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Rabies and Herpes Simplex Virus Encephalitis

An Immunohistological Study on Site and Distribution of Viral Antigens

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Summary. A retrospective study on the frequency, site and distribution of rabies and Herpes simplex virus (HSV) type 1 antigens by means of immunofluorescence (IF) and immunoperoxidase (IP) techniques was performed on routinely processed (formol-fixed, paraffin-embedded) brain autopsy material stored for up to 25 years. In 2 animal and 2 human rabies cases, inclusion bodies in neuronal cytoplasm and processes were brilliantly stained for rabies antigens by IF but were much less prominent or absent in usual histological stains. In 33 cases of histopathologically diagnosed necrotizing encephalitis, HSV antigens were demonstrated in 18 of 26 acute cases; 7 subacute cases (course longer than 4 weeks) were all negative for HSV antigens. Neuronal cytoplasm and nuclear membranes were the main sites of HSV antigens; nuclear inclusion bodies were inconstantly stained. Since most of HSV antigen negative cases also showed intranuclear inclusion bodies in HE stains, such nuclear inclusions are no reliable criterion for an HSV aetiology. On the other hand, their absence does not rule out a herpetic aetiology, but such a constellation is rare (only one of 18 HSV positive cases). Distribution of cells showing a positive reaction for HSV antigens may be patchy and irregular; therefore, a false negative result must be expected if very small tissue samples are examined (e.g, in needle biopsies from temporal lobe). In the leptomeninges, HSV antigen positive cells were found inconstantly and only in small numbers; this finding makes unlikely the possibility of an intravital diagnosis of HSV encephalitis by immunostaining of cerebrospinal fluid (CSF) cell preparations. Both immunohistological techniques applied in this study (IF and IP) gave the same results. Imprint preparations are useful when quick diagnosis is necessary.

Immunohistological investigations are a simple and effective means to demonstrate a viral aetiology even in routinely processed material; the use of such material rules out hazards in laboratories which are not designed to handle highly infectious fresh material.

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Introduction

In recent years, numerous studies have indicated that many antigens remain detectable in tissue sections by immunomorphological techniques, even after routine tissue processing with formol fixation and paraffin embedding. The use of paraffin sections allows the work-up of a great volume of processed and stored material by retrospective immunomorphological methods, in diseases from which fresh material is only rarely available. For the central nervous system (CNS), immunohistological studies centered on plasma proteins have been performed in different conditions (Auff and Budka 1980), especially on immunoglobulins (Esiri 1980) and myelin proteins (Itoyama et al. 1980) in demyelinating diseases or glial proteins in CNS tumors (Duffy et al. 1977). Polypeptides of neurofibrillary tangles have also been studied in this way. (Grundke-Iqbal et al. 1979). There are relatively few studies on viral antigens in routinely processed human autopsy CNS tissue; the reports on measles virus antigens in SSPE (Kumanishi and In 1979; Swoveland and Johnson 1979) and Herpes simplex virus (HSV) antigens in brains from patients with HSV encephalitis (Kumanishi and Hirano 1978; Swoveland and Johnson 1979) are based on investigation of material from single or few cases. Immunomorphological studies of larger series with details of the site and distribution of viral antigens have not been reported. In this paper, a retrospective study of a large series of human necrotizing encephalitis presents data on frequency, site and distribution of HSV antigens traced by immunofluorescence (IF) and immunoperoxidase (IP) techniques performed on formol-fixed, paraffin-embedded autopsy tissue. Rabies virus antigens have not previously been studied in paraffin sections. Investigation of several human and animal rabies cases should clarify if immunohistological diagnosis made by examination of formol material may replace the use of infectious fresh tissue. A brief survey of some of the IF findings has been reported elsewhere (Budka and Popow-Kraupp 1980)

Material and Methods

1 Rabies Investigation

Sections from the ammon's horn region of 2 animal (fox and roe-deer) cases of rabies with many Negri bodies at routine histology, and 3 human autopsy cases were investigated. One case was a typical perivenous "allergic" encephalitis after rabies vaccination. The second patient showed a clinical course typical for rabies after a fox bite in a rabies-infected region and died after 35 days of long-standing coma and ensuing brain death; transmission experiments with autopsy brain tissue were negative but agar-gel immunoprecipitation and IF imprint preparations were positive for rabies virus antigen (Dr. F. Gerstl, Bundesstaatliche Impfstoffgewinnungsanstalt Vienna, Austria). Neuropathological examination revealed extreme autolysis and an advanced stage of a necrotizing encephalitis without cytoplasmic or nuclear inclusion bodies. The third case was probably a rabies-like viral infection imported from Africa to Hungary, showed occasional Negri bodies without marked inflammatory features in the brain and is reported in detail elsewhere (Guseo et al. 1980).

Thin (3–5 μ) sections of formol-fixed, paraffin-embedded brain tissue obtained at autopsy were used after protease treatment (Denk et al. 1977): the dewaxed and rehydrated sections were rinsed in Tris buffer pH 7.5 and incubated for 15 min in a 0.1% protease (Sigma Chemical Company) solution at 37° C. Digestion was interrupted by 2 changes of chilled buffer for 30 min each and a conventional direct IF technique was then performed. The antiserum was produced in hamsters by immunization with the CSV rabies strain, FITC-conjugated and used in a dilution of 1:12 for 45 min. This serum was produced and tested in the Austrian State Institute for Control of Animal Epidemics, Mödling, Lower Austria, where it is routinely used for IF diagnosis of tissue specimens from animals in which rabies is suspected. Controls included sections from normal brains, treatment by heterologous non-immune serum (rabbit) and blocking tests by treatment with unconjugated hamster rabies antiserum (1:12 for 30 min) before incubation with the conjugate. The sera were kindly provided by Dr. F. Gerstl, Bundesstaatliche Impfstoffgewinnungsanstalt Vienna, Austria.

2 HSV Investigation

33 human autopsy cases with a histopathological diagnosis of necrotizing encephalitis were investigated. The material from these patients had been stored in paraffin blocks up to 25 years. There were 26 cases with the classical features of an *acute* necrotizing encephalitis (J. Hume Adams 1976); 7 *subacute* cases had a history longer than 4 weeks but still showed marked inflammatory changes with or without nuclear inclusion bodies and predilection of necroses for the temporal lobe(s). In each case, at least one block was investigated, comprising necrotic areas, border zone and less damaged tissue, mainly from the temporal cortex, and leptomeninges.

2.1 Indirect IF Technique (Performed on All Cases). Protease-pretreated sections (see above) were incubated by rabbit antiserum to HSV type 1 obtained in a working dilution for IF studies (Wellcome Laboratories, London) for 30 min. Specificity of this antiserum was ascertained by IF staining of HeLa cells infected by HSV types 1 or 2; specific fluorescence was obtained only in cells infected by HSV type 1. In the second step, anti-rabbit FITC-conjugated gamma-globulin produced in sheep (Wellcome Laboratories, London) was used; the optimal dilution of 1:15 was determined by chessboard titration of HSV type 1 infected HeLa cells. Controls included sections from normal human brains and brain infarcts, treatment with non-immune heterologous serum from rabbit (there was no IF staining of HSV type 1 infected HeLa cells) as first layer instead of specific antiserum, and two-step blocking experiments: a human blocking serum was followed by anti HSV type 1 rabbit serum and FITC-conjugated anti-rabbit serum. The blocking serum was obtained from a patient with HSV encephalitis and had a HSV CF titer of 1:128; its specificity was tested by IF staining of HSV type 1 infected HeLa cells, and a dilution of 1:15 was used. In addition, adsorption tests were performed by use of the antiserum after adsorption (2 courses) on Vero cells infected with HSV types 1 or 2 according to Rawls (1979); adequacy of adsorption was tested by IF on HSV infected HeLa cells. By this adsorption procedure, the type specificity of the HSV type 1 antiserum was also ascertained (Rawls 1979).

The sections were observed in Leitz or Reichert microscopes equipped with incident mercury vapour lamp illumination and the appropriate IF filter combinations. The results were read blindly and independently by each of the authors. After photographic documentation, the same sections were stained with haematoxylin and eosin (HE) so that identical cells could be compared in IF and HE stainings.

2.2 Peroxidase-Antiperoxidase (PaP) Technique (Sternberger and Cuculis 1969, Performed in 10 Cases). The antisera were diluted in equal parts of Tris buffer pH 7.5 and non-immune porcine serum (Gibco Europe, Glasgow, Scotland); there were 3 rinses of Tris buffer, each at least for 5 min, between the different steps. In short, protease-treated sections (see above) were incubated – after blocking of endogenous peroxidase activity by 0.3% H_2O_2-methanol for 30 min – with the same anti-HSV type 1 rabbit serum as described above in a 1:20 or 1:40 dilution for 30 min, followed by anti-rabbit immunoglobulins produced in swine (DAKO-Immunoglobulins, Copenhagen, Denmark) in a 1:40 dilution for 30 min. PaP complex (soluble complex of horseradish peroxidase/rabbit anti-horseradish peroxidase, DAKO Immunoglobulins, Copenhagen, Denmark), diluted 1:50 in Tris buffer, reacting with the sections for 30 min, was followed by the histochemical

staining with 3,3′-Diaminobenzidine tetrahydrochloride (Fluka AG, Buchs, Switzerland) (0.05% in a 0.033% H_2O_2 solution in Tris buffer) for 5 min. Counterstaining with haematoxylin was optional. Controls were the same as for the IF technique.

Results

Rabies

Both animal cases and the probably rabies-like virus encephalitis (Guseo et al. 1980) showed bright specific fluorescence of globular or ring-shaped inclusion bodies of various size selectively within neuronal perikarya and processes (Fig. 1 A, C). The fluorescence was sufficiently brilliant to permit a diagnosis even at low power (Fig. 1 A). In contrast, Negri bodies and other cytoplasmic inclusions were much less prominent in conventional histological preparations even at higher magnifications (Fig. 1 D). Staining was very weak or absent after pretreatment with the blocking serum. The human case with the prolonged

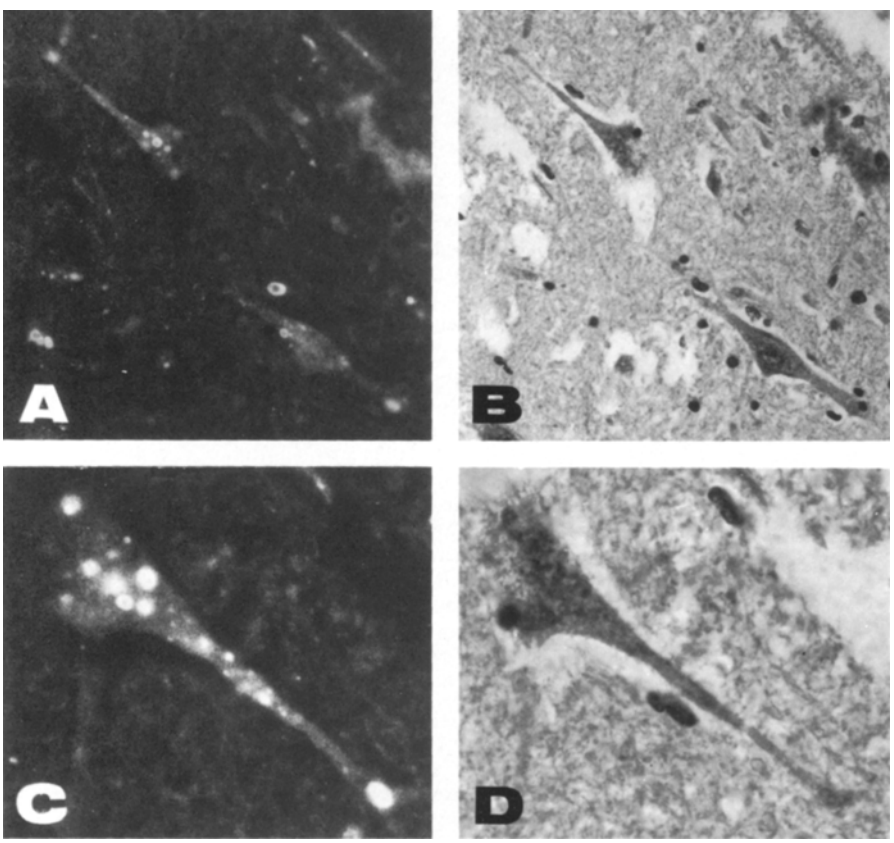

Fig. 1 A–D. Rabies, pyramidal neurons from Ammon's horn (roe-deer). **A** and **C**, direct IF with FITC-conjugated anti-rabies serum, **B** and **D**, same cells in HE stain. Numerous specifically stained inclusion bodies in neuronal cytoplasm and processes clearly visible in IF at low power but rather indistinct in HE stain even at high magnification. **A** and **B** ×250; **C** and **D** ×630

course of 35 days showed some occasional cytoplasmic floccular or globular fluorescence. The material from the perivenous encephalitis patient proved to be negative for rabies antigens.

Necrotizing Encephalitis

Tables 1 and 2 compare basic clinical data of cases positive or negative for the presence of HSV antigens, incidence of nuclear inclusion bodies, and additional virological investigations confirming a HSV aetiology.

Table 1. Basic data of 18 cases of acute necrotizing encephalitis positive for HSV antigen in autopsy brain tissue

No.	NI-No.	Sex	Age (years)	Duration of illness (days)	Nuclear inclusion bodies in HE sections	Remarks
1	224–80	M	6,5	28	+	Only imprint from temporal cortex HSV positive; paraffin sections from frontal cortex and spinal cord HSV-negative (inadequate sampling for histology); serum HSV CF titer rise from 1:16(3rd day) to 1:512(28th day)
2	154–80	F	64	10	+	
3	153–80	F	16	18	+	Brain biopsy positive by IF (5th day), AraA therapy, serum HSV CF titer rise from 1:8 (4th day) to 1:256 (18th day)
4	335–79	M	32	11	+	
5	323–79	M	32	15	+−	
6	57–78	F	17	11	−	
7	128–77	F	55	11	+	
8	573–75	F	11/12	8	+	
9	479–73	M	74	8	+	
10	473–72	M	60	7	+	
11	160–69	F	2	5	+	
12	15–68	M	1/12	5	+−	
13	127–65	F	59	7	+	
14	103–63	M	9/12	6	+	VI (cell culture and egg) positive from autopsy brain
15	25–61	F	18	10	+	VI (cell culture and egg) positive from autopsy brain
16	136–60	M	2,5	ac.	+	
17	140–59	M	60	ac.	+	
18	61–55	F	51	ac.	+−	

\+ = nuclear inclusion bodies unequivocal in routine histological slides
+− = nuclear inclusion bodies suspected in routine histological slides
− = no inclusion bodies in routine histological slides
ac. = acute course, tissue reaction as in cases of 1–2 week course (exact clinical data not available)
VI = virus isolation (HSV)

Table 2. Basic data of 15 cases of necrotizing encephalitis (acute and subacute) negative for HSV antigen in autopsy brain tissue

	No.	NI-Nr.	Sex	Age (years)	Duration of illness (weeks)	Nuclear inclusion bodies in HE sections
Acute:	19	349–77	F	67	2	+
	20	345–72	F	1,2	1	+
	21	132–68	M	0,1	1	+
	22	95–68	F	9	1	+−
	23	67–63	F	2	1	+
	24	56–61	M	3	1	+
	25	92–60	M	4	1	+
	26	58–57	M	50	2	+
Subacute:	27	246–80	F	81	8	−
	28	2–80	M	35	5	−
	29	43–74	F	78	8	+
	30	300–73	F	64	6	+−
	31	70–70	M	9	subac.	−
	32	198–69	M	17	8	−
	33	154–62	F	47	5	+

\+ = nuclear inclusion bodies unequivocal in routine histological slides
+− = nuclear inclusion bodies suspected in routine histological slides
− = no inclusion bodies in routine histological slides
subac. = subacute course, tissue reaction as in cases of 8 weeks duration (no exact clinical data available)

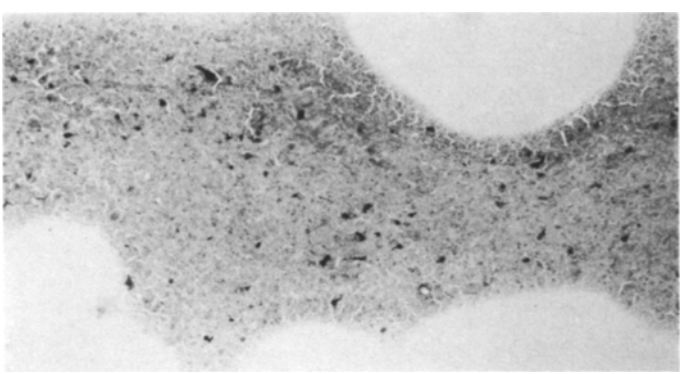

Fig. 2. Imprint preparation from autopsy brain tissue in HSV encephalitis. Numerous cells and processes stained darkly for HSV antigen, diagnosable even at low magnification. PaP technique, no counterstain, ×63

Acetone-fixed imprint preparations from biopsy and autopsy brain tissue of one patient and from autopsy brain tissue of another patient show strong reactivity for HSV antigens which is diagnosable even at low magnification (Fig. 2).

In the paraffin sections, HSV antigens are most prominent in the neuronal cytoplasm, mainly in a finely granular or more coarse, occasionally diffuse

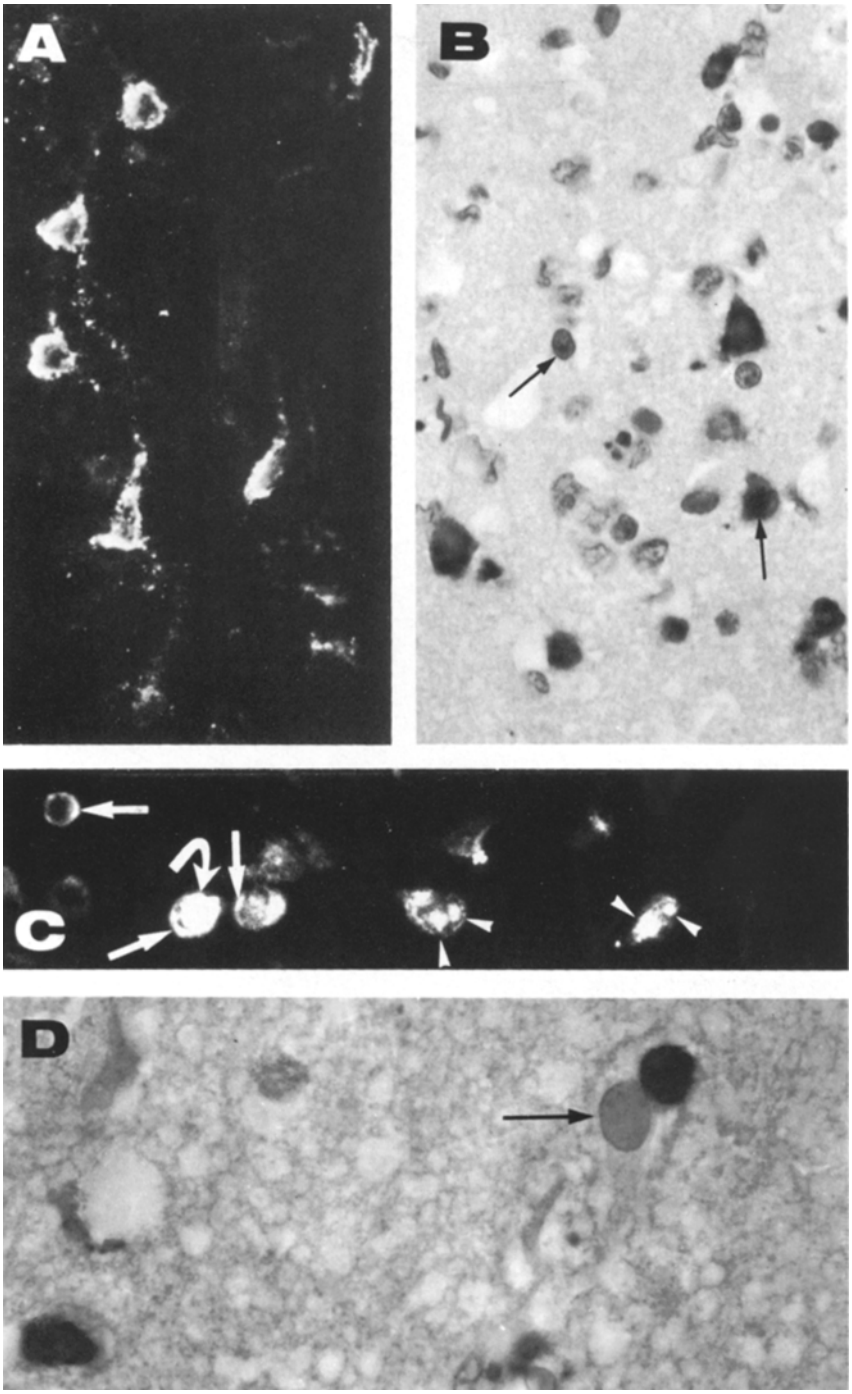

Fig. 3A–D. Herpes encephalitis, temporal cortex (each figure from different case). **A** and **C** indirect IF technique, **B** and **D** PaP technique for HSV antigen, haematoxylin counterstain. **A** and **B**, specific reactivity most prominent in neuronal cytoplasm, less prominent in intranuclear inclusions (*arrows* in **B**). **C**, types of nuclear involvement. Specific fluorescence at the nuclear membrane (*straight arrows*), multiple small inclusions (*arrowheads*) fusing to large single inclusions (*curved arrow*). **D**, strong reactivity of two nuclei filled by large homogenous inclusions; similar adjacent nuclear inclusion (*arrow*) unstained. **A-C**, ×630, **D** ×1,000

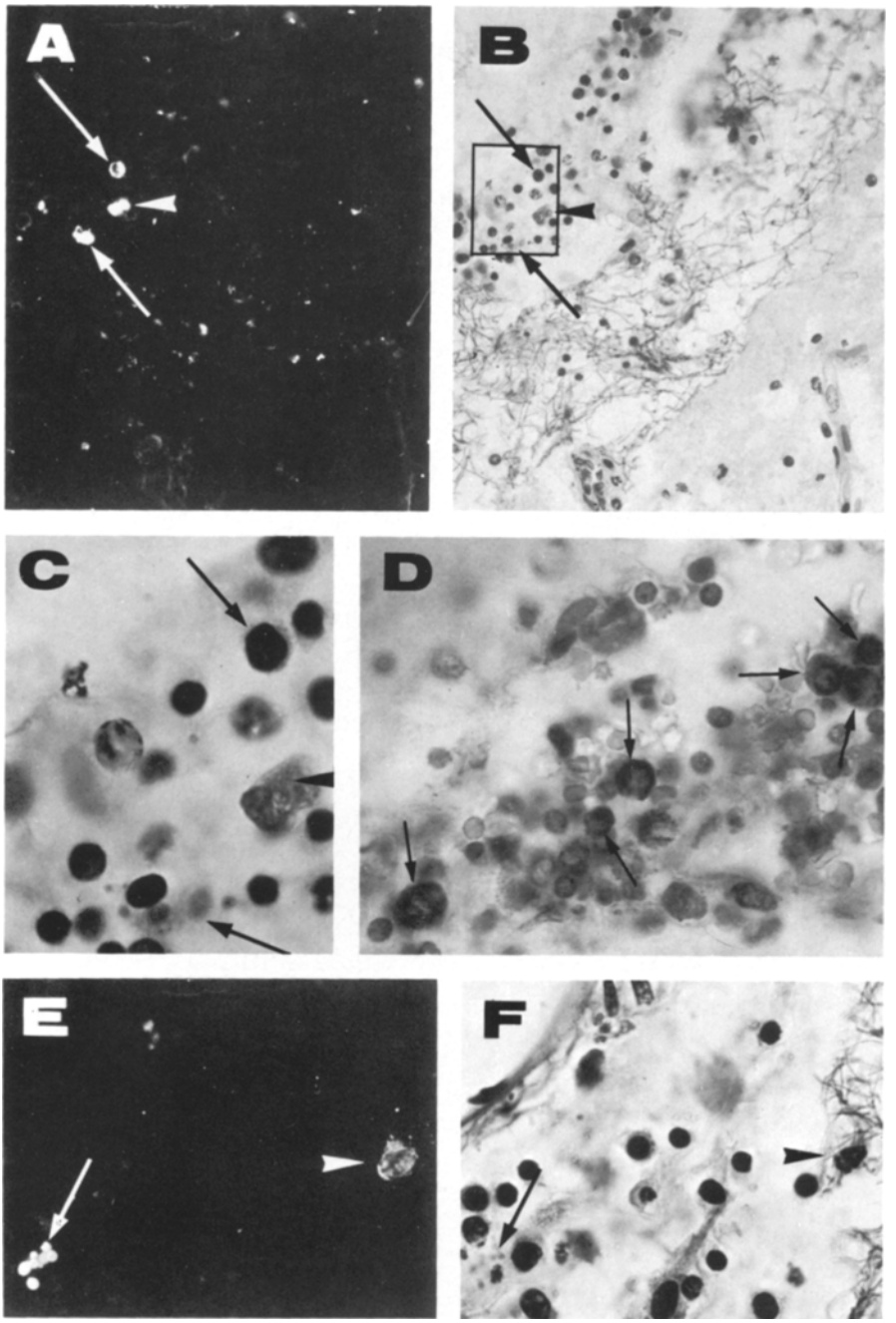

Fig. 4A-F. Herpes encephalitis, temporal leptomeninges (**D** from case different to that of the remaining figures). **A** and **E**, indirect IF technique, **D**, PaP technique for HSV antigen, haematoxylin counterstain. **B** and **F**, HE staining of same cells shown in **A** and **E** by IF. **C**, higher magnification of area squared out in **B. A, B** and **C**, specific fluorescence at the nuclear membrane of a lymphocyte (*upper arrows*), and in homogenous material within a macrophage (*lower arrows*). Autofluorescing lipopigment in a macrophage (*arrowhead*) easily distinguishable on color slides by yellowish colour from specific apple-green fluorescence. **D**, several mononuclear cells reacting mainly in the cytoplasm and at the nuclear membrane. **E** and **F**, specific fluorescence only of a mesenchymal cell (*arrowheads*) and of globular phagocytized material (*arrows*). **A** and **B** ×250, **C** ×1,000, **D-F** ×630

distribution which is discernible with equal contrast both in IF and PaP preparations (Fig. 3A, B). There was no difference in positive or negative results between both techniques. Specific staining was definitely weakened (in blocking tests) or absent in the controls including slides incubated with HSV type 1-adsorbed anti-HSV serum, whereas reactivity remained when type 2-adsorbed antiserum was used. One case, showing bright non-specific nuclear staining of all sections including the controls, was classified as HSV negative. In a given area with positive cells, negative neurones are also dispersed (Fig. 3A, B) and there are, in all positive cases, some variable sized areas lacking any reacting cell. There is no marked difference in frequency of positive cells between necrotic or better preserved areas. In most cases, nuclear involvement is less prominent than that of the cytoplasm (Fig. 3B) and is manifest as reactivity at the nuclear membrane (Fig. 3C, straight arrows, Fig. 4A, upper arrow, Fig. 4D). Staining of intranuclear inclusion bodies is inconstant; sometimes multiple small inclusions (arrowheads in Fig. 3C) fuse to form large single inclusion bodies (curved arrow in Fig. 3C). Histologically similar, immediately adjacent intranuclear inclusions may well show a differing pattern of reactivity for HSV antigens (Fig. 3D). Nuclear involvement is found in neurons, astrocytes and oligodendrocytes, and rarely also in perivascular or leptomeningeal lymphocytes (Fig. 4A–C, upper arrows), or mesenchymal cells (Fig. 4E, F/arrowheads). In only one case are mononuclear cells positive for the presence of HSV antigens shown in larger numbers within the leptomeninges (Fig. 4D). All other cases demonstrated only minor meningeal involvement, sometimes manifest by positively reacting phagocytized globular material, probably nuclear fragments, within meningeal macrophages (Fig. 4A–C lower arrows, Fig. 4E, F arrows). Other cases showed no meningeal involvement. In IF preparations, there is never any difficulty in distinguishing between the bright applegreen specific fluorescence and the yellowish to reddish lipopigment autofluorescence which is troublesome only in black and white prints (Fig. 4A–C).

Discussion

In this retrospective study, immunohistological examination proved to be a simple and effective method to use to determine the aetiology of rabies and HSV encephalitis, even in routinely processed and long-term stored histological preparations. This is most important for nosological classification of infections in which only formol-fixed material is available. Furthermore, any hazard by handling of highly infectious fresh material is avoided by formalin fixation without impairing the possibility of an aetiological diagnosis in many cases. Both immunohistological techniques applied in this study gave the same results; although immunoperoxidase (IP) procedures are much more sensitive than IF (Taylor 1978), we found no case positive in IP but falsely negative in IF. Therefore, wo consider IF methods equally effective for practical neuropathological use in such studies. We preferred IF techniques in the majority of the cases of this study since IF procedures are simpler, cheaper, less prone to technical difficulties and, in our experience, show less non-specific staining than the IP techniques which also have been practised for several years in our labora-

tory (Auff and Budka 1980). Many of the disadvantages of IF methods (Taylor 1978) can be overcome by the use of protease-treated formol-fixed paraffin sections and subsequent staining of the same sections by conventional histological stains (Budka and Popow-Kraupp 1980). Prior treatment with protease (Denk et al. 1977; Auff and Budka 1980) reduces non-specific background staining and enhances specific reactivity in a manner similar to other proteolytic enzymes like trypsin (Swoveland and Johnson 1979).

In human and experimental *rabies*, viral antigens within the CNS are selctively confined to neuronal cytoplasm or processes (Johnson 1965; Sung et al. 1976). Since smaller inclusion bodies may be overlooked in conventional histological preparations, and since the histologically prominent Negri body may be an non-specific lesion (Derakhshan 1975), immunohistology emerges as a necessity in suspected rabies infections especially when only formol-fixed and paraffin-embedded material is available. For animal rabies, sensitivity of IF methods in formol material should be compared to the currently established diagnostic techniques performed on fresh tissues in laboratories which handle daily large numbers of samples from rabies-endemic regions.

In *necrotizing encephalitis*, trials of methods providing an aetiological diagnosis have not been previously reported in a large series. This study that HSV is the causative agent in at least two thirds of the acute cases, whereas all subacute stages were negative. From our studies it cannot be decided whether HSV antigens disappear or become masked during later stages of the disease or if another infectious agent was causative. In the same way, the possibility of false negative results cannot be totally excluded; occasional false negative IF results (when VI is positive) in experimental HSV encephalitis in monkeys were explained by low virus concentrations in tissue (Cho and Feng 1978). However, even the much more sensitive PaP procedure did not demonstrate false negative IF results in our series. It is remarkable that most of the HSV negative acute necrotizing encephalitides occurred in children. If a quick diagnosis is necessary and morphological details on antigen site and distribution are not that important, imprint preparations- if possible from several different regions – may be very useful not only in biopsy but also in autopsy materials.

With regard to the aetiology of non-HSV necrotizing encephalitis, histopathological lesions may be similar in infections by other Herpes viruses, Coxsackie B_5 virus, psittacosis or LCM (Spaar 1976); in only one report were similar changes considered to be caused by HSV type 2 (Manz et al. 1979) although the virological evidence was poor in that case. HSV subtyping by IF is mainly performed on HSV isolates in tissue cultures with a controlled mode of infection and replication cycle (Geder and Skinner 1971; Rawls 1979); however, HSV typing is considered possible in clinical specimens by IF (Nahmias et al. 1971) using appropriately adsorbed antisera as in our investigation. This supports the suggestion that HSV type 1 infection was responsible for the lesions in our series.

Tomlinson et al. (1974) reported that 6 of 12 brains with necrotizing encephalitis were positive for the presence of HSV antigens by IF but give no details on site and distribution of the specific fluorescence. Like Kumanishi and Hirano (1978) and Swoveland and Johnson (1979) we observed both cytoplasmic and

nuclear staining although perikaryal neuronal staining predominated, as in experimental HSV encephalitis (Yamamoto et al. 1965). Prominent nuclear involvement was suggested in conventional histological stains by nuclear inclusion bodies in the majority of cases; however, such inclusion bodies were only inconstantly positive for HSV antigens. This phenomenon has been noted previously and was considered to be due to simultaneous occurrence of various stages in the developmental cycle of the herpetic nuclear inclusion (Spaar 1976). It is important to note that nuclear inclusion bodies in HE stains were found or suspected in 11 out of 15 cases negative for presence of HSV antigens; therefore, they are by no means a reliable criterion for an HSV aetiology. However, one case with detectable HSV antigens did not show nuclear inclusion bodies on HE stains, so a lack of nuclear inclusion bodies does not rule out an herpetic aetiology. Such a constellation, however, will be rare and was previously reported Mannweiler and Colmant (1969) in one case with HSV virions demonstrable by electron microscopic examination, among 3 cases of acute necrotizing encephalitis without intranuclear inclusion bodies.

The patchy distribution of HSV antigen positive cells and the frequent occurrence of larger tissue areas negative for HSV antigens must warn against definite classification of small tissue specimens because of sampling errors. Needle biopsies of the temporal lobe, which were advocated as safe method for rapid intravital diagnosis of HSV encephalitis (Olding-Stenkvist 1974) thus have a high rate of false negative results.

The low frequency of positively stained cells in the leptomeninges of immunohistologically diagnosable HSV encephalitis cases renders any search for HSV antigens in CSF cell preparations of limited use (Dayan and Stokes 1973; Longson et al. 1973), with many false negative samples to be expected.

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