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Murine Cortical Brain Cells are Autoantigenic from a Distinct Developmental Stage Onwards

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Summary

The expression of autoantigens on murine cortical brain cells and their first appearance during development was studied. Autoreactivity was analyzed by weight increase and lymphocyte proliferation in the popliteal lymph node (PLN). Cortical brain cells or defined plasma membrane preparations were injected s.c. without adjuvant into syngeneic recipients. Weak, but significant T cell-dependent PLN enlargement was triggered with brain cells from adult mice. A stronger reaction could be elicited with one defined fraction of purified plasma membranes. The earliest appearance of the antigenic material in the plasma membrane fraction was observed on day 15 after birth. This time point correlates exactly with the completion of the blood-brain barrier in large parts of the central nervous system.

Key words: *Autoantigens – Autoimmunity – Blood-brain barrier – Brain cortex cells – Lymphocyte proliferation – Mouse development – Plasma membranes – PLN assay – T lymphocytes*

Introduction

Large parts of the central nervous system (CNS) are thought to have a special immunological status which is guaranteed by the blood-brain barrier. This barrier is

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characterized by special anatomical structures constructed by endothelial cells of the brain capillaries that prevent entry of antibodies and effector cells. Therefore, in most parts of the CNS immunity operates with certain constraints and the limited capacity of the brain to react immunologically might depend on the integrity of the blood-brain barrier.

The concept of autoantigens secluded by the blood-brain barrier intrigued many investigators for a long time. So far only a few membrane constituents have been described as autoantigens. For example, molecules such as myelin basic protein (MBP), acetylcholine receptor or cerebrosides and gangliosides are targets of the syngeneic immune system under particular pathological circumstances (Lindstrom et al. 1976; Frick 1982; Endo et al. 1984). Many disorders of the CNS are correlated with an increased permeability of the barrier and with abnormal immune response that are autoimmune in nature. For example, T cell-mediated immunity to MBP can result in acute encephalomyelitis and CNS lesions (Lisak et al. 1980). Furthermore, oligoclonal immunoglobulins in the cerebrospinal fluid of multiple sclerosis patients can be detected (Delmotte and Gonsette 1977). The pathogenesis of these diseases is studied in various animal model systems. Autoimmune reactions against neuronal antigens could be produced only by immunization with antigens emulsified in Freund's complete adjuvant (FCA) (Moore et al. 1984; Lebar and Vincent 1984). Thus, T cells sensitized extra-neurally to MBP homogenized in adjuvant have been shown to migrate to neuronal tissue and to generate severe lesions (Ben-Nun et al. 1981; Ben-Nun and Lando 1983).

The question is still open how the recognition of autoantigens takes place. T cells recognize foreign antigens only in the context of self-MHC glycoproteins (Doherty 1985). The fact that most brain cells normally do not express MHC antigens (Wong et al. 1984) raises new aspects about autoantigen recognition and the privileged immunological status of the CNS. There is increasing experimental evidence that under pathological conditions the expression of MHC glycoproteins is induced on neuronal cell populations (Fontana et al. 1984; Wong et al. 1984) which allow T cell recognition of the antigens (Ben-Nun et al. 1981; Watanabe et al. 1983).

The developmental stage is yet unknown at which antigens first appear that are capable of inducing autoimmune reactions. In the present study, purified plasma membrane preparations from murine cortical brain cells of all developmental stages were tested for their ability to generate immune reactions in syngeneic lymphocytes. We have shown that murine brain cells or purified plasma membranes from these cells can induce autoreactivity in the popliteal lymph node but only when the reactive material was derived from animals older than 10 days.

Material and Methods

Animals

Mice. Inbred BALB/c mice were obtained from the Versuchstieranstalt Hannover, F.R.G., or were bred in the animal facility at the Institute of Molecular Biology, Salzburg, Austria. The BALB/c congenic nu/nu mice were purchased from Bomholtgard, Denmark.

Preparation of plasma membrane vesicles

Plasma membrane vesicles were prepared from brain cortex using the modified procedures of Jones and Matus (1974) and Gurd and Mahler (1974). Cortices from mice of various developmental ages were dissected and carefully cleared from meninges and the white matter. The tissues were diced finely and dissociated in a cold isotonic 5 mM phosphate-buffered sucrose solution (0.32 M, pH 7.6) containing 1 mM EDTA. Subsequently, the single cells were broken in a Dounce homogenizer by 20–30 strokes with a tight pestle. After centrifugation of the homogenate ($800 \times g$, 15 min, at 4°C) the nuclear pellet was rehomogenized as described above and the supernatants pooled and spun down at $11\,500 \times g_{av}$ for 25 min. The mitochondrial pellet was washed twice in cold lysis buffer (5 mM Tris-HCl, 1 mM EDTA, pH 8.1 for 30 min). The first purification step of plasma membranes was achieved by density gradient flotation in a SW 28 rotor (Beckman) for 2.5 h at $60\,000 \times g_{av}$. This centrifugation step resulted in 2 layers and the pellet. The layer banding at 1.1 M sucrose (designated as A_2) was further purified by placing the sample on a second discontinuous sucrose gradient with the following densities: 1.2 M, 1 M, 0.8 M, 0.6 M and 0.4 M sucrose in 5 mM phosphate buffer (plus 1 mM EDTA, pH 7.4). This second gradient was centrifuged in a SW 28 rotor for 90 min at $53\,000 \times g_{av}$. Subsequently, fractions were collected, washed in phosphate buffer and after the last washing the pellets were resuspended in sterile distilled water. The samples were either frozen directly or lyophilized and stored at −30°C. Enzyme activities and biological activity remained unaltered by either treatment. The various fractions were designated as follows: the fraction that interphased at 0.6–0.8 M sucrose as B_1, at 0.8–1.0 M sucrose as B_2, at 1.0–1.2 M sucrose as B_3 and fraction 'P' for the pellet. Plasma membranes from adult mouse liver were isolated according to Neville (1968).

Electron micrographs of brain plasma membranes were produced as described previously (Bauer et al. 1979).

Preparation of myelin, MBP and lipid

Myelin was prepared from 5 g of adult BALB/c brain cortices. The tissue was homogenized in cold 0.3 M sucrose phosphate buffer (5 mM, pH 7.4), layered over 0.85 M sucrose solution in centrifuge tubes and spun at $70\,000 \times g_{av}$ for 30 min. The myelin interphase was removed, homogenized with a Dounce homogenizer in cold distilled water, centrifuged, rehomogenized and spun down again. The resulting pellet was resuspended in phosphate-buffered isotonic sucrose solution, homogenized and separated on the discontinuous sucrose gradient described above. Fractions were washed several times, aliquoted and lyophilized. Lipid from 5 g of brain cortex from BALB/c mice was prepared using the protocol of Radin (1969). A crude preparation of MBP was isolated from adult mouse brain according to the method of Deibler et al. (1972). Galactocerebroside Type I was purchased from Sigma

Enzyme assays

NA^+,K^+-ATPase activity in membrane fractions was assayed according to Medzihradsky et al. (1971). The reaction mixture contained 150 mM NaCl, 10 mM

KCl, 3 mM $MgCl_2$, 3 mM ATP (Na salt, Sigma), 50 mM imidazole Cl (pH 7.2), 0.5 mM EDTA and 20 μg membrane vesicles in a final volume of 100 μl. In samples incubated at 37°C the reaction was initiated and stopped at various intervals with cold $HClO_4$ (0.72 M). The samples were centrifuged at $13000 \times g_{av}$ for 10 min. Inorganic phosphate was measured colorimetrically at 700 nm. Lactate dehydrogenase (LDH) activities were determined as described previously (Bergmeyer 1970). Monoamine oxidase (MAO) was assayed using the procedure of Wurtman and Axelrod (1963).

The popliteal lymph node (PLN) assay

Brain cells, plasma membranes (PM) derived from brain cells and liver cells, myelin basic protein, various lipids or phosphate-buffered saline (PBS) were injected into one hind footpad leaving the contralateral side uninjected as control. The popliteal lymph nodes were removed at various time points later and weighed immediately. The strength of the immune reaction was determined by PLN enlargement, the PLN index being defined as the ratio of the lymph node weights from the injected over the uninjected side (Gleichmann et al. 1983).

Injection of ^{51}Cr-labeled syngeneic spleen cells or [^{3}H]thymidine

Lymphocyte trapping was tested according to the method of Emeson and Trush (1973). 10×10^6 ^{51}Cr-labeled syngeneic spleen cells free of red blood cells were injected intravenously into treated hosts 24 h before sacrifice. To determine proliferation of the popliteal lymph node cells, mice were given an intravenous injection of 45 μCi [^{3}H]thymidine and killed exactly 30 min later (Sprent and Miller 1972).

Radiochemical determinations

The popliteal lymph nodes from mice injected previously with ^{51}Cr-labeled spleen cells or with [^{3}H]thymidine were dissolved in 1 ml tissue solubilizer (Soluene 30, Packard) by overnight incubation in a 56°C water-bath. Those labeled with ^{51}Cr were counted in a gamma scintillation counter. The samples labeled with [^{3}H]thymidine were further diluted with Dimilume 30 (Packard) and the radioactivity was determined in a beta liquid scintillation counter. The indices for lymphocyte trapping and for proliferation in the popliteal lymph nodes, respectively, were determined by the ratio of the cpm from the injected side over the uninjected side.

Statistical evaluation

PLN indices are expressed as means and standard errors (SE) or standard deviations as indicated in the Results section. Significance analysis was performed by using Student's *t*-test with a confidence coefficient of 0.95.

Results

Biochemical characterization of the plasma membrane preparations

The composition of cell surface molecules of brain cells from adult mice was analyzed on plasma membrane fractions. The purification included the fractionation

TABLE 1

CHARACTERIZATION OF THE PLASMA MEMBRANE FRACTIONS FROM MURINE ADULT CORTICAL BRAIN CELLS

Material tested	Protein (mg)	(Na^+,K^+-ATPase) (μmol) [a]	MAO (%)	LDH (μmol) [b]
Homogenate	157.5	3.3	100	365
B_1	2.34	10.1	1.04	ND
B_2	2.82	6.4	1.01	ND
B_3	21	10.4	7.03	ND
P	9	ND [c]	ND	ND

[a] Expressed as μmol/mg protein/h.
[b] Expressed as μmol/mg protein/min.
[c] ND, not detectable.

by 2 sucrose gradients, which resulted in the 3 bands (B_1, B_2 and B_3) and the pellet (P) (see Methods). These fractions were defined by marker enzyme activities which are characteristic for the following cell organelles: LDH for cytoplasmic constituents, MAO for mitochondrial organelles and K^+, Na^+-ATPase for plasma membranes. The data are summarized in Table 1. No detectable LDH activity was found in any of the 3 bands or in the pellet. Fractions B_1, B_2 and B_3, but not fraction P exhibited enriched K^+,Na^+-ATPase activity as compared to the cell homogenate. In addition, MAO activity was found in all fractions, indicating that

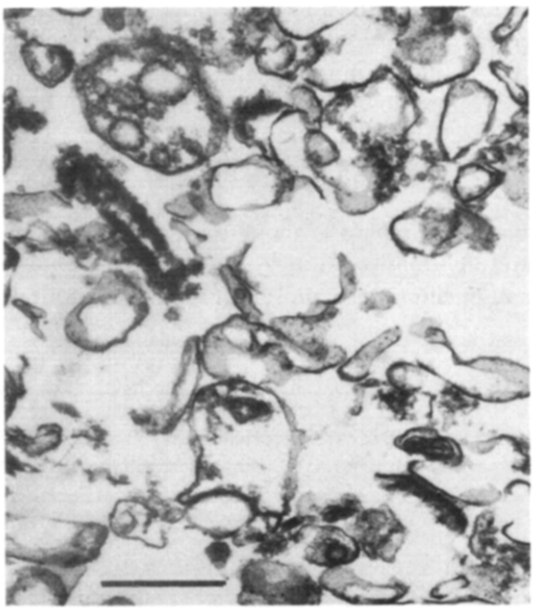

Fig. 1. Electron micrograph of the purified plasma membrane fraction B_2 from adult murine cortical brain cells. The bar represents 250 nm.

the preparations B_1, B_2 and B_3 contain plasma membrane vesicles with minor contaminations of mitochondrial membranes. Among the fractions that were tested for the ability to stimulate the PLN by inducing an enlargement, only fraction B_2 was effective (see data below). Therefore, the purity of this fraction was also monitored electron microscopically as shown in Fig. 1. Fraction B_2 contains membrane vesicles of various sizes, a few synaptosomes and minor mitochondrial and ribosomal contaminants.

Immunological analysis

Brain cell homogenates and plasma membrane fractions were analyzed for the potential to induce immunoreactivity in syngeneic recipients.

The data in Table 2 demonstrate that a preparation of cortical brain cell homogenate induced a weak, though significant PLN enlargement at a concentration of 100 μg protein. The reactivity could not be enhanced further by increasing the injected dosage. After 3 days the lymph nodes decreased to normal size (data not shown).

We assumed that the autoreactive antigens are expressed on the cell surface and therefore plasma membranes were prepared from adult brain cells. After the first sucrose density gradient centrifugation, 2 bands were obtained of which only one (banding at 1.1 M sucrose) exhibited autoreactive activity (data not shown). This fraction (A_2) was further purified on a second sucrose gradient, resulting in 4 protein-containing fractions. Out of the 4 fractions, fraction B_2, B_3 and P were assayed for immunogenicity in the PLN assay. The yield of fraction B_1 was too low to be tested in the biological assay. The results in Table 3 indicate that only fraction B_2 generated PLN enlargement, whereas fractions B_3 and P were ineffective. Dose-response studies showed that B_2 is most immunogenic at concentrations between 20 and 100 μg protein (Fig. 2). Higher dose (500 μg protein) induced PLN enlargement on both sides which, therefore, resulted in reduced PLN indices. An autoreaction was already detected 24 h after injection, peaked between 48 and 72 h and declined after 120 h (Table 4).

Myelin and BMP could be possible contaminants in our active preparations, although the white matter had been carefully removed during dissection of the adult brain cortices. Furthermore, since plasma membranes from brain tissue also contain

TABLE 2

PLN REACTIVITY TO BRAIN CELLS FROM SYNGENEIC ADULT MICE

Dose	PLN index ± SE [a]	n [b]
PBS	1.06 ± 0.04	6
0.05 mg	1.25 ± 0.04	6
0.1 mg	1.70 ± 0.01	5
0.2 mg	1.64 ± 0.15	4
0.7 mg	1.69 ± 0.08	6

[a] Assayed after 96 h.

[b] Number of mice tested.

TABLE 3

PLN REACTIVITY TO DIFFERENT FRACTIONS OF PLASMA MEMBRANE PREPARATIONS FROM ADULT BRAIN CELLS

Mice injected with [a]	PLN index ± SE	*n*
PBS	0.85 ± 0.1	5
Fraction B_2	2.32 ± 0.19	4
Fraction B_3	1.09 ± 0.26	6
Fraction P	1.30 ± 0.18	5

[a] Mice were injected with 40 μg protein in 50 μl into one hind footpad. PLN reactivity was determined 48 h later.

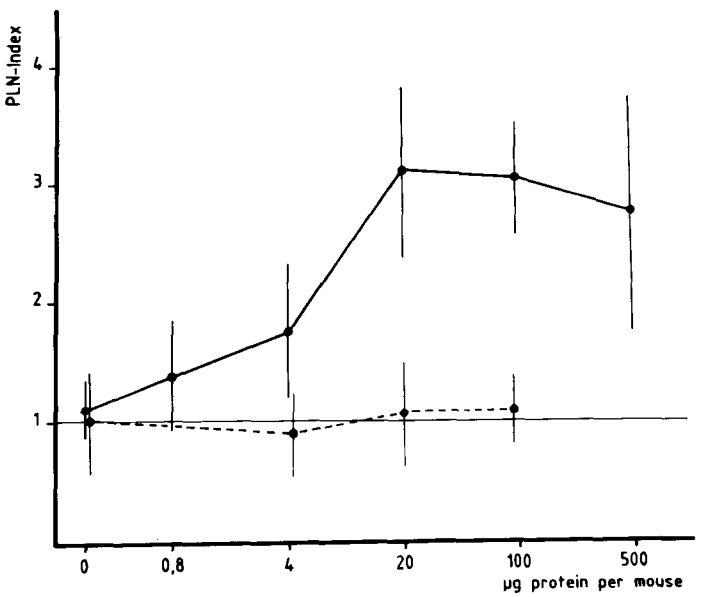

Fig. 2. Dose-response to plasma membrane fraction B_2 from adult murine cortical brain cells (●———●) and of purified plasma membrane fraction from adult murine liver cells (●- - -●). (The straight line at PLN index 1 indicates background level.) Each point represents the mean of 6–8 mice ± standard deviation.

TABLE 4

KINETICS OF PLN REACTIVITY TO FRACTION B_2 FROM ADULT MICE

PLN index tested after (h)	PLN index ± SE after injection of		*n*
	PBS (control) [a]	100 μg B_2	
24	1.10 ± 0.13	1.78 ± 0.22	7
48	NT	2.17 ± 0.22	5
72	1.06 ± 0.08	2.37 ± 0.18	12
96	0.91 ± 0.14	1.81 ± 0.12	4
168	0.97 ± 0.1	1.52 ± 0.09	4

[a] NT: not tested.

TABLE 5

PLN REACTIVITY TO MOUSE BRAIN LIPID, GALACTOCEREBROSIDES, MYELIN AND MYELIN BASIC PROTEIN

Mice injected with	Dose (μg)	PLN index $\pm$ SE [a]	n
PBS	–	1.10 $\pm$ 0.06	8
Lipid	100	1.45 $\pm$ 0.21	8
Lipid	20	1.03 $\pm$ 0.1	8
Myelin	100	1.21 $\pm$ 0.19	8
Myelin	20	1.74 $\pm$ 0.25	8
MBP	100	1.49 $\pm$ 0.03	6
MBP	20	1.2 $\pm$ 0.15	4
Galactocerebrosides	100	1.29 $\pm$ 0.18	4
	20	1.32 $\pm$ 0.07	5
B_2 (adult)	100	2.1 $\pm$ 0.12	6

[a] Assayed after 48 h.

TABLE 6

PLN REACTIVITY TO FRACTION B_2 IN BALB/c AND nu/nu MICE

Mice injected	Dose	PLN index [a] $\pm$ SE	n
BALB/c	PBS	1.05 $\pm$ 0.15	5
BALB/c	100 μg	2.11 $\pm$ 0.18	4
BALB/c nu/nu	PBS	0.79 $\pm$ 0.05	6
BALB/c nu/nu	100 μg	1.08 $\pm$ 0.06	7
BALB/c nu/nu	50 μg	0.95 $\pm$ 0.12	6
BALB/c nu/nu	20 μg	1.04 $\pm$ 0.07	6

[a] PLN reaction was determined after 48 or 72 h.

TABLE 7

PLN WEIGHT AND PROLIFERATION INDEX OF BALB/c TO B_2 FROM MICE OF DIFFERENT DEVELOPMENTAL STAGES

Mice injected with	Dose (μg)	PLN indices $\pm$ SE [a] (weight)	[^{3}H]TdR incorporation [b]	n
PBS	–	1.12 $\pm$ 0.08	1.1 $\pm$ 0.03	8
B_2 18-day-old embryos	20	1.15 $\pm$ 0.12	NT	6
	100	1.42 $\pm$ 0.17	NT	6
B_2 day 10 after birth	4	0.91 $\pm$ 0.07	NT	11
	20	1.17 $\pm$ 0.09	NT	10
	100	1.18 $\pm$ 0.07	1.21 $\pm$ 0.09	11
B_2 day 15 after birth	4	1.17 $\pm$ 0.13	NT	5
	20	2.73 $\pm$ 0.25	NT	7
	100	2.64 $\pm$ 0.17	2.52 $\pm$ 0.07	7

[a] Assayed after 48 h.
[b] NT: not tested.

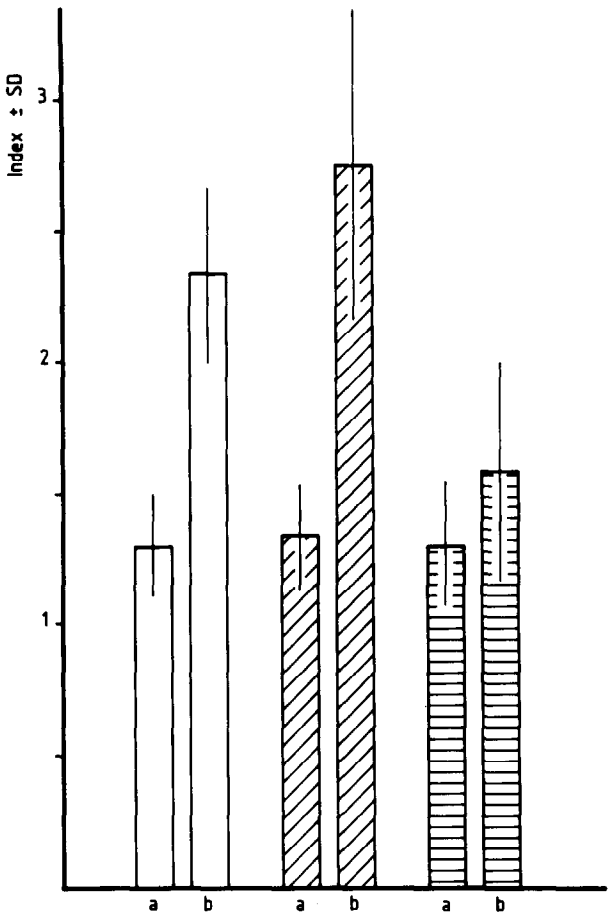

Fig. 3. Weight index (□), [^{3}H]thymidine incorporation index (▨) and trapping index of ^{51}Cr-labeled syngeneic lymphocytes (▤) after injection of 50 μl PBS (columns a) or 100 μg of fraction B_2 (columns b) into the hind footpad. The indices in the popliteal lymph nodes were determined on days 2–3 after injection by calculating the mean ± standard deviation of 6–8 mice per group.

cerebrosides, it was necessary to test stringently whether these lipids could induce PLN reactivity. The results in Table 5 demonstrate that none of the preparations elicited comparable reactivity to fraction B_2 even at the same concentration.

PLN enlargement could be mediated either by lymphocyte trapping, by proliferation, or both. Therefore, BALB/c mice were immunized with 100 μg B_2 injected with ^{51}Cr-labeled syngeneic spleen cells 24 h before sacrifice or with [^{3}H]thymidine 30 min before termination of the experiment. The data in Fig. 3 demonstrate that B_2-induced PLN enlargement is mediated by lymphocyte proliferation. The increase in [^{3}H]thymidine incorporation paralleled exactly the weight increase in the stimulated nodes. This lymphocyte proliferation appears to be T cell dependent, since

only normal BALB/c mice developed PLN enlargement, but not the athymic BALB/c nu/nu mice (Table 6). Lymphocyte trapping (Fig. 3) does not contribute significantly to PLN enlargement.

In further experiments the time point during murine development at which brain plasma membrane preparations could induce PLN reactivity in syngeneic recipients was determined. Plasma membranes obtained from brains of 18-day-old embryos or from pups of 10 or 15 days of age were injected into the footpad of syngeneic, adult recipients. The results are summarized in Table 7. No detectable PLN reactivity was observed with fraction B_2 from embryonic mice nor from mice that were 10 days old. In contrast, B_2 from 15-day-old mice induced PLN enlargement with exactly the same dose-response and kinetics as B_2 from adult mice (see Fig. 2 and Table 4).

Discussion

This study was initiated to investigate novel membrane-associated autoantigens in the murine brain. Our data show that brain cells from adult mice, or alternatively, a distinct fraction of plasma membranes from such cells, can induce T cell-related lymphocyte proliferation in syngeneic recipients. The experimental system employed was the popliteal lymph node assay, which permits rapid and easy detection on an immunogen in vivo. Originally this assay was developed to measure T cell alloreactivity in a graft-versus-host reaction (Rolstad 1976) and more recently, the immunogenicity of chemical compounds (Kammüller et al. 1984). The present results demonstrate that the PLN assay is also a useful technique for the identification of self-antigens in vivo.

The immunogen was applied in the absence of adjuvant in order to avoid all adjuvant-associated reactions. Cortical brain cells from adult BALB/c mice, but not from embryos of neonatal mice (data not shown), then induce a weak but significant and reproducible enlargement of the popliteal lymph nodes in syngeneic recipients. This reactivity could be amplified by the use of a distinct plasma membrane fraction from adult mouse cortical cells (Table 3). The higher reactivity to purified plasma membranes in relation to cortical brain cells could be explained by an increase in the antigen concentration in the plasma membrane fraction. It is also possible that the relatively weak reaction induced by adult cortical brain cells was due to cell damage during the dissection procedure, with consequent release of lysosomal enzymes and destruction of relevant antigens. This problem was circumvented by using a purified plasma membrane fraction, designated as B_2, from brain cortex. The purity of the plasma membranes was documented by electron microscopy (Fig. 1) and by enrichment of the plasma membrane marker enzyme Na^+,K^+-ATPase relative to that in the cell homogenate (Table 1). As little as 20 μg of fraction B_2 was sufficient to induce optimal reaction (Fig. 2).

Plasma membranes contain phospholipids which are the main constituents of artificial liposomes, and it is known that such liposomes possess adjuvant activity (Moore et al. 1984). Therefore, the possibility was considered that PLN reactivity is due to an adjuvant effect mediated by the phospholipid moiety of the plasma

membranes. This, however, is unlikely for the following reasons: only fraction B_2 induced PLN reactivity; fraction B_3 (Table 3), which also was derived from adult brain cells, was ineffective; likewise, B_2 from 10-day-old mice (Table 7), plasma membranes from liver cells of adult syngeneic donors (Fig. 2), lipid vesicles from adult brain and galactocerebrosides were all incapable of inducing PLN reaction (Table 5). Therefore, PLN enlargement cannot be attributed to a mere adjuvant effect of plasma membrane preparations.

MBP and myelin-associated glycoprotein are potent autoantigens in the nervous system and are both associated with myelin (Bernard and Carnegie 1975; Braun et al. 1982). The possibility that a contamination of B_2 with these proteins was responsible for PLN reactivity could be completely excluded because injection of an amount of MBP or myelin corresponding to that of B_2 injected without CFA (which usually has to be present for in vivo immunization (Pettinelli and McFarlin 1981) failed to produce significant PLN enlargement (Table 5). In addition, B_2 from 15-day-old mice was as effective as B_2 from adult mice, although myelinization is only just beginning at 15 days (Schönbach et al. 1968) rendering the contamination of our preparation with myelin rather unlikely.

Interestingly, the first appearance of the potential to induce PLN reactivity (in B_2 from 15-day-old mice) coincides with the establishment of the blood-brain barrier. During postnatal development the barrier is gradually formed and is almost completed between days 10 and 15 (Bradbury 1980). Once the barrier is established various brain cell populations can differentiate in seclusion from the immune system. Consequently, lymphoid cells cannot acquire tolerance to these self-antigens, and may recognize these antigens as foreign under appropriate immunological conditions.

B_2-induced PLN enlargement was mediated by T cell-associated lymphocyte proliferation (Fig. 3). The kinetics of this reactivity (Table 4) exhibited characteristics of a host-versus-graft reaction (HVGR) (Korčáková and Hašcová 1974). HVGRs are mediated by the immune responsiveness of T cells from a homozygous recipient against allogeneic determinants encoded by the major histocompatibility complex (MHC) of the injected F_1 cells. T cell responses against foreign antigens, such as haptens or minor transplantation antigens require the recognition of the antigen in association with autologous class I or class II MHC antigens (Zinkernagl and Doherty 1974). The HVGR type of PLN reactivity to adult brain cells, or to fraction B_2 suggests a similar mechanism. Antigens derived from cortical brain may be degraded, processed and presented by autologous macrophages to T cells, which recognize the antigen in association with self-MHC structures.

In summary, to our knowledge this is the first report providing evidence that immune reactivity to brain cell surface antigens can be induced in syngeneic mice in the absence of any adjuvant. The antigenicity of this material is first detectable at a time point during murine development at which the blood-brain barrier is completed in most parts of the CNS.

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