PL ISSN 0028-3894

ASSOCIATION OF POLISH
NEUROPATHOLOGISTS
and
MEDICAL RESEARCH CENTRE,
POLISH ACADEMY OF SCIENCES

NEUROPATOLOGIA POLSKA

VOLUME 30 1992 NUMBER 1

WROCŁAW · WARSZAWA · KRAKÓW
ZAKŁAD NARODOWY IM. OSSOLIŃSKICH
WYDAWNICTWO POLSKIEJ AKADEMII NAUK

NEUROPATOLOGIA POLSKA

QUARTERLY

VOLUME 30 1992 NUMBER 1

EDITORIAL OFFICE

Medical Research Centre
ul. Dworkowa 3, 00-784 Warszawa, Poland, Phone: 49-54-10

The typescript of the present issue was delivered to the publisher 24.02.1992

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

RAFAŁ KOZIELSKI, PIOTR B. KOZŁOWSKI, HENRY M. WIŚNIEWSKI

MICROGLIAL CELL MARKERS – A REVIEW*

New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, USA

Key words: *microglia, histology, histochemistry, immunohistochemistry, humans, animals.*

The report published by Robertson (1900) directed attention to the previously unknown population of glial cells. Using platinum for impregnation of the neural tissue, Robertson visualized and briefly described the morphology of those cells and introduced the term "mesoglia". These cells were small, with scany cytoplasm and a few processes. Some of them showed phagocytic activity, whereas others appeared closely related to amyloid bodies in "chronic brain degenerations". It was later estimated that Robertson's "mesoglia" consisted of microglia and oligodendroglia (Polak et al. 1977; Imamoto 1981). The method of staining used by Robertson proved to be unreliable, and it never found its way to laboratories outside of Edinburgh (Penfield 1928).

A description of microglia made during the years from 1919 to 1921 by del Rio Hortega initiated broad studies of the subject. Using a newly developed staining method with the use of weak silver carbonate solution, he was the first to describe microglia in detail and to recognize them as an entity within the mammalian CNS.

Since then, micrologia and their function and origin have been studied extensively with the use of a wide spectrum of methods including histology, histochemistry, immunohistochemistry, electron microscopy and autoradiography. With quickly growing knowledge, the number of questions multiplied. The subjects frequently debated used to be the origin, morphology, and nomenclature of these cells. The potential and so-called "metamorphotic capacity" (Streit et al. 1988) of microglia, as well as difficulties in proper defining, seem to be reflected in the multiplicity of terms used over the years to describe microglia, such as "rod cells", "gitter cells", "lattice cells", "lipid cells", "resting microglia", "ramified cells", "cells with dendritic morphology", "activated microglia", "ameboid cells", and "brain macrophages", etc.

---

* Supported by NIH Grant HD 24884 and by the New York State Office of Mental Retardation and Developmental Disabilities.

Investigations in recent years have offered some new suggestions concerning the function of microglia. It appears that their most important function is their unique role in the defense system in the CNS. Microglia may form a network of potential immunoeffector cells, which can be activated by a wide range of stimuli, but the kind of signal that results from the formation of the ramified form into the ameboid one is unknown. They may function as antigen-presenting cells and take part in the inflammatory reaction (Streit et al. 1988) and may also act as the effector cells that link the immune system with brain development and astroglial scar formation (Giulian 1987). Microglia appear to be involved primarily in the presentation of antigens (Poltorak; Freed 1989), and their phenotype, resembling that of Langerhans cells, suggests that they are highly specialized antigen-presenting cells adapted to the microenvironment of the CNS.

Microglia resemble similar cells found in the interstitial tissues of most organs that are thought to have a major role in antigen presentation and initiation of the immune response and that are part of a family of cells with dendritic morphology concentrated mainly in lymphoid tissue (Glenn et al. 1989; Lowe et al. 1989). As the brain macrophages in particular, microglia may be involved in local inflammatory responses of the brain (Streit et al. 1988). Production of superoxide anions by microglia (Colton, Gilbert 1987) led to the hypothesis that microglia are likely to serve an anti-infective function specific to the CNS but similar to the role of neutrophils and monocytes. Cultured microglia also produce tumor necrosis factor (TNF), a monocyte-macrophage-derived protein that induces a cytotoxic effect (Sawada et al. 1989) that might result in microglial involvement un demyelination and denervation. Inhibition of viral replication may be another effect of TNF.

The finding that peripheral nerve injury stimulates increased expression of CR3 antigen by activated microglia led to the notion that these cells may also participate in neuronal repair (Graeber et al. 1988a). Another observation was that in this type of pathology, a marked proliferation of microglial cells occurs in the nucleus of origin. Their role in the process of so-called synaptic stripping is proposed (Streit et al. 1988): microglial cells were shown to strip or disconnect the synaptic contacts from the body of the distressed neuron, possibly in order to allow the neuron to recover. Ameboid microglia respond to nearly every type of brain pathology, including trauma, infection, neoplasm, infarction, and demyelination, by migration, phagocytosis, and proliferation at the site of the injury (Giulian 1987); they also secrete cytokines and cytotoxins (Giulian et al 1991). Microglial cells appear to be involved in the amyloid production in Alzheimer's disease, as well as in experimental scrapie (Mattiace et al. 1990b; Wiśniewski et al. 1990). According to this hypothesis, microglia-macrophages synthesize and secrete the amyloid fibers. Microglia are also direct targets of HIV infection and serve to propagate and amplify the neural HIV infection (Michaels et al. 1988). Microglia and macrophages were demonstrated to be the only cells that harbor HIV (Kure et al. 1990a, b).

Microglia have been studied *in vitro* and *in situ*, in normal and pathological states, and in human brains and a variety of experimental animals. Immunohistochemistry and lectin histochemistry, though relatively new tools, have become widely used to visualize microglia.

CLASSICAL HISTOLOGICAL TECHNIQUES

The histological method with the longest history is metal impregnation. Using silver impregnation, del Rio Hortega was able to give a precise description of the "third element" in the CNS (del Rio Hortega 1917, 1927, 1965). All histological staining methods for microglia have as their basis almost exclusively the impregnation of cells with silver *in situ*, according to the chemical reaction of the reduction of silver salts to metallic silver, and visualization of the cell bodies as well as the processes. Other silver impregnation methods such as those of Cajal, Penfield, Naoumenko, Ibrahim and Howard were used in a wide variety of modifications, but in fact, the prototype technique modified later by others is the method of del Rio Hortega (Penfield, Cone 1950; Naoumenko, Feigin 1963; Ibrahim et al. 1968; Howard, Barnes 1979). Most of these techniques are neither simple nor time-saving, and some of them are not applicable to routinely processed, formalin-fixed/paraffin-embedded, tissues. There are also some divergences of the results of staining in different species. Other methods, including those of Weil and Davenport (1933), Tsuijama (1965), Gallyas (1970), Scott (1971) are improved, more universal techniques (detailed descriptions of all methods mentioned can be found in the literature listed in bibliography).

Impregnation with silver proved to be an excellent technique in electron microscopy thanks to silver's high electron density. Some staining procedures for transmission electron microscopy were developed on the basis of some procedures previously mentioned, including those of Tsuijama, Scott, and Hortega (Scott 1979; Murabe, Sano 1981a; Vogel, Kemper 1962).

HISTOCHEMICAL TECHNIQUES

Identification of microglia in tissue sections and in cell cultures has also been possible by histochemical techniques. These enable detection of cell-specific marker enzymes that transform substrates into color reaction products. Animal cells are among the most often studied subjects. A few histochemical staining methods for human microglia are available, but most of them must be used with fresh tissue. The enzymatic activity of microglia varies depending on functional state investigated. Generally, in the classical staining technique with silver salts, so-called resting microglia, i.e., cells under normal conditions in the developed brain, appear as small cells with small, often elongated nuclei and scanty ramification. Enzymatic activity is rather poor and not specific. Enzymes such as ATPase and UDPase are characteristic for other glia too. Recent studies by Castellano et al. (1991) show that NDPase histochemistry is a specific and sensitive method of visualizing microglial cells *in situ* and *in vitro*. In pathological conditions (in cases of which microglia bear a wide variety of names, e.g., progressive, activated brain macrophages) or in developing brain (so-called nascent microglia), enzymatic activity is quite different in that it is much more intense. Cells in tissue cultures, such as active microglia, are rich in enzymes. Enzymes, which are listed below, were studied in different species. However, the brain tissue from mice and rats were used most frequently. The enzymes studied most often are nucleoside phosphatase, acid

phosphatase, dehydrogenase, esterase, thiaminepyrophosphatase (TPPase), peroxidase, and aryl sulphatase (Ibrahim et al. 1974; Ferrer, Sarmiento 1980a, b; Oehmichen 1980; Murabe, Sano 1981b; Vorbrodt, Wiśniewski 1982; Sanyal, De Ruiter 1985).

LECTIN HISTOCHEMISTRY

Since the 1880's, it has been known that extracts from certain plants can agglutinate red blood cells. Then, in the 1940's, agglutinins were discovered that could select types of cells on the basis of their blood-group activities. Although the term lectin was originally related to agglutinins that can discriminate red blood cells, today the term is used more generally to define sugar-binding proteins from many sources, e.g., plants, viruses, microorganisms, and animals. Their function and nature is not clear. Lectins bind to a well-defined sugar structure and have proved to be a very sensitive tool in morphological studies of microglia. However, there are only a few lectins that positively stain microglia. Because some of these lectin methods are applicable to formalin-fixed and paraffin-embedded tissues, lectins are valuable.

Ricinus communis agglutinin 1 (RCA-1) binds beta-D-galactoside and is considered an established marker for microglia in tissues that are routinely processed (Mannoji et al. 1986; Michaels et al. 1988; Hutchins et al. 1990). *Viscum album* lectin (mistletoe lectin, ML-1) also possesses D-galactose-specific surface-binding site. ML-1, unlike RCA-1, has proved to be an excellent marker for microglia in rodents (Suzuki et al. 1988), with high reproducibility of the results but, like RCA-1, is also applicable to routine histology.

Griffonia simplicifolia lectin (GSA-I-B4, BSA-I-B4) has an exclusive sugar-binding specificity for terminal alpha-D-galactose residues (Streit, Kreutzberg 1987; Ashwell 1990). GSA-I-B4-peroxidase conjugates are used to stain microglial cells in rodent tissue only. New, improved methods of staining have been described recently by Streit (1990).

IMMUNOHISTOCHEMICAL TECHNIQUES

The rapid development of immunohistology resulted in new types of studies of microglia. Highly specific monoclonal antibodies are now used to detect and to visualize specific antigenic determinants that are present on microglial cells. LN-1 is a mouse monoclonal antibody raised against B lymphocytes. It reacts with the proteins of cell membranes and the cytoplasm of the germinal B cells (designated as the CDw73). This antibody was established as a marker for microglia in routinely processed tissues, and a detailed technical protocol can be found in a work of Miles and Chou (1988). Another study of LN-1 reactivity (Dickson, Mattiace 1989) brought some more observations. The LN-1-positive microglia can be detected in tissues fixed for a longer time and in higher concentrations of aldehyde. Shorter fixation gives poorer results in microglial staining, but LN-1-positive astrocytes appear.

HLA-DR is a class-II cell-surface glycoprotein of the human major histocompatibility complex (MHC II). It is expressed on most B lymphocytes, some T lymphocytes, macrophages, thymus epithelium, and other body tissues.

In brain cells bearing the MHC-II, antigens are microglia and some astrocytes. The use of anti-HLA antibodies is restricted mostly to unfixed tissues (Hayes et al. 1987; Lowe et al. 1989). Numerous studies have shown the expression of MHC II antigens by activated microglia in brains of patients with Alzheimer's disease (McGeer et al. 1987; 1988a, b; Itagaki et al. 1989). Examples of other cases in which HLA-positive, reactive microglia were studied are Parkinson's (McGeer et al. 1988a,b), Pick's and Huntington's diseases, among others (McGeer et al. 1988b). Tissues in the above studies were frozen, cryostat-cut, and then fixed shortly before staining. There is also an example of positive staining of microglia in routinely processed tissues (formalin-fixed and Paraplast-embedded) (Grundke-Iqbal et al. 1990). Grenier et al. (1989) performed studies of the expression of MHC-II antigens by microglia *in vitro*; brain tissue for this study was obtained from patients undergoing neurosurgical procedures. Cultured microglia expressed the antigen under normal conditions, and expression was increased after gamma interferon induction. The dependence of HLA-DR expression on technical and clinical factors have been described recently (Mattiace et al. 1990a) and microglial cells have been detected in fixed brains sectioned on vibratome.

HAM-56 is a mouse monoclonal antibody to a macrophage-specific antigen. This antibody reacts with different macrophage populations (e.g., in skin, liver, lymph node, brain) and also with variable endothelial cells. HAM-56 does not react with B and T lymphocytes, but it does react with monocytes. This antibody is mentioned in term of microglial labeling in human brain tissue (Dickson, Mattiace 1989). The pattern of staining with HAM-56 is similar to that with the RCA-1 lectin, which labels both the microglial and endothelial cells. Several recently published works provide detailed studies of HAM-56 reactivity (Adams, Poston 1990; Kure et al. 1990).

Anti LCAg (CD-45) monoclonal antibodies react with the surface molecule of the majority of leukocytes. Microglial labeling with anti LCAg has been successful in different types of pathology such as Alzheimer's disease (Lowe et al. 1989; Mattiace et al. 1990b) and also in the brains of individuals with no neurological pathology. Brain tissue for anti-LCA staining can be used unfixed, cryostat-cut, but also fixed in formalin and embedded in Paraplast, as well as fixed and cut on Vibratome.

EBM/11 is an established microglial marker (Graeber, Streit 1990). This monoclonal antibody was raised against lung macrophages and has been shown to react with cytoplasmic determinants in human macrophages from various organs. EBM/11 labels microglia in normal and diseased human CNS tissues and is applicable to unfixed tissue (Esiri, McGee 1986; Kelly et al. 1988).

The presence of beta-2 integrins on the surface of microglial cells was studied recently (Rozemuller et al. 1989; Akiyama, McGeer 1990). Integrins, which are a super family of cell adhesion molecules, are involved in cell-cell and cell-matrix interactions. Both resting and reactive microglia were found to react with antibodies against all members of the beta-2 integrin family: CD-11a (LFA-1), CD-11b (MAC-1 or CR-3), CD-11c (P150, 95 or CR4), and CD-18 (Beta-2). Frozen cortical and subcortical tissue of Alzheimer disease and non-neurological cases was used. LeuM5 (CD11c) immunoreactivity was found in microglial cells from patients with Alzheimer's disease; the tissue was fixed in

4% paraformaldehyde and in PLP (periodate-lysine-paraformaldehyde) and sectioned on Vibratome (Mattiace et al. 1990b). Another recent report (Mattiace et al. 1990a) says that the best LeuM5 staining was performed in tissue fixed for 10 hours in weak paraformaldehyde solution, whereas prolonged fixation resulted in the complete absence of LeuM5 staining. In tissues from patients with AIDS encephalopathy (Vazeux et al. 1987), LeuM5 stained infected process-bearing and multinucleated cells in addition to noninfected macrophages. In control brains, the LeuM5-positive cells were macrophages with cytoplasmic processes. Tissue for this study was used unfixed, frozen and cryostat cut. LeuM5 has also been used as a microglial marker in cultured cells (Grenier et al. 1989).

Anti-ferritin antibodies are intracellular iron-storage proteins found in most eukaryotic cells. Antisera to ferritin label microglia in routinely processed tissues. An immunohistochemical analysis of brain sections was performed with antisera against holoferritin and a light subunit of ferritin, with similar results (Kaneko et al. 1989). Other authors used rabbit antiserum to ferritin isolated from human brain and liver (Grundke-Iqbal et al. 1990).

The presence of antibodies against alpha-1-antitrypsin, alpha-1-antichymotrypsin, and lysozyme were demonstrated by Esiri and Booss (1984) in the microglial cells within brains of patients who survived up to 13 days after brain injury. In this work, paraffin-embedded tissue was used for staining.

IMMUNOHISTOCHEMISTRY OF MICROGLIA IN EXPERIMENTAL ANIMALS

The biology of microglia in animals, their contribution to the pathology, and their relation to the disease under controlled, laboratory conditions is being extensively studied. The number of microglial markers for humans is higher than for animals. An animal model allows examination of the microglia during ontogenesis and also with or without induced pathology. Stab wound, axotomy, cryoinjury, toxic injuries, kainic acid lesions, viral infection (conventional and unconventional), and experimental allergic encephalitis (EAE) are among the induced types of animal pathology most often studied. Below are some microglial markers that were used in studies of cells *in situ* and in cultures, mostly fresh, but some also routinely processed (formalin-fixed and paraffin-embedded).

F4/80 is the rat monoclonal antibody specific for mouse macrophages. F4/80-positive cells can be detected in such tissues as lymphoid and myeloid, gut, and respiratory tract. F4/80-positive microglia were found in bouth adult and developing mouse brains, but in the latter case, the distribution of positive cells depended on the stage of the ontogenesis. The population of F4/80-positive microglia was visible in larger amounts only after the 20th day of embryonic life and in newborn animals (Perry et al. 1985).
Detection of microglia in an adult mouse brain, with the use of F4/80, 2.4G2, and MAC-1-antibodies showing heterogeneity of distribution and morphology, has been reported recently (Lawson et al. 1990).

2.4G2 rat monoclonal antibody recognizes trypsin-resistant IgG/IgG2b mouse Fc receptor, which can be found on some macrophages, neutrophils, and some lymphocytes. Staining of microglia of both the adult and developing mouse brains with 2.4G2 antibody was positive (Perry et al. 1985).

MAC-1 is the other rat monoclonal antibody against the myelomonocytic differentiation antigen, probably functioning as C3 complement receptor. MAC-1 cross-reacts with human monocytes, granulocytes, and natural-killer cells. MAC-1 reactivity with the microglial population, as has been described in the developing and adult mouse brains, is similar to that of F4/80 (Perry et al. 1985). MAC-1 was also used in the studies of microglia *in vitro*, where it served as a differentiating marker in mixed cell cultures (Frei et al. 1986, 1987). Other authors (Matsumoto et al. 1985) studied MAC-1 reactivity in mouse brains with cold lesions, where they noted an increased number of positive cells.

ED-1 is the monoclonal antibody directed against rat macrophagespecific antigen. When it was used to study brain cell cultures (Gebicke-Haerter et al. 1989), the subpopulation of ED-1 positive cells was recognized as microglia. However, the other authors (Sminia et al. 1987) who examined rat CNS during ontogeny using a panel of three anti-macrophage monoclonal antibodies (ED1, ED2, ED3) called the reactive cells macrophages, whereas in their opinion, microglial cells remained unstained.

OX-6 is a monoclonal antibody against the monomorphic determinant of the rat Ia antigen. OX-6, among other antibodies, was applied to rat brain tissues with developing lesions of EAE (Matsumoto, Fujiwara 1987). The cells with „dendritic morphology", probably microglia, were positively stained with this antibody. Weakly positive staining of microglia was achieved also when OX-6 was applied to the brains of axotomized rats (Streit et al. 1989).

OX-18 monoclonal antibody recognizes the monomorphic determinant of the rat MHC class I antigen. The OX-18 reactivity of microglia has been described in axotomized rats (Giulian et al. 1986) and has also been confirmed by lectin binding of OX-positive cells.

OX-35 and W3/25 are mouse monoclonal antibodies directed against different CD-4 antigen epitopes in the rat brain. When the brains of normal and injured rats were studied for the presence of CD-4 (Perry, Gordon 1987), microglia in the normal CNS stained weakly, whereas a few days after injury, the number of W3/25-positive cells was significantly increased.

OX-1 and OX-30 are monoclonal antibodies directed against the leukocyte common antigen epitopes (LCAg); as in the studies described above, the microglia under normal conditions express the antigen in a very low amount. Only after cortical injury does the number of LCAg-positive cells in the area of the lesion increase.

OX-42 recognized C3b receptor in the rat, which was readily detectable on mature microglia (Perry, Gordon 1987). OX-42 is considered an excellent marker for microglia and also for macrophages.

Antibody against vimentin was suggested to be a marker for activated microglia (including brain macrophages) (Graeber et al. 1988b). The cytoskeleton of the microglia was studied in the rat CNS after axotomy. Vimentin-positive cells can also be labeled with the lectin GSA-I-B4, which is a marker that can discriminate microglia from other glial elements.

Anti-phosphotyrosine antibody is a new marker for microglial cells (Tillotson, Wood 1989). Phosphotyrosine (tyrosine kinase) immunoreactivity has been demonstrated in both types of cells, i.e., in activated/ameboid and in resting/ramified, *in situ* and *in vitro*. Positive staining is preserved in cryosectioned tissue and also after fixation in aldehyde solutions. Authors were unable

to show phosphotyrosine reactivity in paraffin-embedded tissues, because if the usual damage of antigens during routine histological processing.

Also, a lectin obtained from *Griffonia Simplicifolia* (*Bandeieraea Simplicifolia* isolectin B4), GSA-I-B4 proved to stain microglia of the rat brain. GSA-I-B4 peroxidase conjugates have a binding specificity for terminal alpha-D-galactoside residues and have been mentioned already (Streit, Kreutzberg 1987). In the mouse CNS, GSA-I-B4 also detected reactive macrophages in direct injury sites (Schelper et al. 1985).

CONCLUDING REMARKS

The list of immunohistochemical markers above is the result of studies that have greatly altered the methodology for detecting of microglia. Traditional silver impregnation, the only techniques available at the beginning of the 20th century, provides the most detailed and still unsurpassed descriptions of the morphology of microglia, but it is technically difficult. Immunohistology solved that problem to some degree and offered at least a few reliable, reproducible methods for detecting and visualizing the third element. Because the functions of microglial cells in the brain have not yet been characterized, researchers are analyzing the changing "immunophenotype" of these cells in various condition. Immune markers will certainly shed more light on the function of these cells.

Considering the rapidly increasing number of markers for microglial cell available and the heightened interest in the brain's "own" immune system cells, the near future will certainly bring many new findings in the exciting area of human neuroimmunology. Studies of microglial cells may light on numerous aspects of neuroscience, ranging from brain development and regeneration, brain aging, neural infections, neural transplant, and autoimmune diseases.

ZNACZNIKI KOMÓREK MIKROGLEJU

Streszczenie

Autorzy przedstawiają przegląd metod stosowanych do ujawniania komórek mikrogleju w warunkach *in situ* i *in vitro*: od klasycznych metod impregnacyjnych począwszy, poprzez wykorzystanie do tego celu techniki histochemicznej i wreszcie immunohistochemii przy użyciu wysoce swoistych przeciwciał, pozwalających na śledzenie zmian „immunofenotypu" tych komórek.

REFERENCES

1. Adams CW, Poston RN: Macrophage histology in paraffin-embedded multiple sclerosis plaques is demonstrated by the monoclonal pan-macrophage marker HAM-56: correlation with chronicity of the lesion. Acta Neuropathol (Berl), 1990, 80, 208–211.
2. Akiyama H, McGeer PL: Brain microglia constitutively express beta-2 integrins. J Neuroimmunol, 1990, 30, 81–93.
3. Ashwell K: Microglia and cell death in the developing mouse cerebellum. Dev Brain Res, 1990, 55, 219–230.
4. Castellano B, Gonzalez B, Jensen BM, Pedersen EB, Finsen BR, Zimmer J: A double staining technique for simultaneous demonstration of astrocytes and microglia in brain sections and astroglial cell cultures. J Histochem Cytochem, 1991, 39, 561–568.

5. Colton CA, Gilbert DL: Production of superoxide anions by a CNS macrophage, the microglia. FEBS Lett, 1987, 223, 284–288.
6. Dickson DW, Mattiace LA: Astrocytes and microglia in human brain share an epitope recognized by a B-lymphocyte-specific monoclonal antibody (LN-1). Am J Pathol, 1989, 135, 135–147.
7. Esiri MM, Booss J: Comparison of methods to identify microglial cells and macrophages in the human central nervous system. J Clin Pathol, 1984, 37, 150–156.
8. Esiri MM, McGee JO: Monoclonal antibody to macrophages (EMB/11) labels macrophages and microglial cells in human brain. J Clin Pathol, 1986, 39, 615–621.
9. Ferrer I, Sarmiento J: Nascent microglia in the developing brain. Acta Neuropathol (Berl), 1980a, 50, 61–67.
10. Ferrer I, Sarmiento J: Reactive microglia in the developing brain. Acta Neuropathol (Berl), 1980b, 50, 69–76.
11. Frei K, Bodmer S, Schwerdel C, Fontana A: Astrocyte-derived interleukin 3 as a growth factor microglia cells and peritoneal macrophages. J Immunol, 1986, 137, 3521–3527.
12. Frei K, Siepl C, Groscurth P, Bodmer S, Schwerdel C, Fontana A: Antigen presentation and tumor cytotoxicity by interferon-Y-treated microglial cells. Eur J Immunol, 1987, 17, 1271–1278.
13. Gallyas F: Silver staining of micro- and oligodendroglia by means of physical development. Acta Neuropathol (Berl), 1970, 16, 35–38.
14. Gebicke-Haerter PJ, Bauer J, Schobert A, Northoff H: Lipopolysaccharide-free conditions in primary astrocyte cultures allow growth and isolation of microglial cells. J Neurosci, 1989, 9, 183–194.
15. Giulian D: Ameboid microglia as effectors of inflammation in the central nervous system. J Neurosci Res, 1987, 18.
16. Giulian D, Baker TJ, Shih LC, Lachman LB: Interleukin 1 of the central nervous system is produced by ameboid microglia. J Exp Med, 1986, 164, 594–604.
17. Giulian D, Johnson B, Krebs JF, George JK, Tapscott M: Microglial mitogens are produced in the developing and injured mammalian brain. J Cell Biol, 1991, 112, 323–333.
18. Glenn JA, Jordan FL, Thomas WE: Further studies on the identification of microglia in mixed brain cell cultures. Brain Res Bull, 1989, 22, 1049–1052.
19. Graeber MB, Streit WJ: Microglia: immune network in the CNS. Brain Pathol, 1990, 1, 2–5.
20. Graeber MB, Streit WJ, Kreutzberg GW: Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells. J Neurosci Res, 1988a, 21, 18–24.
21. Graeber MB, Streit WJ, Kreutzberg GW: The microglial cytoskeleton: vimentin is localized within activated cells *in situ*. J Neurocytol, 1988b, 17, 573–580.
22. Grenier Y, Ruijs TCG, Robitaille Y, Olivier A, Antel JP: Immunohistochemical studies of adult human glial cells. J Neuroimmunol, 1989, 21, 103–115.
23. Grundke-Iqbal I, Fleming J, Tung YC, Lassmann H, Iqbal K, Joshi JG: Ferritin is a component of the neuritic (senile) plaque in Alzheimer dementia. Acta Neuropathol (Berl), 1990, 81, 105–110.
24. Hayes GM, Woodroofe MN, Cuzner ML: Microglia are the major cell type expressing MHC class II in human white matter. J Neurol Sci, 1987, 80, 25–37.
25. Howard MJ, Barnes PR: A silver carbonate method for cell counts of neurons and glial elements on paraffin embedded brain tissue. Stain Technol, 1979, 54, 299–303.
26. Hutchins KD, Dickson DW, Rashbaum WK, Lyman WD: Localization of morphologically distinct microglial populations in the developing human fetal brain: implications for ontogeny. Dev Brain Res, 1990, 55, 95–102.
27. Ibrahim MZ, Call N, Noden P: Modifications of the Hortega silver carbonate method adapted for celloidin-embedded and frozen sections. Acta Neuropathol (Berl), 1968, 10, 258–260.
28. Ibrahim MZ, Khreis Y, Koshayan DS: The histochemical identification of microglia. J Neurol Sci, 1974, 22, 211–233.
29. Imamoto K: Origin of microglia: cell transformation from blood monocytes into macrophagic ameboid cells and microglia. Prog Clin Biol Res, 1981, 59, 125–139.
30. Itagaki S, McGeer PL, Akiyama H, Zhu S, Selkoe D: Relationship of microglia and astrocytes to amyloid deposits of Alzheimer disease. J Neuroimmunol, 1989, 24, 173–182.
31. Kaneko Y, Kitamoto T, Tateishi J, Yamaguchi K: Ferritin immunohistochemistry as a marker for microglia. Acta Neuropathol (Berl), 1989, 79, 129–136.

32. Kelly PMA, Bliss E, Morton JA, Burns J, McGee JOD: Monoclonal antibody EBM/11: high cellular specificity for human macrophages. J Clin Pathol, 1988, 41, 510–515.
33. Kure K, Lyman WD, Weidenheim KM, Dickson DW: Cellular localization of an HIV-1 antigen in subacute AIDS encephalitis using an improved double-labeling immunohistochemical method. Am J Pathol, 1990a, 136, 1085–1092.
34. Kure K, Weidenheim KM, Lyman WD, Dickson DW: Morphology and distribution of HIV-1 gp-41 positive microglia in subacute AIDS encephalitis. Acta Neuropathol (Berl), 1990b, 80, 393–400.
35. Lawson LJ, Perry VH, Dri P, Gordon S: Heterogeneity in the distribution and morphology of microglia in the normal adult mouse brain. Neuroscience, 1990, 39, 151–170.
36. Lowe J, MacLennan KA, Powe DG, Pound JD, Palmer JB: Microglial cells in human brain have phenotypic characteristics related to possible function as dendritic antigen presenting cells. J Pathol, 1989, 159, 143–149.
37. Mannoji H, Yeger H, Becker LE: A specific histochemical marker (lectin *Ricinus communis* agglutinin-1) for normal human microglia, and application to routine histopathology. Acta Neuropathol (Berl), 1986, 71, 341–343.
38. Matsumoto Y, Fujiwara M: The immunopathology of adoptively transferred experimental allergic encephalomyelitis (EAE) in Lewis rats. Part 1. Immunohistochemical examination of developing lesions of EAE. J Neurol Sci, 1987, 77, 35–47.
39. Matsumoto Y, Watabe K, Ikuta F: Immunohistochemical study on neuroglia identified by the monoclonal antibody against a macrophage differentiation antigen (Mac-1). J Neuroimmunol, 1985, 9, 379–389.
40. Mattiace LA, Davies P, Dickson DW: Detection of HLA-DR on microglia in the human brains is a function of both clinical and technical factors. Am J Pathol, 1990a, 136, 1101–1114.
41. Mattiace LA, Davies P, Yen SH, Dickson DW: Microglia in cerebellar plaques in Alzheimer's disease. Acta Neuropathol (Berl), 1990b, 5, 493–498.
42. McGeer PL, Itagaki S, McGeer EG: Expression of the histocompatibility glycoprotein HLA-DR in neurological disease. Acta Neuropathol (Berl), 1988b, 76, 550–557.
43. McGeer PL, Itagaki S, Boyes BE, McGeer EG: Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology, 1988a, 38, 1285–1291.
44. McGeer PL, Itagaki S, Tago H, McGeer EG: Reactive microglia in patients with senile dementia of the Alzheimer type are positive for the histocompatibility glycoprotein HLA-DR. Neurosci Lett, 1987, 79, 195–200.
45. Michaels J, Price RW, Rosenblum M: Microglia in the giant cell encephalitis of acquired immune deficiency syndrome: proliferation, infection and fusion. Acta Neuropathol (Berl), 1988, 76, 373–379.
46. Miles JM, Chou SM: A new immunoperoxidase marker for microglia in paraffin section. J Neuropathol Exp Neurol, 1988, 47, 579–587.
47. Murabe Y, Sano Y: Morphological studies on neuroglia I. Electron microscopic identification of silver-impregnated glial cells. Cell Tissue Res, 1981a, 216, 557–568.
48. Murabe Y, Sano Y: Thiaminepyrophosphatase activity in the plasma membrane of microglia. Histochemistry, 1981b, 71, 45–52.
49. Naoumenko J, Feigin I: A modification for paraffin sections of silver carbonate impregnation for microglia. Acta Neuropathol (Berl), 1963, 2, 402–406.
50. Oehmichen M: Enzyme-histochemical differentiation of neuroglia and microglia: a contribution to the cytogenesis of microglia and globoid cells. Review of the literature. Pathol Res Pract, 1980, 168, 344–373.
51. Oehmichen M: Are resting and/or reactive microglia macrophages. Immunobiology, 1982, 161, 246–254.
52. Penfield W: A method of staining oligodendroglia and microglia. Am J Pathol, 1928, 4, 153–157.
53. Penfield W, Cone WV: Neuroglia and microglia (The metallic methods). In: McClung's Handbook of Microscopical Technique, 1950, pp 399–431.
54. Perry VH, Gordon S: Modulation of CD4 antigen on macrophages and microglia in rat brain. J Exp Med, 1987, 166, 1138–1143.
55. Perry VH, Hume DA, Gordon S: Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. Neuroscience, 1985, 15, 313–326.

56. Polak M, D'Amelio F, Johnson JE, Haymaker W: Microglial cells origins and reactions. In: Histology and Histopathology of the Nervous System. 1977, pp 481–559.
57. Poltorak M, Freed WJ: Immunological reactions induced by intracerebral transplantation: Evidence that host microglia but not astroglia are the antigen-presenting cells. Exp Neurol, 1989, 103, 222–233.
58. Rio-Hortega del P: Noticia de un nuevo y facil method para la coloracion de la neuroglia y del tejido conjuntivo. Trabajos del Laboratorio de Investigaciones Biologicas de la Universidad de Madrid, 1917, 15, 367–368.
59. Rio-Hortega del P: Innovaciones utiles en la tecnica de coloracion de la microglia y otros elementos del sistema macrofagico. Boletin de la Sociedad Espanola de Historia Natural, 1927, 27, 199–212.
60. Rio-Hortega del P: Microglia. In: Cytology and Cellular Pathology of the Nervous System. Ed: W Penfield. Hafner Publishing Company, New York, 1965, pp. 483–534.
61. Robertson WF: A microscopic demonstration of the normal and pathological histology of mesoglia cells. J Mental Sci, 1900, 46, 724–725.
62. Rozemuller JM, Eikelenboom P, Pals ST, Stam FC: Microglial cells around amyloid plaques in Alzheimer's disease express leucocyte adhesion molecules of the LFA-1 family. Neurosci Lett, 1989, 101, 288–292.
63. Sanyal S, De Ruiter A: Inosine diphosphatase as a histochemical marker of retinal microvasculature, with special reference to transformation of microglia. Cell Tissue Res, 1985, 241, 291–297.
64. Sawada M, Kondo N, Suzumura A, Marunouchi T: Production of tumor necrosis factor-alpha by microglia and astrocytes in culture. Brain Res, 1989, 491, 394–397.
65. Schelper RL, Whitters E, Hart MN: True microglia distinguished from macrophages by specific lectin binding. J Neuropathol Exp Neurol, 1985, 44, 332.
66. Scott T: A rapid silver impregnation technique for oligodendrocytes, microglia, and astrocytes. J Clin Pathol, 1971, 24, 578–580.
67. Scott T: A silver impregnation method for reactive microglia in 1 μm epoxy sections. Acta Neuropathol (Berl), 1979, 46, 155–158.
68. Sminia T, Groot de CJ, Dijkstra CD, Koetsier JC, Polman CH: Macrophages in the central nervous system of the rat. Immunobiology, 1987, 174, 43–50.
69. Stensaas LJ, Reichert WH: Round and amoeboid microglial cells in the neonatal rabbit brain. Z Zellforsch, 1971, 119, 147–163.
70. Streit WJ: An improved staining method for rat microglial cells using the lectin from *Griffonia simplicifolia* (GSA I-B4). J Histochem Cytochem, 1990, 38, 1683–1686.
71. Streit WJ, Graeber MB, Kreutzberg GW: Functional plasticity of microglia: a review. Glia, 1988, 1, 301–307.
72. Streit WJ, Graeber MB, Kreutzberg GW: Peripheral nerve lesion produces increased levels of major histocompability complex antigens in the central nervous system. J Neuroimmunol, 1989, 21, 117–123.
73. Streit WJ, Kreutzberg GW: Lectin binding by resting and reactive microglia. J Neurocytol, 1987, 16, 249–260.
74. Suzuki H, Franz H, Yamamoto T, Iwasaki Y, Konno H: Identification of the normal microglial population in human and rodent nervous tissue using lectin-histochemistry. Neuropathol Appl Neurobiol, 1988, 14, 221–227.
75. Tillotson ML, Wood JG: Phosphotyrosine antibodies specifically label ameboid microglia *in vitro* and ramified microglia *in vivo*. Glia, 1989, 2, 412–419.
76. Tsujiyama Y: Normal and pathological figures of neuroglia stained with Tsujiyama's methods. In: Morphology of Neuroglia. Eds: J Nakai, I Shoin, ChC Tomas, Springfield, Tokyo, 1965, pp 165–192.
77. Vazeux R, Brousse N, Jarry A, Henin D, Marche C, Vedrenne C, Mikol J, Wolff M, Michon C, Rozenbaum W, Bureau JF, Montagnier L, Brahic M: AIDS subacute encephalitis. Identification of HIV-infected cells. Am J Pathol, 1987, 126, 403–410.
78. Vogel FS, Kemper L: Modifications of Hortega's silver impregnation methods to assist in the identification of neuroglia with electron microscopy. In: Proceedings of the 4th International Congress of Neuropathology. Vol. II. Ed: H Jacob. G Thieme Verlag, Stuttgart, 1962, pp 66–71.
79. Vorbrodt AW, Wiśniewski HM: Plasmalemma-bound nucleoside diphosphatase as a cytochemical marker of central nervous system (CNS) mesodermal cells. J Histochem Cytochem, 1982, 30, 418–424.

80. Weil A, Davenport HA: Staining of oligodendroglia and microglia in celloidin sections. Arch Neurol Psych, 1933, 30, 175–178.
81. Wiśniewski HM, Vorbrodt AW, Wegiel J, Morys J, Lossinsky AS: Ultrastructure of the cells forming amyloid fibers in Alzheimer disease and scrapie. Am J Med Genet Suppl, 1990, 7, 287–297.

Correspondence address: Dr. Piotr B. Kozłowski, NYS Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, New York 10314

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

BARBARA GAJKOWSKA, ROMAN GADAMSKI, MIROSŁAW J. MOSSAKOWSKI

INFLUENCE OF SHORT–TERM ISCHEMIA ON THE ULTRASTRUCTURE OF HIPPOCAMPAL GYRUS IN MONGOLIAN GERBIL. III. SYNAPSES IN LATE STAGE OF THE PATHOLOGICAL PROCESS.

Laboratory of Electron Microscopy and Department of Neuropathology, Medical Research Centre, Polish Academy of Sciences, Warsaw

Electron microscope analysis of the CA_1 Ammon's horn sector was performed in Mongolian gerbils three days after an incident of short-term ischemia of the forebrain. CA_1 pyramidal neurons showed advanced disintegration. Some GABA-ergic interneurons revealed ultrastructural alteration of variable degree. The latter finding contradicts the generally helt view on the relative resistance of CA_1 sector interneurons to the ischemic injury. Synapses localized in all cortical layers of the CA_1 sector exhibited ultrastructural abnormalities involving both pre-and postsynaptic parts. They consisted in marked swelling and accumulation of unbound electron dense material, considered as calcium deposits. Presynaptic parts revealed additionally reduced number of synaptic vesicles and their abnormal distribution. Contrary to the early postischemic period, the most severe synaptic alterations appeared in *stratum pyramidale*, *radiatum* and *oriens*, involving both small dendritic branchings and their spines as well as large shafts of both basal and apical pyramidal dendrites.

Synaptic alterations especially features of the postsynaptic damage correspond to those indicating excitotoxic neuronal lesions. Presynaptic alterations may indicate both cessation of neurotransmission function as well as direct ischemic damage. The presence of calcium deposition seems to favour the former possibility.

Key words: *Brain ischemia, hippocampus, CA_1 sector of Ammon's horn, synapses.*

Delayed death of the pyramidal neurons in the CA_1 hippocampal sector after short-term cerebral ischemia is preceded by their hyperactivity (Suzuki et al. 1983; Mossakowski et al. 1989), and accumulation of Ca^{2+} ions in their cytoplasm (Van Reempts et al. 1986; Deshpande et al. 1987). Accumulation of calcium ions leads to severe neuronal damage (Łazarewicz et al. 1975; Siesjö 1981) through stimulation of lipolysis (Strosznajder 1980), proteolysis (Baudry et al. 1981), phosphorylation of proteins (Baudry, Lynch 1980) and to dispersion of cytoskeletal elements (Siesjö, Wieloch 1985).

Numerous experimental data suggest that the delayed death of the pyramidal neurons in the CA_1 sector of Ammon's horn results from the excitotoxic action of amino acid neurotransmitters, mostly glutamate (Kirino

1982; Jörgensen, Diemer 1982; Pulsinelli 1985a). Selective vulnerability of this particular hippocampal area is connected with its specific synaptic organization, namely, with rich innervation of CA_1 pyramidal neurons by glutaminergic Schaffer's collaterals (Collingridge et al. 1983). This hypothesis was convincingly proven in experiments revealing the cytoprotective effect on the pyramidal neurons of both glutaminergic deafferentation of the area (Pulsinelli 1985b) and application of specific blockers of NMDA receptors (Simon et al. 1984).

In the light of this concept an analysis of the synaptic changes in the CA_1 sector resulting from brain ischemia seems to be fully justified.

This study is a continuation of the previously carried out experiments analysing ultrastructural changes in the CA_1 sector of the hippocampus in Mongolian gerbils after an ischemic incident (Gajkowska et al. 1988, 1989), in which detailed morphological evaluation of synapses at an early postischemic period (12 and 24 h) was done. The studies disclosed in all layers of the CA_1 hippocampal sector ultrastructural features of increased synaptic activity of excitatory character with reduced synaptic activity of inhibitory type. These observations inclined us to perform a detailed ultrastructural analysis of synapses of the hippocampal CA_1 sector at a later period after the ischemic insult. The third postischemic day, when most of the pyramidal cells exhibit severe ultrastructural changes, was chosen. All layers of the CA_1 sector of Ammon's horn were analysed.

MATERIAL AND METHOD

Experiments were performed on 10 male, 3-month-old Mongolian gerbils, which were subjected to 5-min forebrain ischemia, produced by bilateral common carotid artery ligation according to the method described previously (Gajkowska et al. 1988). Following the experimental procedure the animals remained in normal laboratory conditions for three days and then were sacrificed under ether anesthesia by transcardiac perfusion with 2% glutaraldehyde. The tissue blocks, containing all cortical layers of Ammon's horn CA_1 sector were taken and processed in a routine way for electron microscope examination. Brains of 5 other animals not subjected to any experimental procedure served as control material.

RESULTS

The ultrastructure of subsequent layers of CA_1 sector of Ammon's horn is described separately (Gajkowska et al. 1988).

Stratum pyramidale. Pyramidal neurons occupying this layer display alterations of various intensity. In most of them there is disintegration of cytoplasm and organelles. Many cellular organelles are greatly swollen. This concerns mostly the Golgi apparatus and mitochondria. The cytoplasm contains large electron-lucent vacuoles, numerous polymorphic lysosomes and a variable number of ribosomes and polyribosomes. Elements of the cytoskeleton are fragmented and haphazardly distributed (Fig. 1). In both neuronal cytoplasm and dendritic processes there are small unbound aggregates of

Fig. 1. Stratum pyramidale. Fragment of cytoplasm of pyramidal neuron containing swollen Golgi complex (AG), mitochondria (M), electron-lucent vacuoles (V) and aggregations of electron-dense material (arrows). The majority of synapses on the neuron and in the neuropil are normal. Occasional astrocytic processes (A) are swollen. × 30 000

electron dense material, identified as calcium deposits. Intercellular spaces are sometimes dilated with loss of synaptic contacts. The remaining neurons of this layer, corresponding to interneurons, generally retain their normal ultrastruc-

ture. Only in few interneurons, swelling of some mitochondria and peripheral portion of the cytoplasm are observed; the number of lysosomes is also increased (Fig. 2). On the surface of pyramidal neurons and interneurons there is a great number of symmetric type synapses with normal ultrastructural

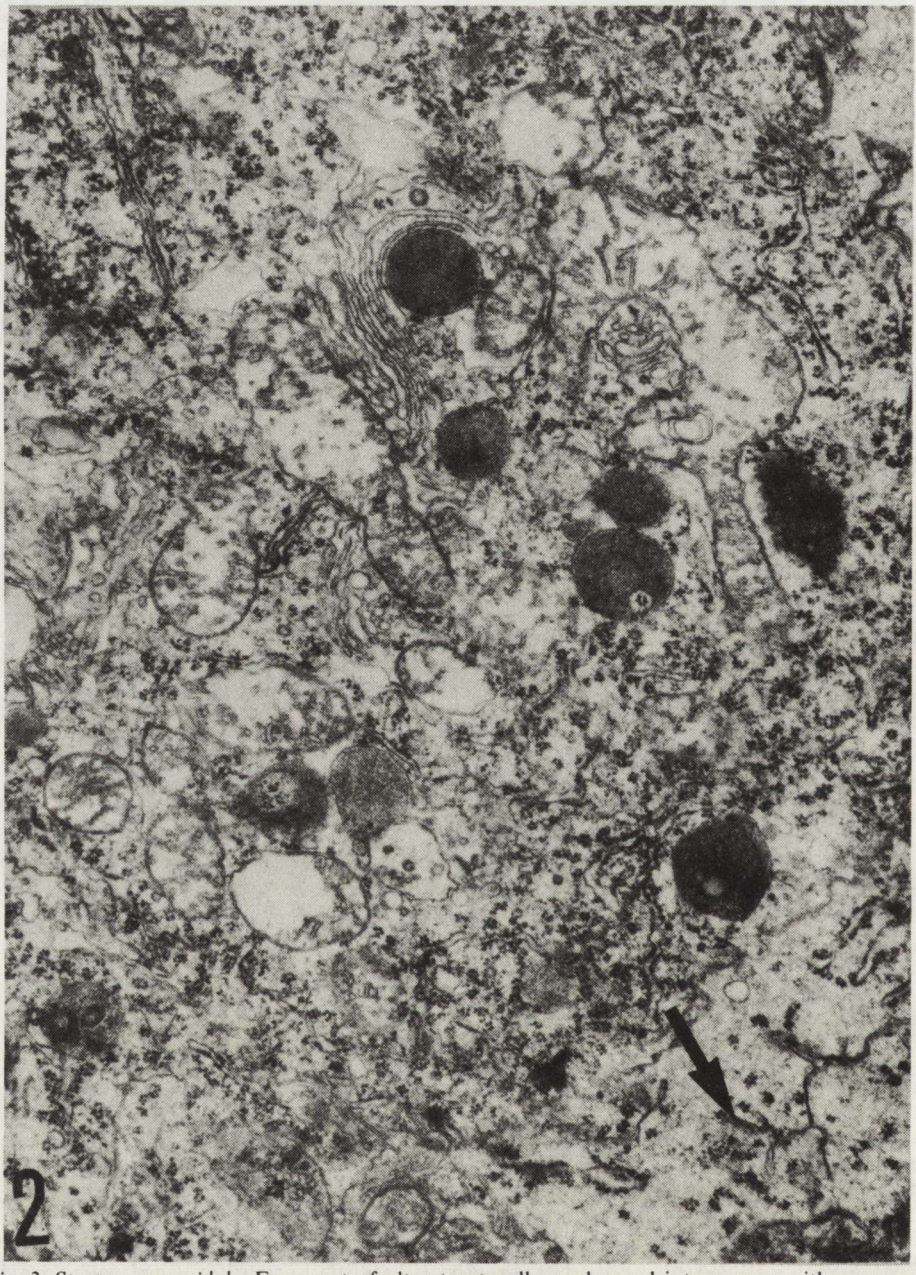

Fig. 2. Stratum pyramidale. Fragment of ultrastructurally unchanged interneuron with numerous lysosomes. Peripheral part of the cytoplasm swollen with reduced endoplasmic reticulum (arrow). × 22 500

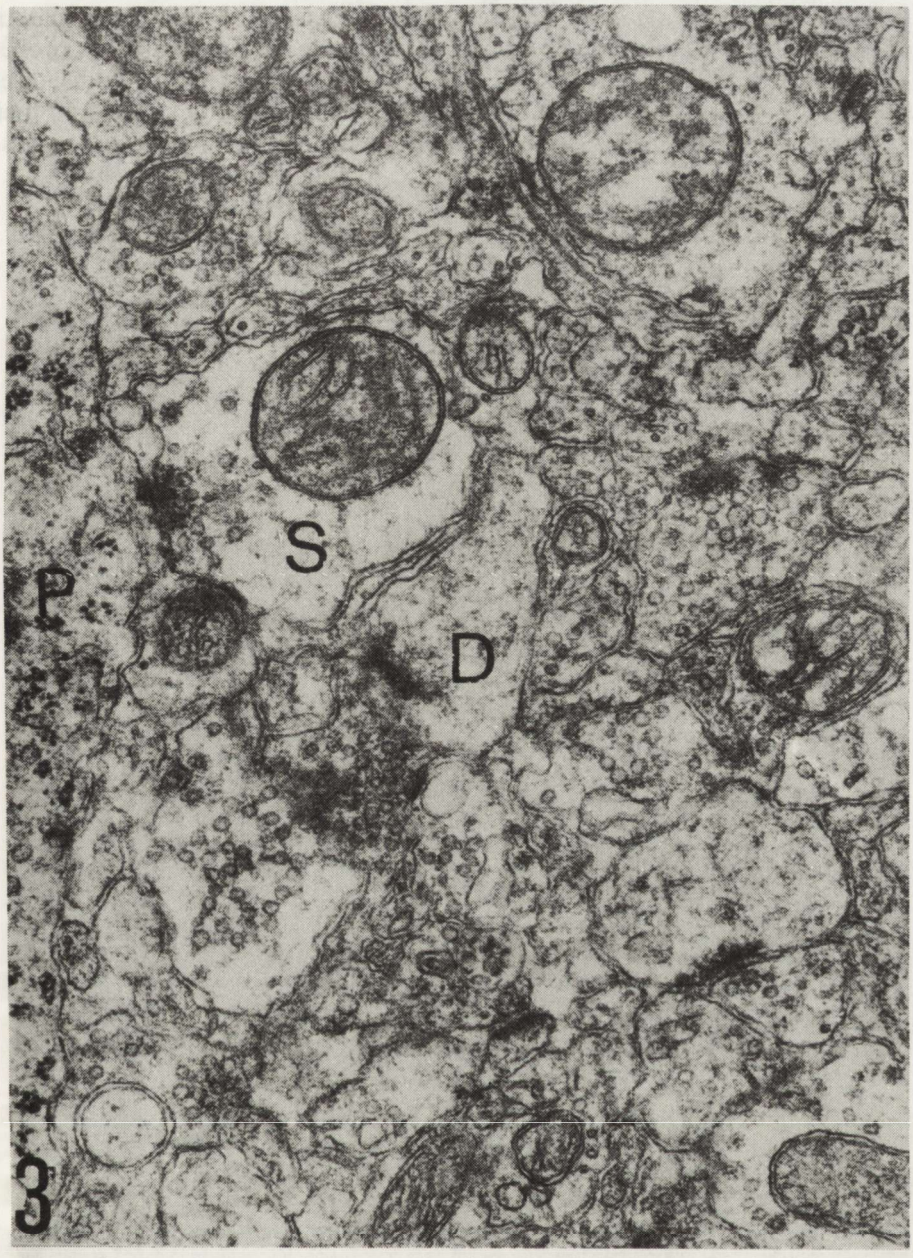

Fig. 3. Stratum pyramidale. Swollen axonal ending (S) contacting surface of the pyramidal neuron (P). In the neuropil some swollen dendritic spines (D) with normal presynaptic parts are visible. × 45 000

appearance (Fig. 1 and 2). A great part of asymmetric synapses, present in the neuropil surrounding pyramidal neurons, reveal severe ultrastructural abnormality, mostly in the form of swelling of the dendritic spines. Synapses are also encountered, although less frequently, which are swollen in the presynaptic

part, with only few synaptic vesicles usually aggregated near the synaptic density (Fig. 3). In the neuropil of this layer there are many greatly swollen dendrites with electron lucent vacuoles, few short, haphazardly dispersed neurotubules and swollen mitochondria (Fig. 4). The synaptic endings on their surface do not display any ultrastructural changes. Swollen astrocytes are also present in the neuropil of this layer (Figs. 1 and 4).

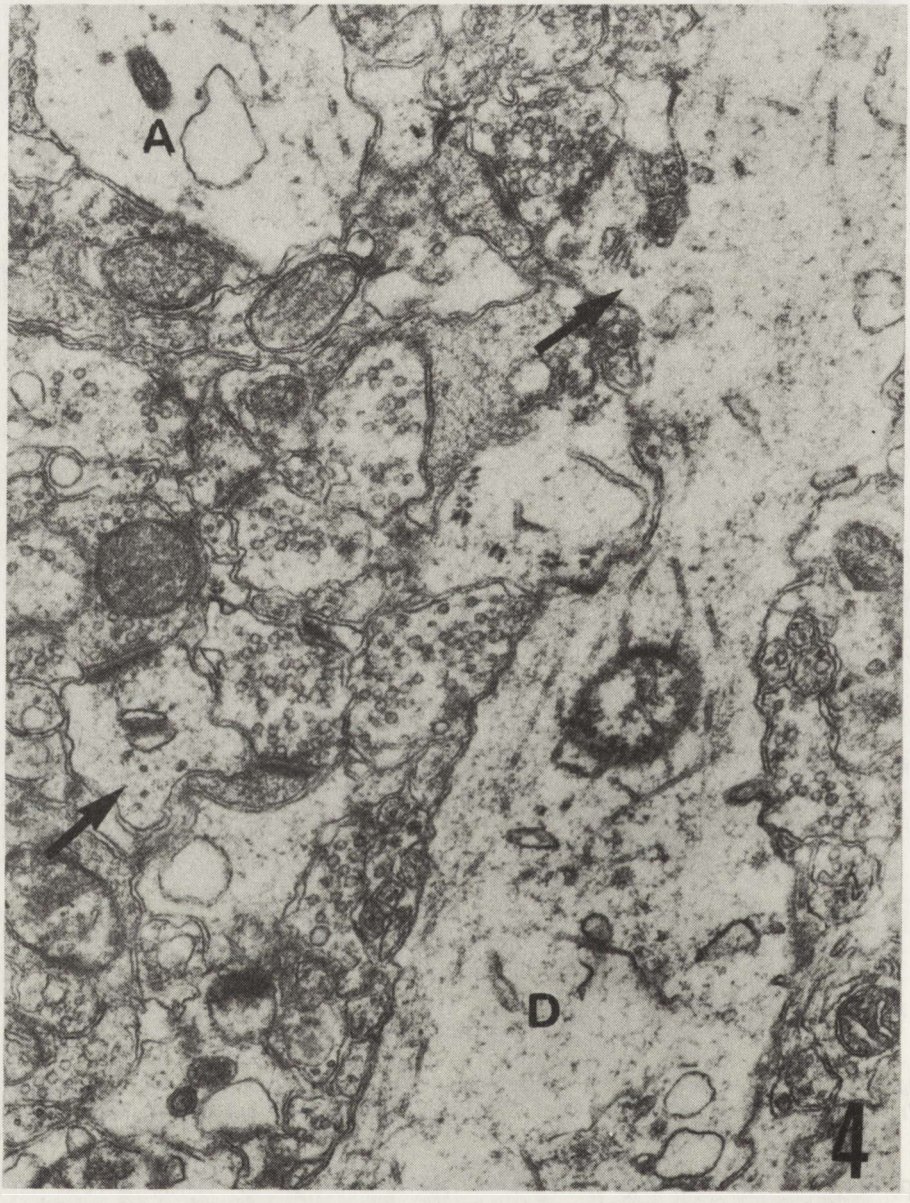

Fig. 4. Stratum pyramidale. Swollen dendrite (D) with fragments of cytoskeletal elements, swollen mitochondria and electron-lucent vacuoles. Note swollen dendritic spine (arrow). Most of the axonal endings are unchanged. Some astrocytic processes (A) are swollen. × 27000

Stratum oriens. In this layer the great majority of synapses contacting basal dendrites of the pyramidal cell represent axonal endings of interneurons and granular cells. Most of symmetrical and asymmetrical synapses preserve normal structure in both pre- and postsynaptic parts (Fig. 6). However, there

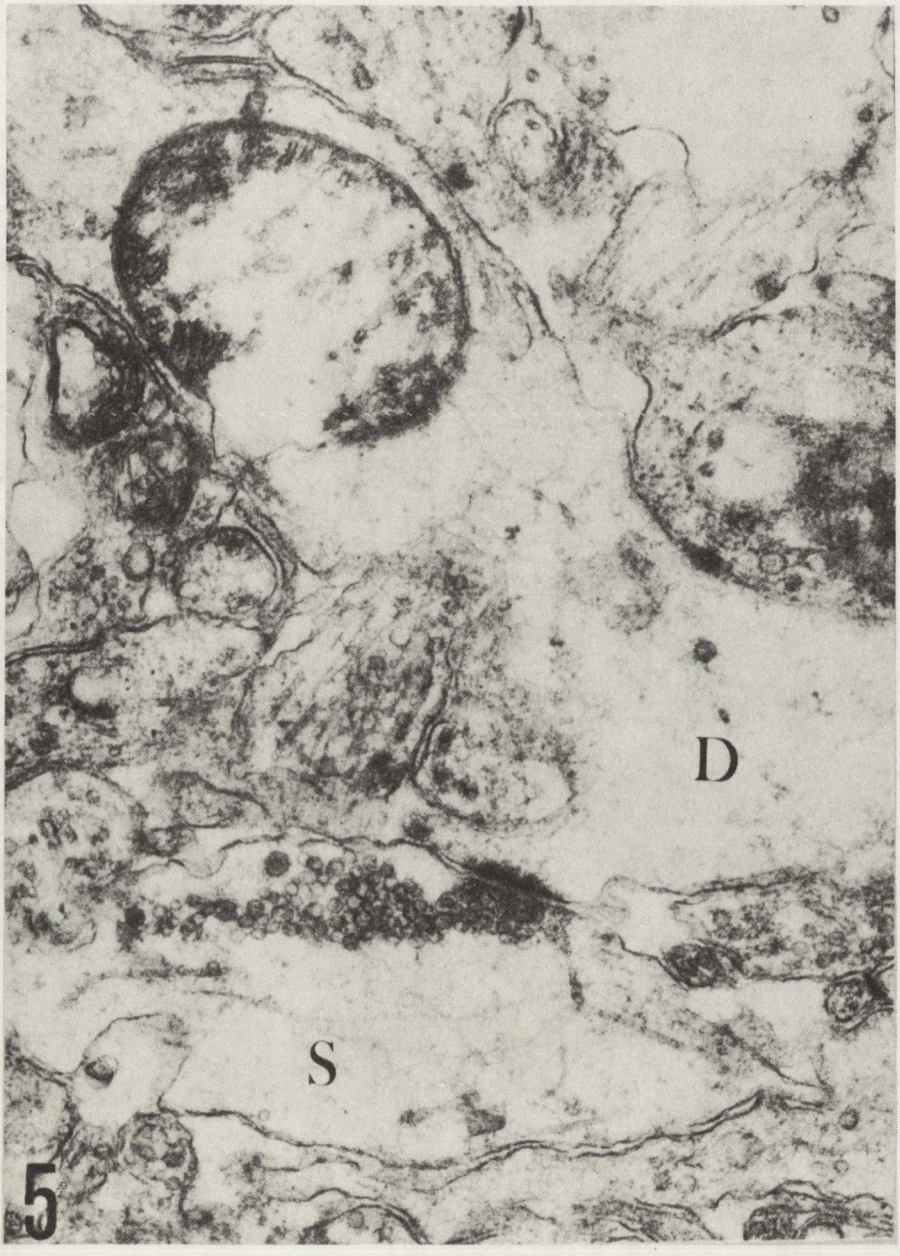

Fig. 5. Stratum oriens. Remarkable swelling of presynaptic (S) and postsynaptic parts (D) of a synapse. Note abnormal clumping of synaptic vesicles in the axonal ending and mitochondrial swelling in the dendrite. × 45 000

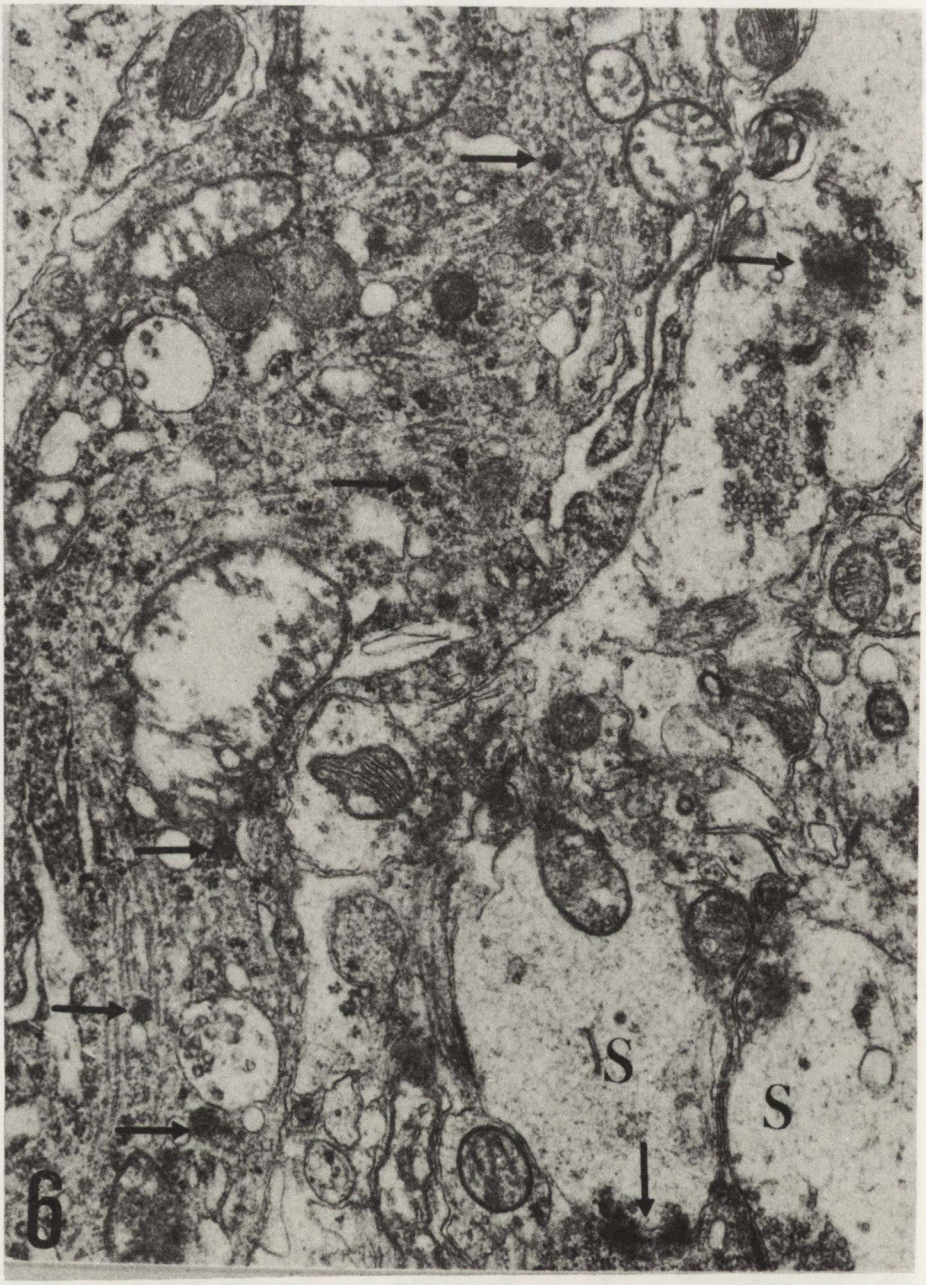

Fig. 6. Stratum radiatum. Fragment of apical dendrite with condensed cytoplasm. Dendritic cytoplasm contains abundant organelles, swollen mitochondria and unbound clusters of electron-dense material (arrows). In swollen presynaptic parts (S) abnormal aggregations of synaptic vesicles and small clusters of dense material (arrows) are present. × 30 000

also are some swollen synapses. Swelling involves both basal pyramidal dendrites and their branchings as well as presynaptic endings in which most of the vesicles accumulate in the vicinity of the active region of the synaptic cleft.

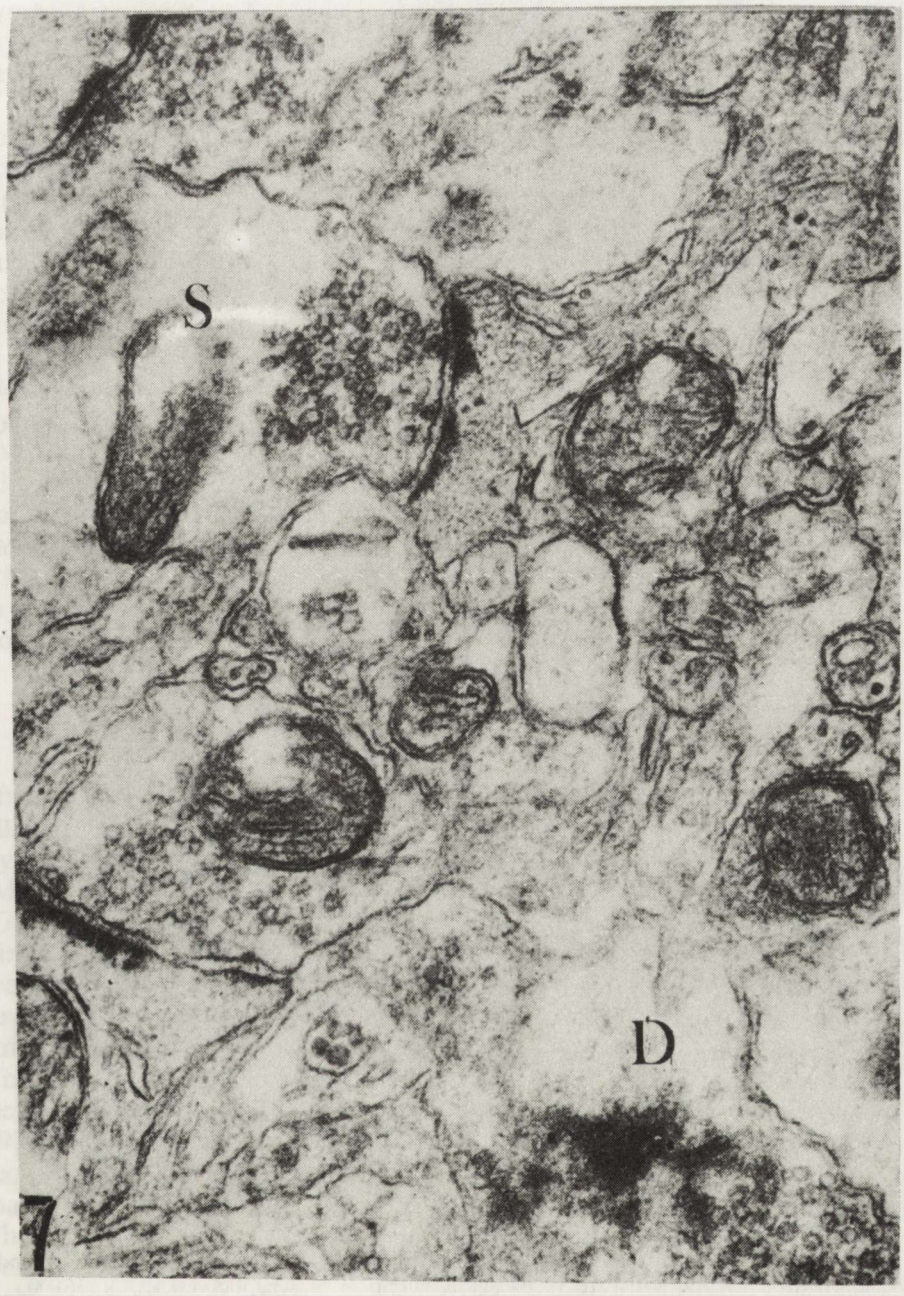

Fig. 7. Stratum lacunosum-moleculare. In the neuropil numerous synapses with swollen both pre- (S) and post-synaptic (D) parts are visible. × 60 000

This concerned the symmetrical as well asymmetrical synapses (Fig. 5). Many astrocytes in this layer display remarkable swelling.

Stratum radiatum. Synaptic contacts present in this layer are mostly formed by nerve endings of Schaffer's collaterals and interneurons as well as nerve

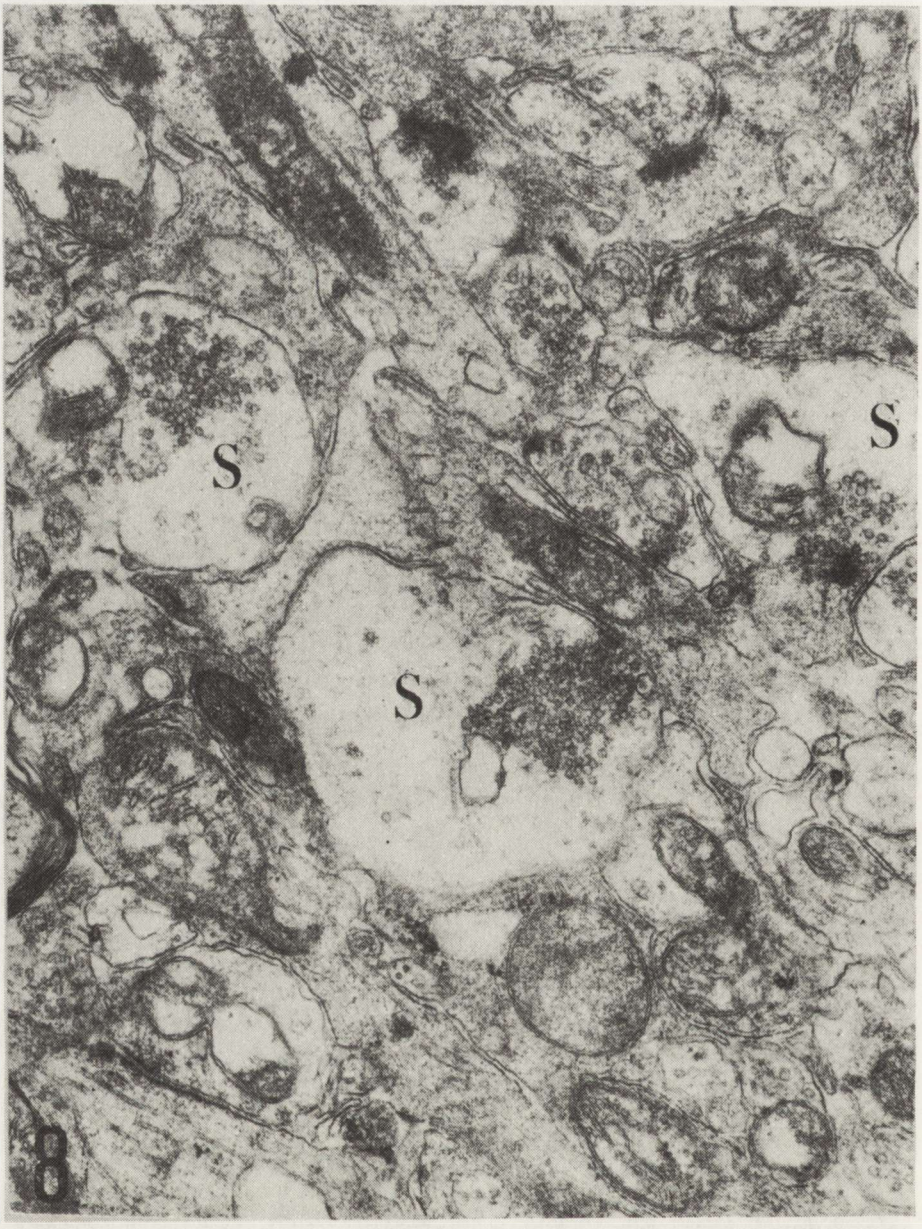

Fig. 8. Stratum moleculare. In the neuropil numerous synapses with swollen presynaptic (S) and unchanged postsynaptic parts are visible. Note clumping of synaptic vesicles. × 36 000

fibers deriving from the dentate gyrus. Apical dendrites of pyramidal neurons, accumulated here usually display some ultrastructural changes. Their condensed cytoplasm contains many organelles and elements of cytoskeleton. Small, unbound aggregates of high electron density are present (Fig. 6). Almost all mitochondria are swollen. Synaptic bulbs contacting such dendrites are also swollen. They contain few synaptic vesicles which accumulate near the active

synaptic region and deposits of electron-dense material (Fig. 6). In the same layer there are also normal synapses filled with a great abundance of synaptic vesicles. Some glial cells also show swelling.

Stratum lacunosum-moleculare. Synapses in this layer are formed by nerve endings of commissural fibers, Schaffer's collaterals and interneurons. They contact here branches of apical dendrites of pyramidal cells. A characteristic feature of the layer are "en passant" synapses formed by fibers deriving from interneurons. Most of the synapses present here, representing both symmetrical and asymmetrical types, usually preserve normal ultrastructure. However, some synapses with remarkably swollen both pre- and postsynaptic part are visible (Fig. 7).

Stratum moleculare. The anatomical composition of this layer is almost identical with *stratum lacunosum-moleculare.* The postsynaptic pool is formed by distal branchings of apical dendrites of the pyramidal cells. The nerve endings contacting them derive from interneurons and Schaffer's collaterals. The additional source of nerve endings consists of fibers originating from the entorhinal cortex. The ultrastructure of this layer is similar to that in *stratum lacunosum-moleculare.* In numerous presynaptic bags swelling of synaptoplasm with abnormal accumulation of synaptic vesicles is observed. (Fig. 8). Changes in postsynaptic parts are less common. In quite a number of otherwise normally looking dentrites clusters of electron-dense material are present.

DISCUSSION

Electron microscopic analysis of the CA_1 sector of Ammon's horn 3 days after the short-term ischemic incident revealed ultrastructural abnormalities in all cortical layers, involving both neuronal perikarya and synapses. Changes of pyramidal neurons were identical with those described in our previous studies (Mossakowski et al. 1989). Comparing with earlier postischemic stages (Gajkowska et al. 1989) they were more advanced and consisted mostly in disintegration of cellular cytoplasm, accompanied by accumulation of electron-dense, unbound aggregates, considered as calcium deposits. Great variance in the intensity of neuronal damage was noted. The ultrastructural picture of small interneurons located in the pyramidal layer deserves a short comment. These GABA-ergic neurons are usually considered as relatively resistant to the ischemic incident (Johansen et al. 1983; Mossakowski et al. 1989; Nitsch et al. 1989). Our previous study, concerning early postischemic changes in the CA_1 sector of Ammon's horn (Gajkowska et al. 1989) showed the appearance of marked, although reversible, alterations of the pyramidal layer interneurons. They consisted in severe swelling of cytoplasm and processes 12 h after the ischemic incident. Their ultrastructural picture returned to normal 24 h after ischemia. Our present study showed that 3 days after short-term ischemia some of the interneurons showed pronounced abnormalities in the form of mitochondrial swelling, an increased number of lysosomes and rarefaction of the peripheral part of their cytoplasm with reduction of endoplasmic reticulum channels, their denudation and decreased number of mono- and polyribosomes. This indicates that, contrary to previous opinions (Francis, Pulsinelli, 1982; Johansen et al. 1983, Mossakowski et al. 1989), interneurons of the CA_1

sector are also sensitive to the ischemic incident. Comparison of their early and late postischemic changes indicate that ultrastructural features of their alteration appear in the early recirculation stage and are less intensive as compared with lesions of pyramidal neurons.

Ultrastructural abnormalities of synapses concern both those of symmetrical type, located mostly on neuronal perikarya and dendritic shafts and asymmetrical type connected first of all with dendritic spines. Pre- and postsynaptic parts are involved, although their alterations vary in intensity, ultrastructural expression and localization. Two ultrastructural features were common for both pre- and postsynaptic parts. These were swelling and appearance of electron-dense unbound material, identified as calcium aggregates. Swollen axonal endings revealed additionally reduced contents of synaptic vesicles, being usually clumped in the vicinity of active zones. Postsynaptic lesions took the form of cytoplasmic swelling of dendrites and/or their spines, with accompanying damage of cytoskeletal structures, mitochondrial swelling and dilatation and fragmentation of endoplasmic reticulum channels. The great variability of the above described synaptic changes is to be stressed. Alongside with normal synapses there were synapses with remarkably swollen both pre- and postsynaptic parts or synapses in which ultrastructural abnormalities appeared only in the presynaptic or postsynaptic parts. In some cases calcium deposits were present in otherwise normally looking synapses.

Abnormalities observed 3 days after the ischemic incident differed from those occurring in the early postischemic period (Gajkowska et al. 1989). At that time swelling of postsynaptic parts with mostly intact axonal endings dominated. This corresponded to axon-sparing synaptic damage, typical for abnormalities resulting from the excitotoxic action of neurotransmitters (Schwarcz et al. 1983). Distribution of the ultrastructural abnormalities observed was also different. In an early postischemic period pathological changes prevailed *in stratum lacunosum-moleculare* and *stratum moleculare*, in which most of the axonal endings of Schaffer's collaterals are located. In the presently examined material abnormalities in these layers, although present were less conspicuous than in the *stratum pyramidale*, *oriens* and *radiatum*. Here, in addition to alterations involving small dendritic branchings and spines, lesions of large shafts of basal and apical dendrites were observed. The nature of the latter differed in both layers. In *stratum oriens* swollen basal dendrites of the pyramidal neurons dominated, while in *stratum radiatum* apical dendrites with condensed cytoplasm prevailed.

In the light of advanced damage of pyramidal neurons in the examined postischemic period, the question arises to what extend dendritic abnormalities, especially those concerning large apical and basal shafts can be considered just as an element of progressing neuronal injury involving both the perikarya and processes and leading to cellular death or as an exponent of the specific mechanism of this damage connected with neurotransmitter toxicity. Delimitation of these two components seems impossible, due to the complexity of structural changes and mechanisms involved. Involvement of both synaptic parts indicates that we are dealing with a process more complicated than pure axon-sparing synaptic damage, related with the excitotoxic action of an amino acid neurotransmitter.

Swelling of nerve endings, changes in the content and distribution of synaptic vesicles may reflect both disturbances in neurotransmission in the sense of exhaustion due to oversecretion or disturbances in membrane permeability resulting from ischemia. The same may concern postsynaptic changes. Mitochondrial abnormality may support the latter mechanism. Horseradish peroxidase studies performed by Diemer and Ekström von Lubitz (1983) revealed abnormalities in synaptic membrane permeability in the CA_1 hippocampal sector in the case of cerebral ischemia. However, the permeability changes described by them concerned mostly postsynaptic membranes with very little involvement of axonal endings and they appeared in much earlier postischemic period.

It seems that the synaptic abnormalities observed in our material correlate well with the general concept of the excitotoxic mechanism of delayed neuronal death. This concerns both elements of synaptic pathology – calcium deposition and swelling of the pre- and postsynaptic parts.

The metabolic cascade initiating delayed death of hippocampal pyramidal neurons begins by a decrease of adenosine triphosphate reserves (Norberg, Siesjö 1975; Onodera et at. 1986), accompanied by accumulation of calcium ions in presynaptic endings and increased production of glutamate (Benveniste et al. 1984). Influx of Ca^{2+} at the nerve terminals triggers synaptic release of neurotransmitter (Rubin 1970). Increased release of glutamate and its reduced re-uptake due to lack of energy resources (Choi 1987) result in an enhanced neurotransmitter content in the extracellular spaces. This phenomenon was observed during ischemia in selectively vulnerable areas of the hippocampus by Benveniste et al. (1984) and Hagberg et al. (1985). Moreover, there seem to exist a close correlation between disorders in calcium homeostasis and the level of excitotoxic amino acids preceding delayed neuronal death in the CA_1 hippocampal sector following cerebral ischemia (Sakamoto et al. 1986; Ogura et al. 1988).

Glutamate accumulated in the synaptic clefts induces activation of glutaminergic receptors, this being accompanied by an increased influx of calcium ions to the postsynaptic part (Sakamoto et al. 1984). Initiated in that way intracellular calcium accumulation activates Ca-dependent enzymes, among others proteases and phospholipase A_1 leading in turn to disintegration of cellular membranes (Abe et al. 1987) and cytoskeletal elements (Yanagihara et al. 1985). Progressing cellular disintegration, connected with calcium accumulation leads finally to neuronal death (Siesjö 1981; Van Reempts et al. 1986; Deshpande et al. 1987). All the above presented data clearly indicate that calcium entry into the cell is a major mediator of the glutamate destructive effect.

Intracellular calcium entry is accompanied by influx of other ions from the extracellular space, mostly sodium and chlorides. This is followed by water redistribution between extra- and intracellular compartments resulting in cellular swelling.

Most probably the process concerns synapses of excitatory character, although it is difficult to exclude synapses of inhibitory nature. Some immunocytochemical studies indicate the coexistence of neurotransmitters of both excitatory and inhibitory character in synapses, deriving from interneurons and contacting the perikarya and dendritic shafts of pyramidal neurons (Somogyi et al. 1984; Kosaka et al. 1985). These observations suggest the need of modification of the traditional morphological classification of hippocampal synapses, even more so, since there are many controversial morphological descriptions of them (Chang, Greenaught 1984). These controversies concern mostly differences in distribution and density of synaptic vesicles considered as morphological exponents of neurotransmission processes (Applegate et al. 1987).

WPŁYW KRÓTKOTRWAŁEGO NIEDOKRWIENIA NA ULTRASTRUKTURĘ ZAKRĘTU HIPOCAMPA U CHOMIKÓW MONGOLSKICH III SYNAPSY W PÓŹNEJ FAZIE PROCESU PATOLOGICZNEGO

Streszczenie

Przeprowadzono elektronowo-mikroskopową analizę sektora CA_1 rogu Ammona u chomików mongolskich po upływie 3 dni od krótkotrwałego niedokrwienia kresomózgowia. Wykazano cechy rozpadu neuronów piramidowych oraz zróżnicowanego nasilenia uszkodzenia interneuronów. Spostrzeżenie to podważa pogląd o oporności na niedokrwienie GABA-ergicznych interneuronów sektora CA_1 hipokampa. Synapsy położone we wszystkich warstwach sektora Ca_1 wykazywały ultrastrukturalne cechy uszkodzenia, obejmujące zarówno ich części przed- jak i posynaptyczne. Wyrażały się one obrzmieniem oraz nagromadzeniem nieobłonionego, elektronowo gęstego materiału, ocenianego jako złogi wapnia. Części presynaptyczne wykazywały ponadto zmniejszenie liczby pęcherzyków synaptycznych i ich nieprawidłowe rozmieszczenie. W odróżnieniu od wczesnego okresu poniedokrwiennego nasilone zmiany synaps występowały przede wszystkim w *stratum pyramidale*, *radiatum* i *oriens* i dotyczyły obok drobnych rozgałęzień dendrytycznych ich kolców, również dużych pni dendrytów podstawowych i szczytowych. Nieprawidłowości obrazu synaps, zwłaszcza przyjmujące postać uszkodzeń postsynaptycznych, mogą stanowić wykładnik ekscytotoksycznego mechanizmu zmian neuronalnych. Zmiany części presynaptycznych mogą zarówno wyrażać zaburzenia procesów neurotransmisji w sensie ich wyczerpania, jak i uszkodzenia związane bezpośrednio z niedokrwieniem. Nagromadzenie w nich złogów wapnia wydaje się przemawiać na korzyść pierwszego mechanizmu.

REFERENCES

1. Abe K, Kogure K, Yamamoto H, Imazawa M, Miyamoto K: Mechanism of arachidonic acid liberation during ischemia in gerbil cerebral cortex. J Neurochem, 1987, 48, 503–509.
2. Applegate MD, Kerr DS, Landfield PW: Redistribution of synaptic vesicles during long-term potentiation in the hippocampus. Brain Res, 1987, 401, 401–406.
3. Baudry M, Lynch GS: Regulation of hippocampal glutamate receptors: evidence for the involvement of a calcium-activated protease. Proc Natl Acad Sci USA, 1980, 77, 2298–2302.
4. Baudry M, Bundmann MC, Smith EK, Lynch GS: Micromolar calcium stimulates proteolysis and glutamate binding in rat brain synaptic membranes. Science, 1981, 212, 937–938.
5. Benveniste H, Drejer J, Schousboe A, Diemer NH: Elevation of the external concentrations of glutamate and aspartate in rat hippocampus during transient cerebral ischemia monitored by intracerebral microdialysis. J Neurochem, 1984, 43, 1369–1374.
6. Chang FLF, Greenaugh WT: Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice. Brain Res, 1984, 309, 35–46.
7. Choi DW: Ionic dependence of glutamate neurotoxicity. J Neurosci, 1987, 7, 369–379.

8. Collingridge GL, Kehl SJ, Loo R, McLennan H: Effects of kainic acid and other amino acids on synaptic excitation of rat hippocampal slices. 1. Extracellular analysis. Exp Brain Res, 1983, 52, 170–178.
9. Deshpande JK, Siesjö BK, Wieloch T: Calcium accumulation and neuronal damage in the rat hippocampus following cerebral ischemia. J Cereb Blood Flow Metab, 1987, 7, 89–95.
10. Diemer NH, Ekström von Lubitz DKJ: Cerebral ischemia in the rat: increased permeability of postsynaptic membranes to horseradish peroxidase in the early postischemic period. Neuropathol Appl Neurobiol, 1983, 9, 403–414.
11. Francis A, Pulsinelli WA: Response of GABA-ergic and cholinergic neurons to transient cerebral ischemia. Brain Res, 1982, 243, 271–278.
12. Gajkowska B, Gadamski R, Wawrzyniak E: Wpływ krótkotrwałego niedokrwienia na ultrastrukturę zakrętu hipokampa u chomików mongolskich. I. Ultrastrukturalna charakterystyka odcinka CA_1 rogu Amona ze szczególnym uwzględnieniem obrazu połączeń synaptycznych. Neuropatol Pol, 1988, 26, 455–476.
13. Gajkowska B, Gadamski R, Mossakowski MJ: Wpływ krótkotrwałego niedokrwienia na ultrastrukturę zakrętu hipokampa u chomików mongolskich. II. Obraz mikroskopowoelektronowy synaps we wczesnym okresie poniedokrwiennym. Neuropatol Pol, 1989, 27, 339–366.
14. Hagberg H, Lehmann A, Sandberg M, Nystrom B, Jacobson I, Hamberger A: Ischemia-induced shift of inhibitory and excitatory amino acids from intra- to extracellular compartments. Cereb Blood Flow Metab, 1985, 5, 413–419.
15. Johansen FF, Jorgensen MB, Diemer NH: Resistance of hippocampal CA_1 interneurons to 20-min transient cerebral ischemia in the rat. Acta Neuropathol (Berl), 1983, 61, 135–140.
16. Jörgensen MB, Diemer NH: Selective neuron loss after cerebral ischemia in the rat: possible role of transmitter glutamate. Acta Neurol Scand, 1982, 66, 536–546.
17. Kirino T: Delayed neuronal death in the gerbil hippocampus following ischemia. Brain Res, 1982, 239, 57–69.
18. Kosaka T, Kosaka K, Tateishi K, Hamaoka Y, Yanaihara N, Wu JY, Hama K: GABAergic neurons containing CCK-8-like and/or VIP-like immunoreactivities in the rat hippocampus and dentate gyrus. J Comp Neurol, 1985, 239, 420–430.
19. Łazarewicz JW, Strosznajder J, Dąbrowiecki Z: Effect of cerebral ischemia on calcium transport in isolated brain mitochondria. In: Prac. VIIth Internat Congress of Neuropathology, Excerpta Medica, Amsterdam, 1975, pp. 605–608.
20. Mossakowski MJ, Gajkowska B, Tsitsishvili A: Ultrastructure of neurons from CA_1 sector of Ammon's horn in short-term cerebral ischemia in Mongolian gerbil. Neuropatol Pol, 1989, 27, 39–53.
21. Nitsch C, Scotti A, Sommacal A, Kalt G: GABAergic hippocampal neurons resistant to ischemia-induced death contain the Ca^{++}-binding protein parvalbumin. Neurosci Lett, 1989, 263–268.
22. Norberg K, Siesjö BK: Cerebral metabolism in hypoxic hypoxia. I Pattern of activation of glycolysis: a reevaluation. Brain Res, 1975, 86, 31–44.
23. Ogura A, Miyamoto M, Kudo Y: Neuronal death *in vitro*: parallelism between survivability of hippocampal neurons and sustained elevation of cytosolic Ca^{2+} after exposure to glutamate receptor agonist. Exp Brain Res, 1988, 73, 447–458.
24. Onodera H, Sato G, Kogure K: Lesions of Schaffer collaterals prevent ischemic death of CA_1 pyramidal cells. Neurosci Lett, 1986, 68, 169–174.
25. Pulsinelli WA: Selective neuronal vulnerability morphological and molecular characteristics. Prog Brain Res, 1985a, 63, 29–31.
26. Pulsinelli WA: Deafferentation of the hippocampus protects Ca_1 pyramidal cells against ischemic injury. Stroke, 1985b, 16, 144–146.
27. Rubin RP: The role of calcium in the release of neurotransmitter substances and hormones. Pharmacol Rev, 1970, 22, 389–423.
28. Sakamoto N, Kogure K, Kato H, Ohtomo H: Disturbed Ca^{2+} homeostasis in the gerbil hippocampus following brief transient ischemia. Brain Res, 1986.
29. Schwarcz R, Whetsell WD, Mangano RM: Quinolinic acid: an endogenous metabolite that produces axon-sparing lesions in rat brain. Science, 1983, 219, 316–318.
30. Siesjö BK: Cell damage in the brain. A speculative synthesis. J Cereb Blood Flow Metab, 1981, 1, 155–185.

31. Siesjö BK, Wieloch T: Cerebral metabolism in ischemia: neurochemical basis for therapy. Br J Anaesth, 1985, 57, 47–62.
32. Simon RP, Swan JH, Griffiths BS: Blockade of N-methyl-D-aspartate receptors may protect against ischemic damage in the brain. Science, 1984, 226, 850–852.
33. Strosznajder J: Role of phospholipids in calcium accumulation in brain mitochondria from adult rat after ischemic anoxia and hypoxic hypoxia. Bull Acad Pol Sci Ser Sci Biol, 1980, 27, 683–692.
34. Somogyi P, Hodgson AJ, Smith AD, Nunzi MG, Gorio A, Wu Y: Different populations of GABAergic neurons in the visual cortex and hippocampus of the cat contain somatostatin or choleceptokinin-immunoreactive material. J Neurosci, 1984, 4, 2590–2603.
35. Suzuki R, Yamaguchi T, Choh-Luh Li, Klatzo I: The effects of 5-minute ischemia in Mongolian gerbils: II. Changes of spontaneous neuronal activity in cerebral cortex and CA_1 sector of hippocampus. Acta Neuropathol (Berl), 1981, 60, 217–222.
36. Van Reempts J, Haseldonickx M, Van Deuren B, Wouters L, Borges M: Structural damage of the ischemic brain: Involvement of calcium and effects of postischemic treatment with calcium entry blockers. Drug Dev Res, 1986, 8, 387–395.
37. Yanagihara T, Yoshimine T, Morimoto K, Yamamoto K, Homburger HA: Immunohistochemical investigation of cerebral ischemia in gerbils. J Neuropathol Exp Neurol, 1985, 44, 204–215.

Authors' address: Medical Research Centre, PASci, 3 Dworkowa Str, 00-784 Warsaw, Poland

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

IRENA NIEBRÓJ-DOBOSZ, AYNALEM G. MARIAM, MIROSŁAWA ŁUKASIUK, JANINA RAFAŁOWSKA

BLOOD-CEREBROSPINAL FLUID BARRIER INTEGRITY IN CEREBRAL INFARCTION*

Deparment of Neurology, School of Medicine, Warsaw

Using a sensitive isotachophoretic technique total cerebrospinal fluid protein, CSF-serum albumin and CSF-serum IgG ratios as indicators of blood-CSF barrier integrity were determined in 35 cases of ischemic cerebral infarction. Since it proved to be changed in about 57% of these patients, the CSF-serum albumin ratio was found to be the most sensitive parameter in evaluating the blood-CSF barrier disturbances.

No clear-cut correlation was found between the age of patients, the clinical course of the disease, different periods after onset of the illness nor the size of the infarction and CSF-blood barrier permeability.

Key words: *blood-cerebrospinal fluid barrier, ischemic cerebral infarction.*

The blood-brain barrier integrity in cerebrovascular diseases, including ischemic infarction, is still a controversial subject (for review see Gumerlock, 1989). Generally the blood-brain barrier disturbances appear within a few hours after acute ischemia (Katzman et al. 1977; Hornig et al. 1983). The most sensitive test indicating the increase in blood-brain barrier permeability proved to be the altered CSF-serum albumin ratio (Al-Kassab et al. 1981; Hornig et al. 1983). However, in about 50% of patients with ischemic cerebral infarction the CSF-serum albumin ratio is found to be normal. It was tried to answer the question in this report whether evaluation of the blood-brain barrier could be improved by using the sensitive isotachophoretic technique for separation of proteins.

MATERIAL AND METHODS

The material consisted of 35 patients, 57 to 87 yrs of age. The diagnosis was established by clinical examination, computed tomography, and in some, by autopsy (5 cases). Ten age-matched patients with no symptoms of central nervous system involvement were used as controls. Lumbar puncture was

* The study was supported by the Polish Academy of Sciences (No. of the agreement: 06.02.II.1.4).

performed as a diagnostic procedure and CSF obtained. Venous blood was drawn at the same time.

The protein composition was evaluated by analytic isotachophoresis using a LKB 2127 Tachophor with UV and thermal detectors, 23 cm length teflon capillary tubing (0,5 mm I.D.), thermostated at 12°C and LKB 2-channel 2210 Recorder (Delmotte 1977). Glycine, valine and beta-alanine were used as amino acid spacers. Two to three μg of serum or CSF proteins were injected. The latter was concentrated 10 to 20 times in vacuum with constant cooling. The area of the isotachophoretic curves was measured by an image analyser system Mini-Mop M1109. Delmotte's formula (1977) to calculate the blood-CSF permeability and Link's index (Link, Tibbling 1977) for quantification of IgG synthesis were used. Total protein content in serum and CSF was estimated by the method of Lowry et al. (1951). Welch's test (1978) was used for statistical analysis as the differences between variances appeared to be significant.

RESULTS

The total protein content was increased in 54.3% of patients, while in 57.1% the albumin ratio, in 28.6% IgA, in 14.3% IgG anodic, in 22.9% IgG fast cathodic and in 8.6% the IgG slow cathodic fractions were changed (Table 1). No clear-cut correlation was found between CSF total protein

Table 1. Total protein content and CSF-serum protein ratios in cerebral infarction

	Cerebral infarction		Normals
Total protein mg %	56.6 ± 23.9	$P < 0.05$	27.3 ± 10.0
Albumin ratio	5.9 ± 4.2	$P < 0.005$	2.6 ± 0.9
IgA ratio	3.7 ± 2.3	n.s.	2.8 ± 0.9
IgG_{anodic} ratio	2.4 ± 1.3	n.s.	1.5 ± 0.8
$IgG_{fast\ cathodic}$ ratio	1.8 ± 1.7	n.s.	0.9 ± 0.7
$IgG_{slow\ cathodic}$ ratio	1.4 ± 0.9	n.s.	0.7 ± 0.6
IgG_{index}	0.6 ± 0.5	n.s.	0.4 ± 0.1

The values are means ± SD

content and the albumin ratio. There was a highly elevated albumin ratio in 22.9% of cases with completely normal CSF protein level, while in 25.7% with normal albumin ratio, the protein content was elevated.

However, a significant correlation was observed between the CSF protein content and IgA ratio ($p < 0.04$). An increase of the albumin ratio was usually followed by an increase of IgA ($p < 0.08$) and the IgG anodic fraction ($p < 0.009$). When the IgG anodic fraction was increased, a rise of the IgG fast cathodic fraction was found ($p < 0.004$). Link's index was found to be increased in 28.6% of cases.

A representative isotachophoretic protein pattern of CSF in cerebral infarction is presented on Figure 1.

No clear-cut correlation between the various CSF-serum protein ratios and the age of the patients, the day of lumbar puncture after stroke onset, the clinical course of the disease and the size of the infarct was observed. Neither was any relation found between the presence of concomitant diseases such as hypertension, diabetes, etc. and CSF-serum protein ratios.

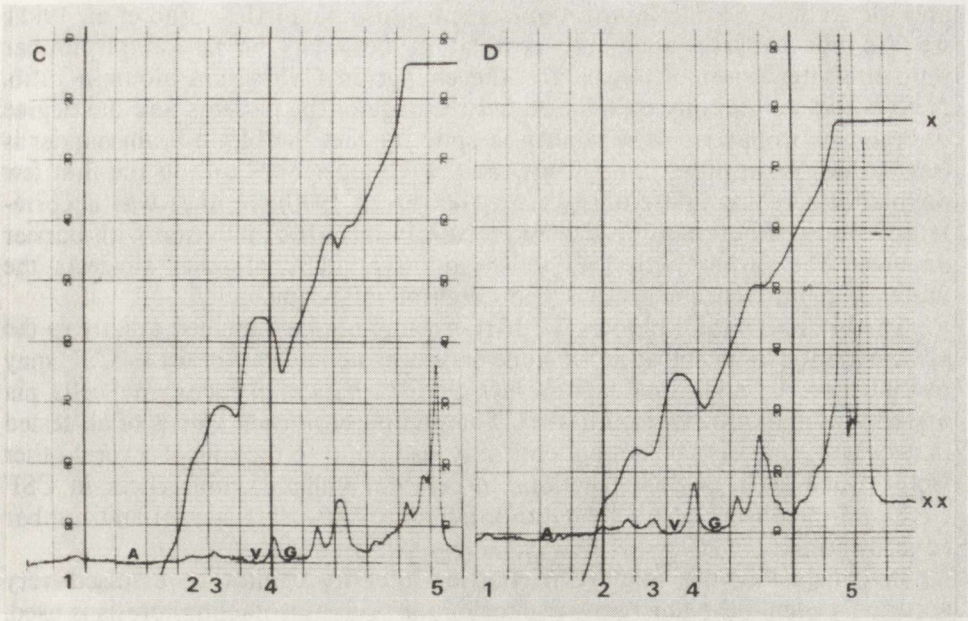

Fig. 1. Capillary isotachophoresis pattern of CSF proteins in patients with ischemic infarction. The isotachophoretic curves were recorded from right to left using UV and thermal detectors. C – normal CSF, D – patient with cerebral infarction. Changes in albumin level are observed. 1 – $IgG_{cathodic\ slow}$, 2 – $IgG_{cathodic\ fast}$, 3 – IgG_{anodic}, 4 – IgA, 5 – albumin, A – β-alanine, V – valine, G – glycine

DISCUSSION

The proper composition of the CSF and also of the brain tissue is maintained by CSF-serum and CSF-brain barriers. The state of these barriers is influenced by several factors such as individual variation in the capacity of CSF protein turnover, the barrier tightness and rate of CSF secretion and drainage. The blood-brain barrier integrity depends also upon age, as it is easily damaged in human foetuses (Gröntoft 1954). When the integrity of these barriers is disturbed, exchange of some components takes place between the brain tissue and CSF and also between serum and CSF. Following blood-brain barrier damage penetration of water, electrolytes, proteins etc. from blood vessels to the surrounding environment occurs (Lee 1982). Two-directional exchange of biochemical components between the brain tissues and CSF can cause an increase of low molecular proteins, such as albumin, in the CSF (Mossakowski 1988). When using a radial immunodiffusion technique and immunoturbidimetric analyses, the CSF-serum albumin ratio appears to be the most sensitive parameter for assessment of disturbed barrier in ischemic cerebral infarction (Hornig et al. 1983). Although the most sensitive marker, the albumin ratio is altered in only 56% of patients, usually in the first two weeks of the disease and in large infarctions.

The results presented here indicate that the most sensitive tests in assessing the blood-CSF barrier in cerebral infarction are the CSF total protein content

and the serum-CSF albumin ratio. Contrary to others (Al-Kassab et al. 1981), we did not observe a definite correlation between the period of lumbar puncture after onset of stroke and the change in CSF-serum albumin ratio. Neither did we find any correlation between age of the patients and the degree of blood-brain barrier permeability, despite the fact the blood-brain barrier is less permeable in older age groups than in younger ones during the first few days after cerebral infarction (Rafałowska et al. 1990). Neither was a correlation observed between the size and localization of the infarction with barrier damage. The rather high IgG index found in 28% of cases suggests the presence of intrathecal synthesis in cerebral infarction.

The brain-CSF and blood-CSF barriers may not be damaged exactly to the same extent. The penetration of some serum proteins from brain to CSF may be influenced by additional factors such as disturbances in ependymal cells, pia mater and glial limiting membranes. The finding of normal values of all tested parameters in nearly 50% of patients may also point to the role of several other factors such as individual reactions to cerebral ischemia, differences in CSF circulation, reabsorption velocity and difference between cisternal and lumbar concentration.

In conclusion, the blood-CSF barrier integrity cannot be assessed very accurately even if the most sensitive technique such as isotachophoresis is used. Since assessment of the status of the blood-brain barrier in cerebrovascular diseases is of importance from the clinical point of view, more accurate parameters and methods in its evaluation should be serched for.

OCENA BARIERY KREW – PŁYN MÓZGOWO-RDZENIOWY W UDARZE NIEDOKRWIENNYM MÓZGU

Streszczenie

W 35 przypadkach udaru niedokrwiennego mózgu określono białko całkowite płynu mózgowo-rdzeniowego. Stosunek albuminy i IgG w surowicy oraz w płynie mózgowo-rdzeniowym oznaczono po zastosowaniu bardzo czułej metody izotachoforezy. Stwierdzono, że w 57% przypadków zmianie ulega stosunek albuminy. Jest to najbardziej czuły wskaźnik zmiany przepuszczalności bariery krew – płyn mózgowo-rdzeniowy. Nie stwierdzono żadnej korelacji między stopniem przepuszczalności bariery a wiekiem chorych, ostrością przebiegu klinicznego, czasem trwania choroby i wielkością zawału mózgu.

REFERENCES

1. Al-Kassab S, Skyhoj Olsen T, Bech Skriver E: Blood-brain barrier integrity in patients with cerebral infarction investigated by computed tomography and serum-CSF-albumin. Acta Neurol Scand, 1981, 64, 438–445.
2. Delmotte P: Analysis of complex protein mixtures by capillary isotachophoresis: Some qualitative and quantitative aspects. Sci Tools, 1977, 24, 33–41.
3. Gröntoft O: Intracranial haemorrhage and blood-brain barrier problems in new-born. Acta Pathol Microbiol Scand, 1954, 100 (suppl), 1–109.
4. Gumerlock MK: Cerebrovascular diseases and the blood-brain barrier. In: Implications of the blood-brain barrier and its manipulation. Clinical aspects. Ed: EA Neuwelt. Plenum Medical Book Company, New York, London, 1989, vol 2, 495–565.

5. Hornig CR, Busse O, Dorndorf W, Kaps M: Changes in CSF blood-brain barrier parameters in ischemic cerebral infarction. J Neurol, 1983, 229, 11–16.
6. Katzman R, Clasen R, Klatzo I, Meyer JS, Pappius HM, Walz AG: Brain edema in stroke. Stroke, 1977, 8, 510–540.
7. Lee JC: Anatomy of the blood-brain barrier under normal and pathological conditions. In: Histology and histopathology of the nervous system. Eds: W Haymaker, RD Adams. ChC Thomas, Springfield, Illinois, 1982, vol 1, 798–870.
8. Link H, Tibbling G: Principles of albumin and IgG analyses in neurological disorders. III. Evaluation of IgG synthesis within the central nervous system in multiple sclerosis. Scand J Clin Invest, 1977, 37, 397–401.
9. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Protein measurement with Folin phenol reagent. J Biol Chem, 1951, 193, 265–275.
10. Mossakowski MJ: Bariera krew-mózg. In: Histologia. Ed: K Ostrowski. PZWL, Warszawa, 1988, 289–290.
11. Rafałowska J, Dolińska E, Dziewulska D, Krajewski S: Human brain infarcts in middle and senile age. I. Blood-brain barrier permeability in immunocytochemical studies (in Polish). Neuropatol Pol, 1990, 28, 1–17.
12. Welch V: Welch's test. In: Grundlage der Statistik. Eds: G Klaus, H Ebner. Volk und Wissen Volkseigener Verlag, Berlin, 1978, pp 212–214.

Authors' address: Department of Neurology, la Banacha Str., 02-097 Warsaw, Poland

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

PAWEŁ P. LIBERSKI[1,2], BARBARA MIRECKA[1], JANUSZ ALWASIAK[1],
RICHARD YANAGIHARA[2], D. CARLETON GAJDUSEK[2]

EXPRESSION OF TUMOR NECROSIS FACTOR-ALPHA CACHECTIN IN PRIMARY BRAIN TUMORS OF ASTROCYTIC LINEAGE

[1]Electron Microscopic Laboratory, Dept. Oncology, School of Medicine, Lodz, Poland
[2]Laboratory of Central Nervous System Studies, National Institutes of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland, USA

The tumor necrosis factor alpha (TNF-alpha) is a cytokine released within central nervous system from activated astrocytes and macrophages and involved in several pathologic processes including AIDS-myelopathy, multiple sclerosis and myelin dilatation in panencephalic type of Creutzfeldt-Jakob disease. We studied the expression of the TNF-alpha in brain tumors. Only tumors of astrocytic lineage like astrocytomas and glioblastomas, or tumors of mixed lineage as oligo-astrocytomas and multipotential primitive neuroectodermal tumors (PNET) expressed TNF-alpha-like immunoreactivity. We conclude that this lymphokine is expressed in neoplastic astrocytes.

We postulate that TNF-alpha may participate in neoplastic transformation of astrocytes via cascade of interactions with receptor for TNF-alpha.

Key words: *tumor necrosis factor-alpha, TNF-alpha, astrocytes, brain tumors.*

The tumor necrosis factor (TNF)-alpha/cachectin is a lymphokine mediating several responses to infection and cancer (Le, Vilcek 1987; Frei, Fontana 1989; Kunkel et al. 1989; Tracey, Cerami 1989, 1990; Tracey et al. 1989; Old 1990; Schleuning, Minker 1990). In the central nervous system TNF-alpha is released from activated astrocytes and macrophages/monocytes (Robbins et al. 1987), and the role of TNF in demyelinating diseases such as multiple sclerosis (Brosnan et al. 1988; Selmaj, Raine 1988; Hauser et al. 1990), and AIDS-related vacuolar myelopathy (Maier et al. 1989; Tracey, Cerami 1989; Grimaldi et al. 1990) has recently been suggested.

Presented in part at the XVII Meeting of the Polish Oncological Society, held in Poznań, September 20–22, 1990 and at the Joint Meeting of the Polish Neurosurgeons and the Association of Polish Neuropathologists in commemoration of Professor Adam Kunicki, held in Warsaw, December 8th, 1990.

Previously we reported the immunohistochemical demonstration of TNF-alpha in astrocytes from mice infected experimentally with the Fujisaki strain of Creutzfeldt-Jakob disease virus, suggesting that this monokine may be the first indentified biologically active molecule mediating the formation of intramyelin vacuoles in this disease (Liberski et al. 1989, 1990). In this communication we report the immunohistochemical evidence of TNF-alpha in human brain tumors of astrocytic origin and suggest its wide role in brain pathologies.

MATERIAL AND METHODS

Material: Fresh samples of 19 brain tumors were obtained during surgery at the Department of Neurosurgery, Regional Multidisciplinary Hospital (Copernicus Hospital, Łódź).

Light microscopy: Surgical specimens were fixed in 10% formalin and embedded in paraffin. To establish the histopathological diagnosis every specimen was stained with hematoxylin and eosin, and for glial fibrillary acidic protein (GFAP) by an immunohistochemical method (*vide infra*).

Immunohistochemistry: Adjacent sections were stained by the avidin-biotin-complex (Vector Laboratories, Burlingame, Calif.) method, using a 1:100 dilution of polyclonal serum against bovine glial fibrillary acidic protein (GFAP) (DAKO, Carpinteria, Calif.) and 1:200 to 1:400 dilution of polyclonal rabbit serum against human TNF-alpha (Genzyme, Boston, USA). Briefly, sections were incubated with 0.3% hydrogen peroxide for 30 minutes followed by 20% bovine serum in Tris-HCL (TBS) for 20 minutes, before incubation with the primary antiserum at 4°C overnight. Sections were then incubated successively with the biotinylated secondary antibody and the ABC reagent for 1 hour at room temperature. Color was developed using 0.05% diaminobenzidine (Sigma, St. Louis) with 0.01% hydrogen peroxide for 5 to 10 minutes. Following a 5 minutes rinse in tap water, the sections were counterstained with hematoxylin for 8 minutes.

To ascertain the specificity of staining, the primary anti-human TNF-alpha antiserum were omitted from the staining procedure or they were replaced with irrelevant antisera.

RESULTS

Results are summarized in Table 1. Only tumors of astrocytic lineage showed expression of TNF-alpha by homogeneous or slightly granular cytoplasmic immunostaining (Figs 1–4). Meningiomas, craniopharyngiomas, neuroblastomas and medulloblastomas were completely immunonegative. Hence, specimens from these types of tumors were further used as negative controls. Fibrillary astrocytomas, gemistocytic astrocytomas and pilocytic astrocytomas were immunopositive in most cells. Glioblastomas were positive in various fractions of cells. It seemed that TNF immunopositivity parallelled

Table 1. GFAP and TNF-alpha immunoreactivity in primary cerebral brain tumors

Tumor type	number of specimens	GFAP	TNF
Fibrillary astrocytoma	1	+	+
Gemistocytic astrocytoma	1	+	+
Pilocytic astrocytoma	1	+	+
Oligo/astrocytoma	1	+	+
PNET	1	+	+
Glioblastoma multiforme	9	+	+
Neuroblastoma	1	−	−
Meningioma	3	−	−
Craniopharyngioma	1	−	−

that of GFAP. In mixed oligo-astrocytomas and primitive neuroectodermal tumor (PNET) presenting features of astrocytic, oligodendrocytic and neuroblastic differentiation, only astrocytes exhibited TNF immunostaining while other cell types remained negative. Furthermore, few astrocytes reactive at the margin of tumors showed weak TNF immunoreactivity.

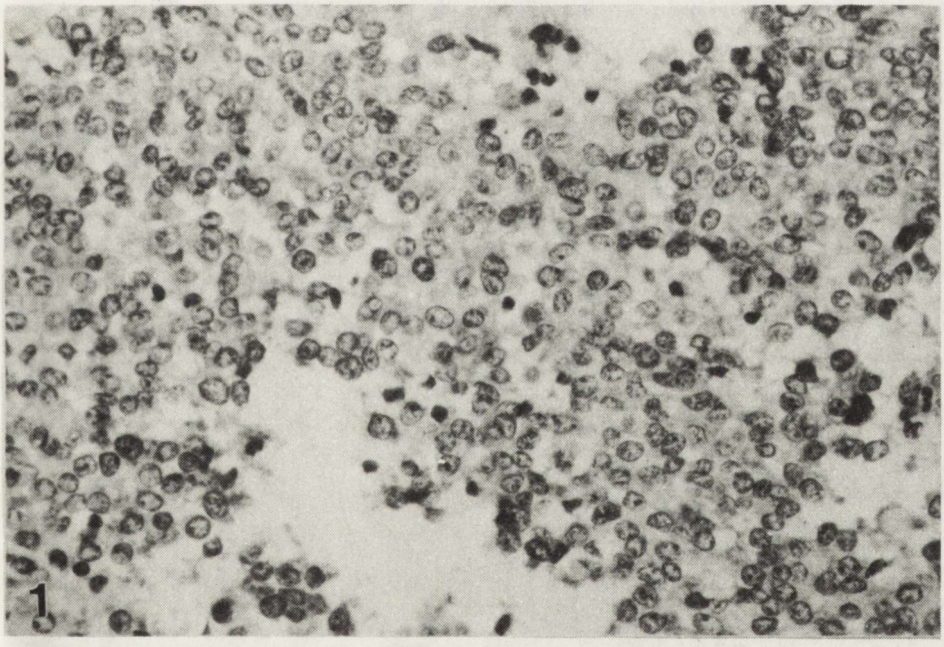

Fig. 1. Neuroblastoma completely negative for TNF-alpha. × 400

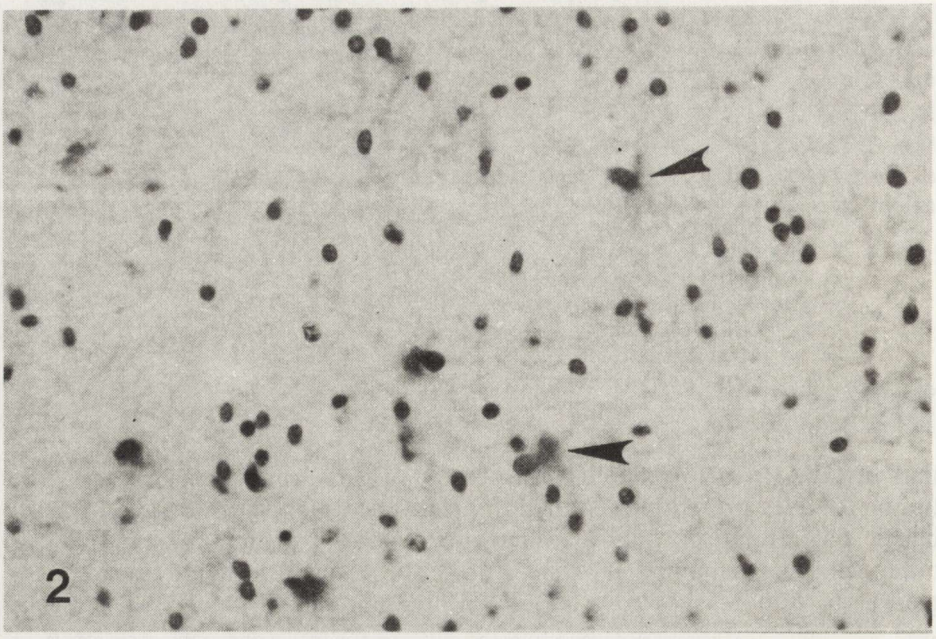

Fig. 2. Glioblastoma multiforme. Brain tissue around the tumor. Few reactive astrocytes (arrowheads) present weak TNF-alpha immunostaining. × 400

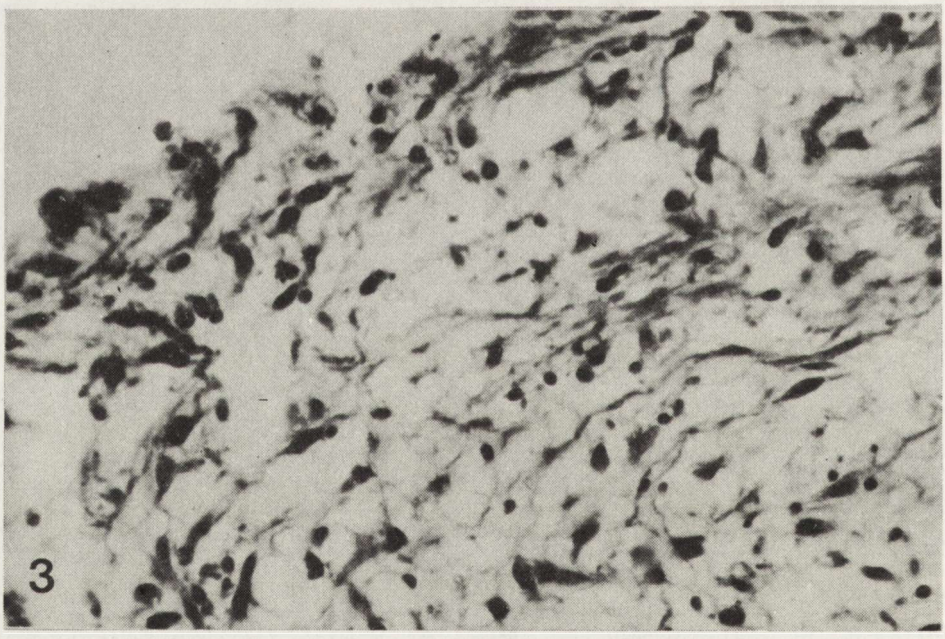

Fig. 3. Pilocytic astrocytoma. Most tumor cells immunopositive for TNF-alpha. × 400

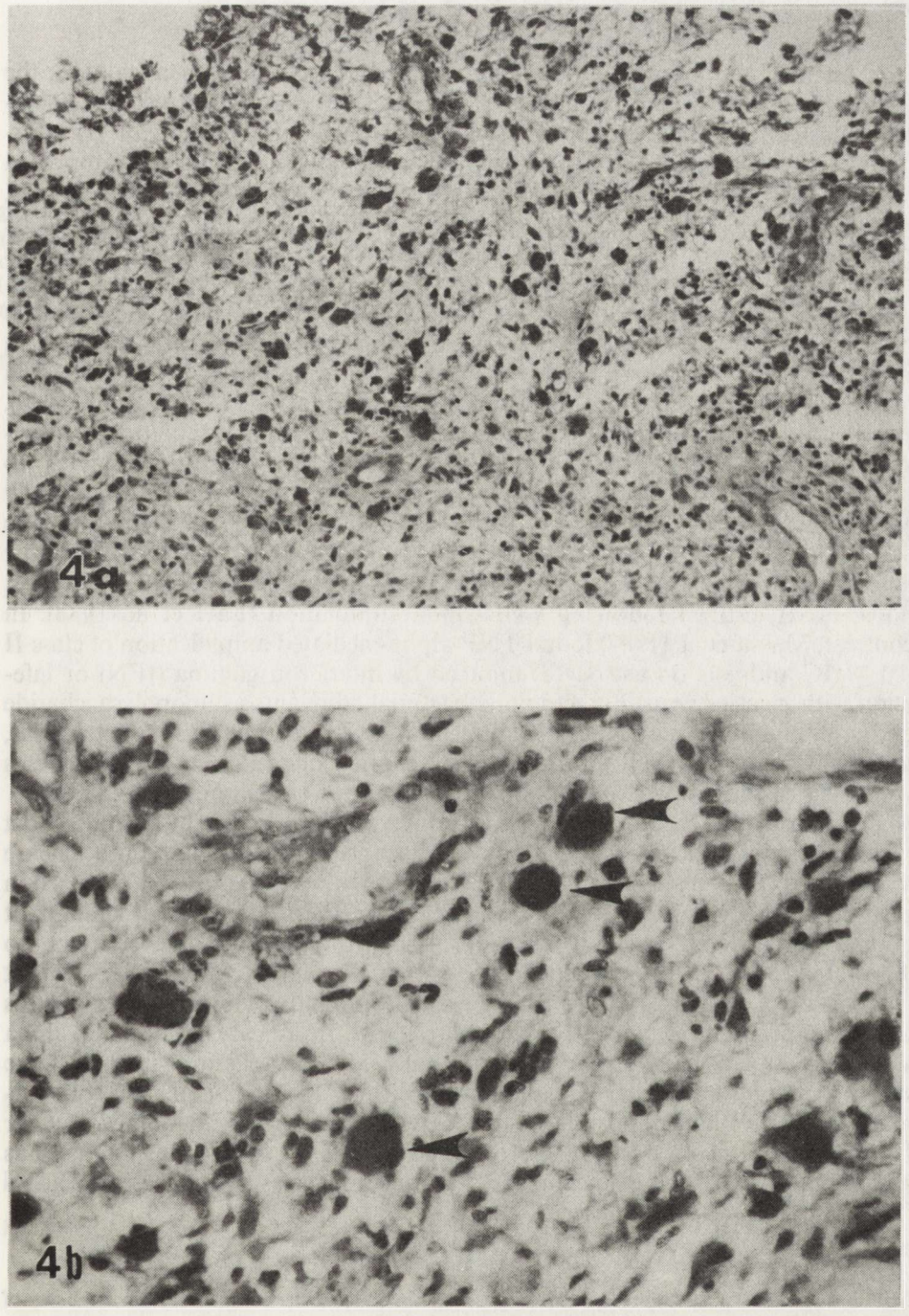

Fig. 4. Glioblastoma multiforme. Few giant cells (arrowheads) showing TNF-alpha immunoreactivity. a – × 160, b – × 400

DISCUSSION

TNF-like immunopostivity is demonstrated immunohistochemically for the first time in reactive and neoplastic astrocytes. This finding suggested a potential role for TNF-alpha in mediation of astrocytic proliferation or transformation or both. The specificity of immunocytochemical reaction was supported by omission and replacement of the primary antibodies but, obviously, Western and Northern blot experiments and *in situ* hybrydization should be performed to prove beyond any doubts the specificity of this reaction and the presence of TNF-alpha peptide and mRNA, respectively (McCall et al. 1989).

The functional significance of TNF-alpha within cells of astrocytic lineage is not clearly understood. TNF-alpha is a proliferative signal for astrocytes *in vitro* as shown by the increase of ^{3}H-thymidine incorporation and by numerical increase of astrocytes after TNF-alpha supplementation (Selmaj et al. 1990). Furthermore, closely related lymphokines, lymphotoxin and macrophage-derived factor IL-6, also stimulate, however, less intensively, astrocytes to proliferate (Selmaj et al. 1990). Astrocytes synthesize and release biologically active substances. Cultured astrocytes released multilineage hematopoietic colony-stimulating activity as recently demonstrated in murine bone marrow colony-stimulating assays (Frei, Fontana 1989) and expressed MHC class I, but not class II antigen following TNF-alpha stimulation (Lavi et al. 1988). In contrast, Massa et al. (1987) found TNF-alpha-mediated amplification of class II (I^{a}) MHC antigens on astrocytes induced by interferon gamma (IFN) or infection with measles virus. Furthermore, bacterial adjuvants as lipopolysaccharide and muramyl dipeptide induced I^{a} antigen on astrocytes, and this induction was, in turn, amplified by TNF-alpha. Lipopolysaccharides and neurotropic viruses stimulate production of TNF-alpha in astrocytes (Lieberman et al. 1989).

The possible role of TNF in brain tumor pathogenesis is intriguing. Most primary brain tumors are of astrocytic lineage and astrocytes both release TNF-alpha (Robbins et al. 1987) and proliferate in response to TNF released in turn from other cells (Selmaj et al. 1990). Furthermore, human malignant glioma cell line (D54-MG) cells respond in a proliferative manner to TNF-alpha and express specific receptors for TNF-alpha (Bethea et al. 1990). It is conceivable that TNF-alpha released from activated astrocytes causes autocrine proliferation of astrocytes which, in turn, release more TNF-alpha acting as signal for further proliferation. Such a reaction may become self-perpetuated and, when escaping control, may lead to neoplastic transformation in a mechanism postulated for some malignancies (Schleuning, Munker 1990). The persistence of an upregulated proliferation-inducing signal has already been suggested as mechanism for cancerogenesis by the Ras oncogenes (Watson et al. 1987).

Acknowledgements: Dr. Liberski is the recipient of a research grant from the Polish Academy of Sciences (VIII/41).

The helpful criticism of Professor Herbert Budka, the Neurological Institute, University of Vienna, is gratefully acknowledged.

Ms. Elżbieta Naganska, Mr. R. Kurczewski, Mr. K. Smoktunowicz and Ms Leokadia Romanska are acknowledged for skillful technical assistance.

EKSPRESJA CZYNNIKA MARTWICY GUZA ALFA/KACHEKTYNY W PIERWOTNYCH NOWOTWORACH MÓZGU POCHODZENIA ASTROCYTARNEGO

Streszczenie

W pracy przedstawiono wyniki badań immunohistochemicznych ekspresji czynnika martwicy guza alfa (TNF-alfa) kachektyny w 19 pierwotnych nowotworach mózgu. Jedynie nowotwory glejowe pochodzenia astrocytarnego (*astrocytoma gemistocyticum*, *astrocytoma fibrillare*, *astrocytoma pilocyticum*, *glioblastoma multiforme*) wykazywały silną ekspresję TNF-alfa. Guzy mieszane (*oligo-astrocytoma* i PNET) wykazywały ekspresję TNF-alfa jedynie w astrocytarnej frakcji nowotworu.

Wysunięto hipotezę, że TNF-alfa bierze udział w patogenezie transformacji nowotworowej poprzez autokrynne pobudzanie receptora dla TNF-alfa na astrocytach.

REFERENCES

1. Bethea JR, Gillespie GY, Chung IY, Benveniste EN: Tumor necrosis factor production and receptor expression by a human malignant glioma cell line, D54-MG. J Neuroimmunol, 1990, 30, 1–13.
2. Brosnan SF, Selmaj K, Raine CS: Hypothesis: A role for tumor necrosis factor in immune-mediated demyelination and its relevance to multiple sclerosis. J Neuroimmunol, 1988, 18, 87–94.
3. Frei K, Fontana A: Immune regulatory functions of astrocytes and microglial cells within the central nervous system. In: Neuroimmune networks: physiology and diseases. Alan R Liss Inc, New York, 1989, pp 127–136.
4. Grimaldi LME, Martino GV, Franciotta DM, Brustia R, Castagna A, Pristera R, Lazzarin A: Elevated alpha-tumor necrosis factor levels in spinal fluid from HIV-l-infected patients with central nervous system involvement. Ann Neurol, 1990, 29, 21–25.
5. Hauser SL, Doolittle TH, Lincoln R, Brown RH, Dinarello CA: Cytokine accumulation in CNS of multiple sclerosis patients: frequent detection of interleukin-1 and tumor necrosis factor but not interleukin-6. Ann Neurol, 1990, 40, 1735–1739.
6. Kunkel SL, Remick DG, Strieter RM, Larrick JW: Mechanisms that regulate the production and effects of tumor necrosis factor alpha. Crit Rev Immunol, 1989, 2, 93–117.
7. Lavi E, Suzumura A, Murasko DM, Murray EM, Silberberg DH, Weiss SR: Tumor necrosis factor induces expression of MHC class I antigens on mouse astrocytes. J Neuroimmunol, 1988, 18, 245–253.
8. Le J, Vilcek J: Tumor necrosis factor and interleukin 1: cytokines with multiple overlapping biological activities. Lab Invest, 1987, 56, 234–248.
9. Lieberman AP, Pitha PM, Shin HS, Shin ML: Production of tumor necrosis factor and other cytokines by astrocytes stimulated with lipopolysaccharide or a neurotropic virus. Proc Natl Acad Sci USA, 1989, 86, 6348–6352.
10. Liberski PP, Nerurkar VR, Yanagihara R, Gajdusek DC: Tumor necrosis factor alpha: cytokine mediated vacuolation in experimental Creutzfeldt-Jakob disease. VIIIth Internat Congress of Virology, 1990.
11. Liberski PP, Yanagihara R, Gibbs CJ Jr, Gajdusek DC: White matter ultrastructural pathology of experimental Creutzfeldt-Jakob disease. Acta Neuropathol (Berl), 1989, 79, 1–9.
12. Maier H, Budka H, Lassmann H, Pohl P: Vacuolar myelopathy with multinucleated giant cells in the aquired immune deficiency syndrome (AIDS). Light and electron microscopic distribution of human immunodeficiency virus (HIV) antigens. Acta Neuropathol (Berl), 1989, 78, 497–503.
13. Massa PT, Schimpl A, Wecker E, ter Meulen V: Tumor necrosis factor amplifies measles virus-mediated Ia function on astrocytes. Proc Natl Acad Sci USA, 1987, 84, 7242–7245.
14. McCall JL, Yun K, Funamoto S, Parry BR: *In vivo* immunohistochemical identification of tumor necrosis factor/cachectin in human lymphoid tissue. Am J Pathol, 1989, 135, 421–425.

15. Old LJ: Tumor necrosis factor. In: Tumor necrosis factor: structure, mechanism of action, role in disease and therapy. Eds: B Bonavida, G Granger. Karger, Basel, 1990, pp 1–30.
16. Robbins DS, Shirazi Y, Drysdale B-E, Lieberman A, Shin HS, Shin ML: Production of cytotoxic factor oligodendrocytes by stimulated astrocytes. J Immunol, 1987, 139, 2593–2597.
17. Schleuning M, Munker R: Tumor necrosis factor: an update on basic research and clinical applications. Klin Wochenschr, 1990, 68, 841–846.
18. Selmaj K, Cannella B, Brosnan CF, Raine CS: Characterization of TCR gamma-delta lymphocytes in patients with multiple sclerosis. XIth Internat Congress of Neuropathology, Kyoto, September 2-8, 1990. Abstr no VI-B-6, p 278.
19. Selmaj KW, Farooq M, Norton WT, Raine C, Brosnan CF: Proliferation of astrocytes *in vitro* in responce to cytokines. A primary role for tumor necrosis factor. J Immunol, 1990, 144, 129–135.
20. Selmaj KW, Raine CS: Tumor necrosis factor mediates myelin and oligodendrocyte damage *in vitro*. Ann Neurol, 1988, 23, 339–347.
21. Tracey KJ, Cerami A: The biology of cachectin/tumor necrosis factor. In: Growth factors, differentiation factors and cytokines. Ed: A Habenicht. Springer, Berlin-Heidelberg, 1990, pp 356–365.
22. Tracey KJ, Vlassara H, Cerami A: Cachectin/tumor necrosis factor. Lancet, 1989, 1, 1122–1125.
23. Tracey KJ, Cerami A: The role of cachectin/tumor necrosis factor in AIDS. Cancer Cells, 1989, 1, 62–63.
24. Watson JD, Hopkins NH, Roberts JW, Steitz JA, Weiner AM: Molecular biology of the gene. Vol II, Specialized aspects. The Benjamin/Cummings Publ Comp, Menlo Prak, 1987.

Correspondence address: Dr. Paweł P. Liberski, Electron Microscopic Laboratory, Dept. Oncology, School of Medicine, 4 Gagarina Str., 93-509 Łódź, Poland

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

ELŻBIETA KIDA[1], MARIA BARCIKOWSKA[1], TERESA MICHALSKA[2], EWA JOACHIMOWICZ[2], ANNA SIEKIERZYŃSKA[3]

PERIPHERAL NERVOUS SYSTEM ALTERATIONS IN SMALL CELL LUNG CANCER. CLINICO-PATHOLOGICAL STUDY

[1]Department of Neuropathology, Medical Research Centre, Polish Academy of Sciences, Warsaw; [2]IInd Department of Neurology, School of Medicine, Warsaw; [3]Institute of Tuberculosis and Chest Diseases, Warsaw

Small cell lung cancer (SCLC) is one of the most malignant tumors, especially often associated with nonmetastatic neurological disorders, corresponding to paraneoplastic neurological syndromes. The pathogenesis of which is unknown, however, mostly attributed to autoimmune processes. The aim of the study was to determine the pattern of the peripheral nervous system damage in SCLC. To provide further data contributing to the pathomechanism underlying these syndromes, immunocytochemical studies were initiated. Autopsy material was collected from 47 cases of SCLC. All these patients were examined clinically. The sections from the cervical, thoracic and lumbosacral segments of the spinal cord with spinal roots and dorsal root ganglia were taken. For immunohistochemistry following antisera were used: GFAP, MBP, IgG, IgM, ferritin, ubiquitin, alpha$_1$-antichymotrypsin, alpha$_2$-macroglobulin, C3 and C5b9 complement fractions. In 18 patients peripheral nervous system disturbances were diagnosed neurologically, 21 of cases presented neuromuscular disorders by emg. Among the nonmetastatic lesions most often a damage of dorsal root ganglia was observed (in 33 cases). Degeneration of the spinal roots was absent only in 8 cases. In 21 cases degenerative changes of motor neurons within anterior horn were present. In no case ubiquitin-positive inclusion bodies within the motor neurons could be found. In 8 cases extravasation of the IgG with diffuse labeling of the grey matter was observed. IgM immunoreactivity was markedly less frequently present, C5b9 complement fraction immunoreactivity was also confined only to cases with peripheral nervous system disturbances. Therefore, our preliminary data seem to confirm the participation of humoral immunity in paraneoplastic syndrome pathogenesis.

Key words: *Small cell lung cancer, paraneoplastic syndrome, peripheral nervous system, immunocytochemistry.*

Small cell lung cancer (SCLC) is one of the most malignant, solid tumors of the adult of still not entirely established histogenesis, exhibiting distinct neuroendocrine component with amine and peptide hormone production and specific enzymes activities (Shimosato et al. 1986). It is well documented, that this particular type of lung tumor is especially often associated with nonmetastatic neurological disorders corresponding to paraneoplastic neurological

syndromes (Henson, Urich 1982). The pathogenesis of paraneoplastic neurological syndromes is unknown, it is, mostly attributed, however, to autoimmune processes, concept strongly supported by the findings of circulating antineural antibodies (CANA), reactive with brain, spinal cord and roots, retina and dorsal root ganglia (Kornguth et al. 1982; Grisold et al. 1987; Anderson et al. 1988; Jeackle, Greenlee 1988). All parts of neuraxis can be affected by pathological process, what determines variety of clinical symptomatology assessed.

The works on peripheral nervous system damage associated with cancer furnished various incidence rates, with distinctly higher figures (up to 50%) when applying electrophysiological criteria which allow to disclose subclinical forms (Campbell, Paty 1974; Terävainen, Larsen 1977). Among various neuromuscular paraneoplastic syndromes peripheral sensory and sensorimotor neuropathies and neuromyopathies, motor neuropathies, subacute sensory neuronopathies, Lambert-Eaton syndrome (LE) and motor neuron disease are distinguished (Lambert, Rooske 1965; Croft et al. 1967; Anderson et al 1988). Previously performed neuropathological studies on peripheral nervous system damage accompanying malignancy disclosed degenerative character of structural lesions, however, usually connected with marked inflammatory component (Denny-Brown 1948; Morton et al. 1966; Croft et al. 1967; Torvik, Slettebo 1980; Vallat et al. 1986). The aim of the present study was to determine the pattern of peripheral nervous system damage in SCLC. To provide further data contributing to the pathomechanism underlying these syndromes immunocytochemical studies were initiated, and their preliminary results will be briefly summarized.

MATERIAL AND METHODS

Autopsy material was collected from 47 cases of SCLC died in the Institute of Tuberculosis and Chest Diseases during the last five years (1985 – 1990). The series consits of 17 women and 30 men aged 35 – 69 (mean age 56.8 years). All these patients were examined clinically by a neurologist after the admission to the Institute, prior to the onset of therapy, and also later on, if necessary (in case of appearance of neurological symptoms or signs). In 32 cases electrophysiological study (emg) was performed prior to the begining of therapy. The patients selected for emg were free of diabetes mellitus, uraemia, alcoholism or other factors affecting peripheral nervous system. This study was done using Disa electromyograph with stimulator and microcomputer Anops. Routinely, the following muscles were evaluated: interosseus I (from the upper extremity), biceps brachii, rectus femoris and tibialis anterior. The nerves investigated with standard methods included: the medianus, peroneus and suralis. Neuromuscular transmission analysis was employed by means of classical electrostimulation method, only in patients with clinical signs of fatiguability. For neuropathological study the sections from the cervical, thoracic and lumbosacral segments of the spinal cord with spinal roots and dorsal root ganglia (except for one) were taken, fixed in formalin and processed to paraffin. The slides were routinely stained with HE, according to Klüver-Barrera or Heidenhain, Bielschowsky or Holmes methods. For immunocytochemistry the

extravidin-biotin-peroxidase complex method was used as the staining procedure with diaminobenzidine in the presence of hydrogen peroxide as a chromogen. The following antisera were used for: GFAP, MBP, IgG, IgM, ferritin, ubiquitin, alfa$_1$-antichymotrypsin, alfa$_2$-macroglobulin. C3 and C5b9 complement fractions. After immunolabeling the slides were counterstained with hematoxylin.

RESULTS

a) Clinical data

The occurrence of subjective symptoms (hands and feet numbness, dysesthesiae, pains, muscular weakness) and neurological signs (reduction or abolition of tendon reflexes, muscular wasting, sensory loss of glove and sock type, sensory ataxia) represented prerequisite to suggest peripheral nervous system damage. Based on these data in 18 patients (56%) already during the first neurological examination, peripheral nervous system disturbances were diagnosed.

Neurological abnormalities were usually mild, confined to slight sensory disturbances of glove and sock type and loss or diminution of tendon reflexes accompanied by subjective complaints. In 2 cases more severe, progressive disability was found with muscular atrophy, pains, disturbances of all modalities of sensation and loss of tendon reflexes preceding for many months the diagnosis of cancer (both men, aged 55 and 60 years). One case (men, aged 61) manifested, besides neuropathy, predominant muscular fatiguability and weakness of occular, bulbar and proximal parts of limbs, with no response to cholinergic treatment. Among 47 cases of the series, 32 cases were investigated by emg, and in 21 of them (65%) diagnosis of neuromuscular disorder was assessed (6 women, 15 men, aged 35–68, mean age 57.8).

The following types of peripheral nervous system disturbances were distinguished on the basis of emg findings: sensory neuropathy (2 cases), motor neuropathy (6 cases), sensorimotor neuropathy (3 cases) neuronopathy (6 cases), neuronopathy with motor neuropathy (Guillain-Barre like) (2 case), neuronopathy with sensory neuropathy (1 case), LE syndrome with motor neuropathy (1 case).

Among 4 untreated patients in the series, 2 cases were not evaluated electrophysiologically, 1 patient showed no abnormalities by emg, and one case exhibited LE syndrome with motor neuropathy.

b) Neuropathological study

Time interval from the neurological examination to death varied from 1 month to 33 months (mean 10.9 months).

Morphological abnormalities were observed in the majority of cases evaluated. Metastatic SCLC lesions within the spinal cord/roots were disclosed in 6 cases. Among the nonmetastatic lesions, most often a damage of dorsal root ganglia was observed (33 cases). Structural changes within dorsal root ganglia were of mild intensity, except for 2 cases diagnosed as subacute sensory neuronopathy (SSN) and described in detail elsewhere. In the majority of cases

degenerative changes of ganglion cells with nuclear pyknosis, vacuolation of the cytoplasm with loss of tigroid and eosinophilia constituted the main alterations. Neuronal loss was small. In the tissue, increased cellularity due to the proliferation of satellite cells and fibroblasts was seen, with occasionally occurring nodules of Nageotte (Fig. 1). Sporadically scarce perivascular infiltrates were found (2 cases, including one with SSN).

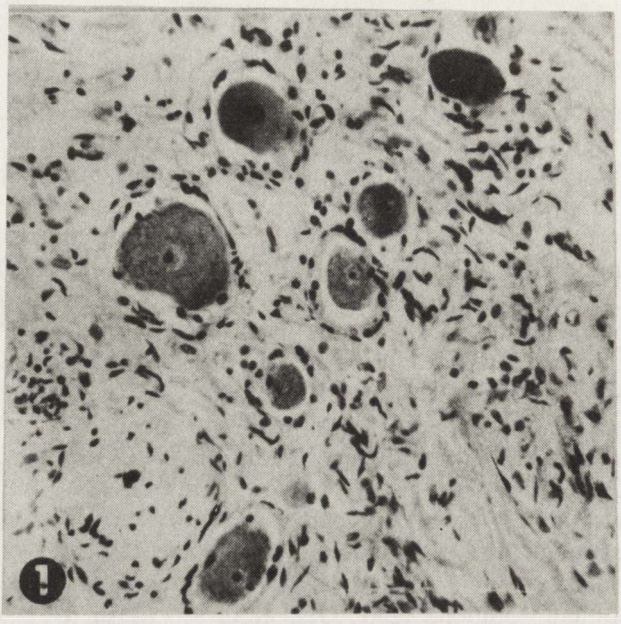

Fig. 1. Dorsal root ganglion. Degenerative changes of ganglion cells and nodule of Nageotte. HE × 200

Another common alteration comprised degeneration of the spinal roots, absent only in 9 cases. However, artifactual postmortem changes within the spinal roots made the final assessment difficult. Helpful were in this respect immunostaining procedures with antisera to MBP and especially to ubiquitin. More often the axon processes were affected than the myelin sheaths, however, Wallerian degeneration was not uncommonly seen. Affected spinal roots showed loss of fibres and structural damage of axons which exhibited segmental swellings and fragmentation visualized with HE, silver impregnation and most easily with anti-ubiquitin sera. Ubiquitin immunocytochemistry allowed for clear visualization of strongly labeled, enlarged, degenerated axons (Fig. 2). Usually posterior roots were more frequently affected than anterior ones. Discrete, perivascular infiltrates of mononuclears were observed within the spinal roots only in 4 cases. One of them represented SSN. The other case showed polineuropathy since the very beginning, with sensory neuropathy accompanied by anterior horn damage by emg study. This case presented besides slight perivascular infiltrates also parenchymal, diffuse proliferation of glial cells, epithelial elements and mononuclears within posterior horn area at the junction with dorsal roots (Fig. 3). Third case, subclinic, with signs of

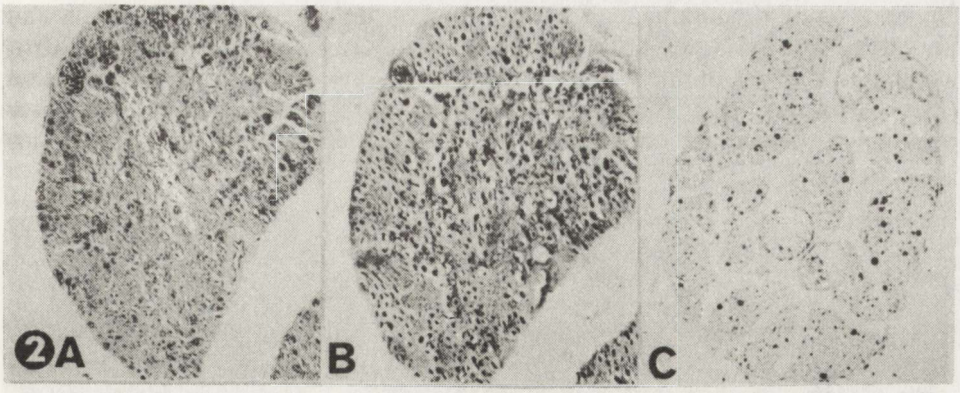

Fig. 2. Dorsal roots. *a.* Loss of myelinated fibres. Heidenhain x 200. *b.* Degeneration of neuritic processes. Holmes × 200. *c.* Damaged neuritic processes. Anti-ubiquitin × 100

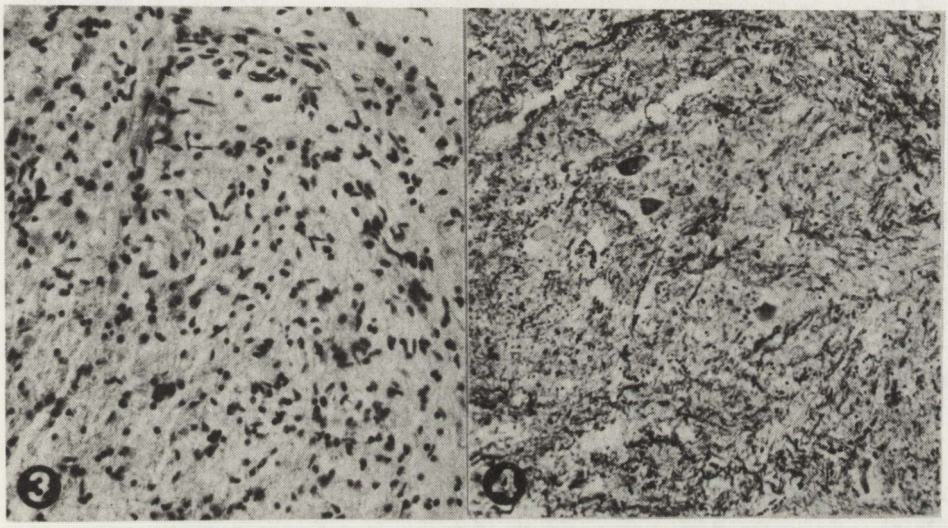

Fig. 3. Posterior horn of the spinal cord. Diffuse mesenchymal cell proliferation. HE × 200
Fig. 4. Anterior horn of the spinal cord. Neuronal loss and degeneration. Klüver-Barrera × 100

neuronopathy by emg, exhibited similar to the former case, but less pronounced, parenchymal lesions within posterior horn. The last case showed LE syndrome with motor neuropathy. Damage of anterior horn of the spinal cord was seen distinctly less frequently. In 21 cases degenerative changes of motor neurons within anterior horn were present, usually slight and confined to fatty degeneration or neuronal atrophic changes, but rarely with marked neuronal loss (Fig. 4). Neuronal loss seen in 12 cases (10 with polineuropathy, 2 asymptomatic cases) was usually mild, accompanied by slight astroglial cells proliferation. In cases with more prominent neuronal loss proliferation of microglial cells was evident. It was mainly focal, most pronounced in the areas of neuronal diminution (Fig. 5). We had the impression that microglial cells better indicated areas of neuronal loss than astroglia. Astroglial cells proliferation was more often observed in the white, than grey matter of the spinal cord.

In 3 cases a marked damage of sensory nuclei within posterior horn was found (2 cases with SSN, 1 case with sensory neuropathy and neuronopathy) accompanied by prominent glial cells proliferation with rod cells participation (Fig. 6). In no case ubiquitin-positive inclusion bodies within the motor neurons of the spinal cord could be found. Degeneration of the posterior

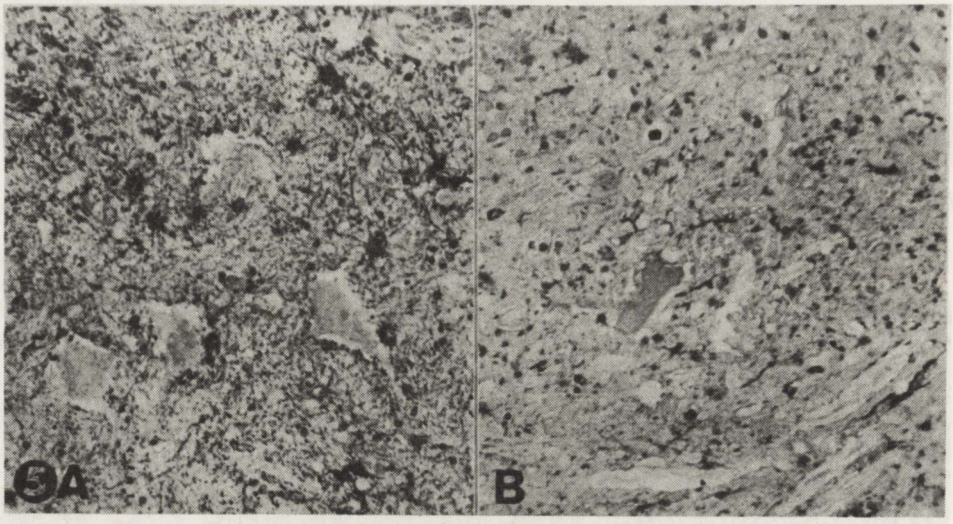

Fig. 5. Glial cells in the anterior horn. *a.* Reactive astrocytes within well preserved anterior horn. GFAP x 200. *b.* Numerous reactive microglial cells in the area of neuronal loss. Ferritin immunohistochemistry × 200

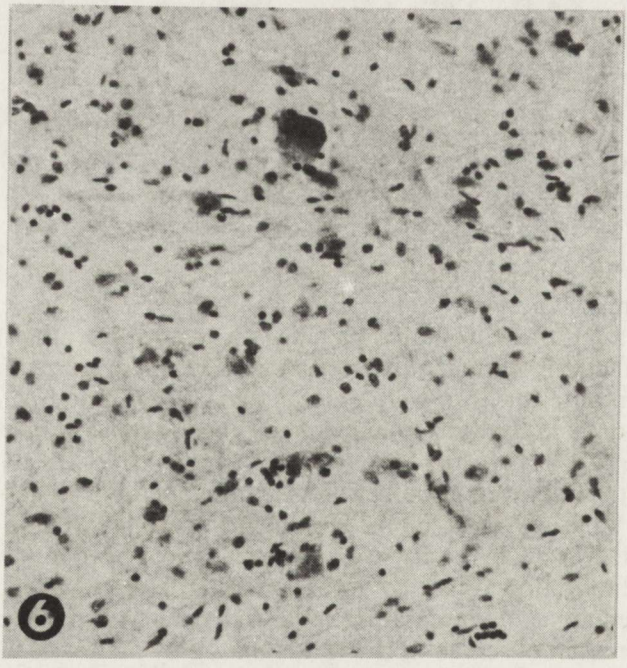

Fig. 6. Damaged sensory nucleus of the posterior horn of the spinal cord. HE ×200

columns of the spinal cord was severely expressed in 2 cases with SSN only. In one case, a damage of posterior column most probably represented a secondary lesion to the presence of metastatic focus in the lumbosacral segments of the spinal cord. Slight degeneration of the posterior columns was also disclosed in 2 other cases (one presenting sensorimotor neuropathy, and the other one sensory neuropathy). In 2 cases recent necrotic areas without glial cells reaction were observed within the junction between the anterior/posterior horn, and in 3 cases large, perivascular hemorrhages were found mostly in the grey matter of the spinal cord.

Immunocytochemical studies showed in 8 cases extravasation of the IgG with diffuse labeling of the grey matter of the spinal cord, slight staining of neurons and strong of astroglia, the latter ones mainly in the perivascular region (Fig. 7). Often ganglion cells of the dorsal root ganglia (including cell

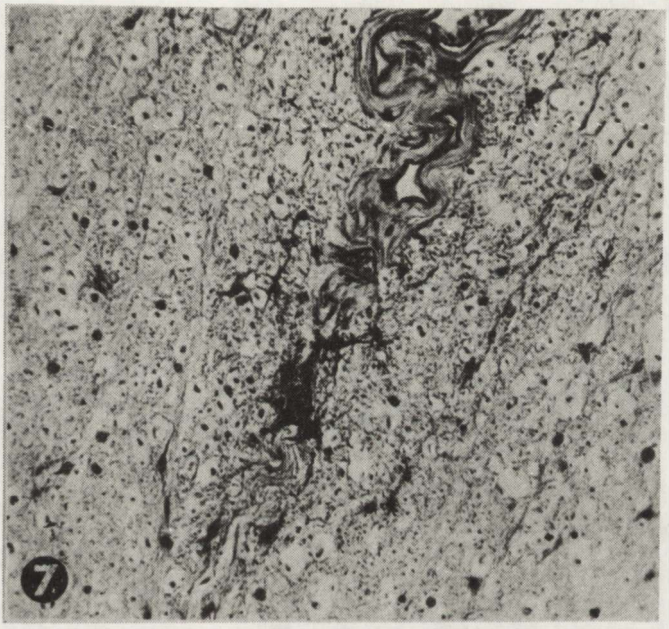

Fig. 7. Extravasation of IgG and numerous progressively changed astroglia labeled. IgG immunostaining × 200

cytoplasm and nuclei) showed labeling, but there was no correlation between the IgG staining and clinical symptomatology. In 22 cases strong IgG labeling of the spinal roots was seen, also without close relationship to the clinical data, however, only occasionally there was lack of IgG deposits in the cases with clinically assessed neuropathy.

IgM immunostaining was markedly less frequently observed than IgG, usually confined to the blood vessel walls. Only occasionally slight, diffuse labeling of the grey matter of the spinal cord was disclosed (5 cases, 3 with neuromuscular disorders). Apart from them, in 8 cases, delicate, diffuse IgM reaction was observed within the spinal roots, showing no correlation with clinical findings. There were no IgM deposits within ganglion cells of the dorsal root ganglia studied. Complement C3 fraction labeling was observed in all the

cases examined within ganglion cells, with variable proportions, from single cells labeled to the positive staining of almost all neuronal population. It is of interest, however, that among 4 cases with the most pronounced C3 staining, 3 cases had clinical signs of neuropathy (Fig. 8). Complement C5b9 fraction immunoreactivity was present within the spinal roots, exhibiting weak expression in 5 cases, but all with clinical signs of neuropathy.

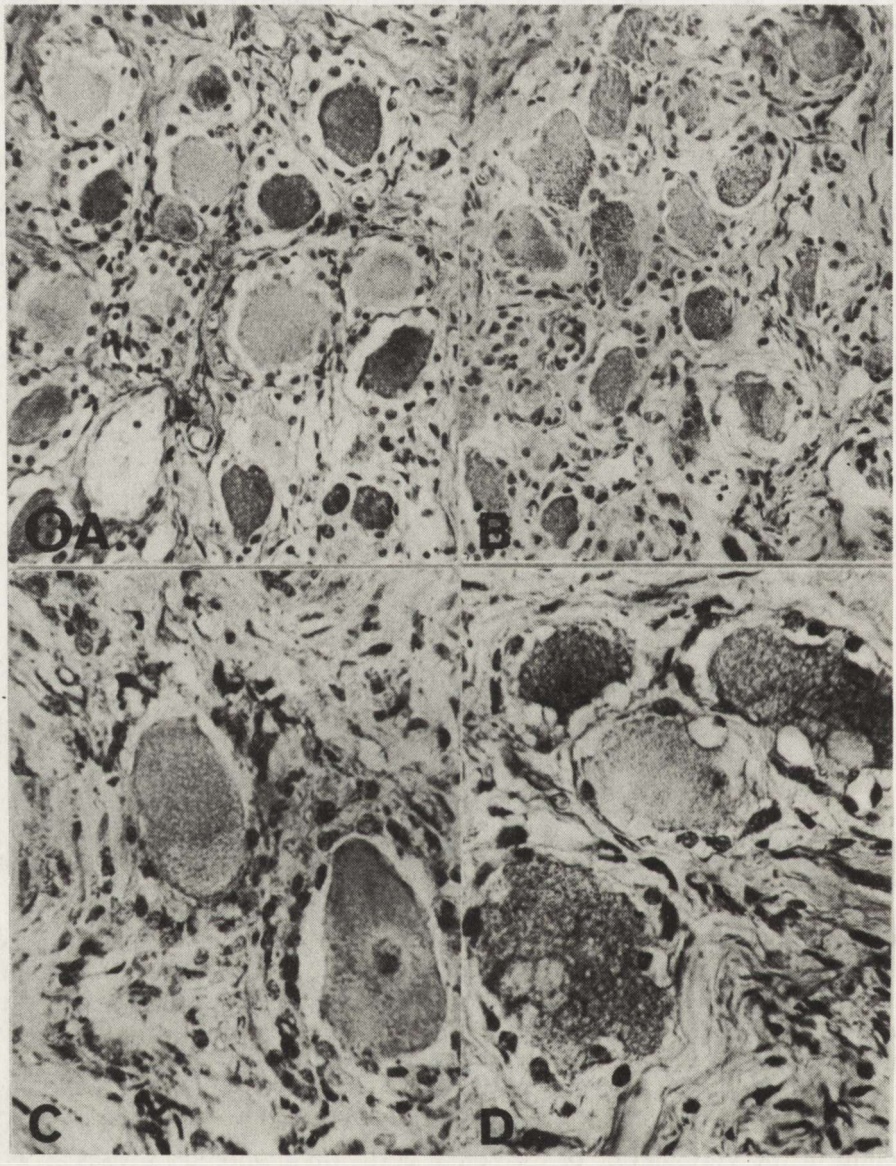

Fig. 8. Immunohistochemistry of the dorsal root ganglia. *a.* C3 complement fraction labeling of numerous ganglion cells. × 200. *b.* C3 complement fraction staining of unaffected case. × 200. *c.* Moderate staining by alfa$_1$-antichymotrypsin of satellite cells. × 200. *d.* Alfa$_1$-antichymotrypsin labeling of degenerating ganglion cells. × 200

Alfa$_1$-antichymotrypsin expression was observed in ganglion cells (cell cytoplasm and nuclei) and satellite cells of the dorsal root ganglia, without close correlation with clinical data. Among 4 cases with the strongest staining of satellite cells, all 4 had metastatic spread. Among 5 cases with the most prominent alfa$_1$-antichymotrypsin within ganglion cells, 3 cases were free of neuromuscular disorders, 2 other cases had clinically assessed neuropathy. However, it is of interest, that among 11 cases with the weakest expression of this serine protease inhibitor, 9 cases had clinically established neuropathy.

Alfa$_2$-macroglobulin activity was observed only within individual ganglion cells in 2 cases, both with neuropathy.

c) Clinico-pathological correlations

Close clinico-pathological correlations are not possible to establish, because of long time interval between the clinical assessments and time of death. Moreover, of great importance is the influence of therapy applied on the neuropathological picture. Patients entered to this study were treated with

Table 1. Clinico-pathologic correlation

PNS emg	Case no.	TI	Therapy	AH	SR	DRG	PC
M	1	16ms	CsP, VP, End, Adr	+L	+	+	−
	2	20ms	VP, Adr, End	+L	++	+	−
	3	11ms	CTX, Adr, VP	+L	+	+	−
	4	6ms	CTX, Vc, MTX, Nt	+L	+	+	−
	5	33ms	CTX, Vc, CCNU, MTX, Adr, Csp	−	−	−	−
	6	15ms	CTX, Vc, MTX, Csp, VP, Adr, CCNU	+−	+	−	−
N	7	10ms	Vp, Adr, Vb, End, CCNU, MTX	+L	+	+	−
	8^M	8ms	VP, Adr, End, Vc	+	+	+	−
	9^M	7ms	Vb, VP, End, CCNU, CsP, MTX	+−	+−	+	−
	10^M	10ms	Adr, VP, Csp, Vc, End, CCNU	+L	++	+	−
	11	10ms	CTX, Adr, VP,	+L	+	+	−
	12^M	18ms	CTX, VP, Adr	+	+	+	−
S/M	13	12ms	Adr, VP, End, Vc, CCNU, MTX	++L	+	+	−
	14	11ms	VP, Adr, End, CTX	−	+	+	+
	15^M	3ms	Adr, End, VP	−	+−	+	−
S	16^M	13ms	Adr, VP, End, CCNU, MTX	−	+	+	+
	17	8ms	Adr, CTX, VP, MTX, Vc, CsP	+	+	+	−
LE/M	18	2ms	−	+−	++	0	−
S/N	19	1m	CTX, VP, Adr	+	+	+	+
N/M	20	9ms	End, VP, Adr	+L	++	++	++
	21	6ms	Adr, VP, CTX, MTX	+L	++	++	++

Abbreviations: M-motor, S-sensory, S/M-sensorimotor neuropathy, N-neuronopathy, LE-Lambert-Eaton, AH-anterior horn, DRG-dorsal root ganglia, SP-spinal roots, PC-posterior columns of the spinal cord. Intensity of changes −/++. TI-time between emg and neuropathological study. L-neuronal loss, M-metastases to the spinal cord

various immunosuppressants (18 cases), often combined with radiotherapy (25 cases). Only 4 cases were not treated. However, when comparing the results of neuropathological study with clinical assessments it became evident, that only 6 cases with marked morphological changes exhibited no clinical abnormalities. In the group of patients without treatment, 2 cases had no remarkable morphological changes, 1 case had discrete structural lesions, and the last patient with LE syndrome and motor neuropathy demonstrated moderate changes with predominant involvement of the spinal roots (dorsal root ganglia in this case were not available). The comparison of the results of clinical and neuropathological studies (confined to the cases with emg) presents Table 1.

DISCUSSION

Presently performed clinico-pathological study confirmed previous observations of the high incidence of neuromuscular paraneoplastic syndromes in SCLC. Most commonly, based on electrophysiological criteria, motor neuropathy and neuronopathy were found (34% of emg diagnosed cases) showing good correlation in the majority of cases with neuropathological findings. Less frequently sensory and sensorimotor neuropathies were disclosed. Such a high rate of motor neuropathies exceeds figures furnished by former studies (Morton et al. 1966; Croft, Wilkinson 1969). It should be stressed, however, that the degree of motor neuron loss within anterior horn of the spinal cord revealed by present neuropathological examination was mild, and in no case could be comparable with neuronal loss encountered in SLA. It might explain slight clinical abnormalities encountered in this group of patients. Characteristic for SLA, filamentous, ubiquitin-positive inclusion bodies within motor neurons (Leigh et al. 1988) were not found in our material.

It is in agreement with previous observations, that neuropathies accompanying cancer usually have benign course. Only 2 cases in our material presented markedly more severe clinical disturbances, and both of them were diagnosed postmortem as SSN. This is always fatal, leading to severe disability, manifestation of peripheral nervous system involvement, observed almost exclusively in SCLC (Anderson et al. 1988). Polyneuropathies associated with cancers are usually axonal, and only sporadical cases with predominantly demyelinative changes were reported (Rae-Grant et al. 1986; Graus et al. 1987), a finding also confirmed by our study. However, morphological alterations were found in our material also in the cases without clinical disturbances. Various factors might contribute to this phenomenon. One of them refers to long time interval between clinical and neuropathological studies. However, the other factors such as drugs, radiotherapy, nutritional deficiencies, metabolic and endocrine disturbances, all might obviously affect tissue structure. Therefore, it seems reasonable to restrict the diagnosis of paraneoplastic neurological syndrome only to those cases in whom secondary damage of the neural tissue caused by the above mentioned factors can be eliminated.

There are many data dealing with the interrelationships between autoimmunity and cancer, and hypotheses of humoral immunity involvement in the pathogenesis of paraneoplastic sydromes. Commonly observed damage of the

dorsal root ganglia in this group of disorders, thus might be explanable easily by the fact that even large molecules such as immunoglobulins could reach their bodies in area lacking the blood-brain barrier (Jacobs et al. 1976). Large molecules can enter neuronal cell body also by retrograde axonal transport (Fabian, Ritchie 1986; Fabian 1988; Loberg, Torvik 1991). Reaching cell surface, immunoglobulins, can cause cell dysfunction or destruction through complement activation, inhibition or activation of cell surface receptors, or become internalized at the presynaptic region and interfere with cell function (Thomas et al. 1989).

Increased anti-GM_1 IgM titres were reported in patients with lower motor neuron disease, sensorimotor neuropathy or motor neuropathy non associated with cancer, and reduction in antibodies concentration was related to clinical improvement observed in some cases (Sadiq et al. 1990). Classical example of paraneoplastic autoimmunity is LE syndrome, in which IgG from the patients sera block the voltage-dependent Ca^{2+} channel on nerve terminals (Kim et al. 1988). It was also shown, that antibodies to myelin-associated glycoprotein can produce central nervous system demyelination *in vivo*, proving, that a single antibody against specific component is able to initiate the pathological process (Sergott et al. 1988). In SCLC complement-fixing polyclonal IgG called "anti-Hu" was disclosed, reacting with the nuclei of neurons in CNS and the dorsal and trigeminal ganglia (Graus et al. 1985, 1986; Anderson et al. 1988). This antibody, reacting also with tumor cells was found in the sera of the majority of patients with SCLC, manifesting SSN, lower motor neuron syndrome or encephalomyelitis, but also in those showing no paraneoplastic syndrome (Graus et al. 1987; Grisold et al. 1987). IgG reacting with CNS tissue was also found in case of sensorimotor axonal neuropathy/myelopathy associated with SCLC (Babikian et al. 1985).

It was suggested, that circulating antibodies might arise in reaction to an antigen shared between SCLC and neuronal nuclei (Budde-Steffen et al. 1988; Grisold et al. 1989). Shared antigenic determinants with myelin-associated glycoprotein were described on the various types of lung cancers previously (Hozumi et al. 1987).

In the present series we had no opportunity to study the patients sera in searching of the CANA. Immunocytochemistry on postmortem material showed a common staining of the ganglion cells with anti-human IgG, however, without close correlation with the clinical data. IgG deposits within ganglion cells in patients with SCLC and SSN were sporadically observed formerly, being confined, however, to the cell membrane or periphery of perikarya predominantly (Grisold et al. 1987; Graus et al. 1990). Based on our observation, we could relate a widespread IgG expression within ganglion cells studied rather to the primary pathology (SCLC) than to the paraneoplastic syndrome.

We could not find any close correlation between immunocytochemically assessed $alfa_1$-antichymotrypsin expression and the presence of peripheral nervous system damage. It was previously shown, that some protease inhibitors are able to cause degeneration of neuronal processes (Takauchi, Miyoshi 1989). However, it was also postulated, that high immunoglobulin titer would be expected to lower the effective concentration of the protease inhibitors, and

therefore produce increased activity of the proteases (Kornguth 1989). In this respect, it is of interest, that in our material the weakest alfa$_1$-antichymotrypsin expression was observed predominantly in cases manifesting clinically peripheral neuropathies.

We observed the expression of alfa$_1$-macroglobulin, another protease inhibitor, only in 2 cases with clinical signs of peripheral nervous system damage. C5b9 complement fraction immunoreactivity, even if weakly expressed, was also confined only to cases with paraneoplastic syndrome. Therefore, our preliminary data seem to confirm the participation of humoral immunity in paraneoplastic syndrome pathogenesis. However, further studies are necessary to explain if it is not only epiphenomenon, or secondary response to as yet unknown pathogenic factor.

USZKODZENIE OBWODOWEGO UKŁADU NERWOWEGO W PRZEBIEGU DROBNOKOMÓRKOWEGO RAKA PŁUC

Streszczenie

Rak owsianokomórkowy płuc (ROP) charakteryzuje się wysokim stopniem złośliwości, bywa także często przyczyną powikłań nieprzerzutowych składających się na zespół paranowotworowy. Patogeneza tych zaburzeń nie jest jasna, chociaż uważa się że ich podłożem są zaburzenia o typie autoimmunizacji. Celem naszej pracy było opisanie zmian w obwodowym układzie nerwowym towarzyszących ROP, a także próba wyjaśnienia ich przyczyny. Przypuszczenia co do patogenezy zespołu paranowotworowego oparto na wynikach badań immunohistochemicznych. Materiał pośmiertny pochodził od 47 chorych zmarłych z powodu ROP, ocenianych uprzednio klinicznie pod kątem obecności odchyleń w obwodowym układzie nerwowym. W każdym przypadku pobrane skrawki rdzenia: szyjnego, piersiowego i lędźwiowego wraz z korzeniami, a także zwoje korzeni grzbietowych wstępnie barwiono i impregnowano rutynowo. Do badań immunocytochemicznych użyto następujących przeciwciał: GFAP, MBP, IgG, IgM, surowic przeciw ferrytynie, ubiquitynie, alfa-1-antychemotrypsynie, alfa-2-makroglobulinie i frakcjom komplementu: C3 i C5b9. U 18 chorych stwierdzono klinicznie cechy uszkodzenia obwodowego układu nerwowego. 21 pacjentów wykazywało zaburzenia nerwowo-mięśniowe w badaniu emg. Pośród nieprzerzutowych zmian najczęściej obserwowano w badaniu histopatologicznym cechy zwyrodnienia w obrębie zwoju korzenia tylnego (w 33 przypadkach). Cech procesu degeneracyjnego w obrębie korzeni nie uwidoczniono tylko w 8 przypadkach. Zanik komórek rogu przedniego stwierdzono w 21 przypadkach. Nie zaobserwowano ubiquitynowych ciał wtrętowych w neuronach ruchowych rdzenia. W 8 przypadkach wykazano obecność wynaczynień IgG wraz z rozlanym zabarwieniem istoty szarej rdzenia. Pozytywne zabarwienie dla IgM występowało nieco rzadziej. Barwienie przy użyciu C5b9 frakcji komplementu było dodatnie w przypadkach, w których stwierdzono kliniczne objawy uszkodzenia obwodowego układu nerwowego. Wydaje się, że uzyskane wyniki potwierdzają udział procesu immunizacji humoralnej w patogenezie zespołu paranowotworowego.

REFERENCES

1. Anderson NE, Rosenblum MK, Graus F, Wiley RG, Posner JB: Autoantibodies in paraneoplastic syndromes associated with small-cell lung cancer. Neurology, 1988, 38, 1391–1398.
2. Babikian VL, Stefansson K, Dieperink ME, Arnason BGW, Marton LS, Levy BE: Paraneoplastic myelopathy: antibodies against protein in normal spinal cord and underlying neoplasm. Lancet, 1985, 2, 49–50.
3. Budde-Steffen C, Anderson NE, Rosenblum MK, Posner JB: Expression of an antigen in small cell lung carcinoma lines detected by antibodies from patients with paraneoplastic dorsal root ganglionopathy. Cancer Res, 1988, 48, 430–434.

4. Campbell MJ, Paty DW; Carcinomatous neuromyopathy. I. Electrophysiological studies. J Neurol Neurosurg Psychiatry, 1974, 37, 131–141.
5. Croft PB, Urich H, Wilkinson M: Peripheral neuropathy of sensorimotor type associated with malignant disease. Brain, 1967, 90, 31–66.
6. Croft PB, Wilkinson M: The course and prognosis in some type of carcinomatous neuromyopathies. Brain, 1969, 92, 1–8.
7. Denny-Brown D: Primary sensory neuropathy with muscular changes associated with carcinoma. J Neurol Neurosurg Psychiatry, 1948, 11, 73–87.
8. Fabian RH: Uptake of plasma IgG by CNS motoneurons. Comparison of antineuronal and normal IgG. Neurology, 1988, 38, 1775–1780.
9. Fabian RH, Ritchie TC: Intraneuronal IgG in the central nervous system. J Neurol Sci, 1986, 73, 257–267.
10. Graus F, Abos J, Roquer J, Mazzara R, Pereira A: Effect of plasmapheresis on serumand CSF autoantibody levels in CNS paraneoplastic syndromes. Neurology, 1990, 40, 1621–1623.
11. Graus F, Cordon-Cardo C, Posner JB: Neuronal antinuclear antibody in sensory neuronopathy from lung cancer. Neurology, 1985, 35, 538–543.
12. Graus F, Elkon KB, Cordon-Cardo C, Posner JB: Sensory neuronopathy and small cell lung cancer. Antineuronal antibody that also reacts with the tumor. Am J Med, 1986, 80, 45–52.
13. Graus F, Elkon KB, Lloberes P, Ribalta T, Torres A, Ussetti P, Valls J, Obach J, Augusti-Vidal A: Neuronal antinuclear antibody (anti-Hu) in paraneoplastic encephalomyelitis simulating acute polyneuritis. Acta Neurol Scand, 1987, 75, 249–252.
14. Graus F, Santamaria J, Obach J, Valls J, Ribalta T, Tolosa T: Sensory neuropathy as remote effect of cancer. Neurology, 1987, 37, 1266.
15. Grisold W, Drlicek M, Popp W, Jellinger K: Antineuronal antibodies in small cell lung carcinoma – a significance for paraneoplastic syndrome? Acta Neuropathol (Berl), 1987, 75, 199–202.
16. Grisold W, Drlicek M, Liszka U, Popp W: Anti-Purkinje cell antibodies are specific for small-cell lung cancer but not for paraneoplastic neurological disorders. J Neurol Sic, 1989, 236, 64.
17. Henson RA, Urich H: Cancer and the nervous system. Blackwell Scientific Publications, Oxford, 1982.
18. Hozumi I, Sato S, Tunoda H, Inuzuka T, Tanaka M, Nishizawa M, Baba H, Miyatake T: Shared carbohydrate antigenic determinants between the myelin-associated glycoprotein (MAG) and lung cancer. J Neuroimmunol, 1987, 15, 147–157.
19. Jacobs JM, Macfarlane RM, Cavanagh JB: Vascular leakage in the dorsal root ganglia of the rat, studies with horseradish peroxidase. J Neurol Sci, 1976, 29, 95–107.
20. Jaeckle KA, Greenlee JE: Immunohistochemical patterns of antibody response in paraneoplastic neurological syndrome correlate with specific syndromes and with tumor types. Ann Neurol, 1988, 24, 121.
21. Kim YI, Neher E: IgG from patients with Lambert-Eaton syndrome blocks voltage-dependent calcium channels. Science, 1988, 239, 405–408.
22. Kornguth SE: Neuronal proteins and paraneoplastic syndromes. New Engl J Med, 1989, 321, 1607–1608.
23. Kornguth SE, Klein R, Appen R, Choate J: Occurrence of antiretinal ganglion cell antibodies in patients with small cell carcinoma of the lung. Cancer, 1982, 50, 1289–1293.
24. Lambert EH, Rooke ED: Myasthenic state and lung cancer. In: The remote effects of cancer on the nervous system. Eds: L Brain, FH Norris. Grune and Stratton, New York, 1965, pp 67–80.
25. Leigh EH, Anderton BH, Dodson A, Gallo J -M, Swash M, Power DM: Ubiquitin deposits in anterior horn cells in motor neuron disease. Neurosci Lett, 1988, 93, 197–203.
26. Loberg EM, Torvik A: Uptake of plasma proteins into damaged neurons. An experimental study on cryogenic lesions in rats. Acta Neuropathol (Berl), 1991, 81, 479–485.
27. Morton DL, Habashi HH, Grimes OI: Non-metastatic neurological complications of bronchogenic carcinoma. The carcinomatous neuropathies. J Thor Cardiovasc Surgery, 1966, 51, 14–29.
28. Rae-Grant AD, Feasby TE, Brown WF, Gilbert JJ, Hahn AH: A reversible demyelinating polyneuropathy associated with cancer. Neurology, 1986, 36, suppl l, 81.

29. Sadiq SA, Thomas FP, Kilidireas K, Protopsaltis S, Hays AP et al.: The spectrum of neurologic diseases associated with anti-GM_1 antibodies. Neurology, 1990, 40, 1067–1072.
30. Sergott RC, Brown MJ, Lisak RP, Miller SL: Antibody to myelin-associated glycoprotein produces central nervous system demyelination. Neurology, 1988, 38, 422–426.
31. Shimosato Y, Nakajima T, Hirohashi S, Morinaga S, Terasaki T, Yamaguchi K, Saijo N, Suemasu K: Biological, pathological and clinical features of small cell lung cancer. Cancer Lett, 1986, 33, 241–258.
32. Takauchi S, Miyoshi K: Degeneration of neuronal processes in rats induced by protease inhibitor, leupeptin. Acta Neuropathol (Berl), 1989, 78, 380–387.
33. Terävainen H, Larsen A: Some features of the neuromuscular complications of pulmonary carcinoma. Ann Neurol, 1977, 2, 495–502.
34. Thomas FP, Adapon PH, Goldberg GP, Latov N, Hays AP: Localization of neural epitopes that bind to IgM monoclonal autoantibodies (M-proteins) from two patients with motor neuron disease. J Neuroimmunol, 1989, 21, 31–39.
35. Torvik A, Slettebo M: Encephalomyelitis with polineuropathy. Acta Neurol Scand, 1980, 61, 287–297.
36. Vallat JM, Lebartet MJ, Hugon J, Loubet A, Lubeau M, Fressinaud C: Acute pure sensory paraneoplastic neuropathy with perivascular endoneurial inflammation: ultrastructural study of capillary walls. Neurology, 1986, 36, 1395–1399.

Correspondence address: Dr. E. Kida, Department of Neuropathology, Medical Research Centre, PASci, 3, Dworkowa Str., 00-784 Warsaw, Poland

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

MARIA DĄMBSKA, DANUTA MAŚLIŃSKA

EFFECT OF SELECTED CYTOSTATIC DRUGS ADMINISTRATION ON THE BRAIN OF YOUNG RABBITS

Department of Developmental Neuropathology, Medical Research Centre, Polish Academy of Sciences, Warsaw

The aim of the study was to compare the effect of two selected cytostatic drugs (cyclophosphamide and vincristine) on the brain of young rabbits. The experimental models were similar to clinical administration of the examined drugs. Cyclophosphamide was given orally from the 5 to the 16th day of life and vincristine in a single intraperitoneal injection on the 8th day of life.

Despite the assumed poor penetration of both drugs througt the blood-brain barrier, both drugs induced changes in perivascular astrocytes. Other structural elements of the CNS exhibited lesions characteristic for the given drug: proliferation of endoplasmic membranes after cyclophlosphamide administration and destruction of microtubules with proliferation of microfilaments after vincristine application.

Key words: *cytostatics, cyclophosphamide, vincristine, rabbit brain.*

The development of chemiotherapy at the time increasing frequency of oncological diseases is connected with the use of a broad spectrum of antimitotic drugs. The choice and/or eventual combination of such drugs has to take into account the side effects connected with this treatment. The problem is particularly important in the pediatric clinic, where the improvement observed after anti-tumor therapy allows to expect a long survival of a greater than before group of young patients.

The maturation of the central nervous system (CNS) is one of the most important processes occurring in infants. Therefore we decided to investigate in experimental models the effect of selected drugs widely used in pediatric oncology. The influence of cyclophosphamide (CP) and vincristine (V) on normal brains of young animals was studied. Various aspects of administration of both drugs have been already presented and partially published (Maślińska et al. 1983, 1991; Iwanowski 1985, 1991; Dąmbska, Maślińska 1986; Dąmbska et al. 1986, 1991; Maślińska 1986). We now consider it useful to compare them.

Cyclophosphamide (Endoxan) is an alkylating drug which in recent years gained increasing application in the treatment of a variety of animal and human tumors (Cox et al. 1975; Colvin 1982) and also of several other processes wirh altered immunoreactivity (Calabresi, Parks 1980; Hommes et al.

1980, 1983). This drug as a water-soluble compound was considered for a long time as unable to cross the blood-brain barrier (BBB). However, recently high doses of CP appeared most effective against neuroblastoma metastases in children's brains (Helson et al. 1976; Allen, Helson 1981), indicating that CP and/or its metabolites may penetrate into the brain.

Vincristine is also very commonly used antimitotic drug known for its neurotoxic activity (Bradley 1970; Casey et al. 1973). The neurotoxic effect was evident in the form of peripheral neuropathy, but changes in the CNS were found only after intrathecal administration of the drug (Schochet et al. 1968). In humans and rabbits the changes were similar and consisted of crystalloid inclusions and agglomeration of neurofilaments in nerve cells and axons (Seil, Lampert 1968). Widely used in clinic, vincristine was considered for many years as not crossing the BBB. Recently some clinical observations suggested its noxious influence on the CNS after intravenous administration (Gennery 1985). In humans and in several species of animals (including rabbits) the BBB matures after birth (Gabryel 1973). Its function could be less effective in this period of life.

We present the changes observed in brains of young rabbits (12 – 16 days old) after administration of both examined drugs given in a schedule similar to routine clinical treatment. We will discuss the observed lesions together with the previously obtained results.

MATERIAL AND METHODS

The experiments were performed on suckling rabbits. The animals with their mothers were housed under controlled conditions. The mothers received a standard diet and water *ad libitum*. Cyclophosphamide (50 mg/kg/day) was administered by oral gavage from the 5 to the 16th day of life, and the animals were sacrificed on that day. Vincristine was administered intraperitoneally to 8-day-old animals in a single dose of 0.15 or 0.3 mg/kg of body weight. The rabbits were sacrificed on the 12th day of life. Each experimental group was compared to age-matched controls.

The animals (4 for each group) were anesthetized by intraperitoneal injection of urethan, perfused with buffered 2.5% paraformaldehyde and 1.5% glutaraldehyde solution (Karnovsky 1965). The slices from cerebral cortex, brain stem and spinal cord were rinsed in cacodylate buffer, pH 7.3, postfixed in 2% osmium tetroxide (4°C), dehydrated in graded alcohols and propylene oxide, then embedded in Epon 812. Ultrathin sections were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate and examined in a JEM-B-100 electron microscope.

Two animals of each group were decapitated for study in the light microscope. Brain slices were stained with cresyl violet and hematoxylin-eosin.

RESULTS

Cyclophosphamide administration resulted in reduced gain of body weight in young rabbits; on the 16th day of life it was significantly lower (56.3%) than in controls. Hair loss was also observed. Brain weight, however, was not

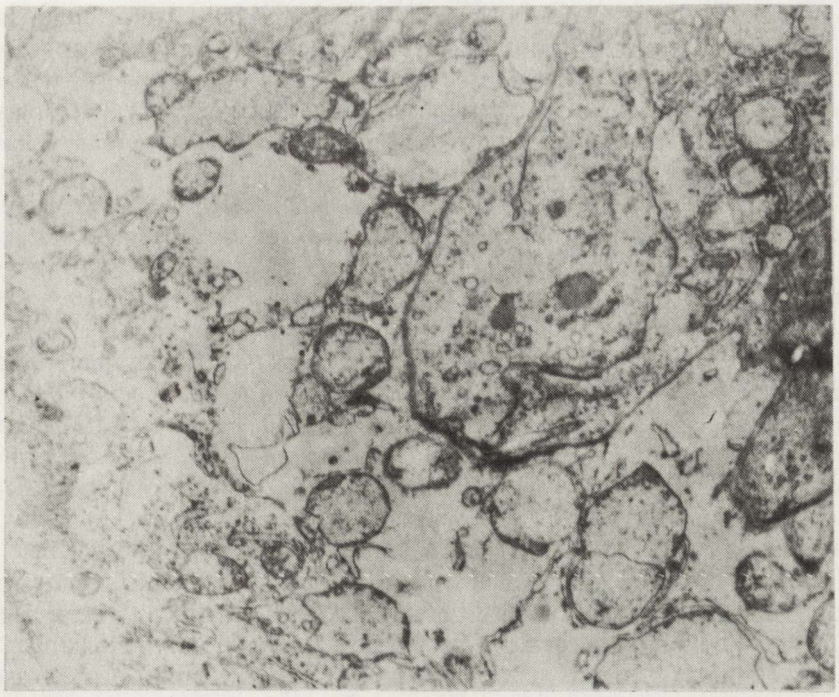

Fig. 1. Swelling of astroglial processes around capillary vessel after CP administration. × 8000

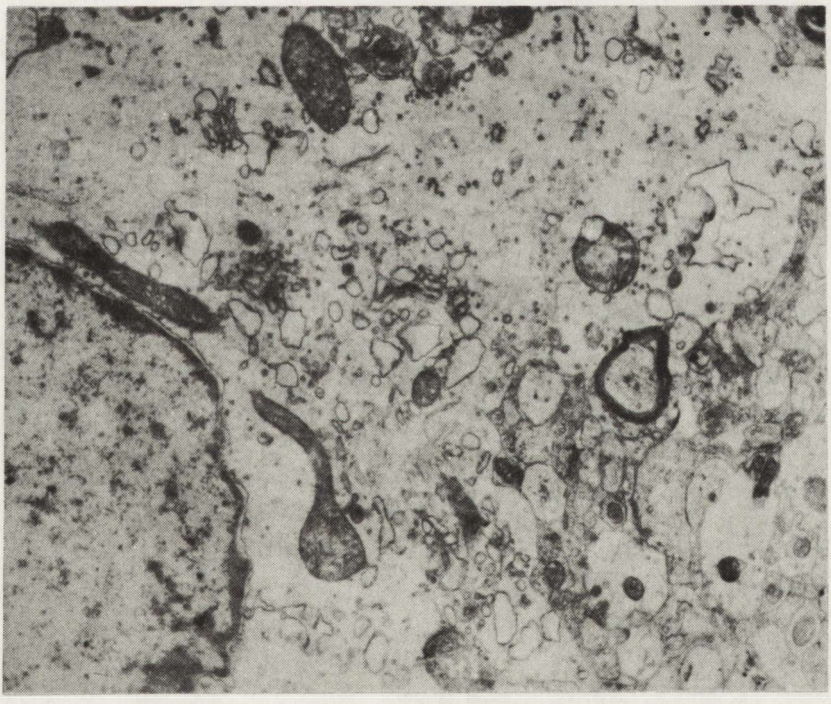

Fig. 2. Channels of endoplasmic reticulum enlarged and electrolucent cytoplasm in an astrocyte after CP administration. × 8000

changed. No neurological abnormalities in those rabbits were found. In the light microscope no CNS lesions were encountered. The changes appeared at the ultrastructural level. They were observed mostly in astrocytes, particularly those localized in the vicinity of capillaries (Fig. 1). The perikaryal cytoplasm of astrocytes was "empty" and only single endoplasmic reticulum channels formed large vacuoles (Fig. 2). Similar vacuoles and proliferation of endoplasmic membranes were found in oligodendroglial cells. Neurons were less affected than glial cells, but in some of them the above described changes were observed. Moreover, in the nuclei of neurons, encapsulated vesicular inclusions were found. The inclusions were variable in size and surrounded by a membrane. In glial nuclei similar inclusions were not observed.

Vincristine was clinically better tolerated than CP, the animals did not differ from controls and no neurological abnormalities were observed. In the light microscope no visible CNS lesions were found. Ultrastructural examination revealed changes in the brain stem and spinal cord. Aggregates of dark mitochondria and between them enlarged channels of endoplasmic reticulum were seen in neurons. In those cells many neurofilaments and only few neurotubules were found. The neurofilaments formed bundles, which were particularly well visible in the neuropil (Fig. 3), both in dendrites and axons, reaching also synaptic endings. Some axons were shrunken, myelin sheaths around them delaminated, which looked as secondary to axonal changes. The changes of glial elements consisted in marked swelling of perivascular

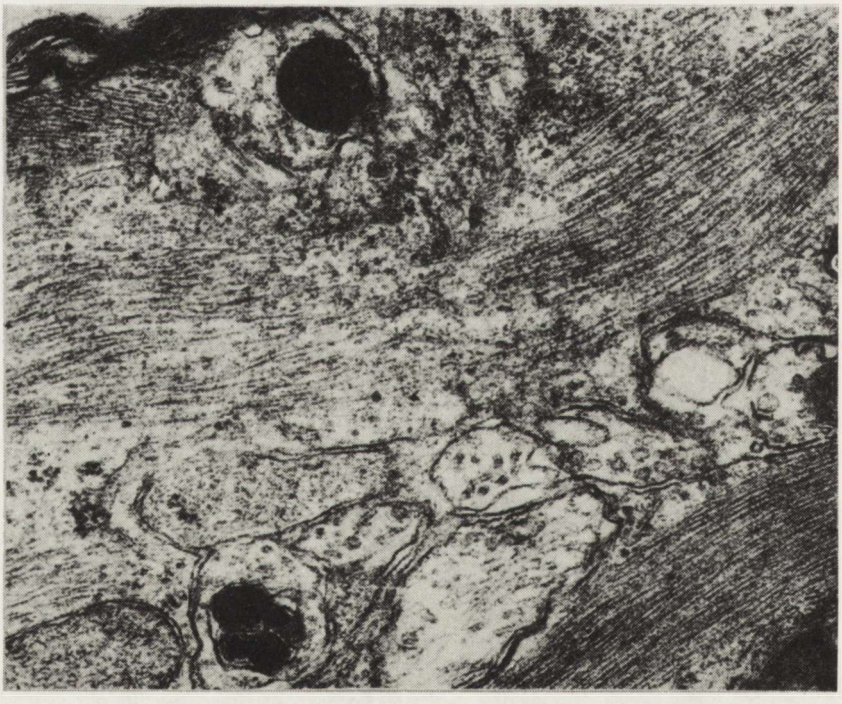

Fig. 3. Neurofibrillary bundles in neuropil after vincristine administration. × 12000

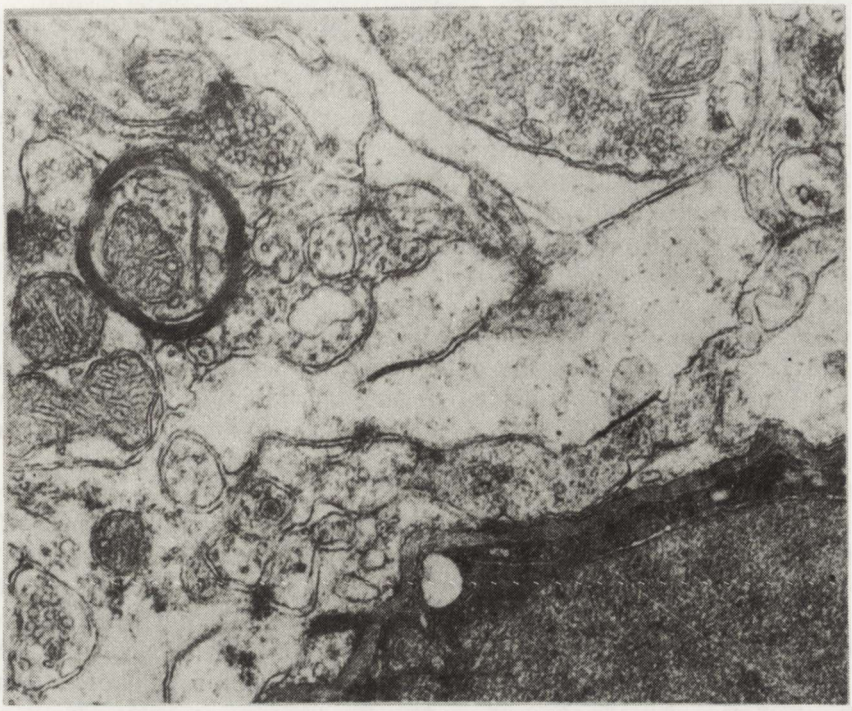

Fig. 4. Swelling of perivascular astroglial processes after vincristine administration. × 8000

astroglial processes (Fig. 4) containing also an increased number of gliofilaments. The intensity of changes in animals treated with 0.15 as well as 0.3 mg/kg of body weight of vincristine was estimated as only moderate.

DISCUSSION

Results of our experiments indirectly indicate that both cytostatics recommended as relatively safe for the CNS because of their poor penetration through the BBB, pass through this maturing barrier, unchanged or as metabolites, in an amount sufficient to induce a moderate but harmful effect on the CNS in experimental animals. In this consists the similarity of the influence of these two quite different cytostatic drugs. According to our observations the animals tolerated CP and V administration relatively well. The side effects after treatment with CP are well known (alopecia, malnutrition) but they never lead to death during the experiments. Therefore, we could assume that the doses used corresponded or, at least, did not exceed those used in clinical treatment. The "sparing" of the brain weight in undernurished rabbits after CP administration has been described earlier (Dobbing, Sands 1971) and is prognostically optimistic for rehabilitation. Nevertheless, ultrastructural lesions found in treated animals demonstrate the harmful influence of CP on the nervous tissue. The higher sensitivity of glial cells than neurons may depend on their

localization in the vicinity of vessel walls. Alkylating metabolites of CP mainly in the liver (Struck et al. 1975; Friedman et al. 1976) are transported with circulation and can penetrate the BBB. Therefore, the perivascular astrocytes could be most exposed to their harmful action. The lesions of endothelial cells in capillary vessels observed in a similar experimental model by Iwanowski (1985) correlate well with perivascular astrocytic changes. According to some hypotheses the vulnerability of cells may depend also on their low ability of detoxication of the alkylating compounds (Connors 1974; Struck et al. 1975). Moreover, still proliferating glial cells are more susceptible for CP acting metabolites than neurons (Connors 1974).

Cytoplasmic vacuoles formed from endoplasmic reticulum channels in glial cells can be connected with the action of CP metabolites (e.g aldophosphamide), since in cells susceptible to cytolysis, the aldophosphamide is cleaved by a beta-elimination reaction, generating of phosphoramide mustard and acrolein, both of which are highly cytotoxic (Connors 1975).

The changes of perivascular astrocytes were observed as changes of long duration (Iwanowski 1985). The neurons presented moderate changes. The intranuclear membraneous inclusions found in some neuronal nuclei we consider as not pathognomonic for any particular etiology, but as the effect of some factors which may distort the homeostasis within the nucleus (Dąmbska, Maślińska 1991). Cyclophosphamide administration may damage the DNA chain and this lead to membraneous inclusion formation (Calabresi, Parks 1980).

Other studies performed on rabbits treated with CP in similar models revealed that the myelination process was not retarded, but disseminated damage of myelinating glia and the activity of the G-6-Phosphatase in those cells could be considered as a feature of pathologic changes within those structures (Dąmbska, Maślińska 1986).

Vincristine given parenterally exerted a harmful effect on the CNS structures. This drug induced swelling of perivascular astrocytic processes, probably due to its penetration from the vascular bed to the brain tissue. This penetration was sufficient to induce changes similar to those produced after its intrathecal administration (Schochet et al. 1968). The abundant agglomeration of neurofilaments in the lower parts of the CNS (brain stem and spinal cord) is known as a result of the specific influence of vincristine. Vinca alkaloids lead to the destruction of microtubules in various types of cells (Kujawa et al. 1980). They block off polymerization of tubuline (Himes et al. 1976). Those changes are responsible for the formation of paracrystalloid structures (Donnoso et al. 1977), impairment of axoplasmic transport and fibrillary changes, well visible in our experimental animals. Further observations in animals with three months of survival testified to the long duration of changes within the cytoskeleton of neurons (Dąmbska et al. 1991) and fibrillary degeneration of perivascular astrocytic processes (Iwanowski 1991).

In conclusion, despite different reactions of various animal species and humans, our results should evoke some caution. The fact that after administration of two different cytostatics pathologic changes were found in the CNS has to be taken into account in therapy planning the composition and doses of the drugs.

WPŁYW WYBRANYCH CYTOSTATYKÓW NA MÓZG MŁODEGO KRÓLIKA

Streszczenie

Porównano wpływ dwóch wybranych cytostatyków: cyklofosfamidu (CP) i winkrystyny (V) na mózg młodych królików. W modelach doświadczalnych starano się zachować sposób podawania leków podobny do stosowanego w klinice. CP podawano zwierzętom doustnie od 5 do 16 dnia życia, winkrystynę – w pojedynczym wstrzyknięciu dootrzewnowym.

Niezależnie od ogólnie przyjętego poglądu na temat złego przenikania CP i V przez barierę krew-mózg, oba leki wywoływały zmiany w okolicy okołonaczyniowej w postaci obrzmienia wypustek astrocytarnych. Inne elementy strukturalne OUN wykazywały zmiany charakterystyczne dla danego leku, a więc rozrost błon endoplazmatycznych po podaniu CP oraz uszkodzenie neurotubul i pomnożenie neurofilamentów po podaniu winkrystyny.

REFERENCES

1. Allen JC, Helson L: High-dose cyclophosphamide chemotherapy for recurrent CNS tumors in children. J Neurosurg, 1981, 55 749–756.
2. Bradley WC: The neuromyopathy of vincristine in guinea pig. An electrophysiological and pathological study. J Neurol Sci, 1970, 10, 133–162.
3. Calabresi P, Parks RE: Antiproliferative agents and drugs used for immunosuppression. In: The pharmacological basis of therapeutics. Eds: AG Gilman, LS Goodman, A Gilman. MacMillan Publ Co Inc, New York, Toronto, London, 1980, pp 1256–1260.
4. Casey EB, Jelliffe AM, Le Quesne PM, Millett YL: Vincristine neuropathy. Clinical and electrophysiological observations. Brain, 1973, 96, 69–86.
5. Colvin M: The alkylating agents. In: Pharmacologic principles of cancer treatment. Ed: BA Chabner. Saunders Co, Philadelphia, 1982, pp 276–308.
6. Connors TA: Mechanism of clinical drug resistance. Clinical Pharmacology Symp Biochem Pharmacol, 1974, 23 (Suppl 2), 89–100.
7. Connors TA: Mechanism of action of 2-chloroethylamine derivatives sulfur mustards, epoxides and aziridines. In: Antineoplastic and immunosuppressive agents. Eds: AC Sartorelii, DG Johns. Handbuch der Experimentallen Pharmakologie, v 38/II, Springer, Berlin, 1975, pp 18–34.
8. Cox PJ, Philips BJ, Thomas P: The enzymatic basis of the selective action of cyclophosphamide. Cancer Res, 1975, 35, 3755–3761.
9. Dąmbska M, Maślińska D: Effect of cytostatic treatment on the brain tissue. Zentralbl Allg Pathol, 1986, 31, 400–401.
10. Dąmbska M, Maślińska D: The effect of some exogenous factors on the formation of intranuclear membranaceus inclusions in the nerve cells. Acta Neurobiol Exp, 1991, subjected.
11. Dąmbska M, Maślińska D, Tubylewicz J: Controversial effect of endoxan administration on maturing rabbit brain. Neuropatol Pol, 1986, 24, 407–416.
12. Dąmbska M, Maślińska D, Tubylewicz J: The elements of cytoskeleton in the central nervous system in rabbits treated with vincristine. Folia Histochem Cytobiol, 1991, subjected.
13. Donnoso JA, Green LS, Heller-Bettinger IE, Samson FE: Action of the vinca alkaloids vincristine, vinblastine and desacetyl vinblastine amide on axonal fibrillar organelles *in vitro*. Cancer Res, 1977, 37, 1401–1407.
14. Dobbing J, Sands J: Vulnerability of developing brain. IX. The effect of nutritional growth retardation on the timing of the brain growth-spurt. Biol. Neonate, 1971, 19, 363–378.
15. Friedman OM, Wodinsky J, Myles A: Cyclophosphamide-related phosphoramide mustards – recent advances and histerical perspective. Cancer Treat Rep, 1976, 60, 337–346.
16. Gabryel P: Bariera krew-mózg, struktura i funkcja. Patol Pol, 1973, 24, 217–235.
17. Gennery BA: Vincristine neurotoxicity. Lancet, 1985, p 385.
18. Helson L, Helson C, Patterson F: A rationale for the treatment of metastatic neuroblastoma. JNCI, 1976, 57, 727–729.
19. Himes RH, Kersey RN, Heller-Bettinger J, Samson FE: Action of the vinca alkaloids vincristine, vinblastine and desacetyl vinblastine amide on microtubules *in vitro*. Cancer Res, 1976, 36, 3798–3802.

20. Hommes OR, Aerts F, Bahr U, Schulten HR: Cyclophosphamide levels in serum and spinal fluid of multiple sclerosis patients treated with immunosuppression. J Neurol Sci, 1983, 85, 297–303.
21. Hommes OR, Lamers NJB, Reekers P: Prognostic factors in intensive immunosuppressive treatment of chronic progressive multiple sclerosis. In: Progress in multiple sclerosis. Eds: H Bauer, S Poser, G Ritter, Springer, Heidelberg, 1980, pp 396–400.
22. Iwanowski L: Ultrastructure of brain capillaries in young rabbits treated with cyclophosphamide. Neuropatol Pol, 1985, 23, 557–563.
23. Iwanowski L: Effect of vincristine on ultrastructure of capillaries of rabbit brain. Neuropatol Pol, 1991, subjected for publication.
24. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol, 1965, 27, 137.
25. Kujawa M, Ochocka M, Moskalewski S: Influence of vincristine on the Golgi complex of leukaemic lymphoblasts. Folia Haematol (Leipz), 1980, 107, 193–203.
26. Maślińska D: Effect of alkylating drugs on rat cerebellum. Folia Histochem Cytobiol, 1986, 24, 47–52.
27. Maślińska D, Dąmbska M, Tubylewicz J: Neurotoxicity of vincristine in rabbits with immature blood-brain barrier. Folia Histochem Cytobiol, 1991, subjected.
28. Maślińska D, Swierczyńska A, Koch I: Cyclophosphamide effect on changes in rabbit peripheral blood in the development period. Acta Physiol Pol, 1983, 34, 85–90.
29. Schochet SS, Capt MC, Usar PW, Lampert PW, Earle KM: Neuronal changes induced by intrathecal vincristine sulphate. J Neuropathol Exp Neurol, 1986, 27, 645–658.
30. Seil FJ, Lampert PW: Neurofibrillary tangles induced by vincristine and vinblastine sulphate in central peripheral neurons *in vitro*. Exp Neurol, 1986, 21, 219–230.
31. Struck RF, Kirk MC, Witt MH, Laster WR: Isolation and mass spectral identification of blood metabolites of cyclophosphamide: evidence for phosphoramide mustard as the biologically active metabolite. Biomed Mass Spectrom, 1975, 2, 46–52.

Authors' address: Department of Developmental Neuropathology, Medical Research Centre, PASci, 3 Pasteura Str, 02-093 Warsaw, Poland

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

MIECZYSŁAW WENDER, JÓZEF SZCZECH, STANISŁAW HOFFMANN*,
WOJCIECH HILCZER*

ELECTRON PARAMAGNETIC RESONANCE ANALYSIS OF HEAVY METALS IN THE AGING HUMAN BRAIN

Department of Neurology, School of Medicine and *Institute of Molecular Physics, Polish Academy of Sciences, Poznań, Poland

Electron paramagnetic studies of heavy metals were performed with the use of a Radiopan spectrometer on autopsy material of 5 aged patients (mean age 80.2 years) and 15 control patients (mean age 29 years). The obtained results lead to the following conclusions:

1. The aging brain is characterized by a tendency to decrease in concentration of isolated Cu^{2+} ions, and a marked decrease in concentration of Fe^{3+} ions as well as of free radicals, whereas the concentration of Cu^{2+} clusters is significantly increased.
2. The cases showing both arteriosclerotic and senile degenerative changes are characterized by higher concentrations of Cu^{2+} clusters than the brains with dominance of the arteriosclerotic process, whereas the concentrations of isolated Cu^{2+} and Fe^{3+} ions as well as that of free radicals do not differ between the two subgroups.
3. The diminished concentrations of Cu^{2+} and Fe^{3+} ions and free radicals observed in brains of old persons and concomitant with increased concentration of multiion aggregates (clusters), more marked in cases of senile atrophy of Alzheimer type, seem to result from some slow-down of metabolic processes in the aging brain.

Key words: *electron paramagnetic resonance, aging brain, heavy metals, copper, iron, radicals.*

The process of brain aging is associated with chemical changes, including deposition and transformation of several compounds which – as lipids and proteins – are essential elements of nervous tissue (Horrocks 1973; Wender et al. 1988, 1991; Svennerholm et al. 1991). In the course of aging some changes take place also in the content of ions, including heavy metals, serving as essential component of several enzymes (Kozik 1978).

The physical method of electron paramagnetic resonance (EPR) introduced broadly in the recent few years in biology, creates new perspectives in the analysis of heavy metals in the central nervous system, showing the significance, not only for its quantitative evaluation, but also for elucidation of the physico-chemical status of the elements in the central nervous system. In this paper we present studies on copper and iron in the aging brain using the EPR method.

MATERIAL AND METHODS

The studies were performed on autopsy material of 5 patients deceased between the ages of 77 and 86 years (mean 80.2 years) with the defined syndrome of organic brain disease. The results were compared with those noted in autopsy material of 15 patients aged from 16 to 38 years (mean 29.0 years), who died of various diseases of the central nervous system. Brain slices were submitted to histological and EPR analysis. The material for morphological evaluation was fixed in formaldehyde and lyophilized as preparation for EPR studies. The following brain regions were examined: parietal cortex, basal ganglia, corpus callosum, centrum semiovale of brain hemispheres and cerebellar white matter.

Electron paramagnetic studies were performed using the spectrometer Radiopan SE/X-2543 in a cylindric resonator of TE $_{OLL}$ type at the frequency of 9.40 GHz. Recording of EPR spectra took place at room temperature and the contents of various kinds of paramagnetic centres were evaluated per 1 g of nervous tissue. The quantity of ions and radicals was estimated using following standards: 1) "weak pitch" 01073A produced by Varian Company, containing 0.00033% P, which corresponds to 10^{13} spins/cm of standard length; 2) freshly prepared solution of diphenolpicrylhydrazyl (DPPH) in benzene, corresponding to 1.275×10^{21} spins.

The quantitative data on the content of ions and radicals in the studied samples were obtained by the method of double integration of EPR curves by comparison to the standard curve with the necessary corrections. The essential methodical questions of the use and significance of EPR in biology may be found in the publication of Heckly (1972).

RESULTS

Morphological characteristics of the presented material are summed up in Table 1. The neuropathological data led to the subdivision of the studied material into group of patients with dominating vascular changes and to the group with mixed changes (i.e. senile atrophy of the Alzheimer type and vascular alterations).

Qualitative analysis of the EPR signals

In all studied samples EPR signals of Cu^{2+} and Fe^{3+} ions as well as those of free radicals were noted. A typical spectrum within the full range of the used magnetic field is presented in Figure 1. An EPR signal with resolved hyperfine lines was characteristic for isolated (mutually nonintteracting) Cu^{2+} ions in octahedral or square-planar coordination. The relatively broad lines of the hyperfine quartet centered at about 300 mT suggested a coordination with atoms containing a magnetic nucleus of ^{14}N. The EPR signal of isolated Cu^{2+} was superimposed on a relatively broad signal from multiion clusters or aggregates of Cu^{2+}.

Table 1. Neuropathological characteristics of the studied material

Age	Diagnosis
	Group A
	Cases with arteriosclerosis* and parenchymatous senile changes**
84	Cerebral arteriosclerosis. Senile atrophy of Alzheimer type.
77	Cerebral arteriosclerosis. Necrosis in the left cerebral hemisphere. Status cribrosus. Senile atrophy of Alzheimer type
	Group B
	Cases with dominant arteriosclerosis
79	Cerebral arteriosclerosis. Status lacunaris.
86	Cerebral arteriosclerosis. Status cribrosus. Singular senile plaques.
83	Cerebral arteriosclerosis. Status lacunaris. Necrosis in medullae spinalis. Singular senile plaques
	Group C
	Cases deceased at age below 40 years (15 cases)
Mean age 29	Different diagnoses: multiple sclerosis, diffuse sclerosis, purulent meningitis, cerebral tumor, subarachnoideal hemorrhage, polyneuropathy

* The term cerebral arteriosclerosis comprises degenerative vascular changes and their ischemic sequelae in the brain

** Diagnosis of senile atrophy is based on the presence of senile plaques and neuron fibrillary tangles

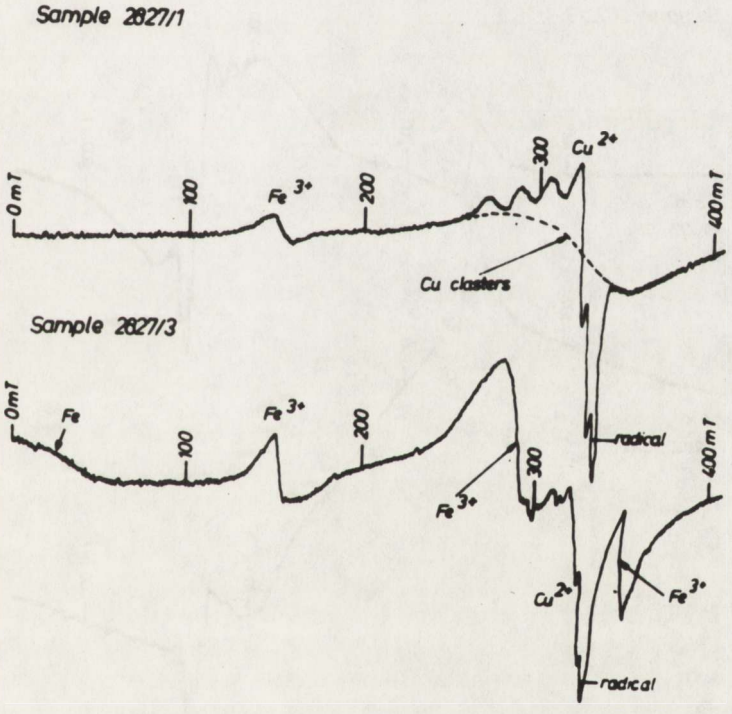

Fig. 1. A typical spectrum within the full range of the used magnetic field. Samples of parietal lobe (2827/1) and corpus callosum (2827/3). Concentrations of ions and radicals, calculated by double integration of the EPR signal, expressed in millitesl (mT). EPR signals of Cu^{2+} and Fe^{3+} ions, clusters of Cu^{2+} and free radicals

In all samples an EPR signal at magnetic field of about 155 mT was observed and was due to isolated iron^{3+} ions, homogeneously distributed over the studied slice and coordinated in a low-symmetry environment. In some samples an additional broad signal of Fe^{3+} was visible in the zero magnetic field and could be related to weakly bounded Fe^{3+} ions or to clusters of Fe^{3+} in the form of dimers or trimers. In a few samples a Fe^{3+} EPR signal was also observed in the form of a single, symmetrical resonance line, changing the position in the magnetic field with a rotation of the quartz tube, containing the sample. The line resulted from crystalline (ferromagnetic) aggregates localized at some points of the studied slice. The existence of iron^{3+} ferromagnetic aggregates was confirmed by EPR spectra recorded at liquid nitrogen temperature (77 K), when a strong and broad "ferromagnetic" signal was observed (Fig. 2).

A free radicals EPR signal at about 333 mT was a single, symmetrical, narrow line slighty disturbed by the Cu^{2+} signal.

Quantitative analysis of Cu^{2+}, Fe^{3+} ions, clusters and radicals content

Concentration of Cu^{2+} and Fe^{3+} ions, clusters and radicals, calculated by a double integration of EPR signals, is presented in Table 2. In Table 3, the

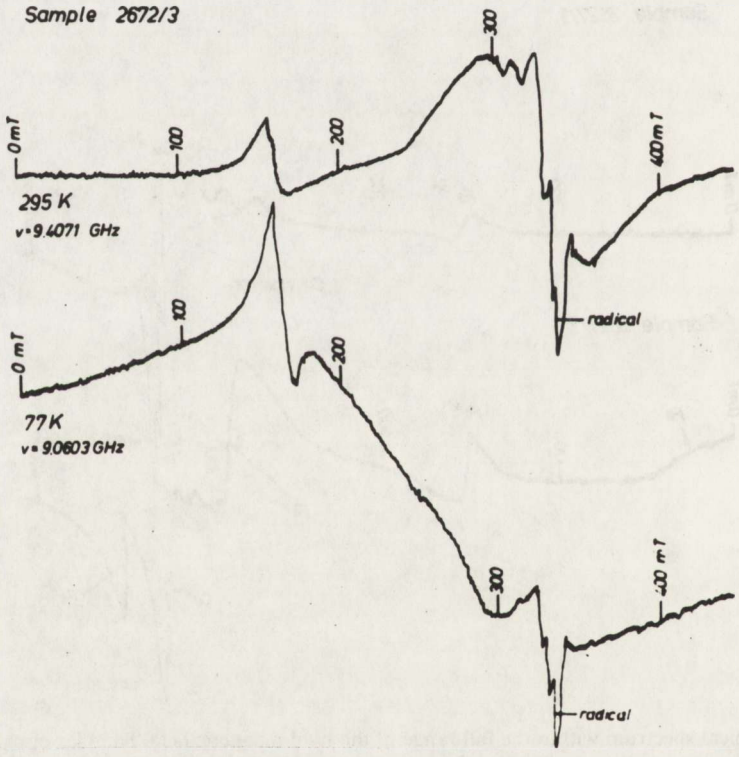

Fig. 2. Effect of temperature on EPR spectrum. EPR spectrum recorded at liquid nitrogen temperature (77 K) shows a strong and broad "ferromagnetic" signal

Table 2. Concentration of ions and radicals in 1 g of brain, calculated by double integration of EPR signals in aged patients

Material	Cu^{2+}	Cu clusters	Fe^{3+}	Radicals
Aged brain (total) (Number 5)	$6.0 \pm 0.68 \times 10^{17}$	$2.3 \pm 0.32 \times 10^{18}$*	$0.9 \pm 0.14 \times 10^{17}$*	$5.0 \pm 1.03 \times 10^{14}$*
Cases with arteriosclerosis and parenchymatous senile changes (Number 3)	$5.5 \pm 0.54 \times 10^{17}$	$2.7 \pm 0.44 \times 10^{18}$*	$1.1 \pm 0.18 \times 10^{17}$*	$4.9 \pm 1.25 \times 10^{14}$*
Cases with dominat arteriosclerosis (Number 2)	$6.5 \pm 0.85 \times 10^{17}$	$1.8 \pm 0.27 \times 10^{18}$	$0.9 \pm 0.09 \times 10^{17}$*	$5.2 \pm 0.80 \times 10^{14}$*
Cases deceased at age below 40 (Number 15)	$7.7 \pm 1.57 \times 10^{17}$	$0.8 \pm 0.45 \times 10^{18}$	$2.4 \pm 0.36 \times 10^{17}$	$10.4 \pm 2.68 \times 10^{14}$
Relative error of estimation	±4%	±8%	±5%	±5%

Mean ± SEM of all studied cases from 5 brain regions (parietal cortex, basal ganglia, corpus callosum, centrum semiovale, cerebellar white matter)

* Differences significant

Table 3. Comparison of ions and radicals concentration in 1 g of brain in aging persons with control material of persons deceased in young age indicated as one

Material	Cu^{2+}	Cu clusters	Fe^{3+}	Radicals
Aged brain (Total) (Number 5)	0.8 ± 0.09	2.8 ± 0.43*	0.4 ± 0.03*	0.5 ± 0.08*
Cases with arteriosclerosis and senile atrophy (Number 3)	0.7 ± 0.08	3.4 ± 0.64*	0.4 ± 0.04*	0.5 ± 0.09*
Cases with dominant arteriosclerosis (Number 2)	0.9 ± 0.1	2.2 ± 0.25	0.4 ± 0.03*	0.5 ± 0.06*
Cases deceased at age below 40 (Number 13)	1.0	1.0	1.0	1.0

Mean ± SEM of all studied cases from 5 brain regions (parietal cortex, basal ganglia, corpus callosum, centrum semiovale, cerebellar white matter)

* Differences significant

relative ion levels in the aging brain are compared with the control material of persons deceased at a young age. The results showed that the aging brain was characterized by a lower concentration of Cu^{2+} ions, which was more pronounced in the group of cases with senile atrophy of Alzheimer type and in the cerebellar white matter. The above mentioned differences were, however, insignificant. We have also noticed a lowered concentration of Fe^{3+} ions in aging brain. However, the lowest levels of the ions were characteristic of cases exhibiting predominance of arteriosclerotic changes (Table 4). The obtained results indicate a severalfold increase in content of Cu^{2+} aggregates (clusters) in the aging brain, the highest one noted in cases with senile changes of Alzheimer

Table 4. Comparison of ions and radicals concentration in 1 g of brain in cases with arteriosclerosis and senile atrophy, with cases with dominant arteriosclerosis only, indicated as 1

Material	Cu^{2+}	Cu clusters	Fe^{3+}	Radicals
Cases with arteriosclerosis and parenchymatous senile changes (Number 3)	0.8 ± 0.05	1.6 ± 0.16*	1.2 ± 0.08*	0.9 ± 0.16*
Cases with dominant arteriosclerosis (Number 2)	1.0	1.0	1.0	1.0

Mean ± SEM of all studied cases from 5 brain regions (parietal cortex, basal ganglia, corpus callosum, centrum semiovale, cerebellar white matter)

* Differences significant

type. The mean value for all studied samples indicated a lower number of radicals in the aging brain in comparison with the control group of patients deceased at an age below 40 years.

DISCUSSION

The exact source of the EPR signal Cu^{2+} ions in the studied material is not clear. However, its comparison with EPR signals derived from various standard substances indicates its similarity to the EPR signal of cytochrome oxidase C after denaturation or a coordination of copper with glycylglycine. The obtained results seem to testify that the aging brain is characterized by a tendency to lowered isolated Cu^{2+} ions, and by a significant increase in multiion copper aggregates. These results are not fully concomitant with the findings of Bonilla et al. (1984) who observed a negative linear regression between age and copper levels in the brain.

In our studies, the most marked signal derived from Fe^{3+} has been observed in the area of about 155 mT. The signal is similar to those of transferrin (Heckly 1972), what might mean that the content of transferrin is lowered in the aging brain. The incorporation of iron into transferrin is preceded by oxidation of Fe^{2+} to Fe^{3+}, the process in which ceruloplasmin, copper-containing protein, takes part. The content of copper ions shows a tendency to decrease in the aging brain.

According to the studies of Espinos et al. (1988) and Connor et al. (1987), an essential role in regulation of the transferrin level in the central nervous system is played by oligodendroglia. In the studies of Goncerzewicz et al. (1989), ultrastructural patterns of oligodendroglia in the aging brain suggested some degenerative changes in their cytoplasm. Also in karyo- and cytometric studies performed in rat brain by Godlewski (1989) and by Wender et al. (1987) in human brain, abnormalities have been found in aging oligodendroglia. Therefore, it seems plausible to suggest that the decrease in Fe^{3+}, observed in all studied brain samples including corpus callosum, might reflect alterations in the function of oligodendroglia.

The EPR shape and parameters of free radicals do not allow to establish their definite origin and significance. First of all, a similar EPR signal was observed in studies on lyophilized material, regardless of its origin, and is known under the term of "lyophilization signal". Even if the latter signal is not

identical with the signal observed in some biological material without lyophilization, one cannot exclude completely that the procedure of freeze drying as well as the influence of atmospheric oxygen on the dry sample exert some effect on the EPR line typical of free radicals in the brain. Therefore, it is only possible to establish that the concentration and total number of free radicals in the aging brain are diminished, without further detailed interpretation of the findings.

CONCLUSIONS

1. Studies of nervous tissue with the use of the EPR method indicate that the aging brain is characterized by a tendency to decreased concentration of isolated Cu^{2+} ions, and a marked decrease in concentration of Fe^{3+} ions as well as of free radicals, whereas the concentration of Cu^{2+} clusters is significantly increased.

2. The cases exhibiting both arteriosclerotic and senile degenerative changes are characterized by higher concentrations of Cu^{2+} clusters than the brains with dominance of the arteriosclerotic process, whereas the concentrations of isolated $Cu^{2+}$and Fe^{3+} ions as well as that of free radicals do not differ between the two subgroups.

3. The diminished concentrations of Cu^{2+} and Fe^{3+} ions and free radicals observed in brains of old persons and concomitant with increased concentration of multiion aggregates (clusters), more marked in cases of senile atrophy of Alzheimer type, seem to result from some slow-down of metabolic processes in the aging brain.

ANALIZA METALI CIĘŻKICH PRZY ZASTOSOWANIU ELEKTRONOWEGO REZONANSU PARAMAGNETYCZNEGO (EPR) W STARZEJĄCYM SIĘ MÓZGU LUDZKIM

Streszczenie

Przeprowadzono badania zawartości metali ciężkich w materiale autopsyjnym 5 starszych pacjentów (średnia wieku 80,2 lat) oraz u 15 pacjentów kontrolnych (średni wiek 29 lat).

Uzyskane wyniki doprowadziły do następujących wniosków: 1) starzejący się mózg charakteryzuje tendencja do spadku stężenia izolowanych jonów Cu^{2+} oraz do znacznego obniżenia stężenia jonów Fe^{3+} i wolnych rodników, podczas gdy stężenie klasterów Cu^{2+} jest istotnie podwyższone; 2) przypadki wykazujące zarówno zmiany miażdżycowe, jak i zwyrodnienie starcze charakteryzuje wyższe stężenie klasterów Cu^{2+} niż mózgi z samym procesem miażdżycowym, podczas gdy stężenie izolowanych jonów Cu^{2+} i Fe^{3+}, a także wolnych rodników nie różni się w obu podgrupach; 3) obniżone stężenie jonów Cu^{2+} i Fe^{3+} oraz wolnych rodników, obserwowane w mózgach starszych osób, równoległe ze wzrostem stężenia wielojonowych agregatów (klasterów), bardziej wyraźne w przypadkach zaników starczych typu Alzheimera, wydaje się następstwem pewnego spowolnienia procesów metabolicznych w starzejącym się mózgu.

REFERENCES

1. Bonilla E, Salazar E, Villasmil J, Gonzales M, Davilla J: Copper distribution in the normal human brain. Neurochem Res, 1984, 11, 1543–1548.
2. Connor J, Phillips T, Lakshman M, Barron K, Pina R, Csiza C: Regional variation in the levels of transferrin in the CNS of normal and myelin-deficient rats. J. Neurochem, 1987, 5, 1523–1529.
3. Espinos-de-los-Montenaros A, Chiapelli F, Ficher E, De Vellis Transferrin: An early marker of oligodendrocytes in culture. Int J. Dev Neurosci, 1988, 2, 167–175.
4. Godlewski A: Karyo- and cytophotometric studies on the oligodendroglia of ageing rat brain. Neuropatol Pol., 1989, 27, 477–490.
5. Goncerzewicz A, Wender M, Adamczewska-Goncerzewicz Z, Hejduk-Hantke H, Szczech J, Godlewski A: White matter of the ageing brain. In: Current topics in clinical and restorative neurology. Eds: G. Nappi, T. Domżał, A. Aguoli, A. Prusiński, P. Pinelli, I. Hausmanowa-Petrusewicz.
6. Heckly R: Free radicals in dry tissues. In: Biological application of electron spin resonance. Eds: H. Schwartz, J. Bolton, D. Borg. Wiley, New York, 1972, pp 197–212.
7. Horrocks L: Composition and metabolism of myelin phosphoglyceride during maturation and aging. In: Neurobiological aspects of maturation and aging. Ed: DH Ford. Prog Brain Res, 1973, 40, 383–395.
8. Kozik M: Laser-spectrographic study on the contents of metals in the brain of patients with arteriosclerotic dementia. Folia Histochem Cytochem, 1978, 1, 31–36.
9. Svennerholm L, Boström K, Helander C, Jungbjer B: Membrane lipids in the aging human brain. J. Neurochem, 1991, 56, 2051–2059.
10. Wender M, Adamczewska-Goncerzewicz Z, Dorszewska J: Myelin proteins in aging human brain. Mol. Chem. Neuropathol, 1991, 14, 1–10.
11. Wender M, Adamczewska-Goncerzewicz Z, Szczech J, Godlewski A: Myelin lipids in aging human brain. Neurochem Pathol, 1988, 8, 121–130.
12. Wender M, Godlewski A, Szczech J: Oligodendroglia of the aging human brain. Karyometric and cytophotometric studies. Neuropatol Pol., 1987, 25, 461–472.

Authors' address: Department of Neurology, School of Medicine, 49 Przybyszewskiego Str., 60-355 Poznań, Poland

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028–3894

JANINA RAFAŁOWSKA, STANISŁAW KRAJEWSKI*, EWA DOLIŃSKA, DOROTA DZIEWULSKA

DOES DAMAGE OF PERIVASCULAR ASTROCYTES IN MULTIPLE SCLEROSIS PLAQUES PARTICIPATE IN BLOOD-BRAIN BARRIER PERMEABILITY?

Department of Neurology, School of Medicine, Warsaw and *Department of Neuropathology, Medical Research Centre, Polish Academy of Sciences, Warsaw

In six young and two senile MS cases perivascular astrocytes within demyelination lesions were evaluated immunocytochemically. The peroxidase-antiperoxidase method of Sternberger et al. (1970) was used for visualization of glial fibrillary acidic protein (GFAP). In all cases very weak immunoreactivity of perivascular astrocytes was noted. Accumulation of perivascular glial fibers, and infrequently their fragmentation were observed both within active and old demyelination plaques. Clasmatodendrosis, Rosenthal's fibers and prominent regressive changes of astrocyte perikarya were found only in old plaques. A lack of immunoreactivity of perivascular astrocytes was noted within old demyelination lesions. The background of the latter was often immunonegative to GFAP. It is suggested that secondary damage of perivascular astrocytes influences vascular permeability within demyelination lesions including old plaques.

Key words: *MS plaques, perivascular astrocytes, GFAP immunoreactivity, vascular permeability.*

Blood vessels permeability disturbances within recent demyelination plaques are known both in experimental allergic encephalomyelitis (EAE) and in multiple sclerosis (MS). However, contrary to literature data, in our material an increase of vascular permeability has been found also within the old demyelination areas, in which exponents of an inflammatory process were absent (Rafałowska et al. 1990). Close structural and functional relationship of astrocytes with the cerebral microvessels is well known. The question arises whether pathological processes involving blood vessels within MS demyelinating plaques reflect on the state of perivascular glia. This question inclined us to perform immunocytochemical assessment of perivascular astrocytes within recent and old MS plaques.

---

* This work was supported by Institute of Psychiatry and Neurology within agreement No. R. 34. I.

MATERIAL AND METHODS

The material studied comprised eight cases of MS. Six of them concerned young patients (from 33 to 42 years) in which the clinical picture and course of the disease were typical. Two other cases concerned old people (76 and 87 years) in which the clinical picture and the disease course suggested the diagnosis of vascular cerebral stroke**. In all the cases autopsy was performed during the first 24 hours after death. Postmortem examination of formalin-fixed brain and spinal cord in young cases confirmed the clinical diagnosis. In senile cases with diagnosis of cerebral stroke MS was diagnosed during gross brain examination which revealed characteristic and typically located periventricular demyelination plaques. This was confirmed by histological examination.

In all cases formalin-fixed and paraffin-embedded slices taken at the level of basal ganglia and parieto-occipital region were investigated immunocytochemically. To evaluate astrocytes the technique of peroxidase-antiperoxidase of Sternberger et al. (1970) was used for visualization of glial fibrillary acidic protein (GFAP). Five to eight μm-thick sections, deparaffinized and pretreated with 0.0125% trypsin for 1 h, were preincubated with 2% normal swine serum diluted with TBS (Sigma) at pH 7.6. Thereafter, they were incubated overnight with primary polyclonal rabbit antibodies against GFAP (Dakopatts), 1:3000. After rinsing of the sections in PBS at pH 7.6, one hour incubation with the following secondary reagents was done: swine antibodies against rabbit IgG (1:50) and rabbit-PAP-complex (1:200), (antisera from Dakopatts). The immune reaction was developed during 15-min incubation in 0.05% 33-Diaminobenzidine tetrachloride (Sigma) with addition of 0.01% H_2O_2. After hemalum counterstaining the sections were dehydrated and mounted with D.P.X.

RESULTS

In areas distant from demyelination foci, in the grey and white matter both astrocyte bodies and their numerous long thin processes were immunopositive to GFAP. Processes of single astrocytes contacted sometimes several neighbouring vessels (Fig. 1). Venous vessels to which processes of a few astrocytes were directed (Fig. 2) were sporadically observed. Pericapillary astrocytes were not a common feature. Sporadically perivascular astrocytes in the superficial cortical layers were stained (Fig. 3).

Within the old demyelination areas and in active plaques the following changes were found: condensation of glial fibers and, infrequently their

---

** The clinical data and neuropathological findings of two senile cases are described in the paper of Rafałowska et al. (1988).

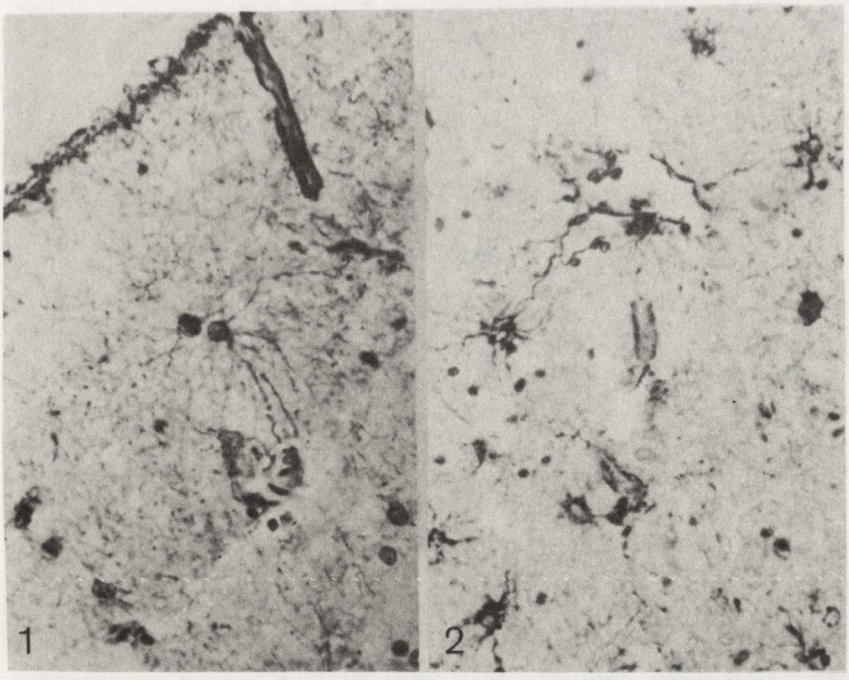

Fig. 1. Cortical molecular layer. Processes of one astrocyte connected with a few vessels. × 900

Fig. 2. White matter. Processes of two astrocytes directed to small venous vessel. × 900

fragmentation; changes of astrocyte perikarya with lesion of their processes in the form of clasmatodendrosis; thick fragments of processes resembling Rosenthal's fibers. In some cases neither astrocytic somas nor their processes were observed in the vascular neighbourhood. Remarkable was scanty perivascular astrocyte reaction.

Perivascular condensation of glial fibers was seen mostly around venous vessels of different sizes within active demyelination fields (Fig. 4) as well as within old plaques (Figs 5 and 6). Sometimes fragmentation of glial fibers surrounding vessels was present (Fig. 4). From time to time reactive astrocyte perikarya significantly changed were found in perivascular location (Figs 4, 7–8). Their processes underwent regressive changes taking the form of clasmatodendrosis (Figs 4, 7) or appeared in the form of thick short fibers resembling Rosenthal's fibers (Fig. 7). Sometimes the perikarya of reactive astrocytes and their processes were totally absent in the perivascular areas. These changes were found both within recent (Fig. 9) and old (Fig. 10) demyelination lesions, but more often within the old ones. The background of the old plaques was very often immunonegative to GFAP (Figs 6, 7).

The accumulation of perivascular glial fibers and their fragmentation were sometimes also seen in areas adjacent to active demyelination. Fragmentation of perivascular glial fibers was found in both types of demyelination, but more often within the active ones. Regressive changes such as clasmatodendrosis,

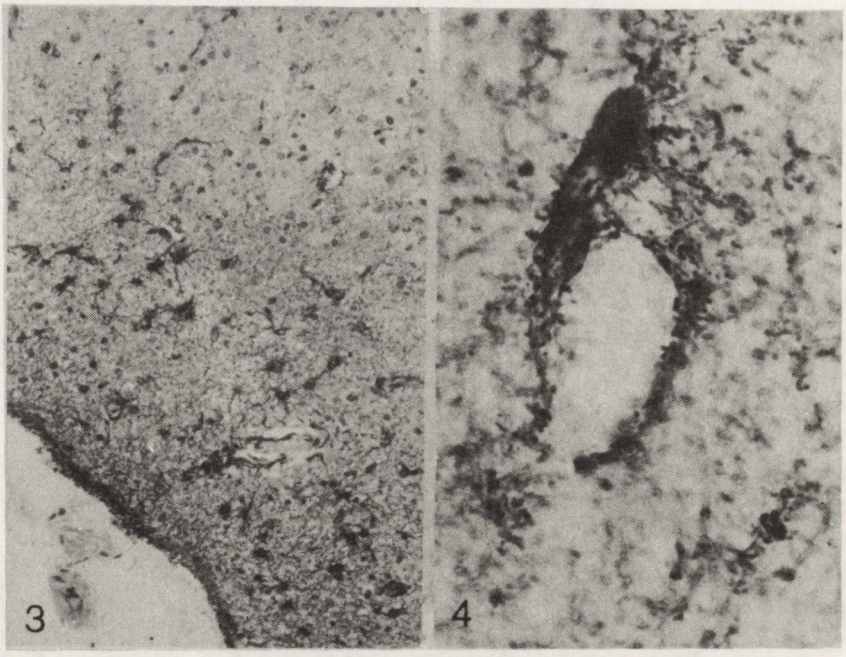

Fig. 3. Perivascular astrocytic proliferation within cortical molecular layer. × 900

Fig. 4. Active plaque. Hypertrophic perivenous astrocyte with short thick processes. Condensation and fragmentation of perivascular glial fibers. × 900

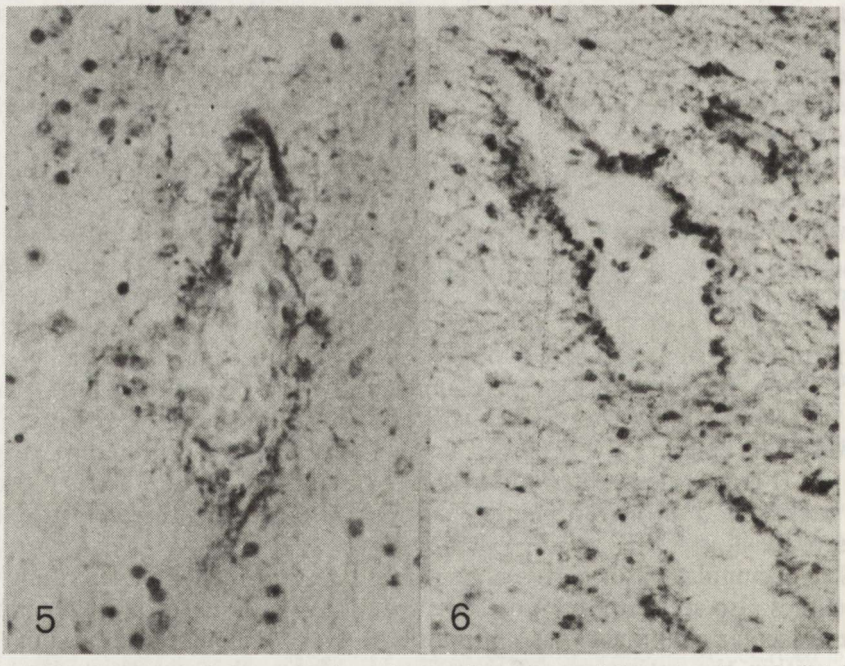

Fig. 5. Old subependymal plaque. Perivascular condensation and fragmentation of glial fibers. × 900

Fig. 6. Old demyelinated lesion. Perivenous condensation and fragmentation of glial fibers. × 448

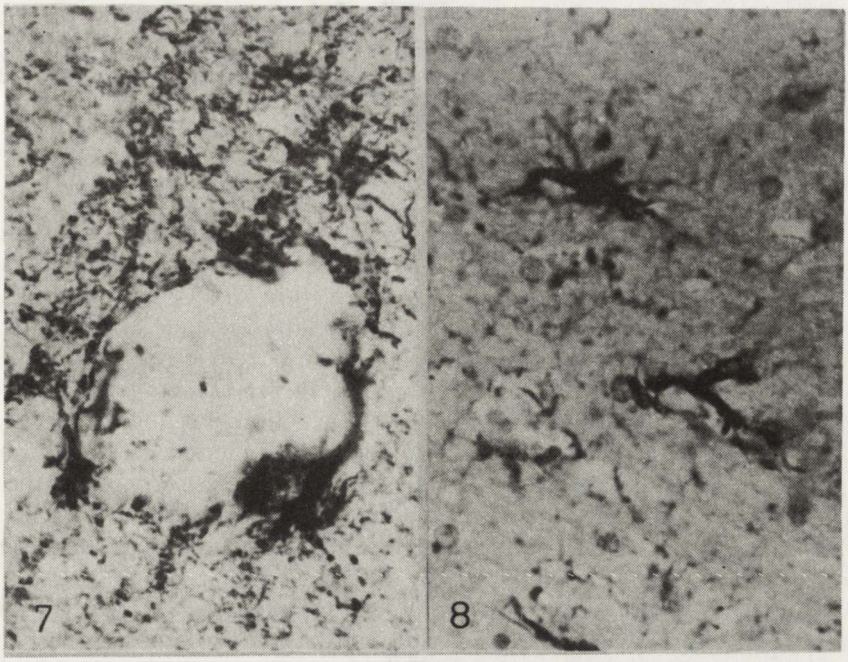

Fig. 7. Old plaque. Lack of gliosis. Perivenous Rosenthal's changes of reactive astrocyte and clasmatodendrosis. × 900

Fig. 8. Old plaque. Marked regressive changes of reactive perivascular astrocytes with thick irregular processes. × 900

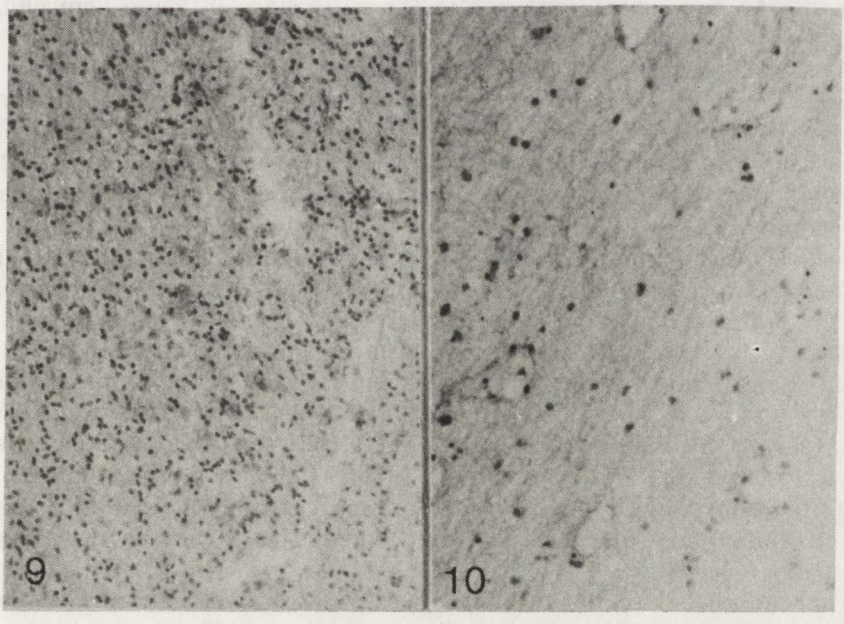

Fig. 9. Recent plaque. Numerous vessels and reactive astrocytes. Lack of perivascular astrocytes. × 448

Fig. 10. Old subependymal plaque. Lack of perivenous immunoreactivity to GFAP. × 448

astrocytic processes resembling Rosenthal's fibers, as well as abnormal astrocytic somas were found only within the old plaques. No distinct differences either in nature or in intensity of changes between senile and middle age cases were observed.

DISCUSSION

Our studies showed that in all 8 cases of MS the perivascular astrocytic reaction within demyelination areas was weak, it was even absent in some demyelination plaques. This morphological picture differs remarkably from that observed in ischemic brain damage. In the latter, islands of less damaged tissue are characterized by a distinct perivascular astrocyte reaction (Rafałowska et al. 1991). In experimental brain injury during the perinatal period in which as a rule there is no noticeable astrocyte reaction, if it does occur it takes the form of perivascular astrocytic proliferation (Janeczko 1988). In brain infarction and brain injury astroglial cells and blood vessels appear in the role of "a bystander" damaged during metabolic disturbances as or like the other brain tissue elements. In inflammatory-demyelinative processes participation of perivascular astroglial cells is probably different.

A very early exponent of structural changes in EAE is penetration of mononuclear cells and serum proteins through the walls of small venous vessels (Lampert 1965; Kristenson et al. 1976). It was demonstrated that both mannitol and serum proteins penetrate through blood vessels with inflammatory infiltrates (Oldendorf, Towner 1974; Ackerman et al. 1981). The increase of blood vessels permeability occurs parallelly with mononuclear phagocyte migration (Simmons et al. 1987). There are a few similar data concerning MS, however, the role of macrophages in the early stage of acute demyelinative process is consistently emphasized (Farrer et al. 1988).

Accumulation of immunological complexes within blood vessel walls both in MS (Campbell et al. 1988) and in experimental demyelinative polyneuropathy (Krajewski, Szablowska-Krajewska 1985) was observed. The above observations indicate that in the demyelinative process various factors may bring about blood vessel wall injury and disturbances in blood-brain barrier permeability.

According to Traugott et al. (1987) presentation of the Ia-determinant of the major histocompatibility complex both by endothelial cells and astrocytes and the presence of gamma-interferon receptors on astroglial cells might be essential in pathologic deteriorations during intercurrent infections e. g. viral ones. It is also known that owing to biochemical similarity, epitopes of various viruses cause a cross-reaction with myelin proteins (Alvord, Ericsson 1988). In some cases of MS, antibodies to endothelial cells and brain vascular smooth muscles were found (Tsucada et al. 1985). The above data indicate that additional nonspecific immunizing factors may cause exacerbation of disease with a subsequent increase of vessel damage and permeability disturbances.

The endothelial cell is the anatomical substrate of barrier mechanisms (Mossakowski 1988). However, capillaries and small blood vessels are very closely connected with astroglial cells through their processes surrounding the

vessels. Possibly a strict anatomical relationship of astrocytes and blood vessels which are pathogenetically involved by the disease process may be responsible for the secondary injury of perivascular astroglial cells observed in our material. Experimental data seem to support this hypothesis. They also show a direct metabolic relationship between astrocytes and small blood vessels endothelium as well as an influence of astrocytes on endothelial cells. Astrocytes are engaged in the synthesis of gamma-glutamyl-transpeptidase by capillary endothelial cells (Maxwell et al. 1986). This enzyme participates in transportation of amino acids through the vessel wall and its regulation (Maker et al. 1976). Astroglial cells influence both the occurrence and localization of Na^+/K^+ -ATPase and alkaline phosphatase activity within a capillary wall. Astrocytes also seem to induce barrier properties in extracerebral endothelial cells (Janzer, Raff 1987). Thus, it seems, that astroglial cells play a very important role in the correct function of barrier mechanisms.

In our material damage of perivascular glial cells might be connected with extension of the pathological process from the blood vessels to the surrounding astrocytes. Persistent pathology of both components of the blood-brain interphase is probably responsible for disturbances of blood vessels permeability within old demyelination plaques in MS.

CZY ASTROCYTY OKOŁONACZYNIOWE BIORĄ UDZIAŁ W ZABURZENIU PRZEPUSZCZALNOŚCI BARIERY KREW-MÓZG W BLASZKACH DEMIELINIZACYJNYCH W STWARDNIENIU ROZSIANYM

Streszczenie

W 8 przypadkach stwardnienia rozsianego w wieku 33—42 lata oraz 76 i 81 lat oceniono astrocyty okołonaczyniowe w obrębie ognisk demielinizacji. Kwaśne włókienkowe białko glejowe (GFAP) uwidaczniano stosując technikę immunoperoksydazy pośredniej według Sternbergera i wsp. (1970). We wszystkich przypadkach stwierdzono bardzo nikły odczyn przynaczyniowych komórek gwiaździstych. Zagęszczenie włókien okołonaczyniowych, niekiedy z ich fragmentacją, obserwowano zarówno w świeżych, jak i starych blaszkach demielinizacyjnych. Klazmatodendroza, włókna Rosenthala oraz bardzo nasilone zmiany wsteczne ciał astrocytów obserwowano wyłącznie w starych blaszkach. Brak immunoreaktywności przynaczyniowych astrocytów stwierdzano również w starych ogniskach demielinizacyjnych. W tych ostatnich obserwowano ponadto brak immunoreaktywności podłoża.

Autorzy sugerują, że głębokie, wtórne uszkodzenie astrocytów okołonaczyniowych wywiera wpływ na przepuszczalność naczyń w obszarach demielinizacji.

REFERENCES

1. Ackerman HP, Ulrich J, Heitz PWU: Experimental allergic encephalomyelitis. Exudate and cellular infiltrate in the spinal cord of Levis rats. Acta Neuropathol (Berl), 1981, 54, 149—152.
2. Alvord EC, Ericsson LH: In search of the human encephalitogenic determinant (s) and at least of the cause of multiple sclerosis. J Neuropathol Exp Neurol, 1988, 47, 322 (Abstr).
3. Beck DW, Roberts RL, Hart MN: Glial cells influence, presence and localization of enzyme activity at the blood-brain barrier. J Neuropathol Exp Neurol, 1985, 44, 338 (Abstr).
4. Campbell AK, Compston DAS, Jasani B, Linington C, Morgan BP, Scolding N, Wilkins P: Complement and multiple sclerosis. J Neuroimmunol, 1988, 17, 255 (Abstr).

5. Farrel MA, Kogan MT, Staunton H: Paucity of plaque lymphocytes in clinically acute multiple sclerosis. J Neuropathol Exp Neurol, 1988, 47, 307 (Abstr).
6. Janeczko K: The proliferative response of astrocytes to injury in neonatal rat brain. A combined immunocytochemical and autoradiographic study. Brain Res, 1988, 456, 280–285.
7. Janzer R, Raff MC: Astrocytes induce blood-brain barrier properties in endothelial cells. Nature, 1987, 325, 253–257.
8. Krajewski S, Szablowska-Krajewska M: Disseminated vasculomyelopathy in the peripheral nervous system mediated by immune complexes (ICs). Immunohistochemical studies of sciatic nerves in chronic serum sickness (CHSS) in rabbits. J Neurol Sci, 1986, 72, 131–145.
9. Kristensson K, Wiśniewski HM, Bornstein MB: About demyelinating properties of humoral antibodies in experimental allergic encephalomyelitis. Acta Neuropathol (Berl), 1976, 36, 307–314.
10. Lampert PW: Demyelination and remyelination in experimental allergic encephalomyelitis. J Neuropathol Exp Neurol, 1965, 24, 371–385.
11. Maker HS, Clarke DD, Lajtha AL: Intermediary metabolism of carbohydrate and amino acids. Basic neurochemistry. Eds: GJ Siegel, RW Albers, R Katzman, BW Agranoff. Little Brown and Co, Boston, 1976, pp 279–307.
12. Maxwell K, Berliner JA, Cancilla PA: Induction of γ-GTP in cultured cerebral endothelial cells by a glial released product(s). J Neuropathol Exp Neurol, 1986, 45, 375 (Abstr).
13. Mossakowski MJ: Bariera krew-mózg. In: Histologia. Ed: K Ostrowski. PZWL, Warszawa, 1988, pp 298–290.
14. Oldendorf WH, Towner H: Blood-brain barrier and DNA changes during the evolution of experimental allergic encephalomyelitis. J Neuropathol Exp Neurol, 1974, 33, 616–631.
15. Rafałowska J, Krajewski S, Dolińska E, Barcikowska-Litwin M: On modification of the histopathological picture of multiple sclerosis in advanced age. Neuropatol Pol, 1988, 26, 211–223.
16. Rafałowska J, Krajewski S, Dolińska E: On the blood-brain barier (BBB) permeability in multiple sclerosis. Neuropatol Pol, 1990, 28, 41–54.
17. Simmons RD, Buzbee TM, Linthicum DS, Mandy WJ, Chen G, Wang C: Simultaneous visualization of vascular permeability changes and leucocyte egress in the central nervous system during autoimmune encephalomyelitis. Acta Neuropathol (Berl), 1987, 74, 191–193.
18. Sternberger LA, Hardy PH, Jr, Cuculis FF, Meyer HG: The unlabelled antibody enzyme method of immunohistochemistry preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antiperoxidase) and its use in identification of spirochetes. J Histochem Cytochem, 1970, 18, 315–333.
19. Traugott H, Lebon P, Raine CS: Multiple sclerosis: possible role of interferon-gamma for reactivation of the disease process. J Neuropathol Exp Neurol, 1987, 46, 366 (Abstr).
20. Tsukada N, Behan WMH, Behan PO: Search for autoantibodies to endothelial and smooth muscle cells in patients with multiple sclerosis. Acta Neuropathol (Berl), 1985, 66, 134–139.

Authors' address: Department of Neurology, School of Medicine, 1A Banacha Str., 02–097 Warsaw

NEUROPAT. POL. 1992, 30, 1
PL ISSN 0028-3894

HANNA DRAC, MAREK BABIUCH

CHRONIC PROGRESSIVE AXONAL POLYNEUROPATHY SIMULATING GUILLAIN-BARRÉ SYNDROME

Neuromuscular Unit, Medical Research Centre, Polish Acedamy of Sciences; Department of Neurology, School of Medicine, Warsaw

Six cases of chronic progressive and/or relapsing polyneuropathy are reported. All cases were idiopathic at the beginning of observation. Electrophysiological examination and biopsy of sural nerve in all cases as well as autopsy of spinal roots S_1 in case 6 showed loss of fibers and axonal degeneration of myelinated fibers but neither active demyelination nor inflammatory cells were observed. Chronic progressive or relapsing idiopathic axonal polyneuropathy appear to be a distinct entity different from GBS. The term "axonal GBS" seems to be questionable.

Key words: *polyneuropathy, Guillain-Barré syndrome, axonal damage*

Guillain, Barré and Strohl (1916) described the syndrome characterized by motor dysfunction, absence of tendon reflexes, glove and stocking type of sensory loss, paresthesia. They stressed the marked increase of protein and slight increase of cellular elements in the cerebrospinal fluid (CSF). The authors concluded that the clinical signs and symptoms reflected an impairment of spinal roots and peripheral nerves suggesting toxic or infectious agents.
In the following years the attention of many authors was attracted by the inflammatory component of the nerve lesion in the Guillain-Barré syndrome (GBS) (Alajouanine et al. 1936; Haymaker, Kernohan 1949; Marshall 1963, Asbury et al. 1969, Prineas 1972). Demyelination (breakdown of myelin sheath) of spinal roots and peripheral nerves due to abnormal immune response was postulated (Arnason 1975).

Precise criteria for GBS were established in 1985 (the Guillain-Barré syndrome study group 1985). In its classical form GBS follows an acute or subacute, monophasic course, but chronic progressive and relapsing remitting forms are encountered (Thomas et al. 1969; Dyck et al. 1975; Prineas, Mc Leod 1976, our own series). Thus, the term chronic relapsing (dysimmune) polyneuropathy (CRDP) for such cases is preferred by some authors (Dalakas, Engel 1981).

Morphological examination of the nerves in cases of GBS reveals first of all foci of primary demyelination associated with infiltrates of lymphocytes and macrophages. Recently, however, descriptions of polyneuropathy called axonal

Table 1. Clinical data

Case no, initials, sex, age (yrs)	Onset of the disease (age) mode	Clinical status during hospitalization	CSF protein mg %	Efect of steroids	Onset to nerve biopsy	Course of the disease
1. P.J. m 25	21 yrs acute	guadriplegia, facial diplegia, muscle atrophy	296 65	beneficial	5 yr	relapsing, Ist episode preceded by gastro-intestinal infection, IInd episode by upper respiratory tract infection
2. J.F. m 27	26 yrs subacute	symmetrical distal motor sensory neuropathy with muscles atrophy	90	marked	1 yr	progressive, marked remission after 2 years
3. R.J. f 38	37 yrs subacute	moderate distal weakness, muscles atrophy, sensory disturbances in upper and lower extremities	142	moderate	1 yr	slowly progressive, remitting; after 1 year: hypertension, renal involvement, increased sediment rate face lipoatrophy skin and subcutaneous tissue involvement
4. W.A. m 55	37 yrs subacute	slight distal weakness of lower limbs with sensory disturbances	97	none	6 months	slowly progressive
5. P.Z. m 65	64 yrs acute	moderate proximal, slight distal weakness of lower limbs	81	much beneficial (recovery)	1 yr	relapsing, complete remission after 2 years since onset of the disease
6. B.W. m 64	63 yrs subacute	asymmetrical distal weakness, atrophy of upper and lower limbs with sensory disturbances	90	moderate	Ist biopsy: 1 year IInd biopsy: 16 months	progressive with some remission followed by relapse, and slight improvement; sudden death due to pulmonary artery emboli

Table 2. Electrophysiological data

Case	Median nerve					Peroneal nerve						Sural nerve			EMG
	MCV M/s	DL ms	A mV	F ms	SCV M/s	DL ms	A mV	MCV M/s	DL ms	A mV	F ms	SCV M/s	DL ms	A mV	
1. P.J.	49	4.8	6	34.8	69	21.0	0.1	45.5	7.9	1.7	40.0	61.5	2.6	0.07	neurogenic
2. F.J.	53.5	3.5	13.0	–	60	3.0	0.07	32.1	7.6	30	–	37.7	4.5	0.01	neurogenic
3. R.J.	41.6	5	25.0	43.0	38.7	4.0	0.04	19.6		0.5	–	not recorded			neurogenic
4. W.A.	48.9	4.9	2.0	32.2	36.2	4.0	0.01	34.3	4.7	4.0	62.4	30.8	3.4	0.06	neurogenic
5. P.Z.	40.0	5.5	20.0	40.2	42.5	3.0	0.03	42.2	5.3	3.0	38.0	52.0	2.3	0.05	neurogenic
6. B.W.	38.0	7.4	15.0	–	not recorded			36.1	4.7	11.0	–	55.0	2.0	0.02	neurogenic

MCV – motor conduction velocity; SCV – sensory conduction velocity; DL – distal latency; A – amplitude; F – wave conduction; EMG – electromyography; – not done

form of GBS appeared (Feasby et al. 1986, 1987; Vallat et al. 1990). Progressive axonal polyneuropathy clinically resembling GBS is also signalled (Julien et al. 1989).

We report on a series of six cases of chronic progressive or relapsing polyneuropathy in which sural nerve biopsy revealed axonal damage of different degree.

MATERIAL AND METHODS

Six patients aged 25 to 65 years suffering from chronic progressive or relapsing polyneuropathy are presented. The patients had been followed for a period from 2 to 4 years. No etiological factor could be established at the beginning of observation. Electrophysiological examination including electromyography, motor and sensory conduction velocity in all cases and F wave in 3 cases were carried out. Cerebrospinal fluid, serum immunoglobulins, protein electrophoresis, antinuclear antibody were assessed. Intoxication by heavy metals, exposure to toxins, neoplasm, systemic disease including diabetes, porphyria, collagen disease were excluded. For clinical data see Tables 1 and 2. Sural nerve biopsy was done in all cases (twice in case 6, which was also autopsied).

The following methods were used:

1. Paraffin sections were stained with hematoxylin and eosin (assessment of infiltrates and vessels).

2. Thick Epon sections were stained with thionine and acridine orange (evaluation of degeneration/regeneration; density of myelinated fibers (mf) per 0.1 mm^2 was calculated from photograms magnified up to 1600 × using a semiautomatic MINI-MOP (Opton) analyzer.

3. Teased fibers were fixed in glutaraldehyde and osmium tetroxide (assessment of pathological process in the nerves: axonal degeneration, demyelination/remyelination).

4. Thin Epon sections stained with uranyl acetate and lead citrate were examined under a JEM 100B electron microscope (blood vessels, unmyelinated fibers, bands of Büngner, infiltrates).

RESULTS

In nearly all cases mononuclear cells dispersed in the endoneurium and around the vessels were visible (Fig. 1, Table 3). On thick Epon sections in three cases fibers undergoing axonal degeneration were observed (Fig. 2). Such fibers were specially numerous in cases 3 and 6 (II-nd biopsy and spinal roots – Fig. 3, Table 4). Regenerated fibers (groups of small fibers present on thick Epon sections) were seen in cases 1, 3 and 6 (Table 4). In most cases loss of myelinated fibers, sometimes quite pronounced was visible (Figs 4a, b, Table 5). In some cases macrophages were present (Fig. 5, Table 4). The teased fibers revealed axonal degeneration (Fig. 6, Table 5) and very few (except case 6 I-st biopsy) remyelinating fibers (Fig. 7, Table 5).

Electron microscopic examination was consistent with the light microscopy findings and did not reveal fibers undergoing active demyelination or demyeli-

Table 3. Morphological changes in sural nerve and spinal roots on paraffin sections

Case	Perivascular infiltrates	Mononuclear cells dispersed	
		around the vessels	in the endoneurium
1. P.J.	–	+	+
2. J.F.	+ –	+	+
3. R.J.	–	+	+
4. W.A.	–	+	+
5. P.Z.	–	+ –	–
6. B.W.			
Ist biopsy	–	+ –	–
IInd biopsy	–	+ –	+
post. root S_1	–	+ –	+ +
ant. root S_1	–	–	+ +

– not present; + – sometimes present; + not numerous; + + numerous

Table 4. Morphological changes in sural nerve and spinal roots on cross Epon sections

Case	Axonal degeneration	Segmental		Regenerated fibers	Macrophages
		demyel.	remyel.		
1. P.J.	+ –	–	–	–	+
2. J.F.	+	–	–	–	–
3. R.J.	+ +	–	–	+	–
4. W.A.	+ –	–	–	+	–
5. P.Z.	–	–	+ –	–	+
6. B.W.					
Ist biopsy	–	–	+ –	+ –	–
IInd biopsy	+ +	–	+ –	–	+
post. root S_1	+ +	–	+ –	–	+
ant. root S_1	+ +	–	+ –	–	+

– not present; + – sometimes present; + not numerous; + + numerous

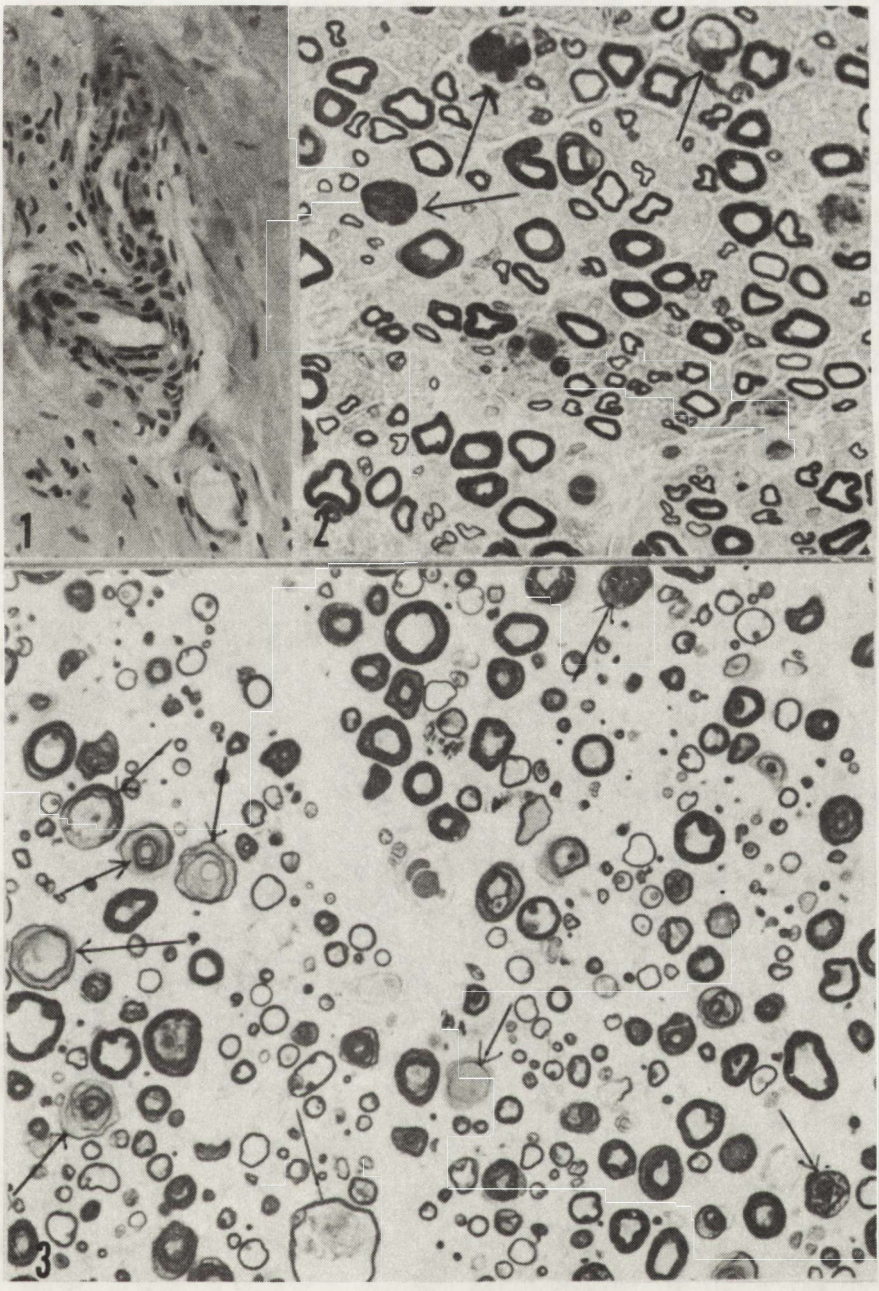

Fig. 1. Sural nerve in case 4. Mononuclear cells dispersed around the vessel. H.E. × 320

Fig. 2. Sural nerve in case 1. Fibers undergoing axonal degeneration (arrows). Cross Epon section × 640

Fig. 3. Posterior spinal root S_1 in case 6. Fibers undergoing axonal degeneration (arrows). Cross Epon section × 640

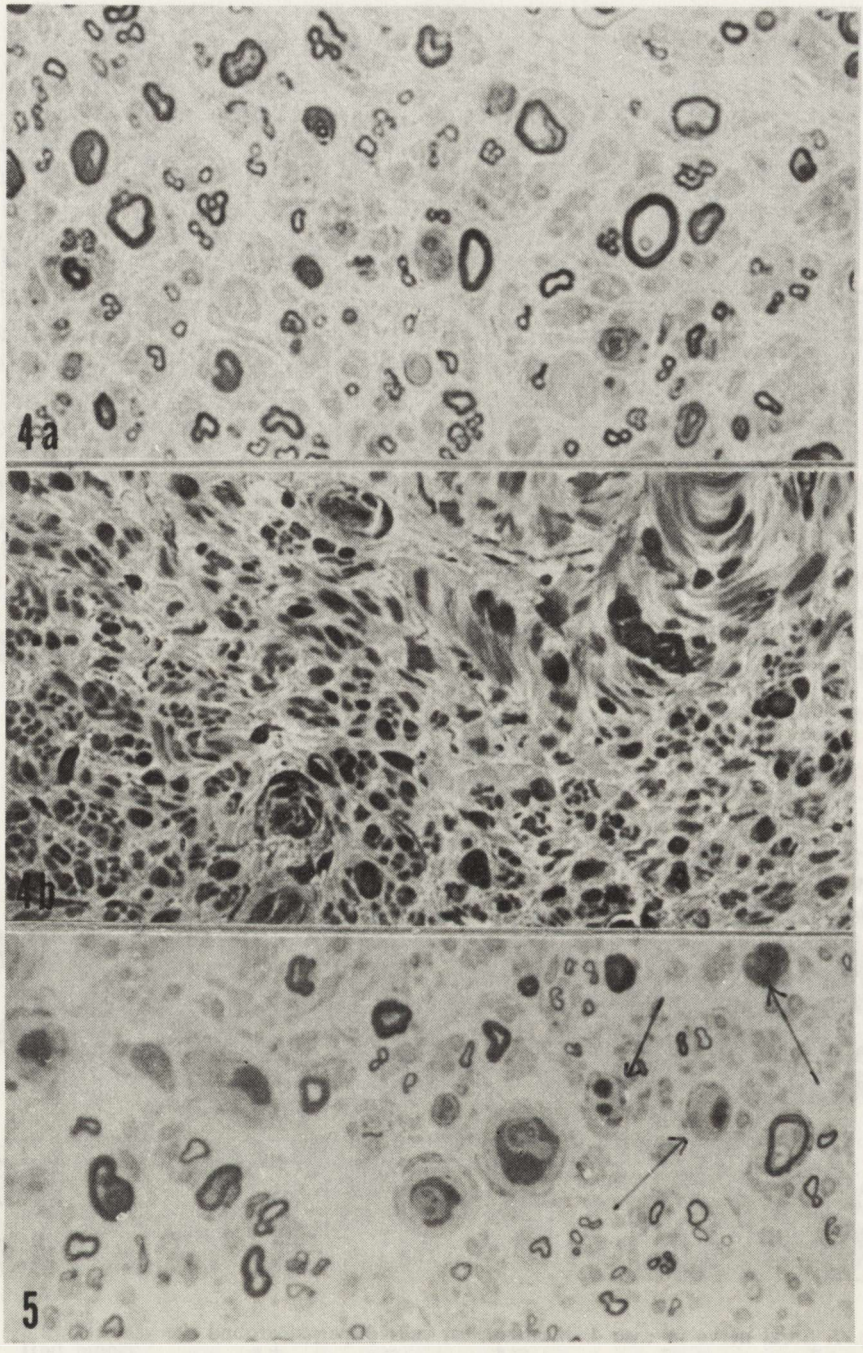

Fig. 4 a, b. Sural nerve in case 3 (a) and in case 2 (b). Note loss of myelinated fibers. Cross Epon section × 640

Fig. 5. Sural nerve in case 1. Note presence of macrophages (arrows). Cross Epon section × 640

Table 5. Qualitative and quantitative data in sural nerve and spinal roots

Case No age (yrs)	Polyeuropathy				Control				
	Density of mf/0.1mm^2	mf with changes %			Age (yrs) n = number of cases	Density of mf/0.1mm^2	mf with changes %		
		demyel. remyel.	axon deg.	total			demyel. remyel.	axon deg.	total
1. P.J. 25	643	1.5	1.0	2.5	25–35 n = 3	836	2.3	3.5	5.8
2. J.F. 27	86	1.0	60.0	61.0					
3. R.J. 38	52	2.2	12.2	14.4	35–45 n = 4	798	7.0	3.4	10.4
4. W.A. 55	243	–	5.0	5.0	50–60 n = 5	748	5.6	4.9	10.5
5. P.Z. 65	344	10.0	5.0	15.0	60–70 n = 3	866	15.0	20.0	35.0
6. B.W.64 Ist biopsy	651	25.0	5.0	30.0					
IInd biopsy	222	–	10.0	10.0					
post. root S_1	–	3.0	80.0	83.0					
ant. root S_1	–	2.0	50.0	52.0					

Fig. 6. Teased fibers from sural nerve in case 2. Axonal degeneration × 160

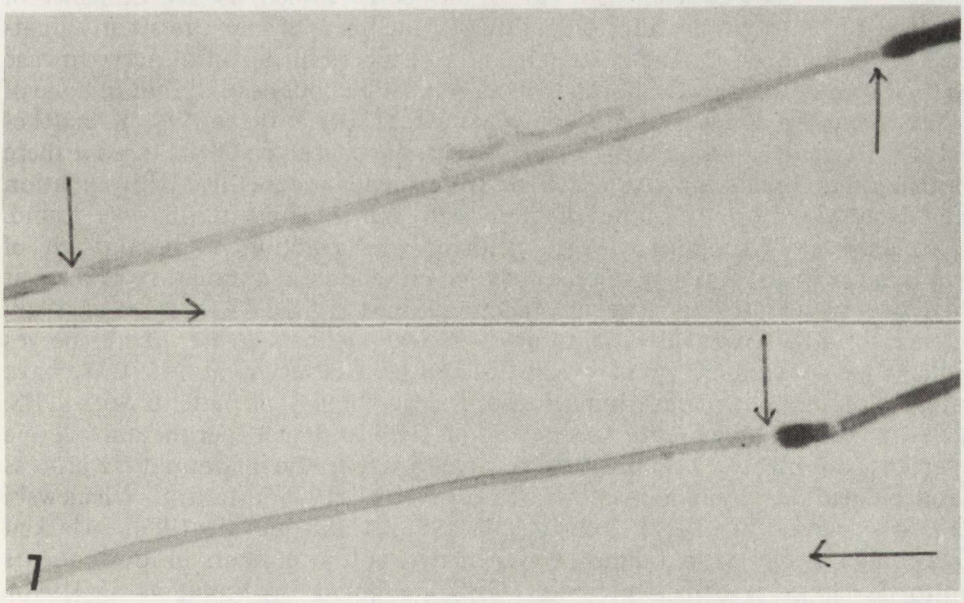

Fig. 7. Remyelinating teased fiber from sural nerve in case 5. Arrows indicate nodes of Ranvier × 160

nated. Normal unmyelinated fibers and bands of Büngner indicating loss of myelinated fibers were found.

Electrophysiological data are presented in Table 2.

DISCUSSION

The diagnostic criteria of chronic progressive or relapsing/remitting polyneuropathy of GBS type for years have been:

1. Presence of sensorimotor polyneuropathy of slow onset, usually over several months.
2. A course that is steadily progressive and/or relapsing/remitting.
3. Elevated CSF protein level.
4. Marked slowing of nerve conduction velocity.
5. Absence of evidence of systemic disease including diabetes or of history of exposure to toxins and heavy metals; in addition serum immunoglobulin electrophoresis should be normal.
6. Presence of segmental demyelination in sural nerve biopsy (Schwartzman et al. 1977; Dalakas, Engel 1981).

The presented cases (except case 3 in which in the course of the observation collagen disease has been found) fulfil four of the above mentioned criteria reserved for chronic or relapsing-remitting polyneuropathy of GBS type. However, the electrophysiological findings were suggestive for axonal damage of the nerve fibers. In all cases in sural nerve biopsy a different degree loss of myelinated fibers, fibers undergoing axonal degeneration i.m. features typical for axonal damage of the nerve were noted. Morphological findings correspond to electrophysiological ones. The percentage of fibers undergoing axonal degeneration in some cases is minimal, though a coexistent loss of myelinated fibers is evident (sec Table 5). This could be explained by the difficulty of isolation of empty tubes after breakdown of the fiber. In four cases remyelinating fibers are present. The percentage of such fibers in the sural nerve in case 6 (first biopsy) exceeds the normal value. It must be underscored that in none of the cases active demyelination characteristic for GBS (Prineas 1981; Krendel et al. 1989) has been found. In spite of the chronic character of the disease there are no onion bulb structures which are the evidence of repetitive demyelination and remyelination. In none of the cases evident infiltration was found. Dispersed mononuclear cells are probably the reaction to breakdown of myelinated fibers. Autopsy in case 6 did not reveal an inflammatory process at the level of spinal roots, contrary to the findings in GBS (Asbury et al. 1969; Prineas 1981). Axonal degeneration of individual fibers in peripheral nerves and spinal roots in GBS was revealed at autopsy (Asbury et al. 1969). We have similar observations concerning the sural nerve biopsy of patients with GBS. Such a kind of damage of the nerves in GBS and in experimental allergic neuritis is thought to be a phenomenon secondary to the inflammatory process and defined as "bystander effect" (Pollard et al. 1975; Madrid, Wiśniewski 1976; King et al. 1977; Said et al. 1981; Hahn et al. 1988). Marked preponderance of axonal damage over demyelination of fibers in four patients who developed a clinically typical GBS are noted by Vallat et al. (1990). Primary axonal lesion in the peripheral nerves and spinal roots without

inflammation or demyelination in one case and electrophysiological findings suggestive of axonal degeneration as the predominant process were observed in four cases by Feasby et al. (1986). The authors consider their cases as an acute axonal form of GBS. The axonal lesion in the distal part of the biopsied nerve could be secondary to demyelinating process affecting the spinal roots. However, in our case 6 the study of the lumbar spinal root did not disclose any demyelination, but only severe axonal damage. This finding might favour an initial axonal attack, possibly caused by a deleterious humoral factor. In most of our cases the effect of steroids was beneficial. This might suggest some immunological (among others) mechanisms of axonal damage of nerves in our material.

The presented cases seem to confirm the existence of relapsing/remitting polyneuropathy clinically immitating GBS, but differing from this syndrome by the axonal character of affection of peripheral nerves. It seems that, in spite of some similarity, such a polyneuropathy is a distinct entity different from GBS. The term axonal GBS seems to be questionable.

Acknowledgement. The authors thank Professor I. Hausmanowa-Petrusewicz for reviewing the manuscript.

PRZEWLEKŁA POSTĘPUJĄCA AKSONALNA POLINEUROPATIA NAŚLADUJĄCA ZESPÓŁ GUILLAIN-BARRÉ

Streszczenie

Przedstawiono sześć przypadków przewlekle postępującej i/lub nawrotowej polineuropatii. We wszystkich przypadkach na początku obserwacji nie udało się ustalić przyczyny choroby. Badanie elektrofizjologiczne i biopsja nerwu łydkowego we wszystkich przypadkach, badanie sekcyjne korzeni S_1 w przypadku 6 wykazało ubytek włókien mielinowych oraz zwyrodnienie aksonalne włókien mielinowych. Nie stwierdzono czynnej demielinizacji włókien ani nacieków w nerwie.

Przewlekła postępująca lub nawrotowa aksonalna idiopatyczna polineuropatia wydaje się być postacią odrębną od zespołu Guillain-Barré. Używany w literaturze termin „aksonalna forma zespołu Guillain-Barré" wymaga zakwestionowania.

REFERENCES

1. Alajouanine T, Thurel R, Hornet T, Boudin G: La polyradiculo-nérvite aigue generalisée avec dipleqie faciale et paralysie terminale des muscles respiratoires et avec dissociation albumino-cytologique: étude anatomique. Rev Neurol, 1936, 65, 681–697.
2. Arnason BGW: Inflammatory polyradiculoneuropathies. In: Peripheral Neuropathy. Eds: PJ Dyck, PK Thomas, EH Lambert. Saunders, Philadelphia, 1975, vol 2, pp 1110–1148.
3. Asbury AK, Arnason BG, Adams RD: The inflammatory lesion in idiopathic polyneuritis: its role in pathogenesis. Medicine, 1969, 48, 173–215.
4. Dalakas MC, Engel WK: Chronic relapsing (dysimmune), polyneuropathy: pathogenesis and t. eatment. Ann Neurol, 1981, 9 (suppl), 134–145.
5. Dyck PJ, Lais AC, Ohta M, Bastron JA, Ozaki H, Groover RV: Chronic inflammatory polyradiculoneuropathy. Mayo Clin Proc, 1975, 50, 621–637.
6. Feasby TE, Gilbert JJ, Brown WF, Bolton CF, Hahn AF, Koopman WF, Lochodne DW: An acute axonal form of Guillain-Barré polyneuropathy. Brain, 1986, 109, 1115–1126.
7. Feasby TE, Gilbert JJ, Brown WF, Bolton CF, Hahn AF, Koopman WJ, Lochodne DW: Acute "axonal" Guillain-Barré polyneuropathy. Neurology, 1987, 37, 357.

Zakład Narodowy im. Ossolińskich – Wydawnictwo. Wrocław 1992.
Objętość: ark. wyd.: 7,50; ark. druk. 5,75+0,13 wkł.; ark. A_1-6,7.
Wrocławska Drukarnia Naukowa. Zam. 2113/92

NEUROPATOLOGIA POLSKA
VOLUME 29, 1991

CONTENTS

AUTHORS INDEX

NEUROPATOLOGIA POLSKA
V. 30, No. 1, 1992

Cena zł 25 000,–

CONTENTS

Indeks 36668