Acta virol. **30** : 149—157, 1986

EARLY INTRATHECAL PRODUCTION OF SPECIFIC IgM AND IgG ANTIBODIES AND ALPHA-INTERFERON IN HERPES SIMPLEX VIRUS ENCEPHALITIS

V. MAYER, V. ZACHAR, H. SCHMIDTMAYEROVÁ, *J. HRÚZIK, J. RAJČÁNI, E. MITROVÁ, V. LACKOVIČ, J. RAUČINA, *J. KOTULIAK, *M. FAYBIK

Institute of Virology, Slovak Academy of Sciences and Joint Virus Research Centre at the *Clinic of Infectious and Parasitic Diseases, Medical Faculty, Komenský University, Bratislava, Czechoslovakia

Received June 7, 1985

Summary. — A complex approach was used in order to establish non-invasively the aetiology in three cases of encephalitis presumptively caused by herpes simplex virus (HSV). As indicative of brain HSV infection was considered the lowered serum to cerebrospinal fluid specific antibody ratio, which also assessed the humoral immune response within the CNS. For this purpose, during ongoing brain tissue infection, the early intrathecal (ITH) production of IgG and IgM antibodies was analysed by a differential enzyme-linked immunosorbent assay (ELISA) along with changing levels of complement-fixing (CF) antibodies in the serum and cerebrospinal fluid (CSF). Antiviral antibody (AA) response was markedly preceded by the appearance of alpha-interferon (IFN) in the serum and CSF. From the CNS biopsy and autopsy specimens one HSV-1 and one HSV-2 strain were recovered.

Key words: herpes simplex virus; encephalitis; intrathecal antibodies; interferon

Introduction

HSV is the most common cause of the sporadic acute focal necrotizing encephalitis. Much remains to be learned about the different pathogenetically important events (primoinfection, recurrence), as well as about triggering and protective mechanisms in this ailment, carrying 40—70 per cent mortality (Barza and Pauker, 1980). The uncertain course and outcome of any individual HSV encephalitis case appeared to be strongly influenced by uncontrolled host and virus factors. The impact modalities of host defenses, namely of mononuclear cells and of AA in the clearance of the CNS infection showing cell-to-cell virus spread, are still insufficiently defined.

The likelihood of correct clinical diagnosis in cases, suspect of HSV encephalitis does not surpass 50 per cent. On the other hand, in a large series of cases, more than 5 per cent of false negative findings in HSV isolation experiments from brain biopsy were found, whereas pitfals of histopathologic

and immunofluorescent methods represented 30—35 per cent (Nahmias *et al.*, 1982). The same authors noted in about 28 per cent from 113 biopsy--proven cases a failure-for unknown reasons —to seroconvert or to seroboost within one month from the onset of the disease, when studied by immunofluorescent and by passive haemagglutination methods. Great individual variations in the humoral immune response stated also Sköldenberg *et al.* (1981, 1984) and Koskiniemi *et al.* (1984). The importance of HSV recovery from brain biopsy for diagnosis of HSV encephalitis has not been questioned, until recently (e.g. Sköldenberg *et al.*, 1985).

New immuno-assays, detecting minute quantities of AA with high sensitivity and specificity, increasing evidence from more carefully studied individual cases, together with the availability of a purine nucleoside analogue of remarkably low apparent toxicity (acyclovir), selectively inhibiting the HSV DNA polymerase (Elion, 1983), seem to lessen the necessity of a virus--positive brain biopsy prior to the institution of antiviral treatment.

In the present report in three cases of HSV encephalitis we analyse the dynamics of ITH immunoglobulin class-specific (IgG, IgM) and alpha-IFN responses together with their concomitant serum levels. The ITH origin of HSV AA in the CSF was proven by parallel investigations of the IgG class AA against an unrelated, reference virus.

Our observations seem to contribute to the concept of a non-invasive alternative in establishing the human CNS infection by HSV.

Subjects, Materials and Methods

Patients. During the 1984 period, three patients with CNS disease, suggestive of HSV encephalitis, came to our attention. 18 CSF samples, collected by lumbar puncture and as many serum samples were obtained from the patients on days 4—58 from the onset of clinical symptoms. In two patients at admission fever, meningeal signs and altered consciousness were found. One patient was admitted deeply comatous on the 12th day of her illness. Neurodiagnostic techniques were in all suggestive of focal lesion(s). The course of the disease is described elsewhere (Hrúzik *et al.*, in preparation).

Control patients. At various intervals after the onset of disease with miscellaneous neurological symtomatology (e.g. tick-borne encephalitis, mumps meningoencephalitis, aseptic meningitis) paired serum and CSF samples were taken from together 12 persons, aged 4—32 years. Some of them showed herpes labialis.

Virological techniques. For HSV isolation experiments, 10 per cent suspensions of the bioptic or necroptic brain tissue were prepared in Eagle's basal medium (EBM) containing 5 per cent of inactivatde calf serum (ICS), and inoculated within two hours after removal. For virus detection primary cultures of human enbryo (HE) skin-muscle cells and intracerebrally administered newborn mice (ICR strain) were used. Virus cytopathic effect (CPE) in HE cell cultures as well as the first signs of experimental encephalitis in mice appeared beginning from the 2nd day after inoculation. Isolated viruses were identified by indirect immunofluorescence staining, using the rabbit FITC-conjugated immune serum to HSV-1 (IMUNA, Czechoslovakia). The virus antigen was detected in both HSV-infected HE cells after fixation with cold acetone and paraffin sections of agonic mice (Rajčáni and Szántó, 1973). The specificity of the virus recovery was, as a rule, confirmed in repeated experiments.

The viral strains, after several passages in HE cells, were typed by neutralization kinetics using monoclonal antibodies in a plaque reduction test performed on VERO cells. A pool of three monoclonal antibodies raised against HSV-1 antigens was used (one of them precipitated

gC, another gD). The ascitic fluid pool neutralized in the presence of complement the HSV-1 („Kupka") strain in dilutions up to 1 : 640—1280, but the prototype HSV-2 in dilution 1 : 80 only.

Viral antibody studies. Individual serum and CSP samples were stored in aliquots at —20 °C. In any sample the presence of the anti-IgM rheumatoid factor was detected using Sevatest RF (SEVAC, Prague).

Complement-fixing (CF) AA were investigated according to Lennette and Schmidt (1969), always simultaneously in paired samples, using the commercial CF HSV-1 antigen (IMUNA). In the CF test, a $\geqq$ 4-fold rise in AA titre was considered significant. If CF antibody were detected, the ratio was calculated for the serum and CSF antibody titres in the corresponding paired samples. The ratio $\leq$ 20 was seen as a presumptive evidence for local antibody synthesis in the CNS (e.g. Koskiniemi *et al.*, 1984).

Virus-neutralizing (VN) antibodies were titrated in VERO cell test tube cultures, using 100—200 CPD_{50} of HSV-1 (Kupka strain) virus. The serum was incubated with the virus for 2 hr at 37 °C in the presence of 128 units of complement. As end-point was considered the dilution of the serum inhibiting the virus CPE by 75 per cent.

ELISA for determination of IgG and IgM antibodies, HSV and control antigens. Monolayers of VERO cells in 1200 ml culture flasks were inoculated with HSV 1 virus (HSZP strain) at the input multiplicity of infection = 1. After 18—20 hr, when the virus CPE was almost complete, washed cells were scrapped into PBS. Pelleted cells, resuspended in bidistilled water, were disrupted by three cycles of freezing and thawing. The resulting suspension was clarified by slight centrifugation and the virus-containing supernate was subjected to centrifugation at 60,000 g /one hr/4 °C. The pellet, resuspended in bidistilled water, was frozen in aliquots. This semipurified native material was used as HSV antigen. Control antigen was prepared similarly from non-infected VERO cells.

Influenza A virus antigen. The 551/A/Bangkok/1/79 strain of influenza virus (H3N2), used as reference virus, was in reproduced eggs. The virus was purified in allantoic sac of embryonated eggs. The virus was purified by a standard procedure from the harvested allantoic fluid by differential centrifugation, followed by equilibrium centrifugation in a linear sucrose gradient (10—55 per cent sucrose).

ELISA procedure. Optimal concentrations of antigens used for coating the test plates and of conjugates (swine anti-human IgG and IgM, heavy chain specific, conjugated with horseradish peroxidase-SwAHuIgG/$P_{\check{x}}$, SwAHuIgM/$P_{\check{x}}$, SEVAC, Prague), were determined by chessboard titration with positive and negative reference sera. Concentrations of 1.7 μg or 2 μg of protein per ml of the HSV-1 and influenza A antigen materials, respectively, were found for most suitable. One hundred μl of this dilution per well, except blanks, was adsorbed to the microtitration plates (ÚMG, Prague) in 0.05 mol/l carbonate-bicarbonate buffer pH 9.6. The coated plates were tightly sealed and stored overnight at 4 °C. Before application of serum specimens, the test plates were washed three times with 200 μl of PBS, containing 0.05 per cent of Tween 20 (PBST). The serum and CFS samples to be examined, were initially diluted 1 : 100 outside the test plates in PBST containing 1 per cent bovine serum albumin for IgG antibody and in PBST containing 50 per cent of ICS for IgM antibody determination. Further serial dilutions were 4^n and 2^n for IgG and IgM antibody level, determinations, respectively. Dilutions of serum and CSF samples were applied in 100 μl amounts and incubated for 2 hr/37 °C in a humid chamber. After another three washes, 100 μl of SwAHuIgG/$P_{\check{x}}$ or SwAHuIgM/$P_{\check{x}}$, appropriately diluted, were added and incubated for 1 hr at 37 °C. The washes were followed by addition of 100 μl of substrate solution, composed of 0.05 per cent of orthofenylendiamin-hydrochlorid in 0.2 mol/l citrate-phosphate buffer pH 5 with 0.05 per cent of H_2O_2. The colour reaction was allowed to develop for 30 min at 37 °C in dark and then stopped with 50 μl of 4 NH_4SO_4. The test plates were measured photometrically. The optical density was determined in a spectral photometer (Minireader, Dynatech), at the wavelength of 489 nm. The end-point titre value was defined as the hignest dilution of specimen investigated, giving in antigen-coated well absorbance exceeding at least twice that in control antigen-coated well and simultaneously, the OD difference between them being at least 0.1. In the assay with influenza virus, essentially the same criteria for the end-point reading were used, but in relation to background values.

Under described conditions of ELISA, a $\geqq$ 4-fold rise in AA titre was considered as significant.

As evidence of ITH specific ELISA AA production, we used criteria, based on earlier observations (Norrby *et al.*, 1974; Sköldenberg *et al.*, 1981, 1984). A reliable demonstration of local antibody synthesis in the CNS demands a comparison of serum/CSF anti-HSV class IgG antibody

ratios with the corresponding ratios of reference virus AA. A difference between the ratios was considered for significant, if the ratio of HSV antibody was at least fourfold lower than the corresponding ratio for the reference AA in the paired samples investigated. The serologic tests used, did not distinguish between HSV-1 and 2 antibodies because of the cross-reaction between the two viral types (e.g. Jeansson *et al.*, 1983).

IFN studies. In the IFN investigations only once-thawed serum and CSF samples were used. Specimens were 2^n diluted in EBM supplemented with 5 per cent of ICS and applied in 60 µl volumes into microplates in duplicates. About 2×10^4 of suspended MDBK cells (sensitive selectively to alpha-IFN, e.g. Gresser *et al.*, 1974) in a 100 µl volume were then added to each well (Lebon *et al.*, 1979). After incubation for 18 hr at 37 °C, the plates were twice washed with EBM and the cells challenged with 30—50 PFU of vesicular stomatitis virus in a 100 µl vol. The plates were read after overnight incubation and the IFN titres were expressed as the reciprocals of the highest dilution of the given materials, inhibiting completely the viral CPE. The IFN-like activity was identified likewise, using also heat inactivation (56 °C/60 min), acid treatment (pH 2/24 hr) and the effect of the sheep anti-human leukocyte IFN globulin (Fuchsberger and Borecký, 1978). In each IFN testing, a sample of standard human alpha-IFN was included as a control.

Histopathologic studies. Serial sections of the brain tissue embedded in paraffin by standard method were stained by haematoxylin-eosin, Luxol fast blue and with cresyl-violet, Masson's green trichrom and impregnated according to Holmes and Cajal. In the autoptic material, the presence of HSV antigen was studied in paraffn sections by indirect immunofluorescence (IF) method.

Results

The results of differential quantification of HSV-specific ELISA IgG and IgM antibody in the serum and CSF, as related to the levels of specific immunoglobulins against reference virus and the CF anti-HSV antibodies are presented in time sequence. The presence of IFN in the samples from the early phase of the disease was also investigated.

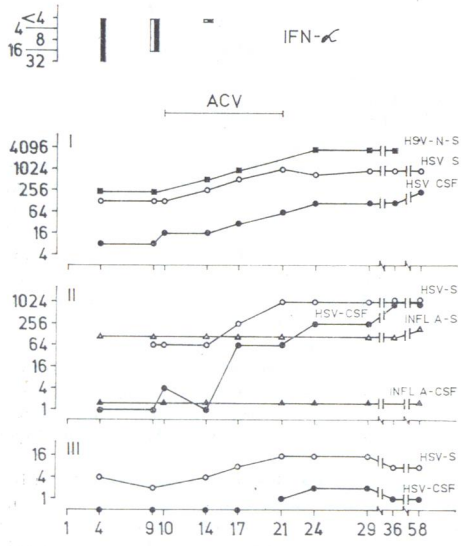

Fig. 1.

Antibodies to HSV and alpha-IFN in the serum and CSF of patient R. R.
I — CF and VN antibodies
II — ELISA test, IgG class antibody (dilution reciprocals × 10^2)
III — ELISA test, IgM class antibody (dilution reciprocals × 10^2)
ACV — acyclovir; S — serum; N — virus neutralizing activity; Infl. A — reference strain A/Bangkok/H3N2; CSF — cerebrospinal fluid.
White columns: IFN in the serum; black columns: IFN in the CSF.

R. R., male, 41 years old

As early as at the 4th day of his disease, high levels of HSV antibodies were detected in the serum and CSF by CF, VN and ELISA tests (IgG) (Fig. 1). Significant rise in the serum antibody titre was observed only during the 3rd week, later reaching the highest levels. Serum VN antibodies attained their peak remarkably late, i.e. during the 4th week. CF antibody levels continued to rise in the CSF until the 58th day showing a fourfold increase between days 9—17. The detection of IgG class AA by ELISA did not hasten the diagnosis, but clearly demonstrated ITH AA production and the undamaged blood-brain barrier (BBB) in this case, based on the unchanged levels of IgG response to reference virus. A constant rise of CSF antiviral IgG was observed until the latest interval investigated (58th day), suggesting a continuous antigenic stimulation, in spite of completed antiviral treatment. The presence of serum IgM preceded in this case (considered as a recurrence of latent HSV infection) the rise of CSF AA. The anti-HSV IgM as detected by ELISA in the CSF became positive during the 4th week after the onset of disease, i.e. relatively late in its course.

The serum/CSF antibody ratios as detected by CF test indicated ITH antibody production from the 9—17 day intervals, this was confirmed by serum/CSF IgG ratio of the HSV AA in comparison to the influenza virus AA ratio. IFN was detected in significant amounts in the serum and CSF already on day 4, but not later than on day 9 from the onset of the disease. The patient died on the 113th day of his disease. Autopsy material (not available for virus study) showed changes, resulting from a preceding acute encephalitis (Dr. J. Valach).

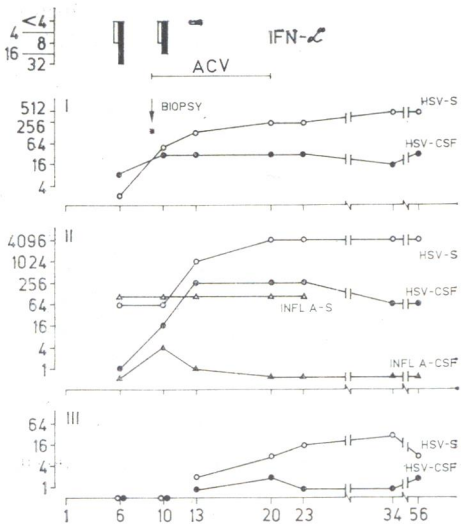

Fig. 2.

Antibodies to HSV and alpha-IFN in the serum and CSF of patient M. D.
I — CF antibodies
II — ELISA, IgG (dilution reciprocals × × 10^2)
III — ELISA, IgM (dilution reciprocals × × 10^2)
Full (●) and empty (○) circles on abscissa indicate non-detectable antibody levels.
For further explanations see Legend to Fig. 1.

M. D., male, 15 years old

Rapid and significant rise of CF AA in the serum and CSF was seen during the first intervals of the acute phase of the disease. On the day 9 from the beginning of ailment, a site-oriented brain biopsy was performed. Considering this moment, ITH AA were studied only at later intervals. The analysis by ELISA of the AA synthesis, confirmed unequivocally the ITH antibody production after the day 20. The CF and ELISA findings from preceding two intervals were obviously influenced by the damage of BBB due to neurosurgery (Fig. 2). A relatively early appearance of ELISA anti-HSV class IgM antibody was noted concomitantly in the serum and CSF. IFN was detected in the serum and CSF on the 6th and on the 10th days, but not at later intervals (Fig. 2).

HSV-1 was isolated from the biopsy in HE cell cultures and in newborn mice. Histology showed acute necrotizing haemorrhagic encephalitis, no inclusions being found. The patient survived, though mentally disabled, up to now.

A. T., female, 19-year-old

On the day 10 of her illness, a craniotomy was performed for a suspect brain tumour. Peroperatively, a presumptive diagnosis of haemorrhagic encephalitis was made (confirmed histologically), brain tissue sample was not available for viral study. Patient was admitted on the day 12 of her illness and died three days later. Only one paired serum and CSF sample (from the 14th day of the disease) was studied. Anti-HSV CF antibody titered in the serum 1 : 16, in the CSF 1 : 8. The serum ELISA anti-HSV class IgG and IgM antibody values attained the levels of 1 : 64 000 and 1 : 200, respectively. In crossed investigations with indirect immunofluorescence, using cell cultures infected with prototype HSV-1 and HSV-2, the

Table 1. Characterization of the IFN-like activity in the CSF from two cases of HSV encephalitis

CSF from the case	Before treatment	Treatment		
		56°C/60 min	pH 2/24 hr	anti-IFN-αIg*
R. R.	16**	16	8	< 4
M. D.	16—32	16	16	< 4
Human leukocyte IFN (reference sample)	64	NT	NT	< 4

* 500 μl of the sheep anti-IFN-α immunoglobulin incubated with the given undiluted specimen at 20 °C for 30 min.

** The reciprocal value of IFN titre.

NT — not tested

IgG antibody titre to the HSV-1 antigen was 1 : 10, but to the HSV-2 1 : 80—160. The IgM antibody levels against HSV-1 were found to be 1 : 10, against the HSV-2 antigen 1 : 80 in the serum sample investigated. The virus isolated from the autopsy specimen (lesion homolateral site) titered 4.2 $\log_{10}$ CPD_{50} units per g of brain tissue. The virus was identified as HSV type 2. No HSV antigen was detected by indirect immunofluorescence in the brain atopsy specimen, from which the virus was isolated. No IFN was detected in the serum and CSF samples.

The IFN-like activities in the serum and CSF of the R. R. and M. D. cases was identified as those of alpha-IFN (Table 1). In none of paired serum and CSF samples from 12 persons with a CNS viral infection, the presence of significant titre or an increase of anti-HSV CF AA was observed. IFN was not demonstrated in either of samples tested.

Discussion

Three cases of HSV encephalitis were investigated. In one of them the final diagnosis was based on the assessment of ITH antibody production (case R. R.), in the second, on demonstration of the ITH antibody production and confirmed by virus isolation from the brain biopsy specimen, and in the third case on HSV-2 isolation at autopsy concordant with demonstration of HSV-2 antibody prevalence.

In the case of R. R., a significant rise in CSF AA levels was not observed until between day 9—17, as confirmed mutually by independent, but in parallel performed methods. The anti-reference virus (influenza antibody level remained stationary) for 58 days. The slow IgM antibody production, being detected only since week 4, might indicate that the HSV encephalitis could have been due to HSV reactivation. This possibility is supported by the marked seropositivity observed in samples taken immediately after admission, but without significant CF or ELISA IgG or IgM in the CSF.

On the other hand, in the case of M. D., the prompt and significant CF antibody response (change from negativity to positivity in the serum) seems to suggest that HSV encephalitis have developed upon primoinfection. The values of CSF antibody titres in samples coming from intervals following immediately this deliberated intervention, do not probably reflect the true situation of ITH antibody production. This is witnessed by the transient increase of the reference virus antibody levels (ELISA IgG) in the CSF. This decisive indicator returned to values observed before biopsy, within 11 days and remained constant until the last interval studied (day 56). The first detection of IgM by ELISA on day 13 was apparently influenced by the BBB damage, because this antibody was not present in the serum and CSF samples coming from other earlier intervals.

The differential analysis of HSV and of reference virus ELISA IgG in the serum and CSF of either studied case demonstrated the validity of approach used in assessment of ITH antibody synthesis. By this means the possible non-invasive diagnosis of HSV was made, provided that a sufficient number of serial paired samples was available for investigation. The serum/CSF CF

antibody ratio ≤ 20 represents a valuable, but rather presumptive indicator of ITH antibody synthesis, warranting a confirmation by more quantitative methods. CSF CF antibody increase was observed — although rarely — also in infections not directly involving the CNS (Koskiniemi *et al.*, 1984). The confirmation by ELISA analysis was needed in our two cases, especially in the first interval samples, when the serum and CSF CF antibody titres are frequently low, easily mimicking falsely low serum CSF ratios.

In the reported cases, the antiviral treatment was started from the 9th to 10 th days of the disease. It did not apparently influence the AA production, namely the IgM appearance. The case M. D. requires a prospective study to confirm persisting (latent) HSV infection of CNS.

Of interest is the very early IFN appearance in the serum and in CSF. IFN was present in considerable amounts at the beginning of neurological symptoms of HSV encephalitis before ITH antibody synthesis became evident (Figs 1, 2). This important issue requires further follow up and it appears to support the diagnosis of HSV encephalitis.

Our results are in agreement with evolving concepts, refraining of the brain biopsy as essential for laboratory diagnosis of HSV encephalitis before starting the treatment with an effective anti-HSV drug. It appears that a multicriterial approach (e.g. ITH antibody synthesis, controlled by reference virus antibody levels, significant rise of CSF antibody levels and the serum/CSF antibody ratios ≤ 20) may — with acceptable degree of sensitivity — elucidate the aetiology of a nonnegligible proportion of CNS involvements, suspected to be caused by HSV. The safety of the antiviral treatment, e.g. with acyclovir, does not hinder to reach eventually an alternative diagnosis.

Acknowledgment. The authors thank Dr. J. Kostolanský from the Institute of Virology, Slovak Academy of Sciences, for providing the purified influenza A virus material, Dr. G. Russ, CSc. and Ing. M. Bystrická, CSc. for the monoclonal antibodies.

References

Barza, M., and Pauker, S. (1980): The decision to biopsy, treat or wait in suspected herpes encephalitis. *Ann. Intern. Med.* **92**, 641.

Elion, G. (1983): The biochemistry and mechanism of action of acyclovir. *J. antimicrob. Chemotherapy* **12**, Suppl. B, 9.

Fuchsberger, N., and Borecký, L. (1978): Quantitative immunoelectrophoresis of human interferon. A new approach to characterization of interferon preparations. *Acta virol.* **22**, 238.

Gresser, I., Bandu, M., Brouty-Boyé, D., and Tovey, M. (1974): Pronounced antiviral activity of human interferon on bovine and porcine cells. *Nature (Lond.)* **251**, 543.

Jeanson, S., Forsgren, M., and Svennerholm, B. (1983): Evaluation of solubilized herpes simplex membrane antigen by enzyme-linked immunosorbent assay. *J. clin. Microbiol.* **18**, 1160.

Koskiniemi, M., Vaheri, A., and Taskinen, E. (1984): Cerebrospinal fluid alterations in herpes simplex virus encephalitis. *Rev. infect. Dis.* **6**, 608.

Lebon, P., Donsot, G., Aicardi, J., Goutiéres, F., and Arthuis, M. (1979): Early intrathecal synthesis of interferon in herpes encephalitis. *Biomedicine* **31**, 267.

Lennette, E., and Schmidt, N. (1969): *Diagnostic Procedures for Viral and Rickettsial Infections* (fourth Edition). American Public Health Association Inc., New York, p. 590.

Nahmias, A. J., Whitley, R., Visintine, A., Takey, Y., Alford, C., Jr. and the Collaborative Antiviral Study Group (1982): Herpes simplex virus encephalitis: Laboratory evaluations and their diagnostic significance. *J. infect. Dis.* **145**, 829.

Norrby, E., Link, H., Olsson, J., Panelius, M., Salmi, A., and Vandvik, B. (1974): Comparison of antibodies against different viruses in cerebrospinal fluid and serum samples from patients with multiplex sclerosis. *Infect. Immun.* **10**, 688.

Rajčáni, J., and Szántó, J. (1973): Pathogenicity of laboratory-manitained and low-passaged strains of herpesvirus hominis in suckling mice. A comparative fluorescent antibody study. *Acta virol.* **17**, 227.

Sköldenberg, B., Kalimo, K., Carlström, C., Forsgren, M., and Halonen, P. (1981): Herpes simplex encephalitis: a serological follow-up study. *Acta neurol. scand.* **63**, 27ě.

Sköldenberg, B., Forsgren, M., Alestig, K. *et al.* (1984): Acyclovir-versus Vidarabine in herpes simplex encephalitis. *Lancet* ii, 707.

Sköldenberg, B., Forsgren, M., Alestig, K. *et ail* (1985): Design of therapeutic studies in herpes simplex encephalitis. *Lancet*, i, 285.

Explanation to Figures 3—6 (Plates XV—XVI):

Patient M. D. (site-oriented brain biopsy tissue).

Fig. 3. Mononuclear infiltration of leptomeninges. (HE, ×450).

Fig. 4. Severe necrotic changes and numerous haemorrhages in the cerebral cortex (HE, ×100).

Fig. 5—I. Numerous small capillary haemorrhages in the cerebral cortex (HE, ×450).

—*II.* Enormously hypertrophic astrocytes in the cortical region. (Cajal method, ×450)

Fig. 6. Focal necrosis with severe oedematous changes and a few degenerated nervous cells (HE, ×450).