed with serum and homogenates of rat kidney and liver. A single precipitation band was obtained in the immunodiffusion reaction with rat brain extract. Previous studies have documented a selective glial immunofluorescence reaction with this serum (Weinrauder, Kraśnicka 1980). Dilution 1 in 20.

RESULTS

The morphological picture of cultures in all experimental groups was identical with that described previously by Mossakowski et al. (1970). All cellular elements of the nerve tissue were present in the cultures, but pathological changes involved selectively astroglia. They were represented by the appearance of hypertrophied astrocytes, on the one hand,

and their degenerating forms, on the other. Hypertrophied astrocytes revealed a great variability of shape, ranging from large typical stellate cells through cells with abundant cytoplasm and scarce, short, layered extensions to bipolar spongioblastic forms. Fragmentation of cellular processes, involving both normal in size and hypertrophied astrocytes was the most common type of degenerative changes. Alongside with astrocytes with fragmented extensions and/or their reduced number, there appeared numerous cells lacking any processes, their irregular outlines only indicated the places of process origin. The process-deprived cells varied greatly in content of cytoplasm, which ranged from abundant to a very narrow, hardly visible rim surrounding enlarged nuclei. There were also cells represented only by large, pale, usually oval nuclei, with sharply outlined membranes. They resembled very much Alzheimer cells, type II. Against the background of the severely damaged glial population a very distinct feature were large, oval or round, usually mononuclear cells with abundant cytoplasm, lacking processes or showing their rudimentary forms. Their morphology corresponded to that of Opalski cells, typical of hepato-cerebral pathology *in vivo*. Careful examination of cultures made it possible to follow subsequent stages of transformation of some hypertrophied astrocytes to formed Opalski cells. Cellular forms, which could be equivalents of Alzheimer, type I cells were not observed in any of the experimental groups.

Despite the common pathomorphological pattern of all experimental groups, there existed essential differences between them as far as intensity of the pathological process is concerned. In cultures grown with exogenous copper and ammonia the pathological changes of astroglia were most intense; this was decisive for shortening of the exposure time to the action of damaging agents to 3 days. In these groups of cultures, especially in that exposed to copper action hypertrophied astrocytes were less numerous; astrocytes with severely damaged processes prevailed. Opalski cells and typical naked nuclei were not a common feature. On the contrary, these cellular types were very numerous in cultures grown with sera from patients with hepatic coma, so were hypertrophied astrocytes appearing alongside with their degenerating forms. The least intense astrocytic lesions were found in cultures with sera of patients with Wilson's disease. There were numerous hypertrophied astrocytes, Opalski cells and naked nuclei, less common were stellate cells showing features of non-specific degeneration.

The immunofluorescent reaction was similar in all experimental groups, revealing an evident relationship between its intensity and the degree of morphological abnormality. Therefore IF-intensity was generally weaker in cultures exposed to the action of copper and ammonia and higher in those grown with sera of patients with Wilson's disease

and hepatic coma. The time of exposure to the action of the damaging factors influenced the immunomorphology of the culture to the same extent as it did to their histology. Cultures exposed for a longer period of time were more severely damaged and revealed weaker immunofluorescence than those with a shorter exposure period. Less differentiated cultures were more sensitive than older ones showing features of more advanced differentiation. Essential differences depended also on the immune sera used. This inclines to present the immunomorphological observations separately for each antiserum.

Anti-GFAP serum

Hypertrophied astrocytes showed a strong immunofluorescent reaction of variable intensity (Figs 1—4). It was strongest in cells showing typical features of large fibrous astrocytes, contrasting with remarkably weaker fluorescence in cells, the morphology of which was indicative of a protoplasmic type of astroglia (Fig. 1). Bright immunofluorescence sharply outlined profiles of cellular processes. It was also more accentuated around cell nuclei (Fig. 2). Sometimes, mostly in hypertrophied astrocytes with abundant irregular cytoplasm and with short layered processes strong fluorescence was connected with bundles and net of fibrillary cytoplasmic structures (Figs 3, 4).

A great variability of intensity of the immunofluorescent reaction was typical of degenerating astrocytes. It ranged from very strong to hardly visible (Figs. 5—8). As a rule it was more intense in those cells which still preserved their, if even rudimentary, processes (Figs 5, 6), although, even in cells with a relatively high intensity of reaction, its connection with intracytoplasmic fibrillary structures was not a feature. In this group of cells a strong dependence of the reaction intensity on the type of damaging factor was very clearly demonstrated. It was highest in cultures with sera from patients with hepato-lenticular degeneration (Fig. 5) and hepatic coma (Fig. 6) and considerably lower in those with exogenous ammonia (Fig. 7) and copper (Fig. 8). The variability of the reaction intensity in different cells led to a mosaic pattern of cultures.

Cells resembling naked nuclei gave a very poor immunofluorescent reaction. Enlarged, oval negative nuclei were surrounded by slightly brighter rims of the residual cytoplasm (Fig. 9). Intense fluorescence around some of them was observed extremely seldom. Sporadically, large astrocytic nuclei were located on a background revealing fluorescent reaction of fibrillary type with ill-defined outlines of individual cells (Fig. 10).

A distinguishing bright reaction was noticed in the cytoplasm of typical Opalski cells (Figs 11, 12). It was either homogeneous or granular

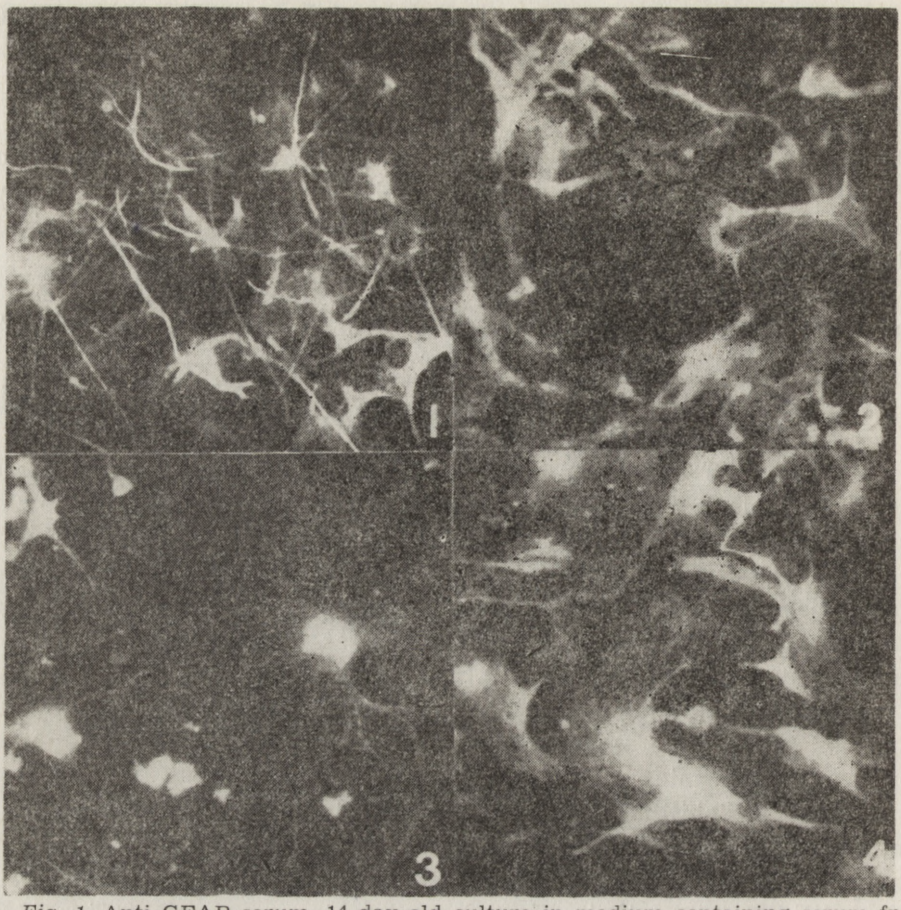

Fig. 1. Anti-GFAP serum, 14-day-old culture in medium containing serum from patient with Wilson's disease (3 days). Strong IF reaction in hypertrophied fibrous astrocytes and weaker in those with features of protoplasmic astroglia. × 250

Ryc. 1. Surowica anty-GFAP. Hodowla 14-dniowa, z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Widoczna żywa reakcja IF w przerośniętych astrocytach włóknistych i znacznie słabsza w komórkach o morfologicznych cechach astrogleju protoplazmatycznego. Pow. 250 ×

Fig. 2. Anti-GFAP serum, 10-day-old culture with medium containing serum from patient with Wilson's disease (3 days). Hypertrophied astrocytes with an intense IF reaction. Strong fluorescence around cell nuclei and in cellular processes. × 250

Ryc. 2. Surowica anty-GFAP, hodowla 10-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Silny odczyn IF w przerosłych astrocytach. Intensywne świecenie wokół jąder i w wypustkach komórkowych. Pow. 250 ×

Fig. 3. Anti-GFAP serum, 14-day-old culture with serum from patient with Wilson's disease (3 days). Strong IF reaction in hypertrophied astrocytes. Intense fluoresence of bundles and network of fibrils within short, distended cellular processes. × 250

Ryc. 3. Surowica anty-GFAP, hodowla 14-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Silny odczyn IF w przerośniętych astrocytach. Zwraca uwagę świecenie sieci i pasm włókienek w cytoplazmie nieprawidłowych, płatowatych wypustek. Pow. 250 ×

Fig. 4. Anti-GFAP serum, 12-day-old culture with exogenous copper salt (24 h). Hypertrophied astrocytes with strong IF reaction. Perikaryal cytoplasm with strongly fluorescent fibrillary network. × 300

Ryc. 4. Surowica anty-GFAP, hodowla 12-dniowa z dodatkiem egzogennej miedzi (24 godz.). Przerosłe, nieprawidłowe astrocyty z silnym odczynem IF. W cytoplazmie perykarialnej sieć włókienek z żywą fluorescencją. Pow. 300 ×

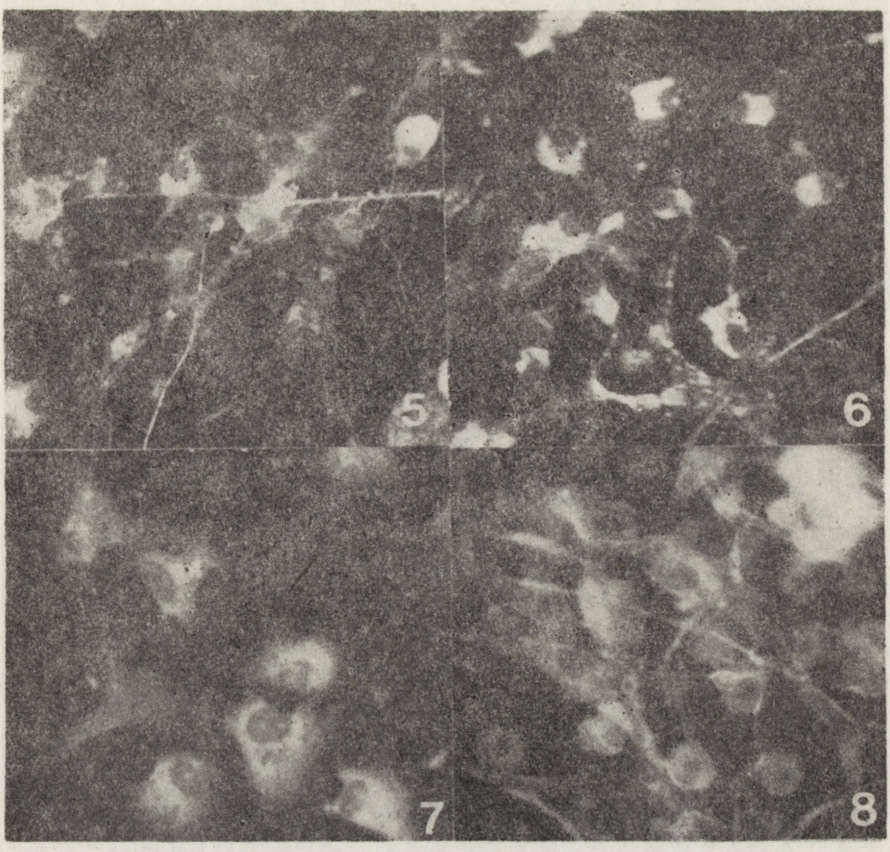

Fig. 5. Anti-GFAP serum, 14-day-old culture with serum from patient with Wilson's disease (3 days). Varying intensity of IF reaction in astrocytes with different degree of structural lesions. Bright fluorescence of preserved cellular processes. × 250

Ryc. 5. Surowica anty-GFAP, hodowla 14-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Zróżnicowana intensywność odczynu IF w astrocytach o różnym stopniu uszkodzenia. Żywa fluorescencja w większości zachowanych wypustek. Pow. 250 ×

Fig. 6. Anti-GFAP serum, 15-day-old culture with serum from patient with hepatic coma (6 days). Variability of IF reaction in degenerating astrocytes. Traces of fluorescence are seen in the remaining cellular processes. × 250

Ryc. 6. Surowica anty-GFAP, hodowla 15-dniowa z surowicą od chorego ze śpiączką wątrobową (6 dni). Zróżnicowany odczyn IF w wyrodniejących astrocytach. Ledwie zaznaczone zarysy nielicznych wypustek komórkowych. Pow. 250 ×

Fig. 7. Anti-GFAP serum, 10-day-old culture with exogenous ammonia (24 h). Weak IF reaction in hypertrophied astrocytes, most of which are lacking processes. × 380

Ryc. 7. Surowica anty-GFAP, hodowla 10-dniowa z egzogennym amoniakiem (24 godz.). Słaby odczyn IF w przerośniętych, głównie bezwypustkowych astrocytach. Pow. 380 ×

Fig. 8. Anti-GFAP serum, 6-day-old culture with exogenous copper (48 h). Most astrocytes, lacking cellular processes exhibit weak IF reaction. Stronger fluorescence is seen in some of the remaining processes. × 380

Ryc. 8. Surowica anty-GFAP, hodowla 6-dniowa z egzogenną miedzią (14 godz.). Większość astrocytów pozbawionych wypustek wykazuje słaby odczyn IF. Żywsza fluorescencja w nielicznych zachowanych wypustkach. Pow. 380 ×

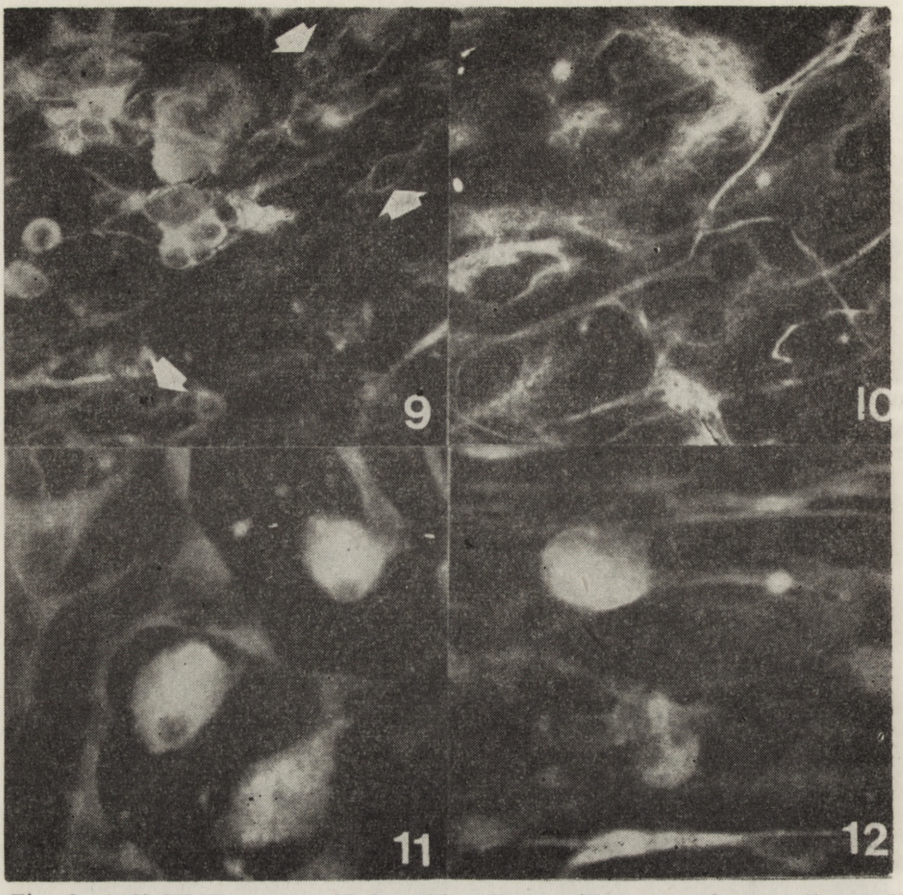

Fig. 9. Anti-GFAP serum, 15-day-old culture with serum from patient with hepatic coma (6 days). Most of the cells represented by enlarged, round or oval naked nuclei surrounded by a narrow rim of weak fluorescence (arrows). Some cells of the same type with a wider cytoplasmic ring with stronger IF reaction. × 250

Ryc. 9. Surowica anty-GFAP, hodowla 15-dniowa z surowicą od chorego ze śpiączką wątrobową (6 dni). Większość komórek reprezentują okrągłe lub owalne nagie jądra otoczone wąskim rąbkiem bardzo słabej fluorescencji (strzałki). Nieliczne — otoczone są obfitą cytoplazmą z żywym odczynem IF. Pow. 250 ×

Fig. 10. Anti-GFAP serum, 15-day-old culture with serum from patient with hepatic coma (6 days). Enlarged astrocytic nuclei against weakly fluorescent background with hardly visible cellular boundaries. Somewhat stronger IF reaction in the remaining cellular processes. × 400

Ryc. 10. Surowica anty-GFAP, hodowla 15-dniowa z surowicą chorego ze śpiączką wątrobową (6 dni). Powiększone jądra astrocytów na słabo świecącym tle ze słabo widocznymi błonami poszczególnych komórek. Nieco silniejszy odczyn IF w nielicznych zachowanych wypustkach. Pow. 400 ×

Fig. 11. Anti-GFAP serum, 15-day-old culture with serum from patient with hepatic coma (6 days). Typical Opalski cells with strong homogeneous IF reaction against background of degenerating astrocytes with weak fluorescence. × 600

Ryc. 11. Surowica anty-GFAP, 15-dniowa hodowla z surowicą od chorego ze śpiączką wątrobową (6 dni). Typowe komórki Opalskiego z silnym, homogennym odczynem IF, na tle zwyrodniałych astrocytów ze słabą fluorescencją. Pow. 600 ×

Fig. 12. Anti-GFAP serum, 14-day-old culture with exogenous ammonia (3 days). Opalski cell with strong cytoplasmic fluorescence against background of weak reaction in damaged glial cells. In the lower part spongioblastic-like cell with strong fluorescence. × 600

Ryc. 12. Surowica anty-GFAP, 14-dniowa hodowla z egzogennym amoniakiem (3 dni). Komórka Opalskiego z żywym odczynem IF w cytoplazmie, na tle słabej fluorescencji pozostałych komórek glejowych. W dolnej części ryciny spongioblastyczna komórka z żywym odczynem. Pow. 600 ×

in nature. No fluorescence connected with fibrillary cytoplasmic structures was seen. The same nature and intensity of the reaction was found in those cells which seemed to represent transitions between hypertrophied astrocytes and typical Opalski cells (Fig. 13).

Anti-GS serum

As in the previous group, the positive immunofluorescent reaction was confined to the astroglial population. Comparing with reaction with anti-GFAP serum, it was different in intracytoplasmic distribution and nature. In no case, even in most hypertrophied astrocytes, was the reaction connected with cytoplasmic fibrillary structures. It was always homogeneous and evenly distributed in the perikaryal cytoplasm, cellular processes and perivascular end feet. The highly positive reaction of hypertrophied astrocytes contrasted with the negative vascular walls (Fig. 14). All cellular equivalents of hepatogenic gliopathy gave positive immunofluorescent reactions. The variability of reaction intensity was lesser than in the previous group. It was the same in large hypertrophied cells (Fig. 15) and in degenerating astrocytes with a varying degree of cellular damage (Fig. 17). A reaction of specially high intensity distinguished Opalski cells from the background of the remaining astrocytic population (Figs 15, 16). So it did in intermediate cells between hypertrophied astrocytes and typical Opalski cells (Fig. 17). The only weak reaction concerned naked nuclei.

Anti-glial serum

The immunofluorescent reaction with anti-glial serum was in general strongest, as compared with other ones. The distinguishing feature was also its appearance in both astrocytic and oligodendroglial populations. Contrary to the control cultures not-subjected to any of the used pathogenic factors, the reaction intensity with antiglial serum was stronger in hypertrophied and degenerating astrocytes (Fig. 18) than in oligodendrocytes (Fig. 19). The outlines of cellular processes were less visible than in cultures with other antisera. The variability of the reaction in different cell types was also less noticeable. Opalski cells showed a high intensity of immunofluorescent reaction in both perikarya and rudimentary processes (Fig. 20). The same was true for intermediate cells. On the contrary, the reaction around the naked nuclei was very weak (Fig. 21).

Control reactions

Control reactions performed either with sera of non-immunized rabbits or with PBS were negative (Fig. 22).

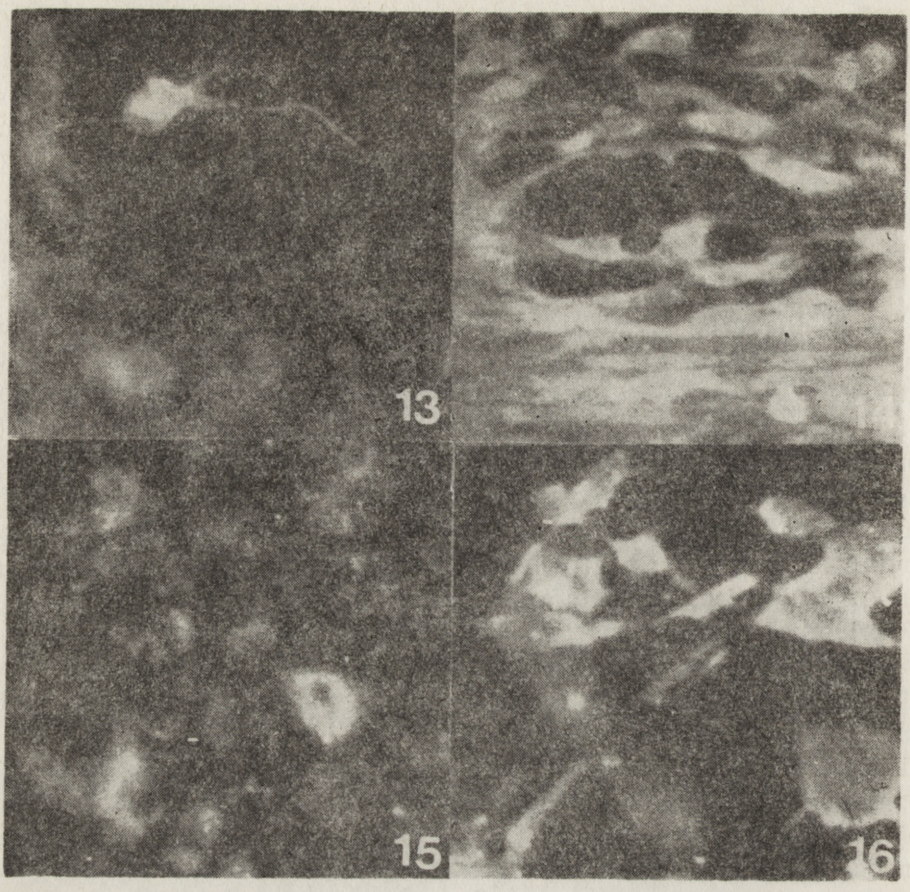

Fig. 13. Anti-GFAP serum, 12-day-old culture with exogenous copper (24 h). Intermediate form between hypertrophied astrocyte and typical Opalski cell with strong IF reaction in perikaryon and residual processes. In the lower portion naked nuclei surrounded by a narrow rim of weak fluorescence and brightly shining Opalski cell. × 600

Ryc. 13. Surowica anty-GFAP, hodowla 12-dniowa z egzogenną miedzią (24 godz.). Postać przejściowa między przerosłym astrocytem a uformowaną komórką Opalskiego z żywym odczynem w cytoplazmie perykarialnej i nielicznych wypustkach. W dolnej części ryciny nagie jądra z wąską obwódką słabej fluorescencji oraz komórka Opalskiego z silnym świeceniem cytoplazmy. Pow. 600 ×

Fig. 14. Anti-GS serum, 15-day-old culture with serum from patient with Wilson's disease (3 days). Strong IF reaction in hypertrophied astrocytes, some of which are lacking cellular processes, contrasting with weak fluorescence of the capillary wall. × 300

Ryc. 14. Surowica anty-GS, hodowla 15-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Żywy odczyn IF w przerośniętych astrocytach, z których część pozbawiona jest wypustek, kontrastujący z negatywnym odczynem ściany naczyniowej. Pow. 300 ×

Fig. 15. Anti-GS serum, 6-day-old culture with exogenous copper (3 days). Against a dense background of astrocytes mostly deprived of processes with high IF reaction, strong cytoplasmic fluorescence of three Opalski cells is discernible. × 400

Ryc. 15. Surowica anty-GS, hodowla 6-dniowa z egzogenną miedzią (3 dni). Na zbitym tle astrocytów pozbawionych w większości wypustek, wykazujących silny odczyn IF, wyróżnia się intensywniejsza fluorescencja trzech komórek Opalskiego. Pow. 400 ×

Fig. 16. Anti-GS serum, 14-day-old culture with serum from patient with Wilson's disease (3 days). Loosely lying and grouped astrocytes with residual pro-

DISCUSSION

The presented results indicate, that the pathological forms of astrocytes obtained in organotypic cerebellar culture preserve their fundamental immunomorphological properties, typical of astroglia in both *in vivo* and *in vitro* conditions. The applied antisera permitted to disclose the presence of: 1) glial fibrillary acidic protein (GFAP), considered as an immunological marker of astrocytes, mostly fibrillary ones but to a lesser degree of protoplasmic ones (Eng et al. 1971; Bignami et al. 1972; Antanitus et al. 1974; Bock et al. 1975, 1977; Ludwin et al. 1976). 2) glutamine synthetase, typical astrocytic enzyme (Martinez-Hernandez et al. 1977; Riepe, Norenberg 1977; Norenberg; Martinez-Hernandez 1979) and 3) a chemically non-identified specific neuroglial antigen, common for both oligodendrocytes and astrocytes (Weinrauder, Lach 1975, 1977; Weinrauder, Kraśnicka 1980). They were present in all types of astrocytes appearing in the experimental cultures, both in those revèaling features of progressive and regressive changes as well as in Opalski cells and naked nuclei. Particular types of cells revealed essential differences in the immunohistochemical reaction, implicating a variable content of antigens. These differences expressed as an increase or reduction of the reaction intensity, seemed to be dependent on the biological state of cells, related with either their activation or damage of varying degree. The most intensive immunofluorescent reaction was observed in hypertrophied astrocytes and in Opalski cells, it was less pronounced, although greatly variable, in degenerating astrocytes with fragmented processes and in the cells deprived of processes, and the weakest in naked nuclei. Scarcity of immunofluorescent reactions in cells resembling Alzheimer cells, type II seems understandable in the light of electron microscope observations on their ultrastructure (Norenberg et al. 1974; Norenberg, Lapham 1974; Ostenda et al. 1976; Norenberg 1977) indicating almost total disintegration of their cytoplasmic structures.

However, evaluation of differences in the intensity of the immunofluorescence reaction, especially in tissue culture conditions, requires great circumspection in view of its tremendous variability and heterogenicity in *in situ* conditions, in the case of all antisera applied (Bignami, Dahl 1974; Weinrauder, Lach 1975; Norenberg, Martinez-Hernandez 1979). On the other hand, it is well known that astrocytes *in vitro*, highly variable in their morphology, differ considerably in their structural and me-

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cesses showing strong IF reaction. Very intense homogeneous fluorescence in the cytoplasm of Opalski cell (right lower corner). × 400

Ryc. 16. Surowica anty-GS, hodowla 14-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Luźno leżące i zgrupowane astrocyty pozbawione wypustek wykazują żywy odczyn IF. Znacznie intensywniejsza homogenna fluorescencja w cytoplazmie komórki Opalskiego (prawy dolny róg). Pow. 400 ×

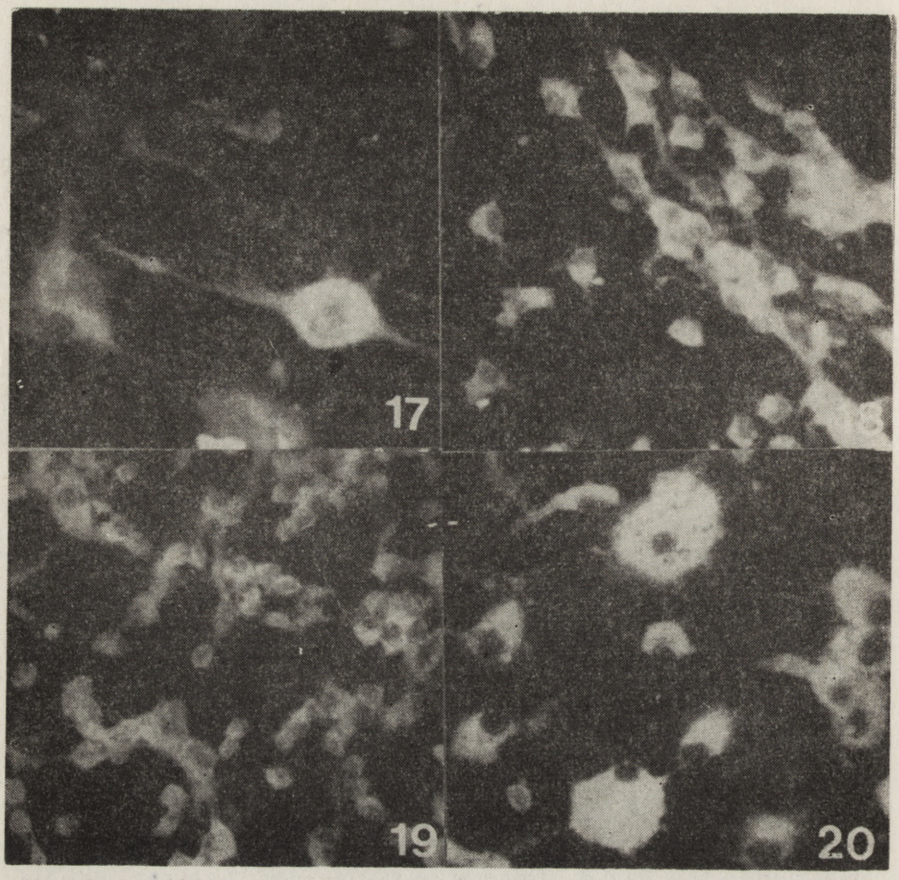

Fig. 17. Anti-GS serum, 6-day-old culture with exogenous copper (48 h). Intermediate forms between hypertrophied astrocytes and formed Opalski cells with strong IF reaction both in perikaryal cytoplasm and in residual processes. × 600

Ryc. 17. Surowica anty-GS, hodowla 6-dniowa z egzogenną miedzią (48 godz.). Przejściowe formy komórkowe między hipertroficznymi astrocytami i uformowanymi komórkami Opalskiego z żywym homogennym odczynem IF w cytoplazmie okołojądrowej i w wypustkach. Pow. 600 ×

Fig. 18. Anti-glial serum, 8-day-old culture with serum from patient with Wilson's disease (6 days). Strong fluorescent reaction in most astrocytes, great part of which shows fragmented processes. × 250

Ryc. 18. Surowica antyglejowa, hodowla 8-dniowa z surowicą chorego ze zwyrodnieniem wątrobowo-soczewkowym (6 dni). Silny odczyn fluorescencyjny w astrocytach, z których znaczna część wykazuje fragmentację wypustek. Pow. 250 ×

Fig. 19. Anti-glial serum, 15-day-old culture with serum from patient with Wilson's disease (3 days). Intense IF reaction in unchanged oligodendroglial population (compare with Fig. 18). × 250

Ryc. 19. Surowica antyglejowa, hodowla 15-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Żywa reakcja IF w populacji niezmienionego gleju skąpowypustkowego (porównaj z ryc. 18). Pow. 250 ×

Fig. 20. Anti-glial serum, 14-day-old culture with serum from patient with hepatic coma (3 days). Intense immunofluorescent reaction in the cytoplasm and processes of Opalski cells (upper one with vacuolar degeneration) and in mostly processless astrocytes. × 380

Ryc. 20. Surowica antyglejowa, hodowla 14-dniowa z surowicą od chorego ze śpiączką wątrobową (3 dni). Żywa reakcja IF w cytoplazmie i resztkowych wypustkach komórek Opalskiego (górna ze zwyrodnieniem wodniczkowym) oraz w bezwypustkowych astrocytach. Pow. 380 ×

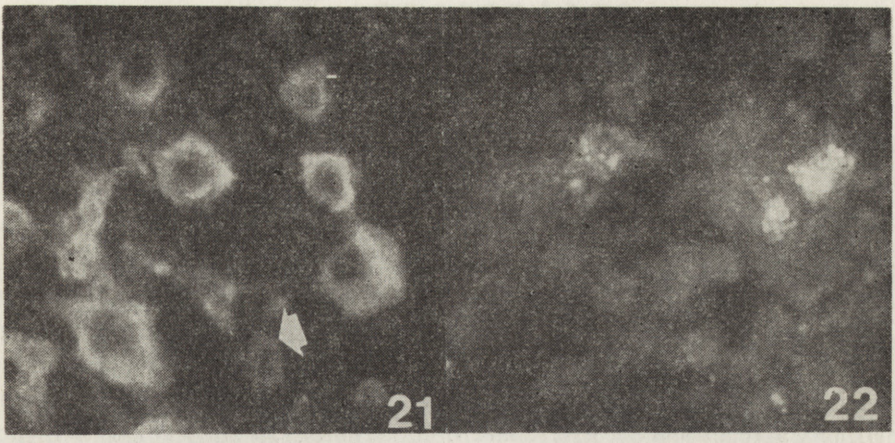

Fig. 21. Anti-glial serum, 12-day-old culture with exogenous copper (24 h). Moderate IF reaction in processless astrocytes and in forming Opalski cell, very weak in the cytoplasmic rim surrounding naked nucleus (arrow). × 400

Ryc. 21. Surowica antyglejowa, hodowla 12-dniowa z egzogenną miedzią (24 godz.). Umiarkowany odczyn IF w astrocytach pozbawionych wypustek i formującej się komórce Opalskiego i bardzo słaby w rąbku cytoplazmatycznym wokół nagiego jądra (strzałka). Pow. 400 ×

Fig. 22. Control reaction with PBS, 15-day-old culture with serum from patient with Wilson's disease (3 days). IF reaction negative. Unspecific granular fluorescence in the cytoplasm of some degenerating astrocytes due to lipid accumulation. × 400

Ryc. 22. Odczyn kontrolny, z użyciem PBS, hodowla 15-dniowa z surowicą od chorego ze zwyrodnieniem wątrobowo-soczewkowym (3 dni). Brak reakcji immuno fluorescencyjnej. Ziarniste świecenie w cytoplazmie pojedynczych komórek ma charakter nieswoisty. Pow. 400 ×

tabolic properties, as shown in histochemical studies, from their counterparts *in situ* (Kraśnicka, Mossakowski 1965, Mossakowski et al. 1966; Kraśnicka, Borowicz 1971). Cultured astrocytes resemble more reactive astroglia than the resting forms *in situ*. Bock et al. (1975, 1980) documented that the GFAP content in astrocytes in organotypic culture is significantly higher than in normal brain astrocytes. The maturation stage of astrocytes has also to be taken into consideration. In our studies newborn rat cerebellar cultures were used. Rat cerebellum at this stage contains a little differentiated glial population, which differentiates and maturates under *in vitro* conditions (Renkawek 1972). This may in an obvious way influence the content of different chemical components of the cell and, therefore, change its immunomorphological picture. Bignami and Dahl (1973) showed that the appearance of GFAP in the ontogeny of astrocytes is connected with an outburst of gliofilaments proliferation in their cytoplasm.

The greatest differences in the intensity of the immunofluorescence reaction were observd in the case of anti-GFAP sera. However, its strong dependence on the degree of structural damage to the cellular population is here a very striking feature. It found also expression in the dependence on the pathogenic factors used. As a rule, the immunochemical

reaction is stronger in cultures grown with sera from patients with Wilson's disease and hepatic coma, in which structural impairment of astrocytes was less advanced, with prevailing features of astrocytic hypertrophy and formation of Alzheimer, type II and Opalski cells. A markedly lower intensity of IF was observed in cultures with exogenous copper and ammonia. These were dominated by non-specific astrocytic degeneration, with much less expressed cellular hypertrophy and appearance of Opalski cells and naked nuclei.

A typical picture of the immunohistochemical reaction with GFAP is characterized by bright fluorescence of fibrillary cytoplasmic structures and astrocytic processes (Bignami et al. 1972). In our experimental material such a type of reaction was only observed in either normal fibrillary astrocytes or in hypertrophied cells. In most of the injured cells, in naked nuclei and in Opalski cells the reaction was homogeneous or granular. This may implicate either the predominance of protoplasmic astrocytes or a possible change of the physico-chemical nature of the antigen substance under the influence of the applied pathogenic factor. Cerebellar culture *in vitro* contains mixed population of fibrillary and protoplasmic astrocytes (Kraśnicka, Borowicz 1971). This would favour the possibility of different cellular reactions. However, the question may arise as to what factors are responsible for the mostly progressive response of fibrillary astrocytes and the predominantly degenerative one of protoplasmic astroglia. The observations of Norenberg et al. (1972), indicating different reactions of astrocytes in various grey matter structures and less involvement of the white matter astroglia in experimental hepatic encephalopathy, may serve as a background for such an assumption. So is the homogeneous reaction in Opalski cells. Opalski (1930) thought that they represent an abnormality of protoplasmic astrocytes. Although this opinion did not find confirmation in further studies (Mossakowski 1966) nevertheless electron microscope observations concerning their structure revealed a striking paucity of gliofilaments in their cytoplasm (Mossakowski et al. 1971). On the other hand, it has to be kept in mind that the nature of the immunohistochemical reaction can be dependent on numerous additional factors such as intensity of fluorescence, blurring its connection with the cytoplasmic fibrillary structures (see processes of fibrous astrocytes), intracellular arrangement and distribution of gliofilaments, their density, degree of cell maturity and differentiation, and probably many others.

The strong immunohistochemical reaction with anti-GS serum indicates a high content of enzyme both in hypertrophied and degenerating astrocytes as well as in Opalski cells. Its reduced content in Alzheimer cells type II can be explained similarly as in the case of GFAP by severe destruction of their cytoplasmic structures. Glutamine synthetase known as a typical astrocytic enzyme (Norenberg 1979; Norenberg, Martinez-Hernandez 1979) seems to be a fundamental factor in ammonia

detoxication in the central nervous system. Although significant increase of glutamine synthetase is not a common feature in various types of hepatogenic encephalopathy (Hilgier 1983), its slightly enhanced content was found in immunohistochemical studies in a limited group of astrocytes (Norenberg 1981). Ammonia is considered to be one of the most probable candidates as the main pathogenetic factor in hepatogenic encephalopathy, the pathological picture of which is dominated by astroglial pathology (Hinfelt 1975; Mossakowski 1978, Norenberg 1981). High glutamine synthetase content in all abnormal astrocytes found in our present studies indicates that they can take part in ammonia detoxication process. This concerns even so severely changed cells as the naked nuclei and Opalski cells.

The high content of the specific neuroglial antigen, common for both oligodendrocytes and astrocytes indicates also the preservation of biological properties in damaged and changed astrocytes. The immunohistochemical reaction, stronger in astrocytes than in oligodendroglia, in contrast to cerebellar cultures grown in standard conditions (Weinrauder, Kraśnicka 1980) may suggest even an increased intracellular production of antigen under pathological conditions.

Our studies make possible the characterization of the histogenesis of Opalski cells. As mentioned above, Opalski (1930) considered them as a specific abnormality of protoplasmic astrocytes. Their astrocytic origin, although not necessarily from protoplasmic astroglia, is also supported by their impregnation abilities and histochemical properties (Mossakowski 1965). However, Kryspin-Exner (1931) believed them to be of microglial origin and Greenfield (1963) considered them as a special form of phagocytes. For Victor et al. (1965) they are degenerating nerve cells. We strongly believe that the content of typical astroglial antigens in their cytoplasm, settles the question of their astrocytic origin.

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IMMUNOMORFOLOGIA GLIOPATII WĄTROBOWEJ *IN VITRO*

Streszczenie

Przeprowadzono badania immunomorfologiczne z użyciem surowic odpornościowych przeciwko GFAP, syntetazie glutaminy (SG) i swoistemu antygenowi glejowemu wspólnemu dla astrocytów i oligodendrocytów, na hodowlach organotypowych móżdżku noworodków szczurzych, w których doświadczalnie wywoływano zmiany komórkowe typowe dla encefalopatii wątrobowej i choroby Wilsona.

Hodowle prowadzono z surowicami od chorych, ze zwyrodnieniem wątrobowo-soczewkowym lub śpiączką wątrobową oraz z dodatkiem egzogennego octanu miedzi, lub chlorku amonu.

Odczyny immunohistochemiczne wykazały obecność GFAP, SG i swoistego antygenu glejowego we wszystkich typach nieprawidłowych astrocytów stanowiących wykładniki gliopatii wątrobowej. Intensywność odczynu immunofluorescencyjnego wykazywała zależność od stopnia strukturalnego uszkodzenia komórek. Była ona najwyższa w przerosłych astrocytach i w komórkach Opalskiego, niższa w wyrodniejących komórkach gwiaździstych, a najniższa w komórkach Alzheimera, typu II. Uzyskane wyniki wskazują, że nawet najbardziej zmienione lub uszkodzone komórki glejowe zachowywały typowe właściwości astrocytów. Wydaje się to szczególnie ważne w przypadku syntetazy glutaminy, gdyż jej obecność w uszkodzonych komórkach glejowych wskazuje na możliwość ich udziału w procesie detoksykacji amoniaku, stanowiącego jak się wydaje podstawowe ogniwo w patogenezie encefalopatii wątrobowej. Obecność swoistych antygenowych znaczników astroglejowych w komórkach Opalskiego może przesądzać o ich astrocytarnym pochodzeniu.

ИММУНОМОРФОЛОГИЯ ПЕЧЕНОЧНОЙ ГЛИОПАТИИ

Резюме

Проведено иммуноморфологические исследования с применением иммунных сывороток против кислого глиозного фибриллярного белка (GFAP), синтетазы глутамина (SG) и специфического глиального антигена общего для астроцитов и олигодендроцитов в органотипичных культурах мозжечка крысиных новорожденных, в которых экспериментально вызывано клеточные изменения типичные для печеночной энцефалопатии и болезни Вильсона.

Культуры были ведены с сыворотками от больных с гепато-лентикулярной дегенерацией или с печеночной комой и с добавкой экзогенного ацетата меди или хлористого аммония.

Иммуногистохимические реакции обнаружили наличие GFAP, SG и специфического глиального антигена во всех типах ненормальных астроцитов составляющих показатели печеночной глиопатии. Интенсивность иммунофлуоресцентной реакции показывала зависимость от степени повреждения структуральных клеток. Она была самой высокой в гипертрофических астроцитах и в клетках Опальского, низшей в вырождающихся астроцитах, а самой низкой в клетках Альцгеймера II типа. Полученные результаты указывают на то, что даже наиболее измененные или поврежденные глиальные клетки сохраняли типичные свойства астроцитов. Это кажется особенно важным в случае синтетазы глутамина, потому что его наличие в поврежденных глиальных клетках указывает на возможность их участия в процессе детоксикации аммиака, составляющей, как кажется, основное звено в патогенезе печеночной энцефалопатии. Наличие специфических антигенных астроглиальных показателей в клетках Опальского может предопределять их астроцитарное происхождение

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