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Comparative immunohistochemical study of herpes simplex and varicella-zoster infections

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Summary. Herpes simplex (HSV) and varicella-zoster (VZV) skin infections share so many histological similarities that distinguishing between them may prove to be impossible. We developed and characterized a new monoclonal antibody, VL8, IgG kappa isotype, directed to the VZV envelope glycoprotein gpI. Immunohistochemistry with VL8 appeared highly sensitive and specific on formalin-fixed paraffin-embedded biopsies and a clear-cut distinction between HSV and VZV infections was possible. The pattern of VL8 immunolabelling in VZV infections was strikingly different from that found in HSV infections studied with polyclonal antibodies to HSV I and II. Double immunolabelling revealed the VL8 positivity of sebaceous cells, endothelial cells, Mac 387- and CD68-positive monocyte-macrophages, and factor XIIIa-positive perivascular, perineural and interstitial dendrocytes. Intracytoplasmic VL8 labelling of endothelial cells and perivascular dendrocytes was found at the site of leukocytoclastic vasculitis.

Key words: Immunohistochemistry – Skin – Herpes – Varicella-zoster – Vasculitis

Introduction

It is often stated in textbooks of dermatopathology that the histological changes seen in primary and recurrent herpes simplex virus (HSV) infections are indistinguishable from those produced by varicella-zoster virus (VZV). Characteristic changes in infected epithelial tissues are "ballooning degeneration", the presence of intranuclear inclusion bodies, and multinucleated giant cells. These criteria, however, do not consider the involvement of endothelial cells, "fibroblasts", Schwann cells and perineurial cells in VZV infection (Aoyama et al. 1974; Orfanos and Runne 1975; Muraki et al. 1992).

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A rapid and precise diagnosis of the type of herpes infection is mandatory in immunocomprised patients, newborns and in pregnancy (Janier et al. 1988; Paquet and Piérard 1992). Tzanck smears examined by optical or electron microscopy do not permit the distinction between HSV and VZV infections. In vitro culture of these viruses requires an average of 7 days for their identification, and is therefore too slow to be clinically useful. Immunofluorescence identification may be performed on frozen, acetone-fixed material (Drew and Mintz 1980; Weigle and Grose 1984a; Cleveland and Richam 1987). Polymerase chain reaction is also a reliable, but expensive and time-consuming procedure applicable to Tzanck smears (Nahass et al. 1991). Another approach uses immunohistochemistry on routinely processed tissue samples (Okuno et al. 1983; Martin et al. 1991; Muraki et al. 1992; Nikkels et al. 1992).

We were interested to revisit, by means of immunohistochemistry, the concept of the similarity between the histological presentation of HSV and VZV cutaneous infections. Therefore we developed a new mouse monoclonal antibody VL8, IgG kappa isotype, directed to the viral envelope glycoprotein I (gpI).

Materials and methods

Biopsy specimens were taken from clinically evident herpes simplex, varicella and herpes zoster lesions (Table 1) present in 33 patients aged 21–82. Eleven of these patients were under immunosuppressive therapy.

Biopsies were formalin-fixed and paraffin-embedded to be routinely processed. Sections were stained with haematoxylin and eosin. Others were used for immunohistochemistry.

Immunostaining was performed using the avidin-biotin system. In brief, the tissue sections were deparaffinized and incubated with a non-immune blocking serum for 20 min. The primary antibodies (Table 2) were applied for 30 min at room temperature. Between incubations, the sections were washed with TRIS-buffered-saline. A biotinylated secondary antibody and the alkaline phosphatase coupled avidin-biotin complex (ABC-AP) were incubated each for 30 min. New fuchsin (Dakopatts) was used as chromogen in the ABC-AP system and diaminobenzidine (DAB) in the ABC horse-

Table 1. Number of skin biopsies

	Stage		
	Erythematous lichenoid	Vesicular spongiotic	Pustular
Herpes simplex	2	10	4
Varicella-zoster	4	10	3

Table 2. Panel of antibodies

Antibody	Clone	Antigen	Dilution	Source
Anti-S-100	–	S100a	Prediluted	Dakopatts
Mac 387	Mac387	L1	1:100	Dakopatts
CD68	KP1	KiM6	1:100	Dakopatts
CD45RO	UCHL-1	–	1:200	Dakopatts
CD45R	4KB5	–	1:200	Dakopatts
Anti-factor XIIIa	)	Factor XIIIa	1:350	Behring
Anti-herpes I	–	–	Prediluted	Dakopatts
Anti-herpes II	–	–	Prediluted	Dakopatts
VL8	–	gpI	1:10	–

radish peroxidase (HRP) system. DAB staining intensity was increased with 0.5% copper sulphate. Sections of normal and inflamed skin served as controls. In other controls from herpes-infected lesions, we substituted non-immune mouse ascites fluid for the primary antibodies.

Double immunostainings were performed by the indirect ABC-AP method for the first antibody, followed by an indirect avidin-biotin peroxidase coupled method. New fuchsin and DAB were used as substrates. Sections were lightly counterstained with Mayer's haemalum and mounted in Glycergel (Dakopatts).

In order to characterise the anti-VZV antibody an isolate of VZV with a restriction endonuclease cleavage pattern of its genome identical to that of strain Ellen was grown in MRC-5 cells (human embryonic lung fibroblasts), in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum (FCS). Infection was transmitted by co-cultivating infected cells with uninfected cells. Cells were harvested 4–6 days after infection by scraping, centrifuged at 2000 rpm and resuspended in 15 ml serum-free medium per 75 cm^2 flask. This procedure was repeated three times and the last suspension was made in 1 ml.

Balb-C mice were infected intraperitoneally with 0.25 ml of freshly prepared infected cell suspension containing 10^5 cells, emulsified in the same volume of Freund's complete adjuvant (Sigma, St Louis, Mo., USA). Fourteen days later, the same inoculation was made with Freund's incomplete adjuvant. One week later, the animals received injections of 10^5 infected cells in the absence of adjuvant. The next day they were killed and their spleens were removed. Splenocytes were harvested, washed and centrifuged at 200 *g* at 20° C; included red blood cells were lysed in ammonium chloride for 3 min at 20° C. Serum-free DMEM was added and cells were washed and centrifuged at 200 *g*.

Sp-2/O-Ag 14 myeloma cells constitutively transformed with the *neo* gene were grown in 45% DMEM, 45% Isove's DMEM, and 10% FCS (Köhler and Milstein 1976). Fusion with spleen cells was performed at 37° C in DMEM without 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) in the presence of 50% polyethylene glycol (Galfre et al. 1977). Myeloma to spleen cell ratio was 1:5. After a 5-min 200 *g* centrifucation at 20° C, pellets were resuspended in and kept in culture in the same medium complemented with hypoxanthine-aminopterin-thymidine (HAT) selection medium (Littlefield 1964). One week later, hybridomas selected in HAT medium were transferred to HT medium. Supernatants were screened first for secretion of immunoglobulins (Ig), then for production of anti-VZV antibodies by enzyme immunoassay (EIA).

Mouse Ig were detected by using 96-well plates coated with rabbit Ig anti-mouse Ig (1:20; Gamma, Liège, Belgium). After saturation with 1% bovine serum albumin, supernatant media of hybridoma cultures were added undiluted for 1 h at 37° C. Rabbit antibodies to mouse Ig coupled to HRP (1:2000; Gamma) were added and kept for 1 h at 37° C. All steps were separated by extensive washings in phosphate buffered saline. Colour development was made with hydrogen peroxide 0.001% and 2-2′-azino di[3-ethylbenzthiazolin sulfonate (6)] 75 mg/ml for 20 min at 37° C. Optical density was measured on a Titertek Multiscan spectrophotometer at 415 nm.

Measurement of anti-VZV antibodies was determined in an EIA performed directly on cell monolayers. Cells were grown in 96-well plates. Half of this material was then infected with VZV. Uninfected and infected cells were fixed in a mixture of methanol and acetone. Samples to be tested were added to the fixed cells and incubated for 1 h at 37° C. Colour development was performed as above.

Supernatants of hybridomas reacting with infected but not uninfected cells were selected and cloned by the limiting dilution method. Three successive clonings were done and verified each time for specificity.

Among the several hundreds of clones obtained, 12 were selected for their VZV specificity. After testing by immunolabelling on deparaffinized sections, clone VL8 was selected for the quality of its staining in such conditions. Its specificity was verified by EIA on uninfected cells or on cells infected with other herpesviridae: herpes virus type I, herpes virus type II, cytomegalovirus and Epstein-Barr virus, towards which it was completely negative. Activity and specificity were also confirmed by cytofluorimetric analysis (Snoeck et al. 1992). VL8 isotype is IgG1k as determined in an EIA by antibody capture on antigen-coated plates.

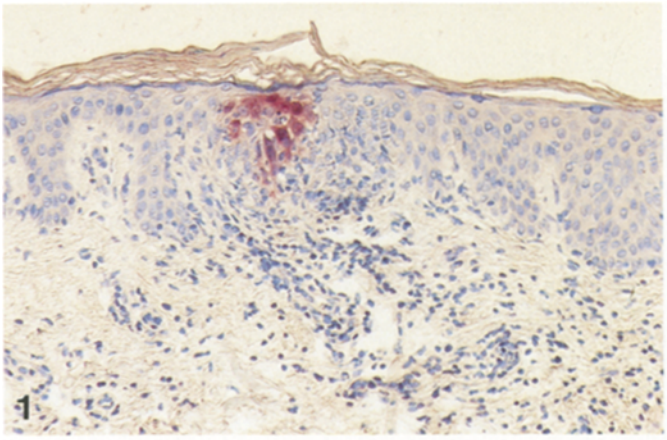
VL8 reacted in Western blotting with bands at 52 kDa, 60 kDa and 72 kDa, a multiple band pattern compatible with gpI. With immunoperoxidase staining of MRC-5 cells infected with VZV, the cytoplasm and plasma membrane were well stained as early as 8 h after infection. Staining became perinuclear after 12 h.

Results

Early signs of HSV and VZV infections were recognized by the presence of keratinocytes with a pale and ballooned cytoplasm. The vesiculous stage was characterized by rupture of ballooned cells and by acantholysis. Multinucleated giant epithelial cells and necrotic keratinocytes were also present. Intraepidermal inflammatory cells consisted mainly of neutrophils. Their accumulation resulted in the formation of pustules.

These classical alterations of the epidermis and hair follicles looked alike in HSV and VZV infections. However, the pattern and density of the inflammatory infiltrate was somewhat different. A deep perivascular and perineural mixed infiltrate was usually more abundant in VZV when compared with HSV infections. Superficial and deep leukocytoclastic vasculitis was only found in herpes zoster with prominent epidermal alterations. All these changes were seemingly more pronounced in immunocompromised patients.

Five among the 33 biopsy specimens were lacking the specific epidermal changes for herpes infection. The correct diagnosis was only reached by immunohistochemistry.



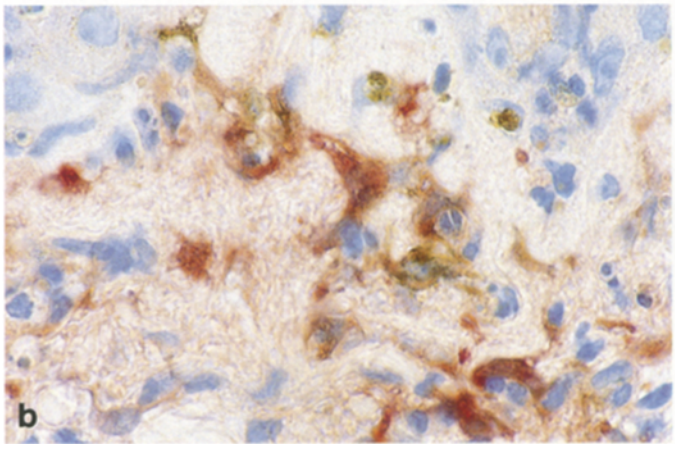
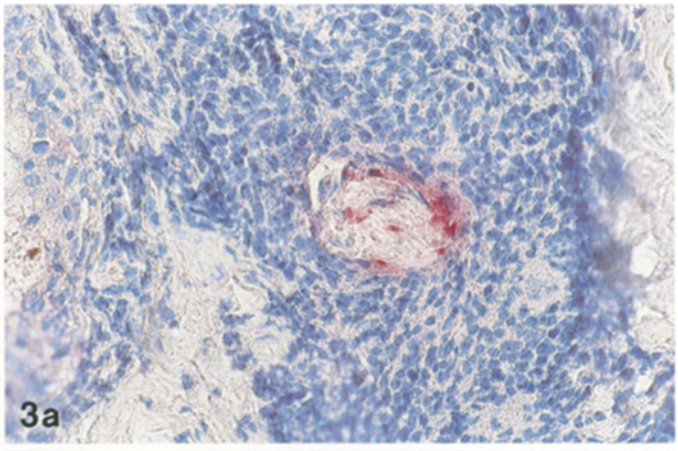


Fig. 1. Spongiotic lesion of herpes simplex virus (HSV) I without presence of ballooning and multinucleated keratinocytes. Immunolabelling anti-HSV I antibody (×250)



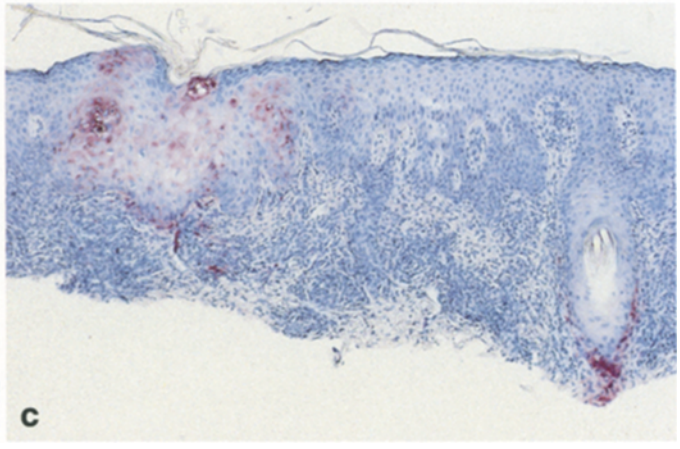


Fig. 3a–c. Herpes zoster. **a** Neural VL8 immunoreactivity (×500). **b** Double immunolabelling revealing VL8 positivity (*brown*) in dendritic factor XIIIa-positive (*red*) dendrocytes (×1000). **c** Spotty VL8 positivity in the epidermis at a site of lichenoid dermatitis clinically diagnosed as herpes zoster (×250)

The immunolabelling with anti-HSV I and II antibodies was always restricted to the epidermis, and in some instances to the upper part of the hair follicles. A cross-reactivity was evidenced between the antibodies to HSV I and II. However, the intensity of the labelling was always different, with predominance of one or the other. No cross-reactivity was found between antibodies to HSV I or II and the antibodiy to VZV.

Three cases of recurrent HSV infection lacked the classical alterations of the epidermis, resembling a spongiotic dermatitis. Immunohistochemistry revealed their viral origin, however (Fig. 1).

VL8 immunostaining disclosed a wedge-shaped distribution of the viral gpI in the skin (Fig. 2a). Single and double immunolabellings revealed the nature of the positive cells, including nuclei, cytoplasm and membranes of epidermal (Fig. 2b, c) and hair follicle keratinocytes, as well as cytoplasm of sebocytes, Schwann cells, and dendritic factor XIIIa-positive cells (dendrocytes), the latter being in subepidermal, periadnexal, perivascular and perineural locations (Figs. 1, 2b, 3a, b). The cell membrane of a few S-100-positive dendritic cells (Langerhans cells) located in the vicinity of blisters were also labelled.

Two cases, clinically diagnosed as herpes zoster, suggested a lichenoid dermatitis histologically without evidence for VZV infection. Scattered keratinocytes proved to be VL8-positive (Fig. 3c).

Among inflammatory cells present inside and underneath the epidermal blisters, the cytoplasm of some CD68-positive and Mac 387-positive macrophages was also VL8-positive. The CD45R-positive lymphocytes and CD45RO-positive lymphocytes remained VL8-negative.

The endothelial cell cytoplasm and perivascular dendrocytes were strongly VL8-positive at the site of discrete leukocytoclastic vasculitis (Fig. 4). Dendrocytoclasis, recognized by the cytoplasmic fragmentation of dendrocytes, was proportional to the extent of vascular alterations.

Discussion

The monoclonal antibody VL8 we have selected to detect the gpI envelope of VZV appears highly specific and sensitive by the series of tests we have reported. It may be added to the list of antibodies created to react

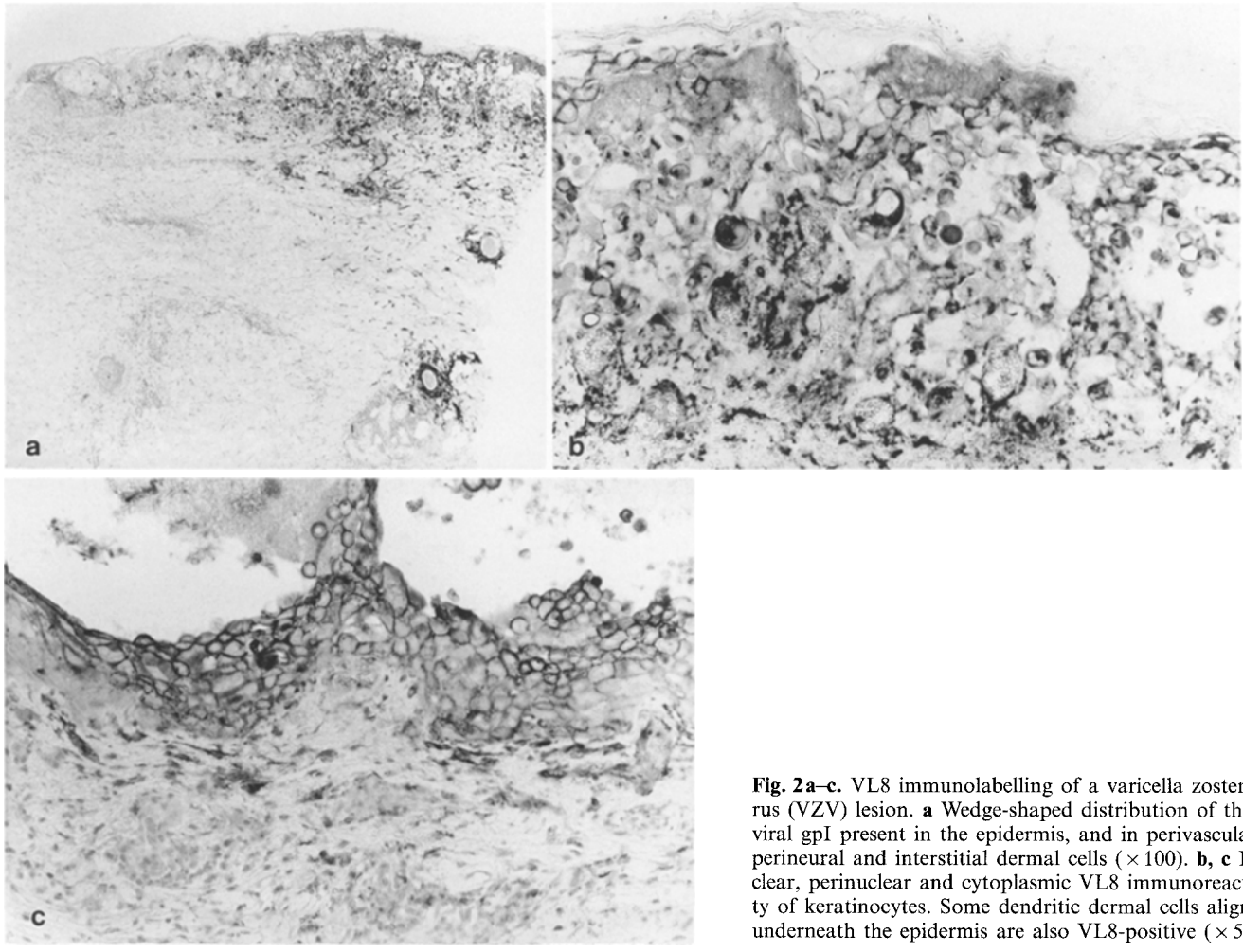


Fig. 2a–c. VL8 immunolabelling of a varicella zoster virus (VZV) lesion. **a** Wedge-shaped distribution of the viral gpI present in the epidermis, and in perivascular, perineural and interstitial dermal cells (×100). **b, c** Nuclear, perinuclear and cytoplasmic VL8 immunoreactivity of keratinocytes. Some dendritic dermal cells aligned underneath the epidermis are also VL8-positive (×500)

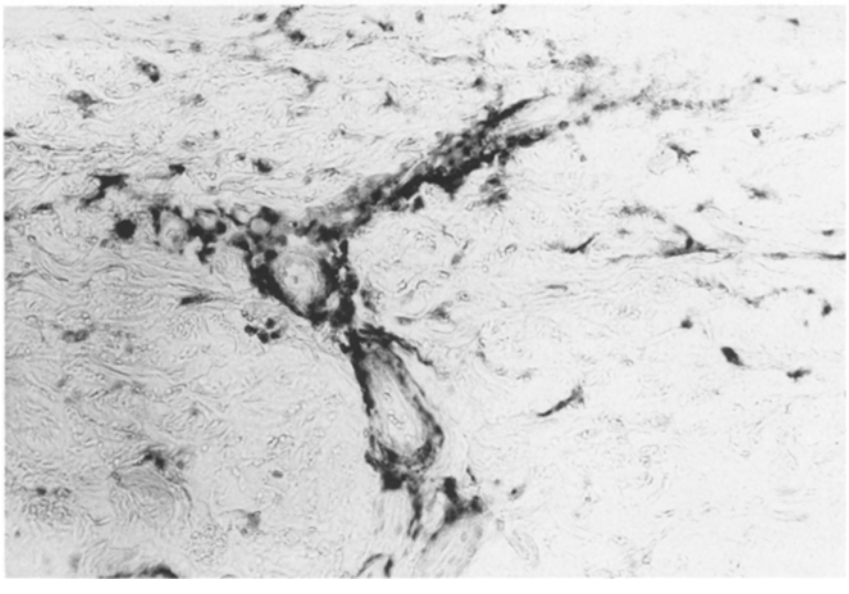


Fig. 4. VZV-related leukocytoclastic vasculitis. Strong VL8 positivity of perivascular dendritic cells (×500)

with VZV (Leonard et al. 1970; Gerna et al. 1977; Drew and Mintz 1980; Schmidt et al. 1980; Schmidt 1982; Grose et al. 1983; Okuno et al. 1983; Forghani et al. 1984; Weigle and Grose 1984a, b; Ozaki et al. 1986; Cleveland and Richman 1987; Landry et al. 1987; Martin et al. 1991; Nahass et al. 1991; Muraki et al. 1992). Our antibody differs from others by the extent of its characterization, including the precise identification of the antigenic target.

We failed to distinguish VZV and HSV infections by standard microscopy. Only subtle clues could be used to suspect herpes zoster histologically, namely a prominent involvement of adnexal structures and the presence of a deep mixed perivascular and perineural infiltrate. Conversely there was excellent clinico-immunopathological correlation in the diagnoses.

The pattern of distribution of the VZV gpI and of HSV proteins were strikingly different. The HSV proteins were only found in the nuclei and cytoplasm of epithelial cells, while the VZV gpI was also present in the cytoplasm of many other cell types that displayed no major primary cytological alterations.

VZV gpI was almost always found in hair follicles and sebaceous glands. HSV proteins were occasionally found in hair follicles, but never in sebocytes. These differences may be related to a preferential spread of VZV from dermal nerve endings through follicular structures, before reaching the epidermis.

The visualization of VZV gpI inside and around nerves was never seen in HSV infections.

VZV gpI was disclosed in mobile macrophages present in the blisters, and this could be related to a possible viral spread of VZV by cells of the monocyte-macrophage lineage. Its presence in factor XIIIa-positive dendrocytes may also be the result of phagocytosis. As these latter cells are probably antigen-presenting cells, they may participate in the initiation of vasculitis. The secondary destruction of endothelial cells and perivascular dendrocytes may further release the intracytoplasmic gpI and increase the inflammatory response. Dendrocytoclasis present in VZV-related vasculitis is not unique for that condition as it has also been reported in other leukocytoclastic vasculitides (Arrese Estrada et al. 1991).

The presence of gpI in dermal cells was limited to a cuneiform space beneath the VZV-involved epidermis. This confirms another observation (Muraki et al. 1992) suggesting that the VZV spreading in the dermis was secondary to keratinocytes infection. These data are, however, at variance with another study (Weigle and Grose 1983) where 62 kDa and 98 kDa glycoproteins, which are constituents of gpI and gpII complexes, were only found in the epidermis. This difference could be due to different technical approaches and/or to distinct sensitivity of antibodies to recognize different epitopes on the gpI complex.

Our findings of a large load of gpI in the microvascular unit during the acute stage of herpes zoster recall previous findings of minute amounts of gpI in the same cells of the site of granulomatous vasculitis occurring later in these patients (Nikkels et al. 1992). In such pathology, other studies have failed to reveal the presence of VZV DNA (Langenberg et al. 1991; Serfling et al. 1991). gpI, which is the most abundant glycoprotein of the VZV envelope, could therefore be one of the main molecules responsible for the local vasculitis.

Immunolabelling with specific antibodies may prove to be useful for the diagnosis of some HSV and VZV infections. It also provides some insight into the pathogenesis of these diseases.

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