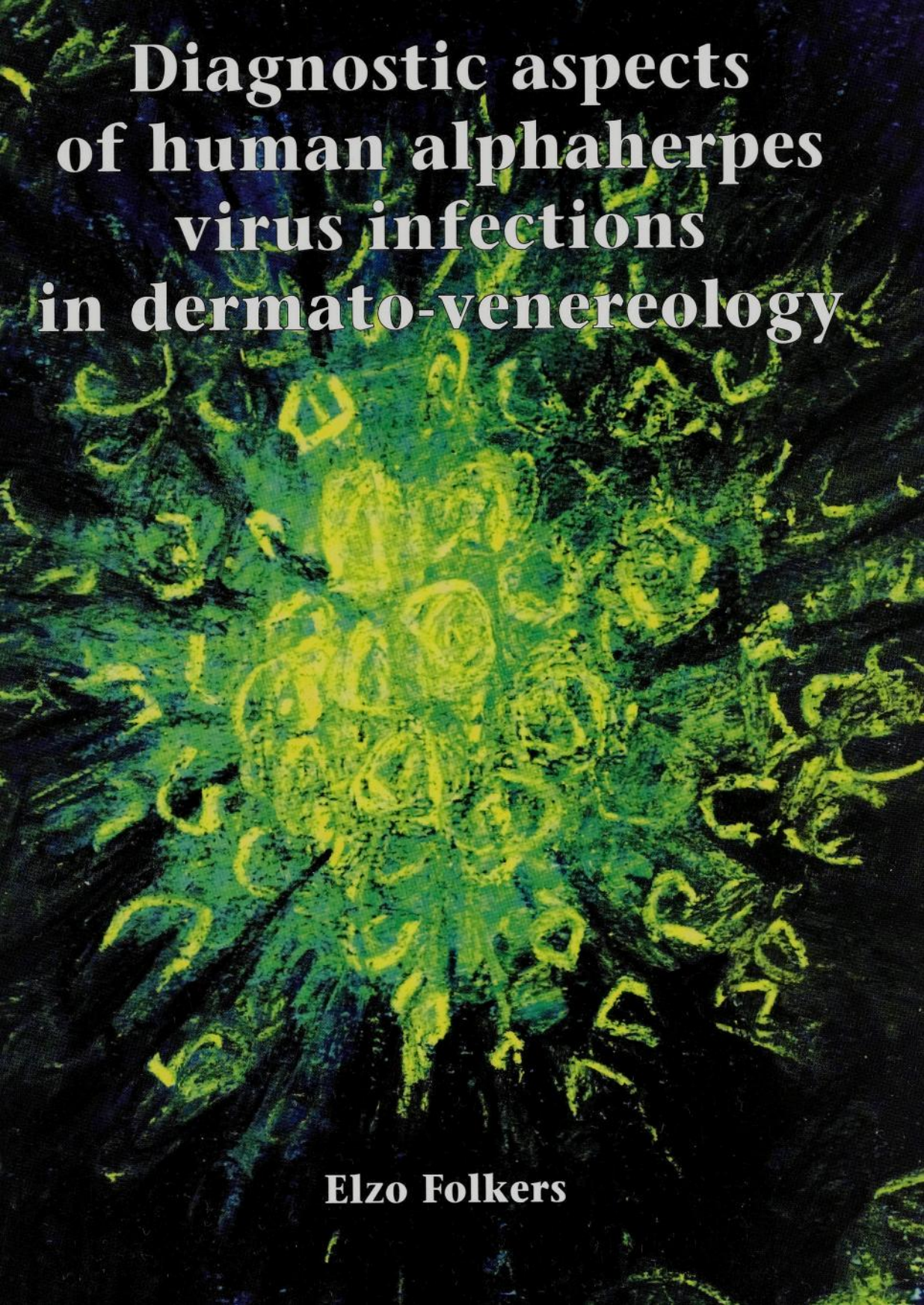
Diagnostic aspects of human alphaherpes virus infections in dermato-venereology



Elzo Folkers


UBA003000190

DIAGNOSTIC ASPECTS OF HUMAN ALPHAHERPESVIRUS INFECTIONS IN DERMATO-VENEREOLOGY

Diagnostic aspects of human alphaherpesvirus infections in dermato-venereology

A C A D E M I S C H
P R O E F S C H R I F T

Ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam,
op gezag van de Rector Magnificus
Prof. Dr. J.J.M. Franse

ten overstaan van een door het
College voor Promoties ingestelde commissie,
in het openbaar te verdedigen
in de Aula der Universiteit

op vrijdag 22 oktober 1999 om 10.00 uur

door

Elzo Folkers
geboren te Willemstad Curaçao

Stellingen behorende bij het proefschrift: Diagnostic aspects of human alphaherpesvirus infections in dermato-venereology

1. Zachte heelmeesters zullen met de Tzanck-test altijd een lagere sensitiviteit bereiken.

2. De klinische microbiologie zou de Tzanck-test als standaardprocedure moeten opnemen en deze test meer bij de diagnose van humane alphaherpesvirussen moeten betrekken.

3. Om onnodige stigmatisering te voorkomen kan de huisarts bij herpes en spitse wratten in de anogenitale regio screening op andere sexueel overdraagbare aandoeningen beter achterwege laten.

4. Een degeneratieve primer is als een sticker met slechte lijm, die alleen blijft zitten als hij optimaal wordt aangebracht (Hoofdstuk 4).

5. Het varicella-zoster virus gedraagt zich ten opzichte van virale antilichamen als een ezel (Hoofdstuk 5).

6. Onderzoeksgelden naar 'stealth technology' voor oorlogsdoeleinden kunnen beter worden aangewend voor onderzoek naar de 'stealth technology' van virussen.

7. Door Turkije als volwaardig lid toe te laten tot de Europese Unie worden de mogelijkheden om de huidige lidstaten tegen exotische infectieziekten te beschermen verruimd.

8. Om in de relationele sfeer besmetting met macrovirussen te voorkomen kan onthouding van digitaal contact overwogen worden.

9. Het democratisch machtsvacuüm rond studenten en hoogleraren is inmiddels opgevuld door niet-democratisch gekozen bestuurders. Dit is een beperking voor de universitaire vorming van de student.

10. Het auteursrecht van wetenschappelijke publicaties moet niet gaan vallen onder het zogenaamde werkgeversauteursrecht, maar dient in handen te blijven van de wetenschappers zolang deze nog voor hun werkzaamheden onderbetaald worden.

11. De enorme toename van industriële bedrijvigheid op het gebied van genetisch gemanipuleerde organismen is verontrustend. Vooruitlopend op nieuwe gevaren die op ons kunnen afkomen kan de definitie 'hazard', die gehanteerd wordt door het Center for Chemical Process Safety (USA), beter worden gewijzigd in 'an inherent physical, chemical *or biological* characteristic that has the potential for causing harm to people, property, or the environment'.

12. De veiligheidsrichtlijnen voor gevaarlijke stoffen die de Europese Unie hanteert voor de chemische industrie, vormen een goed uitgangspunt voor aanvullende richtlijnen voor de pharmaceutische industrietak die belast is met de ontwikkeling en productie van antivirale en anti-bacteriële vaccins.

13. Als je maar vaak genoeg lacht dan krijg je vanzelf rimpels.

14. In muziek kan een uur voorbij vliegen en een enkele maat eeuwig duren.

Elzo Folkers
oktober 1999

Cover illustration: "Corona herpetica" by Elsa J. Loosjes
Production supervisor: Tekstra Grafische Produkties, Jisp
Printed by: PlantijnCasparie Heerhugowaard
Design: Chris van Egmond BNO

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ISBN 90-9013181-7

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Promotiecommissie:

Promotor:	Prof. dr. J.D. Bos
Copromotoren:	dr. A.P. Oranje dr. J. Vreeswijk
Overige leden:	Prof. dr. R.H. Coutinho Prof. dr. J. Goudsmit Prof. dr. R. de Groot Prof. dr. F.J.W. ten Kate Prof. dr. W.A. van Vloten

The studies described in this thesis were conducted at the Department of Mammalian Virology, Institute for Animal Science and Health (ID-DLO), Lelystad;
Department of Dermatology & Venereology, Hospital de Heel, Zaandam;
Department of Dermatology & Venereology, University Hospital Rotterdam, Erasmus University, Rotterdam;
Clinic for Sexually Transmitted Diseases of the Municipal Health Service (GG&GD), Amsterdam, The Netherlands.

Printing of this thesis was financially supported by the University of Amsterdam, Institute for Animal Science and Health (ID-DLO), the Huidstichting Chanfleury van IJsselsteijn,
Glaxo-Wellcome,
Janssen Cilag,
Galderma,
Novartis Pharma.

Additional financial support was provided by:
Abott, Alk Abelló, EuroTec, Hoechst Marion Roussel, INC Pharmaceuticals,
Leo Pharmaceutical Products, Roche Pharmaceuticals, Schering Plough, Tramedico and Yamanouchi Pharma.

Men zou het woord probleem moeten vermijden
om twee simpele redenen:

er zijn oneindig veel voorbeelden van problemen
die er niet zijn – ik kom hier op terug

er zijn even oneindig veel voorbeelden van problemen
die er wel zijn, maar niet zo worden genoemd –
ook hierop kom ik terug.

Rutger Kopland,
uit: Tot het ons loslaat (1997).

Contents

CHAPTER 1

General introduction

1.1 Herpesviruses, important to mankind

The herpesviruses (*Herpesviridae)* belong to a family of double-stranded DNA viruses. The origin and the evolution of herpesviruses is not known but presumably began about 200 Myears before present at the same time as the differentiation of species arose.
An argument in support of this hypothesis is that more than 100 different herpesviruses have been characterized. The host range is confined to eukaryotes and extends from fungi to the invertebrates and the vertebrates. For example herpesviruses have been found in insects, shellfishes, fishes, reptiles, birds and mammals, including primates.[1-5]
The family of herpesviruses is subdivided in three subfamilies. *Alphaherpesvirinae* are rapidly growing cytolytic viruses that have the capacity to establish latent infections primarily but not exclusively in nerve ganglia; they have a variable host range. *Betaherpesvirinae,* which have a restricted host range, are slowly growing "cytomegalic" viruses. Infected cells often become enlarged ("cytomegalia") and cell lysis occurs several days after infection. Secretory glands, lymphoreticular tissue, kidneys and other organ tissue can harbour latent virus. *Gammaherpesvirinae* are growing in lymphocytes and can induce malignant transformation. Their host range is limited to family of the natural host. Some gammaherpesviruses also cause cytocidal infections in epithelial and fibroblastic cells. The virus can be maintained in latent form in lymphoid tissue.[1-3, 5]
Transmission of herpesviruses is generally associated with close contact of skin and mucosal membranes, but droplet infection of the respiratory tract is also common. Many herpesviruses are host specific, but some alphaherpesviruses, such as pseudorabies virus and B virus (*Herpesvirus simiae,* cercopithecine herpesvirus 1), may affect a range of species.[2] Most mammals, except primates and horses, are susceptible to the Pseudorabies virus (suid herpesvirus 1, PRV), which causes severe disease (Aujeszky disease, bulbar paralysis, 'mad itch'). So far it is not certain that man is sensitive to PRV.[6] In primates several species of the genus Macaca act as reservoir for the B virus. The transmissibility of B virus is limited, but it causes a fatal outcome in most cases of B-virus infection. B virus latency in man has been reported: a patient with encephalomyelitis and a ophtalmic zoster-like rash, and recurrent vesicular rash as a consequens of B virus infection.[7-9]
Man is the natural host for at least seven pathogenic herpesviruses: the two types of herpes simplex virus (HSV-1 and HSV-2), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), human herpesvirus-6 (HHV-6), and human herpesvirus 7 (HHV-7).[5, 10, 11] Untill now, no clinical symptomatology has been linked with HHV-7 infection.[10] The newly identified human herpesvirus in Kaposi's sarcoma is called human herpesvirus 8 (HHV-8).[5, 12, 13]
In Table 1 are listed the names of the human herpesviruses according to the Sixth Report of

the International Committee on Taxonomy of Viruses (ICTV),[14] and of the more recently discovered human herpesviruses.[5, 10-13, 15]
How many more HHV's as yet are undiscovered?

Table 1. *International and English vernacular names for human herpesviruses*

FAMILY	***HERPESVIRIDAE***		
TAXONOMIC STATUS	INTERNATIONAL NOMENCLATURE	ENGLISH VERNACULAR NAME	ABBREVIATION
Subfamily	***Alphaherpesvirinae***	***Herpes simplexvirus group***	
Genus	*Simplexvirus*	*Human herpesvirus 1 group*	
Species	Human herpesvirus 1	Herpes simplex virus 1	**HSV-1**
	Human herpesvirus 2	Herpes simplex virus 2	**HSV-2**
Genus	*Varicellovirus*	*Human herpesvirus 3 group*	
Species	Human herpesvirus 3	Varicella-zoster virus	**VZV**
Subfamily	***Betaherpesvirinae***	***Cytomegalovirus group***	
Genus	*Cytomegalovirus*	*Human cytomegalovirus group*	
Species	Human herpesvirus 5	Human cytomegalovirus	**CMV**
Genus	*Roseolovirus*	*Roseolovirusgroup*	
Species	Human herpesvirus 6		**HHV-6**
	Human herpesvirus 7*		**HHV-7**
Subfamily	***Gammaherpesvirinae***	***Lymphoproliferativevirus group***	
Genus	*Lymphocrytovirus*	*Human herpesvirus 4 group*	
Species	Human herpesvirus 4	Epstein-Barr virus	**EBV**
Genus	*Rhadinovirus*		
	Human herpesvirus 8*	Kaposi's sarcoma-associated Herpesvirus	**(KSHV) HHV-8**

* temporarily classified; not assigned in the Family of *Herpesviridae* by ICTV untill now

1.2 Role of human herpesvirus infections in dermato-venereology

Human herpesvirus infections have a wide range of clinical manifestations. The severity of the disease depends on the entry site of the virus, the kind of infection (primary or recurrent), and the state of patient's health (healthy, elderly or immunocompromised).

Alphaherpesviruses

In men and animals many alphaherpesviruses produce localized lesions, particularly of the mucosal membranes of the respiratory and genital tracts or the skin. Viral lesions appear in a specific order. Vesicles transform into pustules, which in turn become crusted or transform into superficial shallow ulcers covered by a pseudomembrane. The lesions usually heal without scar formation in less than fourteen days.[2]

Herpes simplex

The human simplexviruses comprise human herpesvirus 1 (HSV-1) and human herpesvirus 2 (HSV-2). Man is the reservoir and vector of these herpesviruses. Wild and domestic animals are not affected. Herpes simplex viruses are among the most common infectious agents of man. Transmission is generally associated by close personal contact and droplet infection. They can cause a clinical manifest or asymptomatic infection.[16]

The skin, oral cavity, genital mucosal membranes, conjunctiva and nervous system are the most frequent sites affected by HSV infection. However, in newborn infants, in immunocompromised and immunosuppressed patients other organ sites may be involved as well, as a consequence of viremia. Infection with HSV either during delivery (in case of genital herpes of the mother) or more often shortly thereafter by direct contact with persons with labial herpes ("kiss of death") or with herpetic whitlow poses a serious risk to the newborn and carries a risk of mortality.

Acute gingivostomatitis is the most common clinical manifestation of a primary herpes simplex virus type 1 infection. A primary HSV-1 infection of the mouth is sometimes accompanied with (primary) herpetic lesions on other body sites. Primary herpetic dermatitis, nasal herpes, ocular herpes, herpetic whitlow and genital herpes can be complications of acute gingivostomatitis. Recurrent herpetic lesions appear at or near the site of primary eruptions.[16] The body sites commonly involved in human herpes simplex virus infection are listed in table 2.[1]

The characteristic vesiculobullous eruptions of HSV can resemble those of other viral and non-viral skin infections.[17-20] For instance impetigo caused by *streptococci* and *staphylococci*, and herpes simplex may have very similar clinical appearances. Eczema herpeticum, most commonly seen in patients with atopic dermatitis, but also reported in patients with Darier's disease, resembles secondary bacterial infected eczema.[21] Disseminated HSV infection can mimic varicella and dermatomal distribution of HSV lesions resembles herpes zoster. In case of intra-oral ulcers besides herpes simplex other ulcerous diseases, especially aphtous ulceration, must be considered. In patients with genital ulcers syfilis, ulcus molle, Behçet's syndrome, lymphogranuloma venereum, granuloma inguinale, candida infections, trauma, and toxic ulceration as side-effect in chemotherapy, must be considered in addition to HSV infection.[16] Without the appearance of characteristic vesicles cutaneous HSV infection can be difficult to diagnose. Especially immunosuppressed patients often show atypical mucocutaneous manifestations of (persistent) HSV infection.[22-24]

Erythema multiforme is considered to be a immunologic reaction to many exogenous agents, most commonly to recurrent herpes simplexvirus infections and drugs.[25, 26]

Table 2. *Herpes simplex virus infections: commonly involved body sites and type of infection*

Involved sites	HSV type most usually found	Usual type of infection	Subclinical manifestation
Mucous membranes:			
oral cavity	HSV-1	Primary	Frequently
lips	HSV-1	Recurrent	
penis	HSV-2	Recurrent	
vulva	HSV-2	Recurrent	
vagina	HSV-2	Recurrent	Frequently
cervix	HSV-2	Recurrent	Frequently
Eyes:			
cornea	HSV-1	Recurrent	
conjunctiva	HSV-1	Primary	Frequently
Skin:			
above the waist	HSV-1	Recurrent	
below the waist	HSV-2	Recurrent	
hands	HSV-1 & 2	Recurrent	
Sensory ganglia:			
trigeminal	HSV-1	Recurrent	
sacral	HSV-2	Recurrent	

Varicella-zoster

The alpaherpesviruses VZV, pseudorabiesvirus (Suid herpesvirus I), equine abortion virus (Equid herpesvirus I), and simian varicella virus (Cercopithecine herpesvirus 9) are members of the genus *Varicellovirus* . Varicela-zoster virus (human herpesvirus 3, VZV) also belongs to the *Alphaherpesvirinae* subfamily. Man is the natural host of VZV, and is the etiological agent of varicella (chickenpox) and herpes zoster (shingles). Varicella is caused by primary infection and herpes zoster is the recurrent manifestation of VZV infection. Varicella is mainly transmitted by the respiratory route. The appearance of successive crops of skin lesions all over the body indicates a systemic infection with secondary intermittent viremia and the skin as the ultimate target organ. Primary VZV infection may involve any organ, mostly without clinical manifestation, except for the skin and occasionally the lung and brains. The initial skin lesions are macules and papules which rapidly become vesicular. The vesicles, surrounded by a small erythematous area, become rapidly crusted. The lesions heal usually within ten days, leaving sometimes small shallow scars. The lesions appear in successive crops so that all stages can be observed all over the body at any one time. The most common complication of varicella in otherwise healthy children is secondary bacterial infection of the lesions, which sometimes can lead to staphylococcal scalded skin syndrome (SSSS), and/or acute bacterial sepsis. Cerebellar ataxia, meningoencephalitis and encephalopathy (Reye's syndrome) may occur as neurologic

complication. Viral pneumonia in varicella can occur in previously healthy individuals, particularly in adults, but is mainly seen in immunocompromised patients. Recurrent varicella has been described in children infected with HIV. Varicella can be severe in pregnancy, and poses a high risk of complications to the mother and the fetus. VZV infection contracted before the 20th week of pregnancy can cause the rare congenital varicella syndrome of the baby, with scarring of the skin, musculoskeletal defects, cerebral and ocular damage. VZV is known to cross the placenta very late in pregnancy. If the mother contracts varicella within a week before the baby's birth, or if the child is infected before it is one week old, the infant may suffer from neonatal varicella.[27, 28]

Herpes zoster results from the reactivation of latent varicella-zoster virus in a single sensory ganglion. It typically affects a single dermatome of the skin and the siting of the skin lesions depends on the involved ganglion. Especially in immunocompromised patients the virus can spread from the affected dermatome to infect the skin or other organ at some distal site, and in case of extensive dissemination the appearance can resemble varicella.[29]

In most patients a VZV infection is diagnosed from the typical clinical features. Clinical diagnosis of herpes zoster in immunocompromised patients can be difficult, especially in the early stages of the disease, when only a few herpeticlike vesicles are present. These may not have the typical dermatomal localization.[30] In addition, due to acyclovir resistant VZV patients with acquired immunodeficiency syndrome may develop unusual cutaneous patterns like disseminated ecthymatous lesions[31, 32], hyperkeratotic nodules an crusted verrucous lesions [33-38], pox-like lesions[39], and disseminated pinpoint-sized erythematous papules[40]. Zoster-like cutaneous manifestations can develop in other diseases giving cause to faulty clinical VZV-diagnosis.[41-43]

Several types of cutaneous lesions have been reported at the sites of resolved cutaneous herpes zoster lesions. These comprise comedones, xanthomatous changes, sarcoidal and tuberculoid granulomas, granulomatous vasculitis and unclassified granulomaous dermatitis, nodular solar degeneration, pseudolymphoma,, psoriasis, lichen planus, morphoea, lichenoid chronic graft-versus-host disease, eosinophilic dermatosis, acquired reactive perforating collagenosis, lymphoma, leukemia, Kaposi's sarcoma, angiosarcoma, basal cell and squamous cell carcinoma, and cutaneous metastases from internal carcinoma.[44]

In immunosuppressed patients chronic lichenoid dermatoses may be associated with a chronic, non-cytolytic, low-productive VZV infection.[24, 45]

Betaherpesviruses

Cytomegalovirus

Human cytomegalovirus (human herpesvirus 5, CMV) belongs to the *Betaherpesvirinae* subfamily. Primary infection with CMV can be acquired intrauterine transplacentally especially from the primary infected mother. Perinatal infection is acquired mainly from infected maternal secretions or breast milk. The postnatal routes of transmission of CMV infection are salivary transmission, blood transfusion, and organ transplantation. Urine is an additional source of infection in children. Semen may contain virus so that it can be spread via sexual contact. The incubation period of CMV is unknown, but is estimated to

be four to eight weeks. After a clinically silent infection of unknown host cells probably at the site of infection, for example the upper respiratory tract, the virus spreads locally to lymphoid tissues and then systemically infects circulating lymphocytes and monocytes to involve lymph nodes and the spleen. The infection then localizes in epithelial cells in salivary glands, in kidney tubules, in cervix, testes and epididymis, from whence virus shedding occurs. In infected individuals CMV has been demonstrated in tissues of epithelial origin (kidney, liver, bile ducts, salivary glands, gut, lung, pancreas) as well as in endothelial cells. In vitro CMV replication is only possible in human fibroblasts.[46, 47]

More than 90% of congenital infected babies have no symptoms at birth. The classic symptoms at birth are intra uterine growth retardation, jaundice, hepatosplenomegaly, trombocytopenia and central nervous system involvement. Purpura can be present due to the trombocytopenia. Other complications are pneumonitis and myocarditis. Perinatal and postnatal CMV infection is mostly clinically not apparent. Also, recurrences are in general mostly asymptomatic.[46, 47]

In immunocompromised and AIDS patients CMV disease is a more serious threat. CMV infection, usually as reactivation of latent virus, is one of the AIDS-defining opportunistic infections. Retinitis in patients with AIDS is the most frequent manifestation of CMV infection, which can also cause gastrointestinal disease, hepatitis, encephalopathy and pneumonia. Skin lesions caused by CMV are not frequently seen in these patients. Vasculitis, hyperpigmented nodules and plaques, papular exanthema, warty hyperkeratotic lesions, and (perineal) ulcers are reported. Concurrent skin infections of HSV and VZV with CMV are described, but these are probably due to CMV viremia without specific cutaneous involvement.[48-56]

Human herpesvirus 6

Human herpesvirus 6 (HHV-6) has two variants, A or B (HHV-6A and HHV-6B). This virus is a mature T-cell lymphotropic virus and shares genetic homology with human cytomegalovirus.[11, 57] HHV-6B is the causative agent for exanthema subitum (roseola infantum).[58, 59] However, the etiologic role for HHV-6A has not yet been clarified. The full clinical spectrum of diseases caused by HHV-6 infection has not yet been confirmed. Because of the high incidence of infection in childhood primary HHV–6 infection of adults is rare. HHV-6 infection in adults can lead to hepatitis, and mononucleosis–like illness with prolonged lymphadenopathy.[11, 60-63]

The characteristic symptoms of exanthema subitum (ES) are prodromal high fever persisting 3-5 days, followed by the disappearance of the general symptoms and the onset of a maculopapular rash, that lasts 1–2 days. The rash is generally localized on the trunk and the neck, but also on the lower part of the face and the extremities. This syndrome of fever, defervescence, and rash occurring in infants is not always caused by HHV-6, but also occurs in infections such as with HHV-7 and enteroviruses.[64, 65]

Human herpesvirus 7

In 1990 another human herpesvirus, HHV-7, has been isolated, from CD-4 positive T-cells.[66] Infection, as determined by seroconversion, occurs in most children, but at later age than in the case of HHV-6. The virus persists, and is present in the majority of normal adult saliva samples. The role of HHV-7 in human disease is unclear.[10] HHV-7 might be a

co-factor in HHV-6 related syndromes. The primary infection with HHV-7 is linked to febrile illness with or without rash that resembles ES.[65] Association between reactivation of HHV-7 infection and pityriasis rosea has recently beeen published.[67-69] Interaction among HHV-7 and EBV in mononucleosis symdrome has been suggested.(70)

Gammaherpesviruses

Epstein-Barr virus

Epstein-Barr virus (human herpesvirus 4, EBV) belongs to the *Gammaherpesvirinae*. EBV infects squamous epithelial cells and B lymphocytes where it remains latent after primary infection. Exposure to EBV is often asymptomatic, and is only apparent from seroconversion in most individuals. However, primary infection may be symptomatic. The most common presentation of primary EBV infection is infectious mononucleosis (IM) in adolescents. Up to 10% of patients with IM may develop dermatologic manifestations: jaundice, maculopapular exanthema (especially related with penicillin and ampicillin administration), palatal petechiae and cutaneous hemorrhage (in cases with marked trombocytopenia), and occasionally urticarial eruptions. Recurrent infection by reactivation of latent virus is seen in immunocompromised patients, resulting in recurring chronic fevers, weight loss, lymphadenopathy, hepatosplenomegaly, interstitial pneumonia and uveitis. In patients with HIV infection a distinctive lesion in the mouth is known as oral hairy leukoplakia.[71-75]

In Africa EBV is associated with Burkitt's lymphoma in areas where malaria is endemic. In certain areas of China, South-East Asia and North Africa, EBV is associated with nasopharyngeal carcinoma, probably due to genetical predisposition of the population. Lymphoproliferative disorders such as Hogdkin's disease, non-Hodgkin's lymphoma may result from EBV infection in immunocompromised patients and males with the X-linked lymphoproliferative syndrome.[71, 72] A case of chronic bullous disease of childhood following EBV seroconversion has been reported.[76]

Human herpesvirus 8

The newly identified human herpesvirus 8 (HHV-8) associated with Kaposi's sarcoma shares limited homology with EBV.[12, 13, 15] HHV-8 resembles EBV in that it has a tropism for epithelial and B-cells, it is kept under immunological control, and only presents a problem during immunosuppression. An AIDS–related body cavity–based lymphoma has recenty been linked to HHV-8.[77]

Summary

Dermatologic manifestations caused by human herpesviruses are listed in table 3.

Table 3. *Dermatologic manifestations of human herpesvirusinfections*

VIRUS TYPE	TYPE OF DISORDER
HHV1 (HSV1)	mucocutaneous lesions (gingivostomatitis, cold sores); ophtalmic infection (keratoconjunctivitis).
HHV2 (HSV2)	mucocutaneous lesions (ano-genital herpes, cutaneous herpes);
HHV3 (VZV)	varicella (chickenpox), zoster (shingles); ophtalmic infection (ophtalmic zoster).
HHV4 (EBV)	maculopapular rash (especially related to antibiotic administration), facial oedema; oral hairy leukoplakia (immunocompromised host).
HHV5 (CMV)	purpura (congenital infection) / vasculitis, hyperpigmented nodules and plaques, papular exanthema, warty hyperkeratotic lesions, and (perineal) ulcers (immunocompromised host).
HHV-6A	a clear etiologic role has not yet been identified.
HHV-6B	exanthema subitum; in adults: hepatitis, mononucleosis-like illness.
HHV7	mononucleosis-like illness (interaction among other human herpesviruses?); exanthema subitum; pityriasis rosea;
HHV8	Kaposi's sarcoma; primary effusion lymphoma (body cavity-based lymphoma).

1.3 Microbiology of herpetic infection

All members of the Herpesviridae have an identical morphology of the virus particle (virion). By negative stain transmission electron microscopy the herpesviruses show a pleomorphic appearance, but this is artificially induced during processing. Pseudo-replica electron microscopy reveals the most accurate representation of the virus particle.The overall diameter of the complete enveloped herpesvirus particle is between 120 and 300 nmm and depends upon the particular virustype.(3, 5)

The herpesvirus has four basic structures: 1.) an electron opaque core, 2.) an icosadeltahedral capsid enclosing the core, 3.) an amorphous electron-dense material (tegument) surrounding the capsid, and 4.) an outer membrane (envelope) which surrounds the nucleocapsid and tegument. The herpesvirus envelope contains numerous glycoprotein protrusions (spikes), which are important antigenic determinants of the herpesviruses. The herpesvirus core is composed of linear double-stranded DNA packaged in the form of a torus.[3, 5]

The various herpesviruses cannot be differentiated on the basis of the virion morphology. The general features of their replicative cycle is basically also the same.To initiate infection, the virus must attach to cell receptors. Initial attachment and penetration of the host cell is mediated by the glycoprotein spikes on the surface of the envelope. After apposition of the virion envelope and the host cell plasma membrane fusion occurs, which results in the introduction of tegument proteins and nucleocapsid into the host cell cytoplasm.

Tegument proteins induce a decrease of host DNA synthesis, protein synthesis, ribosomal RNA synthesis and glycosylation of host proteins. The nucleocapsid is transported through the cytoplasma to a nuclear pore. At the nuclear pore the capsid degrades and viral DNA is released into the cell nucleus. The transcription of viral DNA takes place in the nucleus. Mature nucleocapsids bud through the nuclear membrane and acquire their tegument and envelope. By electron microscopy the virus envelope shows a typical trilaminar appearance and it seems to be derived from patches of altered nuclear membranes, modified by insertion of virus glycoprotein spikes. The virion egresses from the infected cell by transit through the cisternae of the rough endoplasmatic reticulum of the Golgi apparatus and via cytoplasmatic transport vesicles. Viral replication finally results in the destruction of the host cell.[16, 78]

In cells with active herpesvirus replication typical intranuclear inclusions are formed, representing crystalline arrays of virus particles. These Lipschütz (Cowdry type A) intranuclear inclusion bodies are characteristic for the cell pathology all herpesvirus infections, but in histopathological examination they cannot be used to differentiate the human herpesviruses from each other. Herpesviruses induce fusion of the cells they have infected. This will generate multinucleated giant cells (polykaryocytes) that can be demonstrated in affected tissues by direct light microscopic examination.[16, 78]

Alphaherpesvirinae can establish latent infection. The site of virus latency is related to the site of primary infection. During the primary infection epithelial receptors of local sensory nerves in the affected skin or mucous membranes come into contact with the virus, which penetrates the nerve and moves by axoplasmatic flow within the nerve to the perikaryon. The pathogenetic mechanism in herpesvirus latency, and the mechanism of reactivation of latent virus in recurrent infection, has not been solved yet. The virus egress from the latently infected sensory nerve and infects adjacent epithelial cells causing recurrent infection.[16, 78]

At present it is not yet clear that a recurrent human cytomegalovirus infection is caused by reactivation of latent virus, or by reinfection.[46]

The Epstein-Barr virus probably induces latently infected long-living memory B-lymphocytes.[71, 79].

1.4 Methodology for diagnosing herpetic infections

Cleator stated that "To achieve meaningful diagnosis a close collaboration between clinic and laboratory is necessary".[16]

As with all viruses, there are two potential strategies for providing a laboratory diagnosis: detection of virus or the demonstration of a specific immune response. The aim of this chapter is to provide a general overview rather than a complete, indepth analysis of diagnostic methods. Each test has advantages and disadvantages.

Diagnosis of HSV infection[16, 80-87]

The diagnosis of mucocutaneous manifestations of HSV infection is predominantly clinical. Laboratory diagnosis may be required to confirm the clinical diagnosis, to look for asymptomatic viral shedding, and to demonstrate HSV infection in HSV-atypical lesions in the immunocompromised patient.
In HSV and VZV infection, the virus-infected cells are located in the base of the herpes lesions and the virus is released into the vesicle. The roof of the vesicle will not contain virus, and, therefore, this material is not suitable as specimen for herpes diagnosis. The appropriate specimen should be taken from the vesicular fluid and the infected cells from the base of the lesion. Virologic methods for HSV diagnosis include direct demonstration techniques (light and electron microscopy), detection of viral antigens in specimens by immunoassays (immunofluorescence cytology, immunosorbent immunoassays), virus culture, detection of viral DNA (hybridization techniques, polymerase chain reaction) and serology. These methods are summarazid below.

Direct demonstrating techniques

Light microscopy

Source: cells derived from mucocutaneous papulovesicular, vesicular, bullous, pustular, and ulcerous lesions, scraped from the base of the lesion (*Tzanck smear*); tissue biopsy and necropsy specimens.
Detection: multinucleated giant cells; intranuclear inclusion bodies.
Sensitivity: acceptable only with typical herpetiform lesions, high sensitivity in vesicular stage (*subject of study in this thesis*).
Advantage: rapid assay (minutes), easy to perform, inexpensive and suitable for office diagnosis.
Disadvantage: cannot differentate between HSV-1, HSV-2 and VZV; inadequate sensitivity for cervical lesions and asymptomatic shedding.

Electron microscopy

Source: vesicle fluid; cells derived from papulovesicular, vesicular, bullous, pustular, and ulcerous lesions, scraped from the base of the lesion; crusted lesions; biopsy and necropsy specimens; serum (*detection of serum HSV antibodies, subject of study in this thesis*).
Detection: herpesvirus particles, and herpesvirus induced antibodies.
Sensitivity: relatively insensitive assay (a specimen must contain at least 106 particles Pro millilitre); sensitivity increases by ultracentrifugation methods and immunological capture assays; acceptable only with typical herpetiform lesions; highest sensitivity in the vesicular stage.
(*Subject of study in this thesis*).
Advantage: all type of lesions can be used for virus detection (subject of study in this thesis); rapid with standard negative staining technique (within an hour of receipt of the specimen in the laboratory), and several hours with immune

EM techniques to type HSV and VZV virus particles; serology by EM can be combined with virus detection in the same EM session (*subject of study in this thesis*).
Disadvantage: not widely available; laborious; expensive.

Immunoassays

Immunofluorescence microscopy
Source: cells derived from papulovesicular, vesicular, bullous, pustular, and ulcerous lesions, scraped from the base of the lesion; tissue biopsy and necropsy specimens.
Detection: viral antigens.
Sensitivity: sensitive assay, comparative with virus culture.
Advantage: rapid results (few hours), specific detection of HSV, low cost.
Disadvantage: less sensitive for cervical lesions and asymptomatic shedding.

Immunosorbent immunoassays
Source: herpes simplexvirus-infected cells.
Detetction: viral antigens.
Sensitivity: sensitive assay; radio- and enzyme-immunoassays achieve not the same sensitivity as virus culture for infectious virus.
Advantage: rapid in comparison with virus culture; can detect non-infectious virus, and viral antigens.
Disadvantage: use of radioisotopes in radio-immunoassays.

Virus culture

Source: vesicle fluid, swabs or scrapings from the base of the lesion, saliva, urine, cerebro-spinal fluid (CFS), tissue biopsy and necropsy specimens. Sensitivity of virus culture depends upon the preservation of virus infectivity within the clinical specimen. To reduce loss of infectivity during transportion to the laboratory a suitable transport medium is needed. If transportation takes more time and/or tempory storage is needed, the material should be maintained at +4°C; in case of prolonged/final storage immersion in liquid nitrogen (at -196°C), or, when not possible, at least kept at -70°C. CFS, biopsy and necropsy specimens should be transported in dry sterile containers and maintained at +4°C.
Detection: infectious viruses.
Sensitivity: high sensitivity, with a theoretical detection limit of one infectious particle (golden standard), but not as sensitive as was believed on theoretical grounds; sensitivity can be increased by centrifugational methods.
Advantage: allows virus typing and drug sensitivity testing.
Disadvantage: virus is rarely isolated from crusted lesions.

Viral nucleic acid detection

Hybridization techniques

Source: biopsy or necropsy material (in situ hybridization); clinical samples other than tissue sections (dot-blot hybridization).
Detection: viral DNA.
Sensititivty: sensitive assay.
Advantage: rapid compared to virus culture.
Disadvantage: expensive.

Polymerase chain reaction
(PCR subject of study in this thesis)

Source: all clinical samples.
Detection: viral DNA
Sensititivty: highly sensitive detection.
Advantage: rapid compared to virus culture
Disadvantage: false positive reactions by external contaminant DNA; fals negative results in cerobrospinal fluid by contamination of hemoglobin or the presence of inhibiting factors; expensive (special laboratory, logistics, specialists).

Serology

There is no standard serological technique for HSV diagnosis. Commonly used techniques are ELISA (enzyme-linked immunosorbent assay), CFT (complement fixation test), Western-blot and immunodot techniques.

Source: serum (to demonstrate intrathecal synthesis of HSV antibodies in case of central-nervous-system infection HSV IgG antibody and albumin in serum are compared with the titers in the cerebrospinal fluid).
Detection: HSV-specific IgA, IgG, and IgM class antibodies.
Sensitivity: high, except in recent infection; depends largely on the applied method (CF is relatively insensitive).
Advantage: useful in epidemiological seroprevalence studies; investigation of paired sera demonstrates recent acquired infection. (EM serological diagnosis is subject of study in this thesis)
Disadvantage: IgM antibody response does not allow reliable differentiation of primary infection and virus reactivation, except for neonatal HSV infection.

Diagnosis of VZV infection[27, 28, 83-88]

Varicella and herpes zoster are usually diagnosed from the clinical picture. Laboratory diagnosis is generally required when there is doubt about the clinical diagnosis, especially important in case of atypical lesions in immunocompromised patients. Confirmation of VZV infection is also needed for the treatment of patients at risk of contracting the severe forms of VZV infection, and for treatment in pregnancy.
Virological methods for VZV diagnosis are about the same as for HSV: demonstration of specific cytological changes in herpesvirus-infected cells, detection of viral proteins in specimens using immunologic reagents, virus culture, detection of viral DNA (e.g. by dot–blot, in situ hybridization, and polymerase chain reaction), demonstration of herpesvirus particles by EM and virus typing combined with immunological techniques, and serological diagnosis (immuno–EM is subject of study in this thesis).
Virus culture of VZV is less sensitive than HSV virus culture, mainly due to temperature inactivation of VZV during storage and transportation to the laboratory. Samples not inoculated shortly after sampling will not yield good growth of virus. The VZV particle is degradated by prolonged storage at temperatures of -10°C or above.[89] If transportation and/or storage takes more than a few hours, the specimen should be kept on dry ice, or immersed in liquid nitrogen (or, when not possible, at least kept at -70°C). To obtain optimal results from virus culture, the specimen should be inoculated as soon as possible.
VZV grows slowly compared to HSV. The average time for VZV isolation from clinical specimens is 7 days. Therefore, VZV culture is not suitable for rapid confirmation of clinical diagnosis. Before cytopathic effect becomes apparent, it is possible to detect VZV viral antigens with monoclonal antibodies. This can reduce the time to diagnose VZV infection considerably. Positive HSV and VZV cultures differ in growth characteristics, cell line sensitivity and the virus specific cytopathological effect. For additional prove monoclonal antibodies are now commonly used.

Diagnosis of human cytomegalovirus infection[46, 47]

Congenital or neonatal CMV infection must be differentiated from toxoplasmosis, rubella, herpes simplex and varicela-zoster virus infection. CMV infection in the immunocompromised host is severe and frequently life-threatening. To reduce the incidence of CMV infection following organ transplantation, the donor and recipient must be screened for their CMV status.

The following laboratory tests can been used for detection CMV infection:

Direct demonstration techniques

Light microscopy

Source: tissue specimens.
Detection: swollen CMV infected cells have large nuclei containing intranuclear inclusions wit the characteristic 'owl's eye' aspect.
Sensitivity: not sensitive.
Advantage: assessment of CMV infection in diseased organs (detection of viraemia cannot prove this!).

Electron microscopy

Source: urine (samples from congenitally infected infants ususally contain high titers of CMV, not detected in urine samples from adults).
Detection: herpesvirus particles.
Sensitivity: sensitive in case of infected infants.
Advantage: rapid assay (which only takes a few hours). Specific in case of infected infants (no other herpesvirus causes such high titers in urine from infants).
Disadvantage: genital herpes, frequently seen in patients with AIDS, can cause viral shedding in urine. With negative staining no discrimination is possible between the human herpesviruses; immune EM can differentiate between HSV and CMV virus.

Immunoassays

Source: tissue specimens, bronchoalveolar lavage fluid.
Detection: CMV infected cells; early viral antigens by leucocyte antigen detection.
Sensitivity: high.
Advantage: rapid assay.

Virus culture

Source: urine, saliva, unseperated heparinized blood, tissue specimens. Samples should be send fresh to the laboratory as soon as possible (or on wet ice without freezing , if a delay of more than a few hours is expected).
Detection: infectious cytomegalovirus; rapid diagnosis by detection of early antigen fluorescent foci (DEAFF).
Sensitivity: high.

Viral nucleic acid detection

Polymerase chain reaction

Source: all clinical samples.
Detection: viral DNA
Sensititivty: very sensitive.
Advantage: rapid assay compared to virus culture.
Disadvantage: detection of clinically not relevant low virus quantities and latent virus(without prognostic value). False negative results by interference of unknown tissue factors can occur.

Serology

Many laboratory techniques have been described for the detection of CMV induced antibodies.

Source: serum.
Detection: CMV specific IgG and IgM class antibodies. IgM antibodies cannot be found in immunocompetent patients with a recurrent infection. Renal transplant Patients can produce IgM antibodies during recurrent infection.
Immunocompromised patients with a primary infection sometimes fail to produce specific IgM antibodies. Seroconversion of specific IgG antibodies points to an acute infection. Rising titers of IgG antibody without seroconversion may be caused by a primary or recurrent infection. Increase of both CMV specific IgG and IgM antibodies can occur in bone marrow transplant recipients, but this not necessarily means that the patient will get the disease.
Sensitivity: depends on the method in use.
Disadvantage: the rheumatoid factor interferes with detection of CMV-specific IgM antibodies.

Diagnosis of epstein-barr virus infection[71-74]

The diagnosis of infectious mononucleosis (IM) is based on the clinical picture, supported by the presence of 'atypical' blood lymphocytes, and confirmed by serology. The heterophil antibody test (e.g. Paul–Bunnel test) is the test of choice for diagnosis of IM and detects an antibody which causes haemagglutination of non-human erythrocytes. Specific EBV antibodies to viral capid antigens (IgM VCA and IgG VCA) and Epstein-Barr nuclear antigens (EBNA) can be detected, and, except for IgM VCA, persist lifelong. Early antigen antibodies to EBV (Anti–D and Anti–R) persist from several months up to several years. Specific antibodies to soluble complement–fixing antigens (Anti–S) and neutralizing antibodies also persist lifelong. Determination of EBNA and Anti–S antibodies may be helpful in diagnsosis in cases with a negative heterophil antibody test.

Diagnosis of human herpesvirus type 6 infection[11, 90]

The diagnosis of exanthema subitum is primarily clinical. HHV-6 can be identified by virus culture, viral antigen detection, and in the convalescent phase of exanthema subitum by antibody detection. Virus isolation is attempted from peripheral blood of patients with manifest exanthema subitum, and from blood of severly immunocompromised immunocompromised patients.

Diagnosis of human herpesvirus type 7 infection[10, 90, 91]

Virus isolation, serology, PCR, and electron microscopy have been used to detect HHV–7 infection. The role of HHV–7 in human disease is still unclear. However, some investigators claim the etiologic role of HHV-7 in pityriasis rosea.[67-69]

Diagnosis of human herpesvirus type 8 infection

Diagnosis of HHV-8 related diseases has not yet passed the research state.[13]

1.5 Aim and outline of this thesis

The diagnosis of varicella, herpes zoster and herpes simplex can be made on the basis of physical examination and clinical history. Because vesiculobullous eruptions of other viral and nonviral skin infections can resemble those of HSV and VZV, the infection can be misdiagnosed, if not confirmed by laboratory tests. This can be a hazardous situation for some group of patients. A rapid conclusive diagnosis is very important for adequate antiviral therapy. Delay in treatment will diminish the effectiveness of the antiviral drugs. In many hospitals in Holland an adequate laboratory facility for viral culture is not available. This is also the case for more sophisticated viral diagnostic techniques, like PCR. Specimens must be sent to specialized virological laboratories elsewhere, and therefore the processing of viral specimens will be delayed. Especially VZV can loose infectivity very reapidly when kept in transport medium for longer periods. Even under optimal laboratory conditions, VZV isolation is substantially less sensitive than those for HSV.
The principal aim of this thesis is to inform the dermato-venereologist on expectations and pitfals of diagnostic methods available for human alphaherpesviruses. This was done by applying old and newly developed methods on well described clinical samples (*Chapter 2, 3, and 4*). This thesis also aims to get more insight into the virus induced immuneresponses taken place at the level of the skin, and how these processes and serum antibody responses cohere (*Chapter 5*).

Chapter 2.1 describes a rapid diagnostic test (Tzanck test) to exclude herpesvirus infections in vesicles, blisters and pustules, especially useful for office diagnosis. The diagnostic value of this test in herpetic and non-herpetic vesicular and bullous skin disorders in pediatric practice is evaluated in *chapter 2.2* . The value of the Tzanck smear in comparison with virus culture in diagnosis of anogenital lesions suspected of HSV infection is described in *Chapter 2.3*.
Chapter 2.4 emphazises the need for investigating every neonate with pustules to exclude herpetic infections. A systematic diagnostic approach of pustular eruptions in the neonate is proposed.
Electron microscopy can be applied as a rapid method for virus diagnosis, and can be used in validation studies. *Chapter 3.1* reports on colloidal gold immunoelectron microscopy for rapid diagnosis of VZV infections by discrimination between VZV, HSV-1 and HSV-2. *Chapter 3.2* describes improved methods for detection of HSV by electron microscopy in clinical specimens. *Chapter 3.3* outlines the sensitivity and specificity of the Tzanck preparation in comparison with viral culture and electron microscopy. *Chapter 3.4* reports on a case of human T-cell lymphotropic virus type 1-positive leukemia complicated by atypical multidermatomal herpes zoster. Where standard tests failed, cytodiagnosis (Tzanck test), and immuno-electron microscopy unmistakably have proven VZV infection. As *addendum* to chapter 3 we describe an easy method of storage of EM-grids, applied to this study.
The continuous need for more specific and sensitive diagnostic methods lead to the introduction of polymerase chain reaction (PCR) in HSV and VZV diagnosis. In *chapter 4* PCR for VZV, based on degenerative primers, was used to diagnose clinical specimens. Because degenerated primers were used in PCR, glycoprotein B DNA could be amplified

from all alphaherpesvirus field strains, present in clinical samples. The amplification of glycoprotein B allowed virus typing of VZV, HSV–1 and HSV–2 using restriction enzyme digestion on the PCR-products.

Human immunologic responses to HSV and VZV infections comprise humoral and cell mediated immunity, together with other nonspecific host responses.VZV immune serum globulin prophylaxis, still applied by high-risk patients, reduces the attack rate and the severity of primary VZV infection. How this is reached is still not clear. Anti-VZV immunoglobulins (VZIG) play a role in the lysis of infected cells by antibody-mediated cellular cytotoxicity. It is still not known whether VZIG can abort viral replication at the initial sites of infection (i.e. the regional lymph nodes) during a primary VZV infection, can protect against further spread of the infection via the blood (viraemia), or is effective at both levels of infection. Serologic procedures, although giving an incomplete picture of immunity to VZV, are used to assess the immune status of the patient. However, serological cross-reactivity between HSV and VZV may cause a false positive outcome of serological test. *Chapter 5* describes studies on serologic cross reactions between VZV and HSV. The results of serology by IEM are compared with those obtained by techniques employing fluorescent antibody to membrane antigen (FAMA), carried out on infected cell monolayers. The influx of T- and B-lymphocytes, and the presence of complement factors in herpetic skin lesions, studied with immunohistology, are compared with the presence of virus and the appearance of virus-immune complexes *in vivo* in relation to anti-VZV serum antibody titers. Activation of immune effector mechanisms against VZV and differences in the immune response in varicella versus herpes zoster are discussed.

In *chapter 6*, the findings of this thesis are summarized and discussed.

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CHAPTER 2

Tzanck smear and viral culture in diagnosis of herpes simplex virus and varicella-zoster virus infection

Summary

Herpes simplex virus and Varicella-zoster virus infections usually present a characteristic clinical picture. Tzanck smear and viral culture are just two of several laboratory tests to confirm the clinical diagnosis. The Tzanck smear especially is usefull for office diagnosis in dermatovenereological practice. The results of our investigations on this subject are described is this chapter. In clinical diagnostic work, sensitivity and specificity are key assay features. A systematic diagnostic approach of pustular eruptions in the neonate is proposed.

2.1 A rapid diagnostic test (Tzanck test) to exclude herpesvirus infections in vesicles, blisters and pustules

In 1948 Tzanck described a microscopic test for the identification of skin disorders. He used skin scrapings of the affected skin.[1] Over the years several modifications of the Tzanck test have been described, which allow cytologic differentiation between a herpetic infection and other skin disorders which involve vesicles, blisters and pustules.[2,3] When there is suspicion of herpetic infections, for example neonatal herpes, eczema herpeticum or generalized herpes zoster, a rapid diagnosis can be of utmost importance to differentiate between bacterial and yeast infections, especially in newborns, in immunocompromised or immunosuppressed patients, or in patients with severe malignancies.
Referring to a few case histories of children the speed, efficacy and simplicity of this method will be demonstrated. It can be stated that, in a large number of cases, not performing the Tzanck test can be considered a grave omission.

Folkers E [1], Oranje AP [2]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[2] Department of Dermatology and Venereology, Erasmus University, Rotterdam, The Netherlands
(Published in: Ned Tijdschr Geneesk 1985; 129: 241-243. This article was originally written in Dutch.)

Case A was born at night after a normal pregnancy. Skin lesions were noticed at birth, consisting of erythema, vesicles, pustules and extensive scaling. A herpes infection was suspected and aciclovir medication was given intravenously. The next morning doubt rose about the diagnosis that was made. Skin scrapings for cytologic examination and skin cultures were taken. The Tzanck test proved negative, which means that no abnormal epidermal cells were observed. Spores and pseudomycelia were seen in the Gram-preparation, which led to the clinical diagnosis of candidiasis. The aciclovir medication was stopped and local antimycotic treatment with miconazol cream was initiated. The culture for Candida albicans proved later to be positive, confirming the clinical diagnosis. The skin lesions healed quickly with the topical antimycotic treatment.

Case B was a 2 year old boy suffering from atopic eczema. He was admitted with a generalized vesiculo-pustular eruption and based on the clinical picture eczema herpeticum was suspected. Giant cells, characteristic for herpetic infection, were observed in the Tzanck smear. He was treated intravenously with aciclovir. The virus culture from the pustules proved later to be positive for herpes simplex virus type I. The patient continued to be extremely ill and developed a sepsis, probably of bacterial origin. Blood cultures proved positive only for *Staphylococcus epidermidis*. Supplementary treatment was given intravenously with dicloxacillin and gentamicin. His condition slowly improved; the fever subsided, but rose again sharply, attended by diarrhoea. Gastroenteritis was diagnosed and *Salmonella typhimurium* was isolated from faeces. By that time the skin lesions were healing, the vesicles were dried and no recurrence was noted.

Case C was an eight day old male baby. The first days after birth there were no medical problems; he drank good and grew at a normal rate. On the sixth day after birth he developed diarrhoea, he vomited and became pale. A sepsis was supposed, he was therefore transferred to our hospital. On the seventh day he developed several pustules with underlying redness scattered over the body; some had spontaneously disappeared leaving behind a small ulcus. On the left wing of the nose was a group of three pustules. Material for cytologic smears and viral culture was taken. The Tzanck test demonstrated beside numerous granulocytes, macrophages and a few lymphocytes, numerous epithelial cells with monstrously swollen, often multilobed nuclei, and frequently with multiple nuclei. In the Gram-preparation some Gram-positive cocs were found, but no yeast. Herpetic infection was diagnosed. The child died on the ninth day after birth. (At that time, treatment with aciclovir was not possible.) Afterwards herpes simplex virus type I was isolated from the pustules. Post mortem viral culture and immunohistochemical examination from the heart muscle and liver tissue revealed herpes simplex virus type I as well.

With these three case histories we have demonstrated the value of the Tzanck test. We consider it therefore important to discuss with you how to carry out the test.

It is very important to obtain the material for cytologic examination in the correct manner. The vesicle, blister or pustule in question must be opened using a vaccinostyle or small lancet. The content is used for cultures and microscopic examination for the presence of

bacterias, yeasts or fungi. Using the curved side of the vaccinostyle or lancet, material is obtained from the surface of the bottom of the lesion, smeared on a glass slide and air dried. The preparation is then coloured and examined under the microscope using a 25x and 40x magnification. A herpes positive preparation demonstrates a specific cytological picture: epithelial cells with monstrously swollen nucleus, often multilobated or multinucleated, surrounded by a perinuclear halo (fig.1). Cytologic differentiation between herpes simplex, herpes zoster and varicella is not possible.

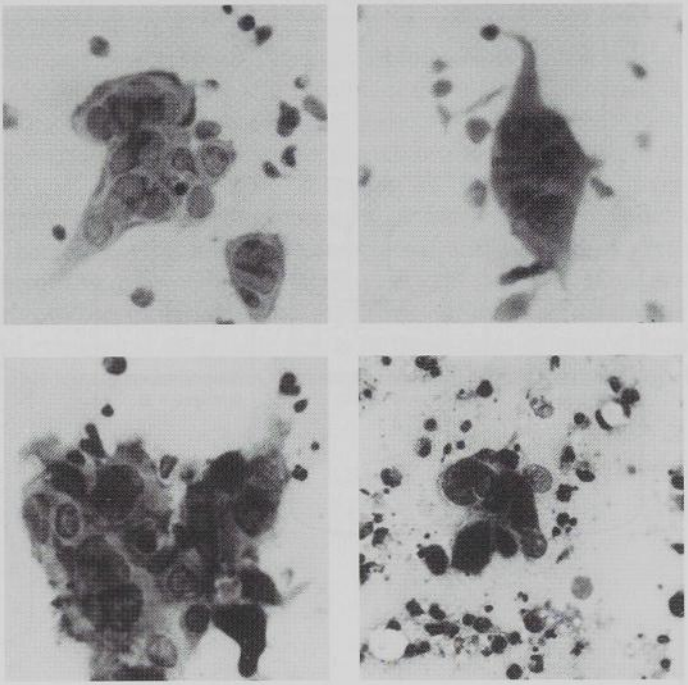

Fig. 1. Tzanck smears coloured with Hemacolor®. Epithelial cells with monstrously swollen, lobated nuclei and perinuclear halo. (magnification 400x)

Several colouring methods have been used for the Tzanck test. Most commonly used are the methods according to Giemsa, Wright, and Papanicolaou, and colouring with haematoxylin-eosin and methyleneblue. The Giemsa and Papanicolaou methods and haematoxylin-eosin colouring are very suitable for the Tzanck test, but time consuming. They are therefore less suitable for consulting-room diagnostics. The Wright and methyleneblue colouring methods consume little time, but give for the Tzanck test less clear results. The Giemsa colouring method was modified to give faster results, but the smears obtained are less preservable.
The Paragon colouring method[2] gives fast results and is therefore suitable for the Tzanck test, but is not available in our country. Therefore we have searched for another rapid

colouring method for the Tzanck. We now use a rapid haematologic colouring method (Hemacolor; fig. 2), making it possible to examine the smear within a few minutes. This method makes use of a fixing solution (methanol pro analyze), a red colouring agent (eosin solution) and a blue colouring agent (thiazine solution). Buffered, distilled water at pH 7.2 is used as a rinsing agent. Approximately half a minute is all that's needed for this colouring.

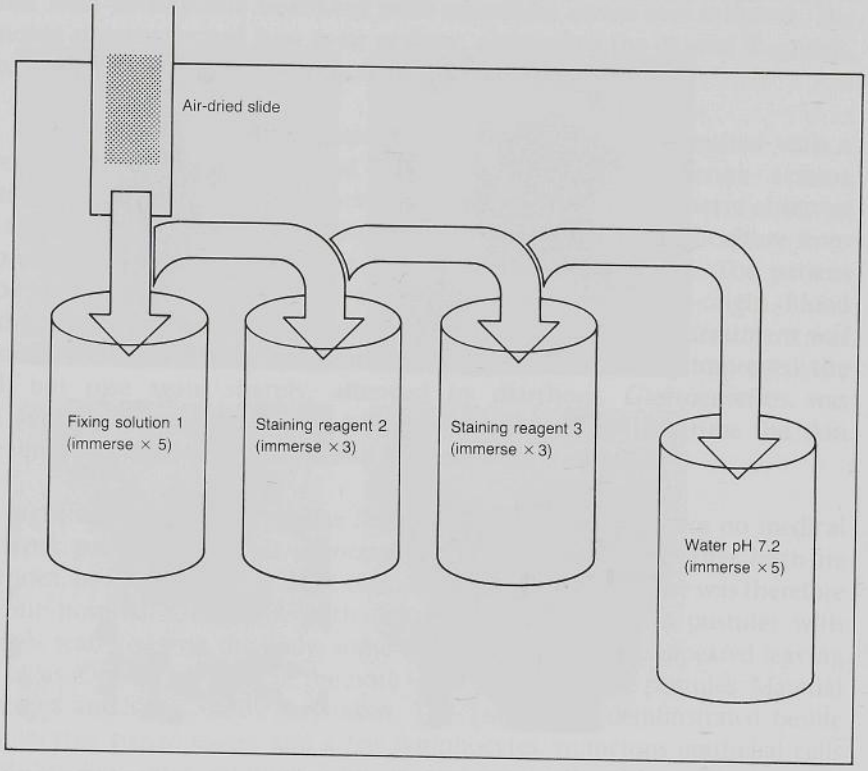

Fig.2. Hemacolor® rapid blood smear staining suitable for the Tzanck test. Immerse the air-dried smear as many times as specified for about 1 second in the 3 solutions. Drain off, rinse with buffer solution pH 7.2 and allow to dry.

If the epitheloid shapes are difficult to discern as a result of overcolouring, in most cases the swollen nuclei often with several lobes or multinucleation can still be observed with stronger light (see fig. 1). If only one smear is available as a result of scarcity of lesions, the already coloured preparation can be decoloured using hydrochlorate alcoholic solution (℞/acid. hydrochloricum 25%; alcohol ketonatus 95% ad 100 ml). The decoloured preparation can then be reused with other colouring methods.

Because of its simplicity, the Tzanck test can easily be performed in the consulting-room of the general practioners and dermatovenereologist. Its sensitivity has been found to be high enough in comparison with the sensitivity of the virus culture. The results shall depend on the experience of the investigator with cytologic examination, for example the obtaining of material, preparing the slide and interpretating it, and depend on the duration of the lesion. We have observed that in cases of herpetic infection treated with aciclovir, the Tzanck test will be positive for herpetic infection for a certain amount of time. In cases which were previously diagnosed as herpetic infection based on the clinicical picture, and later proven incorrectly diagnosed based on the Tzanck test, we deemed it responsible to stop the aciclovir medication.

For optimal diagnostic results, both cytologic examination and virus culture should be performed; false negative results are still possible if the material obtained is of insufficient quality.

Beside the afore mentioned diseases is the Tzanck test also valuable for the diagnosis of other skin disorders accompanied by acantholysis, for example discerning between pemphigus vulgaris and pemphigoid.[5] Namely in newborns with vesicles, blisters and pustules, as in bacterial, viral and yeast infections, and in case of erythema toxicum neonatorum, incontinentia pigmenti, staphylococcal scalded skin syndrome, toxic epidermal necrolysis or miliaria, is an extensive differential diagnostic examination required; a rapid cytological diagnostic test is in these cases of utmost importance.[6] This was reason enough for us to bring this, for many of you, unfamiliar diagnostic test to your attention.

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2.2 Diagnostic value of Tzanck smear in herpetic and non-herpetic vesicular and bullous skin disorders in pediatric practice

Abstract

The diagnostic value of the Tzanck smear was investigated in 76 patients of a pediatric hospital population suffering from vesicular, erosive or bullous skin disorders. Examination took place by two investigators together (AB), besides the smears were examined by two others (C and D) double blind. Sensitivity for patients with clinical herpetic infections was >80%, specificity for those without herpetic infections was >90%. These figures are higher than expected from literature. Reliability was also high: between the three investigators no significant differences were found. The Tzanck smear is simple, inexpensive, easy to perform and rapid: it does not require a specialized laboratory, but experience and correct technique of sampling is required.

Oranje AP [1], Folkers E [2], Choufoer-Habova J [3], Duivenvoorden JN [4]

[1] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University, Rotterdam, The Netherlands
[2] Department of Virology, Erasmus University, Rotterdam and Academic Hospital Rotterdam/Sophia Children's Hospital, Rotterdam,The Netherlands
[3] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[4] Department of Pathological Anatomy (Cytology), Hospital 'de Heel', Zaandam, The Netherlands

(Published in: Acta Derm Venereol (Stockh) 1986; 66: 127-133)

Introduction

In 1948 Tzanck[1] introduced a test as a diagnostic aid in order to identify vesicular, bullous and erosive dermatoses using scrapings from diseased skin lesions. During the next decades several modifications of this microscopic test, known as the Tzanck smear, have been described[2,3]. The Tzanck smear is used above all in the diagnosis of herpetic infections[4,5]. It is of value for the diagnosis of eczema herpeticum. neonatal herpes, but also for varicella or herpes zoster. It is of greatest importance in the newborn, in pregnant women and immune compromised hosts and it is also applicable in other skin diseases as pemphigus vulgaris, pemphigoid, staphylococcal scalded skin syndrome, toxic epidermal necrolysis and other vesicular, bullous and erosive skin diseases[6]. The test is simple, inexpensive, easy to perform and rapid.
In this paper we present the results of a study performed in a pediatric hospital population (including children, parents and hospital personnel) thus illustrating the sensitivity, specificity and reliability of this test.

Material and methods

Patients

From July 22, 1983 to March 31, 1985 samples were obtained from vesicular, bullous and erosive skin diseases from totally 76 patients (66 children aged 0–18 years, 3 parents and 7 hospital personnel. The children (of whom 15 infants) were hospitalized in the Sophia Children's Hospital (Rotterdam, The Netherlands) or attended the Outpatients Department of Pediatric Dermatology. The investigated population was assigned to the following groups:

Patients with herpetic infection (n=41)
- suffering from herpes simplex infection (n=25)
- suffering from herpes zoster infection (n=6)
- suffering from varicella (n= 10)

Patients without herpetic infection (n=35)
Detailed diagnoses are listed in Table I. From each patient single specimen for culture and smears were taken.

Viral cultures

From vesicular or bullous diseases a lesion was opened using a vaccinostyle, the content was taken on a swab, that was placed and shaken into 3 ml transport medium (Dulbecco's modification of Eagles medium with 10% fetal bovine serum and antibiotics). From erosive lesions a swab was taken and treated as described above. Each specimen was inoculated into tube cultures of HEL (Human Embryonal Lung) fibroblasts (0,2 ml/tube, 2 tubes/specimens) within 1/2 hour after collection. Virus isolation was attempted on these HEL cells at 37°C for maximal 2 weeks stationary and daily scored for cytopathic effect. Identification of isolated viruses was performed in immunofluorescence tests with monoclonal antisera to herpes simplex viruses and human antiserum to

varicella/zostervirus. In the case of negative results a blind passage was made for another 2 weeks.

Tzanck smear

From the base of the vesicles, bullae or erosions scrapings for the Tzanck test were smeared on a slide and air dried. After drying the material was fixed in methanol and stained within 1/2 minute with Hemacolor® (Merck). Briefly this method includes dipping 5 times in methanol, 3 times in a red fluid (eosine) and again 3 times in a blue reagent (thiazine). After this procedure the slide was washed in buffered distilled water (pH=7·2) and was ready for microscopic examination (ocular 10^x, objective 10^x and 40^x) [7].

Table I. *Clinical diagnoses in investigated population (n=76) of pediatric practice supported by viral and bacterial cultures or/and Tzanck smear*

	n	
Patients with clinical herpetic infections	41	
Neonatal herpes simplex	2	
Labial herpes simplex	8	(3 culture negative)
Cutaneous herpes simplex	11	
Eczema herpeticum	4	
Varicella	10	(4 culture negative)
Herpes zoster	6	
Patients without clinical herpetic infections	35	
Hand, foot and mouth disease	4	
Impetigo	6	
Staphylococcal scalded skin syndrome	3	
Infected eczema	4	
Toxic erythema of newborn	4	
Miliaria	4	
Other diseases	11	

Criteria for microscopic diagnosis of herpetic infection

Epidermal cells with characteristic and typical herpetic changes were scored as positive (Figs 1 and 2). These nuclear changes include enlargement, multinucleation and crowding resulting in moulding of adjacent nuclei. In the nuclei chromatinic margination beneath the nuclear membrane is typical. If the nuclei are enlarged, the content can be more coarse or show an opaque homogenization (ground-glass aspect). Also inclusion bodies, surrounded by a halo, can be visible in most or some of the nuclei.

Investigation of Tzanck Smears

Slides were examined by investigators AB (A.P.O. dermatologist and J.C. virologist) and later on double blind by investigators C (E.F. dermatologist) and D (J.N.D. cytotechnologist).

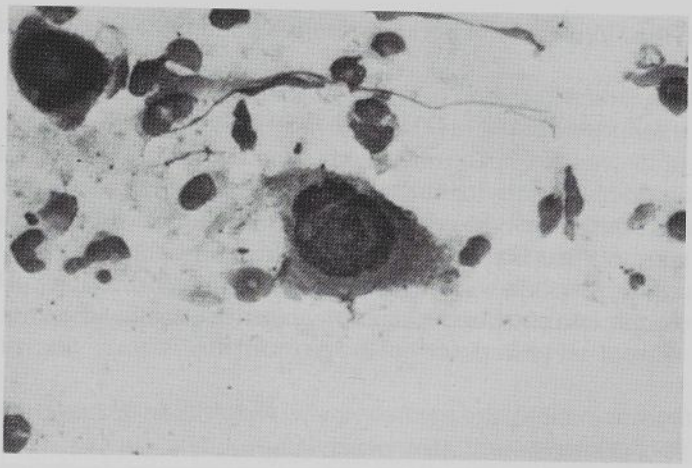

Fig. 1. Early stage of nuclear enlargement. Note coarse nuclear content (x330)

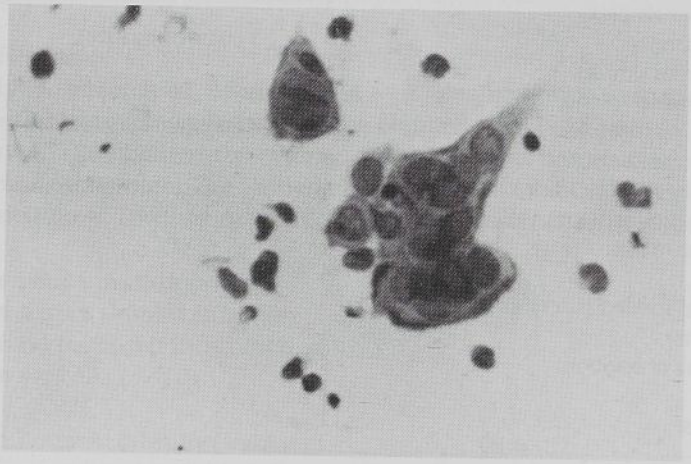

Fig. 2. Multinucleated cells typical of herpetic infections (x330)

Definitions[8]

$$\text{Sensitivity} = \frac{\text{diseased persons with a positive test}}{\text{all diseased persons tested}} \times 100\%$$

$$\text{Specificity} = \frac{\text{non-diseased persons with a negative test}}{\text{all non-diseased persons tested}} \times 100\%$$

Predictive value of a positive test = PV+:

$$\text{PV+} = \frac{\text{number of diseased persons with a positive test}}{\text{total number of persons with a positive tested}}$$

Predictive value of a negative test = PV–:

$$\text{PV–} = \frac{\text{number of non-diseased persons with a negative test}}{\text{total number of persons with a negative tested}}$$

Statistical analysis

Mc Nemar's test[9] was used to compare the percentages of positive and negative results obtained by different investigators (AB, C, D) from the same patients.

Results

Patients with herpetic infections (n=41)

Out of 25 patients with clinical herpes simplex virus infection, culture was positive for herpes simplex virus type 1 in 22 cases (sensitivity culture=88%). In three cases of recurrent labial herpes simplex the cultures were negative (Table I); two of them were in a late disease stage (crusts).

Out of 16 patients with clinical herpes zoster/varicella infections, culture was positive in 12 cases (sensitivity=75%). In four cases of varicella (two in early stage) the culture was negative. Table II_A lists the percentages of positive results by microscopic examination in patients with herpetic infections obtained by investigators AB, C and D. Based on clinical picture and culture separately sensitivity is calculated. Primary screening done by investigators AB achieved a sensitivity of 81% (clinical picture) and 86% (culture proven). Investigator C reached a sensitivity of 88% (clinical picture) and 92% (culture proven), D 83% and 86% respectively.

The differences in sensitivity obtained by the different investigators AB, C and D were not significant (Mc Nemar's test).

Summarized Tzanck smear sensitivity versus clinical picture is >80%, versus culture proven herpetic infection >85%.

Table II. Comparison of Tzanck smear sensitivity obtained by investigators AB, C and D

	Investigators: AB	C	D
A. *Patients (n=41) with herpetic infection*			
Sensitivity (%) versus clinical picture	81	88	83[A]
Sensitivity (%) versus culture proven herpetic infection	86	92	86[A]
B. *Patients (n=35) without clinical herpetic infection*			
Specificity (%)	100	97	91[A]

[A] Using McNemar's test no significant differences were obtained between the investigators ($p>0.1$)

Patients without herpetic infections (n=35)

Table II_B lists the percentages of negative results in patients without herpetic infections obtained by investigators AB, C and D. Investigators AB achieved a specificity of 100%, B 97%, and C 91% respectively.

The differences in specificity obtained by the different investigators AB, C and D were not significant. Summarized specificity is >90%.

Discrepancies between clinical diagnosis and cultures in comparison with Tzanck smears

Table III lists results of Tzanck smears in patients with clinical herpetic infection without positive cultures.

In two cases of culture-negative varicella the Tzanck smear is considered positive by all three investigators. In three cases (varicella -early stage-, herpes simplex -late stage-, and labial herpes simplex) all three investigators found no herpetic changed cells in the smears. In the two other cases one of the three investigators considered the smear as positive. An early or late stage of disease represented the majority of the cases in which a discrepancy was found.

Predictive value of Tzanck smear in investigated population

Predictive values of a positive and negative smear are calculated, when sensitivity is considered as >80% and specificity as >90%. The prevalence of herpetic infections in the investigated population, decribed in this article, is about 50%.

For this study predictive values can be calculated as followed: In this kind of population (fictive n = 1000) 500 persons will have a herpetic infection; the Tzanck smear will be positive in >400 and false negative in <100 persons. Also 500 persons will not have a herpetic infection: the Tzanck smear will be negative in >450 and false positive in <50 persons. The predictive value of a positive scored Tzanck smear:

(PV+) is >0,88 $\left(\frac{400}{400+50}\right)$ and of a negative (PV-) >0,82 $\left(\frac{400}{400+50}\right)$.

Table III. *Results of Tzanck smears in patients with clinical herpetic infection without positive cultures*

	Tzanck smear result obtained by investigators		
Diagnosis (culture negative)	AB	C	D
1 Varicella, early stage	–	–	+
2. Varicella, early stage	+	+	+
3. Varicella	–	–	–
4. Varicella	+	+	+
5. Labial Herpes simplex, late stage	–	–	–
6. Labial Herpes simplex, late stage	–	+	–
7. Labial Herpes simplex	–	–	–

– = Negative, + = positive

Discussion

Herpes simplex, herpes zoster or varicella will be diagnosed easy on clinical aspects in most of the cases. In difficult diagnostic instances confirmation by laboratory test, e.g. culture, will be necessary.

Several (most quick) tests have been developed recently. Herpes enzyme (commercially ELISA test), Micro trak (commercially immunofluorescence test) both for herpes simplex virus type 1 and 2. Monoclonal antibody assays in immunofluorescence tests are available for herpes simplex virus 1, 2 and varicella. Those tests are however expensive and need sometimes a specialized laboratory.

Direct and quick confirmation of herpetic infection, though not specific for herpes simplex type 1 or 2, or varicella, is possible by the Tzanck smear and by direct electron microscopy (negative staining). Especially the Tzanck smear, as allready stated, is simple, inexpensive, easy to perform and rapid; this test is suitable (in experienced hands) for usage in the office practices, but second screening is an important supplementary diagnostic procedure.

Solomon et al[10] studied the results of Tzanck smears and viral cultures in 30 patients (32 examinations) with clinical cutaneous herpes simplex. Cultures were positive in 78% and Tzanck smears in 53%. The sensitivity of the culture was 78% and of the Tzanck smear 53%. They concluded that the Tzanck smear looses its sensitivity as the herpetic lesions age.

Veien and Vestergaard[4] compared viral cultures. Indirect immunofluorescent staining and Tzanck smears from 32 patient with clinical cutaneous herpes simplex. The three tests were almost equally sensitive (>63%). It was of great interest to observe that the results of viral culture and Tzanck smear both were negative in herpetic infections of longer duration (about 9 days).

Our study indicates a higher sensitivity of the Tzanck smear (>80%) and specificity (>90%) than described in these previous studies[4,10]. In our studied population, the predictive values of Tzanck smear are satisfactory high, PV+ >0.88 and PV– >0.82. Besides these results were achieved after rescreening the smears twice; no significant differences between investigations AB, C and D were obtained. This indicates a high reliability of the Tzanck smear if performed by experienced specialists. Rescreening of smears is considered as an important quality control procedure in cytopathology in some or other form[12].

Next to 25 herpes simplex virus infections we also studied 16 herpes zoster/varicella cases. Almost all herpes simplex lesions were cutaneous, only 8 were located on the lips. From these labial herpes simplex infections three were culture negative (two of them were in a late stage). It is well known, that varicella cultures are prone to failure; in our material four cultures were negative of whom two were in a very early stage of disease.

The cytologic features of herpes zoster/varicella and herpes simplex are basically and morphologically the same[11]. It is not possible to distinguish different types of herpes simplex virus 1 and 2, or varicella-zoster virus infection from each other by cytopathology. Probably the lesions of herpes zoster/varicella show less cell destruction and inflammation in early stages than those of (primary) herpes simplex, although this is doubtful and needs further confirmation.

The Tzanck smear is probably more sensitive in cutaneous herpetic infections than in infections of the mucous membranes. In genital herpes Moseley[13] achieved with the Tzanck smear a sensitivity of 38%. Further study is needed to evaluate the value of this test in these types of infection. Preliminary results (Folkers et al, unpublished data) also indicate a high sensitivity and specificity in herpetic infections of the mucous membranes.

In summary our findings suggest a high sensitivity and specificity of the Tzanck smear in herpetic infections. Diagnosis of herpetic infections is not always confirmed in early and late disease stage by both culture and Tzanck smear. The Tzanck smear results, obtained by us, show a higher sensitivity and specificity than expected from literature[4,10]. Our results indicate, that the Tzanck smear is a quick and reliable test for the diagnosis of herpetic infections. It is easy to perform and does not require specialized laboratory equipments. It does however, require experience.

Acknowledgement

The authors are indebted to H.J.A. Schouten of the Department of Biostatistics for statistical advices.

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2.3 Tzanck smear in diagnosing genital herpes

Summary

In 126 patients with anogenital lesions, in which herpes simplex virus (HSV) infection was suspected or included in the differential diagnosis, the results of cytodiagnosis of herpetic infection (Tzanck smear) were compared with virus culture. Cervical lesions were excluded from this study.

HSV infection was proved by culture in 78 patients and was absent or non-active in 41 patients. Excluded from this study were seven patients who did not yield the virus on culture but had positive Tzanck smear results from three investigators. The characteristic cytopathic effect of herpetic infection was found in 56 patients who yielded HSV on culture. Tzanck smear sensitivity for skin lesions was 79% and for mucous membrane lesions was 81% in men and 52% in women. Tzanck smear specificity for the 41 patients without herpetic infection proved by virus culture was 93%. Differences in sensitivity and specificity between the results found by three investigators (double blind screening) were not significant. The Tzanck smear is reliable, inexpensive, and easy and quick to perform; it is suitable for office diagnosis because it does not require a specialised laboratory.

Folkers E [1], Oranje AP [2], Duivenvoorden JN [3], van der Veen JPW [4], Rijlaarsdam JU [4], Emsbroek JA [5]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[2] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University, Rotterdam, The Netherlands
[3] Department of Pathological Anatomy (Cytology), Hospital 'de Heel', Zaandam, The Netherlands
[4] Department of Dermatology, Free University, Amsterdam
[5] Clinic for Sexually Transmitted Diseases of the Municipal Health Service (GG&GD), Amsterdam,The Netherlands

(Published in: Genitourin Med 1988; 64: 249-254)

Introduction

Herpetic infections of the skin and adjacent mucous membranes can be diagnosed by clinical features in most cases. In patients with an inconclusive clinical diagnosis, in whom herpetic infection is suspected, confirmation by laboratory tests will be necessary.[1] Virus culture remains the standard method, but direct and quick confirmation of these herpetic infections can be obtained by the Tzanck smear.

In previous studies, we reported our experience with a cytopathological test based on the Tzanck smear in diagnosing herpetic and non-herpetic vesicular and bullous skin disorders in paediatric practice[1] and in varicella-zoster virus infections (Folkers E *et al* unpublished observation)[2]. We obtained a sensitivity of the Tzanck smear in herpetic infection of more than 80% and a specificity of at least 90%. We confirmed previous reports, that the Tzanck smear is a useful tool in diagnosing cutaneous herpetic infections.[3,4]

In this study we describe the application of the Tzanck smear for anogenital lesions suspected of being caused by herpes simplex virus (HSV) infection, and we compare the results with those of virus culture.

Patients, materials, and methods

Patients

From May 20 to December 31 1986 at one of the outpatient clinics for sexually transmitted diseases (STDs) of the Municipal Health Service in Amsterdam we collected Tzanck smears from 126 patients with anogenital lesions, in which HSV infection was suspected or included in the differential diagnosis. Lesions of the skin (n = 46) and mucous membranes (n = 80) were classified as being vesicular, pustular, or ulcerous. Cervical specimens were excluded as cervical cytology is hard for inexperienced observers to judge.

Tzanck smear

A Tzanck smear was routinely taken first, followed by a swab for virus culture. Scrapings from the edge and base of each vesicle, pustule, or erosion were smeared on to a glass slide, air dried, stained with Diff-Quik (Merz-Dade AG, 3186 Düdingen, Switzerland)[5] according to the manufacturer's instructions, after which the smear was examined by light microscopy (ocular 10^x, objectives 10^x, 25^x, and 40^x magnification).

All Tzanck preparations were examined first by one investigator (EF, dermatovenereologist) and then double blind by two others (APO, dermatovenereologist, and JND, cytotechnologist).

Criteria for microscopic diagnosis of herpetic infection

Epithelial cells showing characteristic and typical herpetic changes were classified as positive. These changes include enlargement, multinucleation and crowding of the nuclei with nuclear "moulding", different stages of peripheral margination of the nuclear chromatin, and alteration of the ground substance, which can be more coarse or have an opaque ("ground glass") appearance. As well as the nuclear changes described above, mononucleate, nongiant cell virocytes can also be seen (figs 1-4). Sometimes intranuclear inclusions surrounded by a prominent halo are recognisable.[6,7]

Virus culture

After a sample had been taken for Tzanck preparation, the base and edge of each lesion were swabbed vigorously, and the specimen was transferred immediately into a viral transport medium. Virus culture and HSV subtyping were carried out according to standard virological procedures for HSV diagnosis.

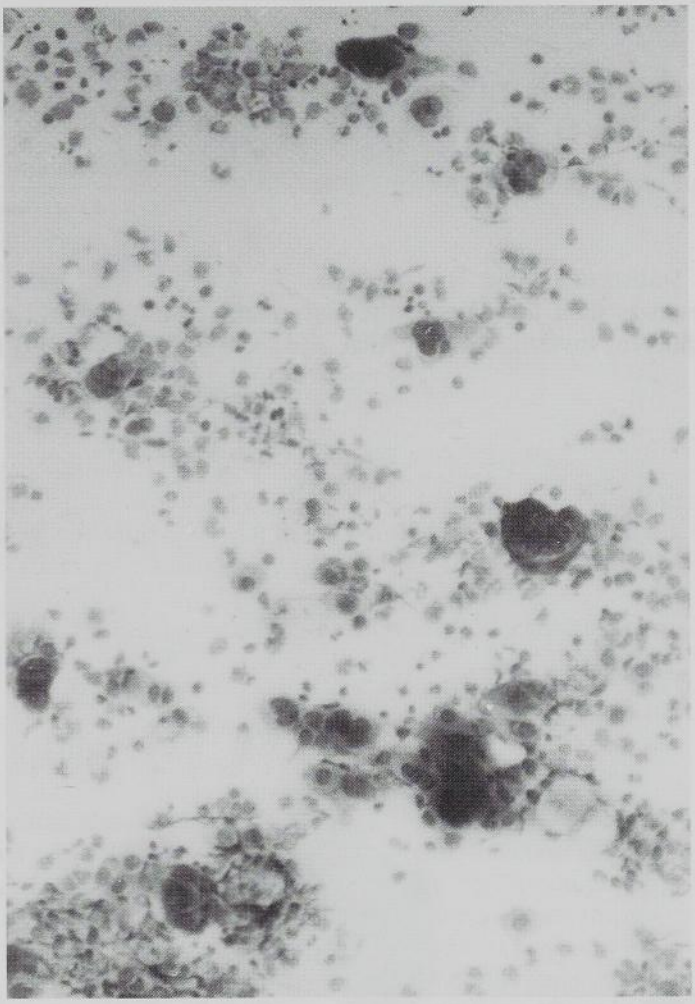

Fig.1. Tzanck smear of genital mucosal ulcer showing multiple different multinucleated epithelial giant cells, abundant erythrocytes, and sporadic leucocytes (Diff-Quik stain)

Definitions

Definitions of sensitivity, specificity, and the predictive value of a positive or negative result of Tzanck smear. were as described previously.1

$$\text{Sensitivity} = \frac{\text{No culture and Tzanck test positive}}{\text{No culture positive people tested}} \times 100\%$$

$$\text{Specificity} = \frac{\text{No culture and Tzanck test negative}}{\text{No culture negtive people tested}} \times 100\%$$

$$\text{Predictive value of a positive test (PV +):} \quad PV+ = \frac{\text{No culture and Tzanck test positive}}{\text{No Tzanck test positive}}$$

$$\text{Predictive value of a negative test (PV –):} \quad PV- = \frac{\text{No culture and Tzanck test negative}}{\text{No Tzanck test negative}}$$

Statistical analysis

McNemar's test was used to compare the percentages of positive and negative results obtained by different investigators (EF, APO, and JND) of Tzanck smears from the same patients.[8]

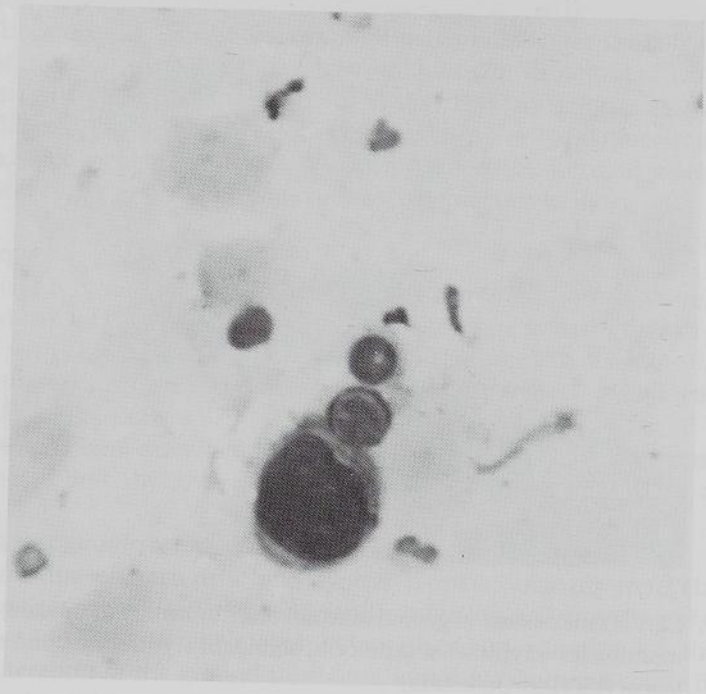

Fig.2. Mononucleated epithelial giant cell (Diff-Duik stain)

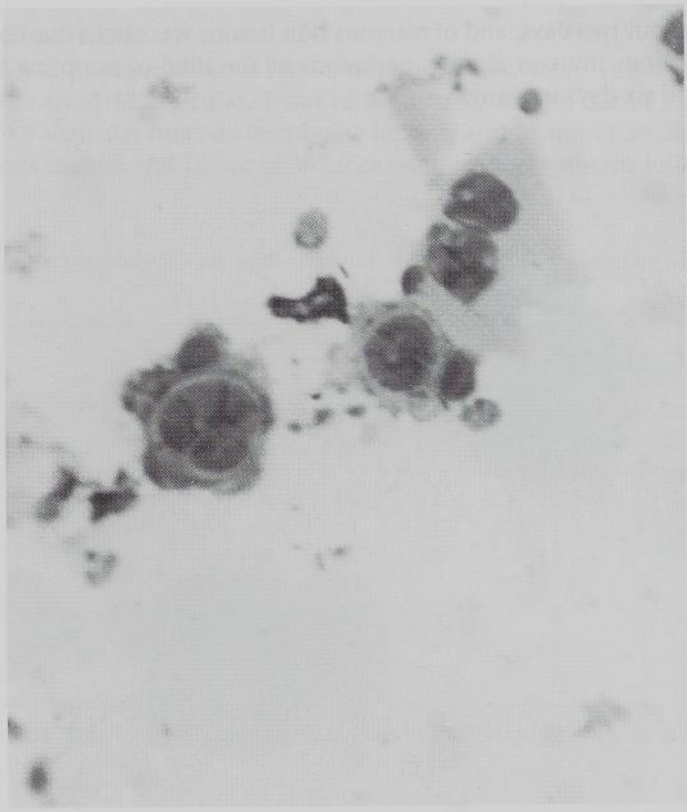

Fig.3. Epithelial giant cell with multinucleation (Diff-Quik stain)

Results

Clinical features

Of 126 patients with clinically overt genital herpetic infection or genital herpes in the differential diagnosis, 78 yielded the virus on culture. HSV could not be cultured from 48 specimens: in two men syphilis was diagnosed, in seven patients the diagnosis of herpetic infection was maintained because of positive Tzanck smear results by all investigators, and in 39 patients lesions were finally classified as "ulcers of unknown cause".

The 46 skin lesions were found on the penis (30), scrotum (1), vulva (1), perineum (2), anorectal region (5), buttocks (2), pubic region (3), and groins (2). In men, Tzanck smears were taken from mucous membrane lesions on the glans penis, coronal sulcus, or subpreputial sac (35), and from the anorectal mucous membrane (6). In women, Tzanck smears were taken from mucous membrane lesions in the vestibule or vagina (39). More lesions were ulcerous (99) than vesicular (25) or pustular (2). The average duration of the vesicular lesions of the skin and mucous membranes of men and women at the time of

sampling was about two days, and of ulcerous skin lesions was about five days; the average duration of ulcerous mucous membrane lesions at the time of sampling was about four days in men and six days in women.

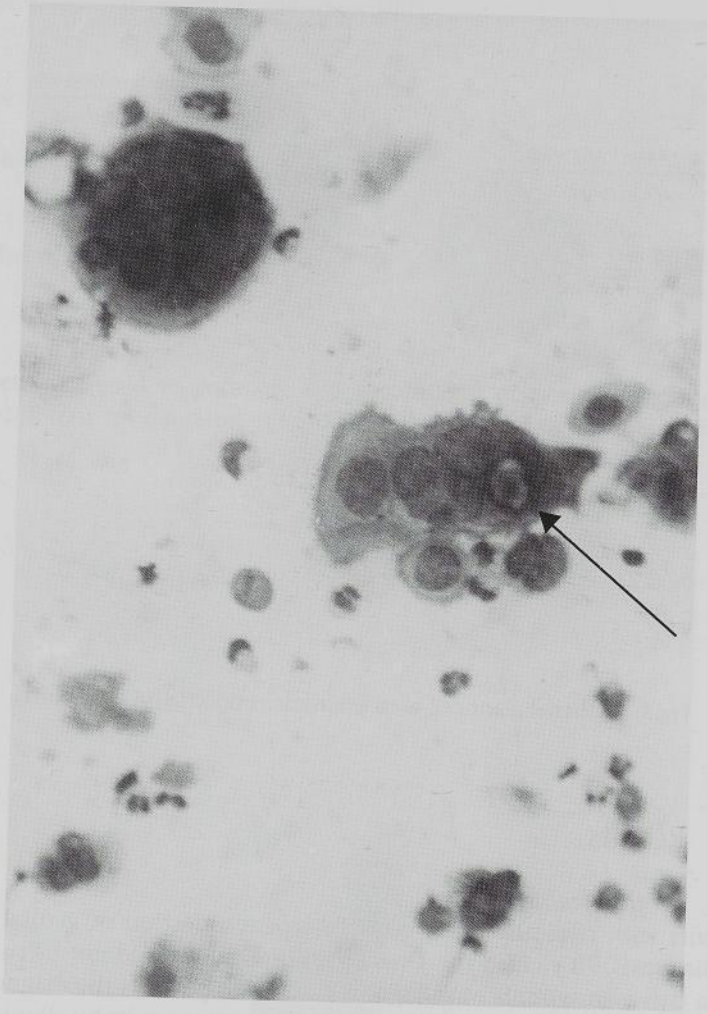

Fig.4. Multinucleated epithelial giant cells, one with an inclusion body (arrowed) (Diff-Quik stain)

No laboratory investigations were undertaken to classify the basic type of the herpetic episodes in the population investigated. Based on each patient's history, 14 were experiencing an initial episode and 28 a recurrence of genital herpes. Insufficient data were available to classify the basic type of herpetic episode experienced by the remaining 84 patients (including 41 finally regarded as having no or non-active herpetic infection).

Virus culture

HSV 2 serotype was found in 76 and HSV 1 in two isolates. Table 1 shows that HSV was isolated from 12 out of 13 vesicular, 1 out of 2 pustular, and 16 out of 26 ulcerous skin lesions; 8 out of 9 vesicular mucous membrane lesions; and 25 out of 39 ulcerous mucous membrane lesions in men and 16 out of 30 ulcerous mucous membrane lesions in women.

Table 1. *Tzanck smear sensitivity in 119* patients with culture proved genital infection with herpes simplex virus (HSV)*

Location lesions	Sex of patients	Stage of lesions	No	HSV positive by: Culture (n=78)	Tzanck smear (n=56)**	Sensitivity of %
Skin	Men and women	Vesicular	13	12	10	75
		Ulcerous	2	1	1	80
		Ulcerous	26	16	12	44
Mucous membrane	Men and women	Vesicular	9	8	6	75
	Men	Ulcerous	39	25	20	80
	Women	Ulcerous	30	16	7	44

* Excluding seven patients not yielding virus on culture but with unanimously positive Tzanck smear results.

** Results obtained by APO.

Tzanck smear

The most important factors in obtaining a positive Tzanck smear were the stage of lesion development at the time of sampling and whether it was located on skin or mucous membrane. These data are also listed in table 1.

Tzanck smear sensitivity was measured against positive HSV culture results, and was 72% with APO, 77% with EF, and 81% with JND (data not given). Using McNemar's test these differences were not significant ($p>0.1$). Tzanck smear sensitivity for all skin lesions (in men and women together) that yielded HSV on culture was 79% (23/29). Mucous membrane lesions of men (one vesicular, 25 ulcerous) and women (seven vesicular, 16 ulcerous) yielding HSV showed Tzanck smear sensitivities of 81% (21/26) in men and 52% (12/23) in women.

Table 2 shows a discrepancy between unanimously positive Tzanck smear results and negative virus culture in seven patients. These patients were diagnosed as having genital herpes, despite their negative culture results, but were not included in calculations of the sensitivity and specificity of the Tzanck smear.

All 41 patients with no (or late non-active) herpetic infection yielded negative results to the Tzanck smear when tested by EF and APO (specificity 100%); positive results were

obtained only by JND (cytotechnologist) in two men with penile skin ulcers and in one woman with a mucous membrane ulcer (combined specificity 93% (38/41)).

Table 2. *Discrepancies between Tzanck smear results in 10 patients with genital lesions in which genital herpes was suspected or included in differential diagnosis but yielding negative culture results*

Location	Stage of lesion	Duration of clinical symptoms (days)	Tzanck smear by:		
			EF	APO	JND
Penile skin	Vesicular	2	+	+	+
Penile skin	Vesicular	?	+	+	+
Penile skin	Ulcerous	5	+	+	+
Penile skin	Ulcerous	1	+	+	+
Penile skin	Ulcerous	1	+	+	+
Penile skin	Ulcerous	?	–	–	+
Penile skin	Ulcerous	?	–	–	+
Penile mucous membrane	Vesicular	1	+	+	+
Vulval mucous membrane	Ulcerous	2	+	+	+
Vulval mucous membrane	Ulcerous	?	–	–	+

? = data not known. Results + (positive), - (negative)

Table 3 lists the Tzanck smear sensitivities and specificities and the predictive values of positive (PV+) and negative (PV–) results in active herpetic lesions at different clinical stages and locations. We calculated predictive values of positive and negative Tzanck smears for the total study group on the basis of 72% sensitivity and 93% specificity. The prevalence of active genital herpes (proved by virus culture) in the study population was about 65% (78/119). The predictive value of a positive Tzanck smear (PV+) was 0·95 and of a negative Tzanck smear (PV-) was 0·64.

Table 3. *Sensitivity, specificity, and predictive values of positive (PV +) and negative (PV–) results of Tzanck smears obtained from lesions at different stages and locations in patients with herpetic infection proved by virus culture*

Location	Stage of lesion	Sex of patients	Sensitivity %	Specificity %	Prevalence %	PV+	PV–
Skin Mucous membrane	Ulcerous or pustular	Men and women	81	100	88	1	0.42
Skin	Ulcerous	Men and women	75	92†	62	0.94	0.69
Mucous membrane	Ulcerous	Men	80	92†	64	0.95	0.72
Mucous membrane	Ulcerous	Women	44	92†	53	0.86	0.59
Total			72	93	65*	0.95	0.64

* Prevalence of culture proved herpetic infection in lesions of patients suspected of having genital herpes or in whose differential diagnosis it was included.

† Summarized specificity obtained in all ulcerous lesiosn (no clear differences obtained between men and women).

Discussion

In treating genital herpes, confirmation of the clinical diagnosis of HSV infection is desirable. Acyclovir, the preferred drug for antiviral treatment, is more effective the earlier it is given.[9] As swift diagnosis can therefore lead to more effective treatment, the advantage of a rapid diagnostic test is obvious.

Several rapid direct diagnostic tests using monoclonal antibody immunofluorescence or immunoperoxidase techniques have been developed recently, but these do not yet show acceptable sensitivity and specificity compared with virus culture.[10] Culture is still the standard verification method for HSV infections, and it can confirm the clinical diagnosis within 24 hours.[10,11] It does not, however, reach 100% sensitivity even in clinically typical cases.[3] HSV culture commonly gives positive results in the vesicular and pustular stages of the infection, but its sensitivity decreases considerably when lesions are old and crusted or in the ulcerous stage.[3,12]

Direct and rapid confirmation of herpetic infection, though not specific for HSV 1, HSV 2, or varicellazoster virus, is possible with the Tzanck smear, using rapid staining techniques.[5,13] Our previous study, which was conducted in a paediatric clinic and focused on herpetic and non-herpetic vesicular and bullous skin disorders, indicated the high sensitivity (80%) and specificity (90%) of the Tzanck smear. The Tzanck smear as a diagnostic tool can reliably support a clinical diagnosis of herpetic skin infections.[1-4]

Clinicians can easily obtain experience in using the Tzanck smear effectively in office practice with the help of supplemental screening by a cytopathologist or cytotechnologist. We do not consider routine Tzanck smear examination to be suitable for office diagnosis of cervical lesions, because examining cervical smears is complex, takes time, and demands cytopathological experience. Cervical lesions were therefore excluded from this study. Nevertheless a positive Tzanck smear of cervical specimens can support the diagnosis of HSV infection.

The two most important factors in obtaining positive Tzanck smear results were the stage of the infection at the time of sampling and the location of the lesion. Moseley *et al* reported a Tzanck smear sensitivity of 38% in genital herpes.[14] Our study, however, indicated a higher sensitivity of the Tzanck smear. The Tzanck smear sensitivity in skin lesions of our patients yielding virus on culture was 79%. In vesicular lesions of skin and mucous membranes, the Tzanck smear reached a sensitivity of 81% in patients with culture proved genital herpes infection. Tzanck smear sensitivity was 80% in men and 44% in women with HSV positive ulcerous mucous membrane lesions. In the total group of patients with culture proved genital herpes the summarised Tzanck smear sensitivity compared with virus culture was 72%.

A discrepancy between a unanimously positive Tzanck smear result and negative culture results was found in seven patients. These can probably be considered as culture failures. These patients were finally assigned a clinical diagnosis of genital herpes, but they were not included in the calculations of this study. As already mentioned, the sensitivity of HSV culture is not 100%, but is presumably more than 90%.

The results showed reduced Tzanck smear sensitivities with differing sites of infection and stages of lesions. The average duration of ulcerous skin lesions was about five days and of ulcerous mucous membrane lesions about four days in men and six days in women.

Viral shedding decreases with the duration of herpetic lesions. A decrease in viral shedding from herpetic lesions of longer duration may correlate with the lower sensitivity of the Tzanck smear. The speed with which cell cultures develop a cytopathological effect (CPE) can be used to estimate roughly the amount of herpes virus antigen present in the lesions investigated. We found no appreciable difference, however, in average time to developing CPE in herpetic mucous membrane lesions between men (7·7 days) and women (7·4 days). Necrotic syncytial giant cells can be found in herpetic lesions that are more than 72 hours old. The characteristic cytomorphological features of herpetic infection are obscured by the rapid loss of nuclear details in cells infected for longer.[6] The longer average duration of ulcerous mucous membrane lesions in women in this study compared with the other herpetic lesions investigated might be a cytomorphological explanation for the lower Tzanck smear sensitivity.

In vesicular lesions, a Tzanck smear specificity of 100% was obtained by all investigators. This study showed a summarised specificity of at least 93% for the Tzanck smear in genital herpes with lesions of skin and adjacent mucous membranes. By using McNemar's test, statistical analysis showed no significant differences between the Tzanck smear sensitivity and specificity obtained by EF, APO, and JND. With the aid of sensitivity, specificity, and prevalence figures, the predictive values for positive (PV+) and negative (PV–) results were calculated. The best PV+ was attained in vesicular lesions, the best PV– in mucous membrane ulcers of men.

Positive and negative predictive values depend on the prevalence of the disease in the population investigated. With lower prevalence figures the PV+ decreases, but the PV– increases. For example, a prevalence of 50% would give a PV+ of 0·91 and a PV–of 0·77, whereas a prevalence of 10% would give a PV+ of 0·5 and a PV– of 0·97. In the population attending this STD clinic the prevalence of herpetic infections proved by virus culture of ulcerous genital lesions is about 50%.

The usefulness of a test with a given sensitivity and specificity depends on the purpose it

has to be used for. For screening in high risk populations, such as prostitutes, tests with a high sensitivity giving minimum false negative test results are desirable, in which case a high PV– will be obtained. Cytodiagnosis of herpetic infection cannot respond to criteria of a routine screening test excluding herpetic infection; the Tzanck test is therefore not suitable for antenatal screening for HSV infection. On the other hand positive Tzanck smear results are very reliable in confirming the clinical diagnosis of herpetic infection.
In cytodiagnosis PV+ and PV– values depend on the skill and experience of the observer. Application of the Tzanck smear routinely in herpetic infections has the advantage of helping observers to gain and maintain experience with cytodiagnosis, so that they can rely on observation and interpretation of the test in cases with inconclusive clinical diagnosis. A positive Tzanck smear result in patients with equivocal clinical features may even obviate the need for virus culture, thus saving cost.
Immunofluorescence cytology using monoclonal antibodies potentially increases the specificity and sensitivity of cytological preparations, but this method is time consuming and therefore not particularly suitable for office diagnosis.[4,9] Further investigation of this technique is required.
This study, conducted in an STD clinic, shows Tzanck smear results of comparable sensitivity and specificity to those of our previous investigation[1] (with the exception of the results of Tzanck smear sensitivity obtained in women with ulcerous mucous membran lesions). Our results show highly reliable positive Tzanck smear results in venereological practice. Moreover this test is inexpensive, quick, and easy to perform, which makes it suitable for office diagnosis.
In dermatological and venereological practice, positive Tzanck smear result can lead to rapid confirmation of the diagnosis of herpetic infection, prompt treatment with acyclovir, if necessary, and greater assurance of the drug's therapeutic effect. Confirmation of clinically diagnosed herpetic infection is possible with the Tzanck smear, even when virus culture fails.

Acknowledgements

We thank H.J.A. Schouten, Department of Medical Informatics and Statistics, University of Limburg, Maastricht, for statistical advice, and the staff of this clinic for their part in this investigation, particularly Sylvia Bos, Adrienne Flipse and Joyce Noordhoek Hegt-de Graaf for technical help, and Wies van Bolderik and Yvonne Creusen for supplying data on patients.

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2.4 Diagnosis and treatment of pustular disorders in the neonate

Abstract

The diagnosis of a pustular dermatosis ocurring during the first months of life is usually based on clinical findings. However, some cases may require simple investigations including microscopic examination of pustular content, cultures, and skin biopsies. The main benign transient neonatal types of pustulosis include erythema toxicum neonatorum, infantile acropustulosis, transient neonatal pustular melanosis, and neonatal acne. The most common causes of infectious pustular skin lesions include bacterial infections, which may be initially localized (*Staphylococcus aureus*) or septicemic (with *Listeria monocytogenes* as the leading causitive agent); viral infections (*herpes simplex, varicella zoster, and cytomegalovirus infections*); fungal infections (candidiasis); or parasitic disorders (scabies). The main objective of this review article is to propose a systematic approach of pustular eruptions in the neonate. The need for investigating every neonate with pustules for an infectious disease, is emphasized. The Tzanck smear, the Gram stain and a potassium hydroxide preparation are the most important quick diagnostic tests. The Tzanck smear is a very easy, rapid and sensitive test for detection of an a herpetic infection (multinucleated giant cells) as well as noninfectious pustular eruptions (eosinophils, neutrophils). Therefore, the Tzanck smear should be the first test to be performed. Moreover, a Gram stain and potassiumhydroxide preparation should be performed in cases of neonatal pustular disorders to detect bacterial and fungal infections. The goal of this diagnostic approach is to spare a healthy neonate, with a benign transient condition, an invasive evaluation for sepsis, potentially harmful antibiotic therapy, and prolonged hospitalization with its own inherent morbidity.

van Praag MCG [1], van Rooij RWG [1], Folkers E [2], Spritzer R [3], Menke HE [1], Oranje AP [4]

[1] Department of Dermatology, Sint Franciscus Hospital, Rotterdam, The Netherlands
[2] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[3] Department of Pediatrics, Sint Franciscus Hospital, Rotterdam, The Netherlands
[4] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University and University Hospital Dijkzigt/Sophia, Rotterdam, The Netherlands

(Published in: Pediatr Dermatol 1997; 14: 131-143)

Introduction

In the first 4 weeks of life (defined as the neonatal period), the infant is extremely vulnerable to bacterial, viral and fungal infections. Therefore, the presence of pustular lesions in the neonatal period evokes justifiable concern in the clinician caring for these infants. Pustular eruptions in neonates present a diagnostic challenge to the skilled dermatologist and pediatrician (Table 1). Simple diagnostic techniques can differentiate transient benign pustular eruptions from serious and life-threatening conditions that require immediate therapy. In this way a healthy neonate, with a benign transient condition, can be spared an invasive evaluation for sepsis, a potentially harmful antibiotic therapy, and prolonged hospitalization with its own inherent morbidity.We are aware of the fact, that a strict distinction between pustular disorders and vesiculobullous dermatoses in the neonate is rather artificial, because vesicles are frequently seen as precursor lesions in pustular dermatoses. However, primary vesiculobullous diseases in the neonate, such as miliaria cristallina, acrodermatitis enteropathica, epidermolysis bullosa, epidermolytic hyperkeratosis, herpes gestationis, pemphigus vulgaris, and urticaria pigmentosa, fall beyond the scope of this article.

The discussion below will provide information on history, physical and laboratory findings that help to distinguish between transient benign disorders, mild infections, and serious infectious conditions that all can occur during the neonatal period with pustular eruptions. The histopathology of the pustular dermatoses will be discussed, if relevant for establishing a diagnosis. Very rare neonatal pustular eruptions will be only briefly discussed here. Further information on issues not covered in detail here may be found in more exhaustive sources, such as Hurwitz[1] and Schachner and Hansen[2]. Information about therapeuticintervention will be included where appropriate. The main objective of this review article is to propose a practical approach of neonatal pustular dermatoses in clinical practice.

Noninfectious neonatal pustular eruptions

Erythema toxicum neonatorum

Erythema toxicum neonatorum is a benign, self-limited neonatal eruption seen in approximately one-third of all full-term newborns[3]. Black and white infants are affected equally. The condition usually appears after 24 to 72 hours of life although it has been reported at birth as well[4,5]. The etiology of erythema toxicum is unknown.

The lesions evolve from poorly defined erythematous macules to red, white, or yellow papules to a vesicular and, more rarely, pustular eruption on an erythematous base. They are asymptomatic and evanescent, and may disappear within hours after eruption. The sites of predilection are the forehead, face, chest, trunk, and extremities (Fig. 1). It is unusual to see lesions on the palms or soles.

Table 1. *Neonatal pustular eruptions*

NONINFECTIOUS

- Erythema toxicum neonatorum
- Infantile acropustulosis
- Transient neonatal pustular melanosis
- Neonatal acne
- Pustular miliaria
- Eosinophilic pustular folliculitis of infancy
- Incontinentia pigmenti
- Congenital self-healing Langerhans cell histiocytosis

INFECTIOUS

Bacterial

- Staphylococcus aureus (bullous impetigo)
- Listeria monocytogenes
- Streptococcus (group B ß-hemolytic)
- Pseudomonas aeruginosa
- Hemophilus influenzae

Viral

- Herpes virus infections
- Herpes simplex
- Varicella zoster
- Cytomegalovirus

Fungal

- Candidiasis
- Congenital
- Neonatal
- Pityrosporum folliculitis

Parasitic

- Scabies

Laboratory findings in erythema toxicum may include eosinophilia up to 18% in as many as 15% of the cases[4]. Otherwise there are no abnormal hematologic findings. Microscopic examination of pustular content is essential to confirm the diagnosis. A Tzanck smear (Giemsa, Wright, or Hemacolor® stain) or a Gram's stain of lesional content demonstrates numerous eosinophils. Histopathologic examination reveals intrafollicular, subcorneal pustules with a dense accumulations of eosinophils. Eosinophils also infiltrate the outer root sheath of the pilosebaceus unit proximal to the sebacious duct. More macular lesions may present with sparse perivascular accumulations of eosinophils in the dermis[6,7].
Erythema toxicum will resolve spontaneously without residua within days to weeks, the usual duration being less than 1 week. Therefore, the diagnosis is based on the clinical

appearance and evanescent nature of the lesions, as well as their eosinophil content. No treatment is neccessary other than reassurance to the parents.

Infantile acropustulosis

Infantile acropustulosis is a relatively uncommon disorder first reported by Kahn and Rywlin[8] and Jarratt and Ramsdell in 1979[9]. It occurs primarily in black infants and in boys[8]. Outside the United States, the preponderance in blacks has not been observed[10]. The condition may begin during the neonatal period and continue throughout infancy and early childhood[8].

Clinical manifestations are limited to the skin, and affected neonates are healthy otherwise. The appearance of lesions is accompanied by pruritus, making the neonate irritable and restless. The lesions begin as small red papules, which evolve within 24 hours into vesicles and pustules several millimeters in diameter. The pustules are intensely pruritic, last for 7 to 10 days, and appear in crops every 2 to 3 weeks. They are found on the hands and feet predominantly and occasionally on the scalp, face and trunk. Mucosal surfaces are not reported to be affected, and sites of involvement can heal with slight residual pigmentation and scaling.

Laboratory studies are usually normal, but peripheral blood eosinophilia has been reported[11]. A Tzanck smear (Giemsa, Wright, or Hemacolor® stain) or Gram's stain of pustular content reveals numerous neutrophils, occasionally eosinophils, and no bacteria[11,12]. On histopathologic examination well-circumscribed, subcorneal pustules with polymorphic neutrophils and occasional eosinophils are seen. The underlying dermis is edematous and has a perivascular, mainly lymphocytic infiltrate. No correlation can be found between blood eosinophilia, composition of cutaneous infiltrate, age of the infant, and course of eruption[10].

Therefore, diagnosis is based on distribution of lesions, their pattern of recurrence, and appropriate stains of the pustules. The etiology of infantile acropustulosis is unknown. It is a self-limited disease, with exacerbations and remissions over 2 to 3 months and afterwards complete resolution. It is frequently worse in the summer months. Topical corticosteroids may be valuable. Antihistamines may provide relief of itching in older infants but are contraindicated in neonates because of the undesirable side effect of sedation[10]. In severe cases dapsone at a dose of 1 to 2 mg/kg/day may be effective[8].

Transient neonatal pustular melanosis

Transient neonatal pustular melanosis (TNPM) was first described by Ramamurthy *et al.* in 1976[13]. This disorder is present at birth in 4% to 5% of black infants and in 0.1% to 0.3% of white infants[14]. Males and females are affected equally. The etiology of TNPM is unknown, although an increased incidence of placental squamous metaplasia has been reported in mothers whose infants develop this dermatosis[15].

Cutaneous lesions consist of vesiculopustules without surrounding erythema (Fig. 2). These are noted at birth or during the first day of life. They rupture easily, with the formation of pigmented macules that are surrounded by a characteristic collarette of scales. The macules may persist for several months but usually fade spontaneously within 3 to 4 weeks. Most commonly affected areas include the chin, neck, upper chest, lower back, buttocks, abdomen, and thighs, but all areas may be affected. Palms and soles are often

involved (Fig. 2). No systemic symptoms are associated with the lesions[16].
A Tzanck smear (Giemsa, Wright, or Hemacolor® stain) or Gram's stain of pustular content in TNPM demonstrates polymorphic neutrophils and occasional eosinophils. On histopathologic examination, intracorneal and subcorneal pustules with collections of polymorphic neutrophils and a few eosinphils are seen under a thickened stratum corneum[7,13]. The dermis is generally uninvolved although in a later stage postinflammatory changes and melanophages may be seen[17].
Thus, the diagnosis of TNPM is suggested by vesiculopustular neutrophilic lesions in association with hyperpigmented macules that are present at birth. No specific therapy is recommended for TNPM since it resolves spontaneously. Parents should be informed that the hyperpigmented macules will disappear within several weeks to months.

Neonatal acne

Mild acne is fairly common in the newborn[18]. The typical eruption consists of closed comedones on the nose, forehead, and cheeks. Open comedones, inflammatory papules and, pustules, may also occur. Although the etiology is not clearly defined, neonatal acne appears to result from stimulation of sebaceous glands by maternal and infant androgens. Lesions spontaneously resolve within 1 to 3 months as the sebaceous glands involute, and scarring is absent[18]. Most cases of neonatal acne do not require treatment. If necessary, neonates can be treated with a 2.5 % benzoyl peroxide lotion[19]. As therapy of choice, we would suggest 1 % salicylic acid, 1 % resorcin in a cream or 2% erythromycin in an alcoholic solution.

Miliaria

Miliaria is often seen in the first weeks of life. It is a manifestation of sweat retention due to occlusion of the immature eccrine sweat ducts resulting in rupture of the ducts with escape of sweat into the surrounding epidermis. There are three types of miliaria: crystallina, rubra, and profunda. These disorders may be distinguished by the level of obstruction within the ducts[20].
Miliaria crystallina is the most superficial, and is caused by subcorneal obstruction of the eccrine ducts, whereas miliaria rubra is intermediate in depth and results from intraepidermal duct obstruction, with outpouring of sweat into the lower epidermis. The lesions of miliaria crystallina consist of asymptomatic, clear, thin-walled, easily ruptured vesicles that appear in a generalized distribution, with an increase in intertriginous areas. Miliaria rubra consists of itchy, small, red papules or papulovesicles ("prickly heat"). The lesions are frequently grouped and extrafollicular in location, making the distinction between acneiform lesions possible[1]. In neonates the face, neck, and trunk are most commonly involved[21]. Miliaria rubra may progress to pustular lesions (pustular miliaria or miliaria profunda), particularly in climates with high temperature and humidity or during treatment with occlusive dressings or ointments. Miliaria profunda is rare in neonates. Microscopic examination of miliaria profunda shows a mononuclear infiltrate and eccrine obstruction at the dermo-epidermal junction, with disruption of the dermal eccrine system[7].
The diagnosis of miliaria is usually made on clinical observation. The treatment of choice is to reduce the infant's environmental temperature and clothing. Cool soaks or shake lotions are helpful. Appropriate medication should be used to treat any suspected bacterial or candidal infection superimposed on miliaria.

Eosinophilic pustular folliculitis of infancy

Eosinophilic pustular folliculitis was first described in adults by Ofuji *et al.* [22]. Additional cases has been described recently[23,24,25]. This rare disorder shows male predominance and can be present at birth[25]. It is characterized by eosinophilic infiltration of hair follicles, resulting in pruritic grouped papules and pustules. In infants, unlike adults with eosinophilic pustular folliculitis, the lesions appear in a perifollicular pattern on the scalp, hands, and feet. They occur as recurrent crops of 1 to 3 mm white to yellow pruritic pustules on a erythematous base. They can also be present on the trunk. Many lesions have secondary crusting. There are no signs of systemic disease.

A Tzanck smear (Giemsa, Wright, or Hemacolor® stain) or Gram stain of pustular content demonstrates numerous eosinophils. Some patients have eosinophilia as well as leucocytosis on blood counts obtained during outbreaks[23,26,27]. Biopsy of the pustules show eosinophils and eosinophilic spongiosis in the epidermis, with a dense dermal perifollicular infiltrate of eosinophils, histiocytes, and lymphocytes [23,26,28].

Diagnosis is based on the distribution of the pustules, appropriate stains of pustular content or biopsy revealing an eosinphilic infiltrate, and the presence of peripheral blood eosinophilia. The etiology is unknown although the possibility of an immunologic abnormality[23] or an exaggerated allergic response to insects, mites [26], or parasites[29] has been raised. More recently an association with human immunodeficiency virus has been reported[30,31]. Many other diagnoses can be considered in the differential diagnosis of eosinophilic pustulosis of the scalp, in particular Langerhans cell histiocytosis[25].

In adults, the course is variable with exacerbations and remissions. In infants the total duration varies from 3 months to 5 years[25]. Topical treatment with corticosteroids may reduce the pruritus and may attenuate the recurrences[23]. Systemic antibiotics, antihistamines, and other topical agents are ineffective. Sulfonamides, sulfones, and systemic steroids have been used in adults with severe eosinophilic pustular folliculitis with variable success[26,27,32,33]. Similar therapeutic experience is not available in infants.

Incontinentia pigmenti

Incontinentia pigmenti (Bloch-Sulzberger syndrome) is an uncommon and unusual genodermatosis that is believed to be transmitted as an X-linked dominant trait, lethal in most males; primarily female infants are affected. This disease is a multisystem syndrome having dermatologic, neurologic, skeletal, ocular, and dental manifestations. Classically, the infant manifests characteristic linear vesicular lesions that evolve into verrucous lesions within a few weeks, to be followed by a peculiar, swirled pigmentation that lasts for several weeks[34]. In the early vesicular stage, which is rarely pustular, one can find microscopically intraepidermal, spongiotic blisters containing mainly eosinophils[34-36].

Congenital self-healing Langerhans cell histiocytosis

Congenital self-healing Langerhans cell histiocytosis (CSHLCH) is rare condition initially seen at birth or in the neonatal period with generalized papules, vesicles, pustules, or nodules[37,38]. The characteristic features of CSHLCH appear to be an otherwise healthy infant with no or mild systemic symptoms, histopathology demonstrating a Langerhans cell infiltrate, and spontaneous involution of skin lesions[39]. Immunohistochemically Langerhans cells can be identified by demonstration of important markers, such as CD1a

(OKT6), and the S-100 protein, and electronmicroscopically by the presence of Birbeck granules[40]. No histopathologic features and immunohistochemical characteristics seperate definitively CSHLCH from malignant forms of Langerhans cell histiocytosis (LCH). The ultrastructural findings of CSHLCH differ only in degree from those other forms of LCH by fewer cells with Birbeck granules and more with dense bodies, multivesicular bodies, and "comma-like" or "worm-like" bodies[38,41].

At the present time, the clearing of skin lesions and failure to develop other organ involvement over the ensuing months is the only definitive way to be sure that an infant has congenital self-healing type of LCH rather than a malignant form of LCH [39]. Although CSHLCH is usually a benign, self-limited condition, careful evaluation for systemic disease must be performed. Long-term follow-up for evidence of relapse or progression of disease is essential[38,42].

Infectious neonatal pustular eruptions

Bacterial infections

Impetigo bullosa

Certain strains of *Staphylococcus aureus* (*e.g.*, phage group II, commonly lysotypes 3A, 3C, 55 or 71) have the ability to produce an exfoliative exotoxin [43,44]. When the infection and toxin remain localized, focal *S. aureus* infection results (impetigo bullosa). When the toxins enter systemic circulation, there is potential involvement of the entire cutaneous surface. This disorder is called the staphylococcal scalded skin syndrome[45].

Impetigo bullosa may appear as early as the second or third day of life. It consists of vesicles, pustules or bullae on erythematous bases. Typical bullae are flacid, filled with clear yellow or turbid fluid and rupture easily, leaving a narrow rim of scale at the edge of a shallow, moist erosion (Figure 3). These lesions reepithelialize rapidly and do not result in scars. The diaper region and intertriginous areas of the body, such as the axilla and the neck, are commonly involved[43].

Polymorphic neutrophils and gram-positive cocci in clusters are seen on a Gram's stain of lesional contents. Cultures will grow *Staphylococcus aureus*. Histopathology demonstrates subcorneal pustules with gram-positive cocci and polymorphic neutrophils.

The infection is chiefly limited to the epidermis and does not usually produce systemic manifestations. Localized infections can be treated with a topical antibiotic such as mupirocin or fucidic acid. More widespread lesions require a systemically administered antbiotic for seven days: a penicillinase-resistant penicillin (*i.e.*, flucloxacillin, cloxacillin), macrolides (i.e., erythromycin, clarithromycin), or cephalosporins (*i.e.*, cephalexin, cefprozil)[43,46]. If fever, irritability, or other signs of systemic illness are present, the neonate requires admission to the hospital for a complete evaluation for sepsis and intravenous antbiotics. The hospital at which the infant was born should be notified, as the infection may indicate nursery contamination.

Other bacterial infections

Bacterial infections can be acquired prenatally, during delivery, or after birth. Group B ß-hemolytic streptococcus, *Listeria monocytogenes*, *Hemophilus influenza*, and *Pseudomonas aeruginosa*, are bacterial pathogens that may produce pustules and sepsis in the neonate[47]. The diagnosis of these bacterial infections requires the isolation of the organism by cultures.

Neonatal *ß-hemolytic streptococcus group B* infection was uncommon, until the 1960s. For unknown reasons the incidence of the disease has increased, and ß-hemolytic streptococcus group B now being the most common agent responsible for bacteremia or meningitis in the first month of life[48]. The optimal antimicrobial therapy is benzylpenicillin.

Listeriosis is a septicemic or meningitic disease, caused by a gram-positive bacteria, *Listeria monocytogenes*. The organism is isolated from soil, animals, and transmission is mainly foodborne. Two clinical forms of neonatal listeriosis have been described, an early and a late onset form. Early onset neonatal listeriosis occurs in infants infected in utero, most often infants of mothers who experienced the bacteremic, flulike prodrome before onset of labor. Widely disseminated granulomas are characteristic of severe disease in the newborn who is infected in utero, which is apparent either at birth, within a few hours after birth, or within a few days of life. In this disorder, lesions are most common in the liver and placenta, but can also be found in the skin, brain, adrenal glands, spleen, kidney, lungs and the gastrointestinal tract. There may be little clinical evidence to distinguish early-onset neonatal listeriosis from other forms of neonatal sepsis, but placental and posterior pharyngeal granulomas, as well as multiple small granulomas on the skin, can be early clues to the diagnosis[49].

Late-onset neonatal listeriosis usually affects full-term infants of mothers who have experienced uncomplicated pregnancies. These infants are usually healthy at birth, and manifestations of the infection are apparent several days to several weeks after birth. The clinical manifestations of infection in this group are more likely to be related to meningitis than to sepsis. The characteristic skin eruption, which can occur in the early onset as well as in the late onset listeriosis, consists of a grayish-white maculopapulovesicular or pustular rash[36]. The treatment of choice is the combination of amoxicllin and tobramycin.

Hemophilus influenza is a small gram-negative coccobacillus and the main cause of meningitis in young children[2]. Cutaneous manifestations can be variable: patients with fulminant sepsis may have a petechial rash and occasionally bright pink tender macules, papules or pustules on the extremities and the trunk[2,36]. As treatment amoxicillin (ß-lactamase negative H. influenza) and cefuroxim (ß-lactamase positive H. influenza) are recommended.

Sepsis caused by *Pseudomonas aeruginosa* occurs most commonly in the child with an underlying illness. It is seen in children with immune deficiency due to cancer chemotherapy, in those with malnutrition, and in those with extensive burns. Premature infants exposed to excessive humidity are also at risk for its development. The cutaneous manifestations of *Pseudomonas* sepsis are erythematous macules and petechiae, but the disease may also present with small, discrete nodules, vesicles and bullae. The classic skin lesion of *Pseudomonas* sepsis is termed ecthyma gangrenosum[50]. Although these lesions were once thought to occur in patients with bacteremia, ecthyma gangrenosum may

appear without evidence of hematogenous spread[51,52]. The lesions typically begin as erthymatous or purpuric macules that become indurated and progress to hemorrhagic blue-black bullae. The bullae rupture, leaving a central area of necrosis. The progression of the lesions is rapid, evolving in 12 to 24 hours. Moist areas of the body are most frequently the sites of these infections, especially the groin, axillae, and perianal areas. The lesions rarely occur on the mucous membranes, palms, or soles[50]. Therapy with the combination of ceftazidim and tobramycin should be initiated immediately. Supportive therapy for the cause of the predisposing illness must also be started.

Viral infections

Herpes simplex virus infection

Most (70%) neonatal herpes simplex virus (HSV) infections are due to herpes simplex type 2 (HSV-2)[53]. HSV-2 may be acquired by the neonate transplacentally by viremia during gestation, intranatally by passage through an infected birth canal, or postnatally by direct contact with infected humans. It is becoming clear that 16-30% of woman in the USA are HSV-2 seropositive, and 0.3-2.0% of woman shed HSV from the vagina at the time of delivery[54]. The incidence of neonatal HSV-2 infection is dependent upon the type of maternal infection. In the case of primary HSV infection approximately 30-60% of neonates will be infected, compared to only 1-3% if the genital infection is a recurrence[55]. Approximately 5% of neonatal HSV is a truly intrauterine infection, resulting in a baby infected at birth[56]. Since the primary period of viral inoculation is intrapartum, and given the variable incubation time, neonatal HSV may be present any time in the first 4-6 weeks of life. However, the majority of cases will present in the first week of life[57]. Up to one-quarter of infected neonates have signs of infection on the first day of life[58].

The initial symptoms of disseminated HSV infection are lethargy, hypo- or hyperthermia, irritability, and poor feeding. Cutaneous findings are the first visible sign in about two-third of neonates infected with HSV[54]. Grouped or single vesicles or pustules on erythematous bases appear in crops on the skin and mucous membranes (Fig. 4). The eyes may also be affected. Vesicles can coalesce to form bullae. Mucosal vesicles may quickly erode or may become pustular and crusted. Neonatal herpes may spread rapidly to involve the central nervous system and/or multiple internal organs. This may progress to rapid deterioration of the neonate's condition[47,54].

A Tzanck smear (Wright, Giemsa or Hemacolor®) of vesicle bases reveals ballooned and multinucleated giant epithelial cells, indicating a herpes infection (Fig. 5). The virus itself can be demonstrated by culture, direct immunofluorescent testing and immunoelectron microscopy[59] of material obtained from a herpetic lesion or a conjunctival swab. In the future the polymerase chain reaction may facilitate early diagnosis. Histopathologically an intraepidermal vesicle produced by ballooning and reticular degeneration of epidermal cells is seen. Marked acantholysis is present. Multinucleated cells and eosinophilic inclusion bodies can be seen. In the dermis an inflammatory infiltrate is present[7].

If left untreated, disseminated HSV infection is fatal in many cases[47]. Early antiviral therapy may reduce overall mortality from 65 to 25% [60]. Survivors often have severe developmental and neurologic deficits.

Although it has been demonstrated that vidarabine is as effective as acyclovir in the HSV infected neonate[56], most experts utilize acyclovir in these patients because of relative ease of administration[54].
If active genital herpetic lesions are present in a pregnant woman at the time of labor, nearly all experts recommend cesarean section if the fetal membranes have been ruptured for less than 6 hours[54]. Prevention is the best treatment, and newborns should be protected from exposure to HSV whenever possible.

Varicella

Varicella-zoster (VZ) virus is the agent responsible for varicella (chickenpox) and herpes zoster (shingles). Neonates can only develop varicella, as the development of herpes zoster would require prior exposure to the virus. Neonatal varicella occurs when a pregnant woman develops chickenpox during the last 2 or 3 weeks of pregnancy or the first few days post partum. In such instances, the timing of the onset of disease in the mother and her newborn are critical. If the disease onset in the mother is 5 or more days before delivery or in the newborn during the first 4 days of life, the infection usually results in no major sequelae for mother, fetus, or newborn. In contrast, however, congenital VZ infection acquired from 5 days prior to delivery until 3 days postdelivery may result in a neonatal varicella of increased morbidity and mortality due to insufficient passive transfer of maternal antibody and insufficient active development of neonatal antibody to varicella. Those neonates acquiring chickenpox after the third day of life tend to have a benign course[47,48,57].
The incubation time for chickenpox is approximately 2 weeks (range 10 to 23 days). Mild prodromal symptoms like fever, malaise, and upper respiratory symptoms may precede the onset of rash by 1 to 2 days but are generally absent in neonates. The skin lesions erupt in crops over 1 week and evolve within 12 to 24 hours of eruption from small red macules to papules to vesicles and pustules on erythematous bases. The lesions are typically described as umbilicated and appear to sit on top of the skin. Crusting occurs 1 to 3 days following eruption. Viral transmission is possible from 1 day before the rash erupts until all lesions are crusted over[47].
Diagnosis may be aided by the finding of multinucleated giant cells on a Tzanck smear of the base of an intact vesicle. Histopathology is identical to that seen in herpes simplex infections. Final prove is established by a positive VZ culture or immunoelectron microscopy[61,62].
Treatment of varicella in uncomplicated cases is supportive and symptomatic. Immunocompromised exposed newborns or neonates who were infected within 5 days prior to or several days after delivery should receive 125 to 250 units of zoster immune globulin (ZIG) from convalescing zoster patients or varicella-zoster immune globulin (VZIG) from high-titered normal adults intramuscularly as soon as possible. If ZIG or VZIG is not available, 1.3 ml/kg* of regular immune serum globulin may be used[58]. In addition, acyclovir needs to be given (preferably within the first 24 hours after delivery) in severe neonatal varicella infection and when zoster immune globulin is not available or is too late to use effectively.

Cytomegalovirus infection

Although Cytomegalovirus (CMV) infection in neonates is generally transmitted from a pregnant mother with inapparent infection across the placenta to the fetus late in gestation, it can also be transmitted by passage through an infected maternal genital tract

at the time of delivery or postnatal CMV-seropositive bloodtransfusion. Approximately 90% of congenital CMV infections are asymptomatic. The remaining 10% may have mild to severe, and occasionally result in fatal, cytomegalic inclusion disease[1]. CMV may directly invade fetal organs and results in defects in organogenesis[63]. Skin findings include petechiae and purpura, a generalized maculopapular eruption, and in some instances, a generalized papulonodular eruption with blueberry muffin lesions similar to those seen in infants with congenital rubella and neonatal toxoplasmosis[1]. Although vesicles and pustules are unusual in congenital CMV infection, the disorder must be considered in the differential diagnosis of cutaneous vesiculopustular lesions in the neonate[47,64].
Diagnosis is often based on the finding of intranuclear inclusions in epithelial cells of urinesediment, which accounts for the name cytomegalic inclusion disease. CMV may also be isolated from placental tissues, amniotic fluid, blood, and cerebrospinal fluid (CSF). There is no effective therapy, and prognosis for the infant with severe involvement is poor.

Fungal infections

Candidiasis

Candidiasis exists in two forms, congenital and neonatal. Congenital candidiasis is an intrauterine infection, while neonatal candidiasis is acquired as the infant passes through a contaminated vagina. In both forms the causative organism is *Candida albicans*, a pathogen found in the vaginal canal of 20% to 25% of pregnant women[65]. A possible way in which intrauterine infection may occur include *Candida* organisms ascending via the vagina and crossing ruptured or intact fetal membranes[16].
In congenital candidiasis lesions are present at birth or usually within 12 hours following delivery[65]. The rash is usually diffusely scattered over the whole body, incuding the face, chest, back, and extremities. Oral and diaper area involvement is generally absent. The congenital form usually starts as erythematous macules and papulovesicles. Over the next 4 to 7 days the lesions become pustular. A pronounced desquamation follows the acute phase with exfoliated crusted lesions. Signs of systemic disease and hematological abnormalities are generally absent. Stool cultures at birth are normally sterile[66,67].
Neonatal candidiasis is usually seen after the seventh day of life by oral thrush and lesions confined to the diaper area. Pustules and vesicles arising from the perianal area erode and spread peripherally with satellite lesions. The intergluteal fold, perineum, genitalia, suprapubic area, buttocks, and inner thighs are frequently involved. In these areas candidiasis evolves into scaling confluent plaques of a beefy red color, with distinct pustular and vesicular satellite lesions at the periphery of the plaques. Constitutional symptoms are absent. In neonatal candidiasis, *C. albicans* can often be isolated from the feces.
Diagnosis of candidiasis is made by finding pseudohyphae and spores of a potassium hydroxide preparation of a pustule or scale. *Candida albicans* may be cultured from the vesicles and pustules.
Candidiasis is treated topically with imidazole derivatives, such as miconazole, clotrimazole or ketoconazole cream. Lesions last approximately 2 weeks, desquamate, and resolve without residua. Thrush is treated by oral nystatine[67].
Disseminated systemic candidiasis may occur rarely and is primarily an infection of

preterm, low birth weight infants, immunologically compromised patients and neonates requiring intensive care, with invasive procedures[67]. It may affect the lungs, bronchial tree, meninges, kidneys, bladder, joints, and, less commonly, the liver, myocardium, endocardium, and eyes[1]. Disseminated candidiasis is associated with significant morbidity and mortality. The spreading of a candidal diaper rash to the trunk and extremities is indicative of a localized infection beginning to disseminate. Intermittent, spiking, therapy resistant fever, with cutaneous candidal lesions or cellulitis at the site of an intravenous catheter, and persistent candidemia or candiduria, even in the absence of skin findings or systemic symptoms, indicate the presence of disseminated disease[47,67].
Confirming the diagnosis of suspected disseminated candidiasis is difficult. Widespread infection despite negative cultures is common[67]. The diagnosis is confirmed by isolating *Candida albicans* from blood, abcesses, urine, or other body fluids, or by demonstration of the organism in a cutaneous biopsy or other surgical specimens. The early institution of treatment is the critical prognostic factor. Amphotericin B or 5-flucytosine intravenously are the drugs of choice[68,69]. The use of these medications requires careful monitoring as their side effects are considerable[67].

Pityrosporum folliculitis

*P*ityrosporum yeasts (*Malassezia furfur*) are the cause of pityriasis versicolor, which is usually seen as a disorder of adolescents and young adults, but may be a very rare cause of folliculitis in neonates[36,70]. Cutaneous lesions consist of follicular papules and sparse pustules on the face and scalp. The diagnosis is based on direct microscopy (potassiumhydroxide preparation) and culture of pustular contents.
Pityrosporum folliculitis can be succesfully treated topically with imidazole derivatives, such as miconazole, clotrimazole or ketoconazole cream[71].

Parasitic infections

Scabies

Scabies is a contagious disorder caused by *Sarcoptes scabiei*, a parasitic mite, which invades the stratum corneum. After an incubation period of 3-6 weeks, an extremely pruritic dermatitis develops[72,73]. If infestation occurs soon after delivery, the disorder may be seen in the neonate[73]. Scabies is a distinct clinical eruption characterized by pruritic papules, vesicles, and linear burrows mixed with excoriations, eczematization, crusting, or secondary infection. The clinical pattern of scabies in newborns differs from that seen in older infants, children, and adults. In older children and adults most of the lesions are concentrated on the finger webs, wrist, axillae, arm flexures, beltline, perineum and genitals. In infants and young children, the infestation rapidly becomes more generalized, usually involving the palms, soles, head, neck, and face[73]. Vesicles are common in neonates, and there is tendency is to pustule formation early in the course of the infestation (Fig. 6). Irritability, poor feeding, and failure to gain weight are also quite characteristic. A careful history and examination of the baby's caretakers will frequently disclose a history of pruritus and/or typical scabies lesions. Frequent maternal sites of lesions are periareolar regions of the breasts, as well as the wrists and fingers.

Definitive diagnosis is made by microscopic examination of scrapings from unexcoriated lesions in a potassium hydroxide or mineral oil preparation. The presence of the adult mite, ova, and/or larva confirms the diagnosis.
The treatment of choice appears to be permethrin 5% cream, which must be applied from scalp to toes for six hours before rinsing with soap and water[72]. When permetrin 5% cream is not available, neonates can also be treated with 5% precipitated sulfur in petrolatum[73]. Sulfur-containing preparations are messy, staining, and odoriferous, and must be applied for three nights[74]. All family members need to be treated at the same time. Clothing and bedding should be washed in high temperature water[75].

Direct preparations for establishing a diagnosis

Definitive diagnosis is usually obtained with smears and stains. Pustular eruptions of infectious etiology, caused by herpes simplex and varicella zoster virus, or cytomegalovirus must be ruled out with a Tzanck smear; bacterial infections, such as impetigo with routine Gram stain; candidiasis with routine potassiumhydroxide (KOH) preparation, and scabies with appropriate mineral oil or KOH preparations. A Giemsa, Wright, or Hemacolor® stained Tzanck smear is useful in identifying cell types in noninfectious pustular eruptions, especially eosinophils[76]. For the Tzanck smear, a scalpel is used to carefully scrape material from the base of a fresh vesicle or pustule and the scraping is smeared on a slide and air dried. After drying, the material is fixed in methanol and stained preferentially by us with Hemacolor® (Merck), because the staining can be done within one minute. Briefly this method includes dipping 5 times in methanol, 3 times in eosine and again 3 times in thiazine. After this procedure the slide is washed in buffered distilled water and ready for light microcopic examination[77]. A herpes positive preparation demonstrates a specific cytological picture: epithelial cells with monstrously swollen nucleus, often multilobated or multinucleated, surrounded by a perinuclear halo. Cytologic differentiation between herpes simplex, herpes zoster and varicella is not possible. If the epitheloid shapes are difficult to discern as a result of overcolouring, in most cases the swollen nuclei often with several lobes or multinucleation can still be observed with stronger light (Fig. 5). If only one smear is available as a result of scarcity of lesions, the already coloured preparation can be decoloured using acid hydrochloride 25% in ethyl alcohol 95%. The decoloured preparation can then be restained according to other colouring methods, for example Gram's stain.

Discussion

It is essential to investigate every neonate presenting with pustules for an infectious disease. A rapid differentiation between benign transient noninfectious pustular eruptions and such diseases as sepsis and herpes infections can be life saving. In few other conditions maternal history is important, since maternal infection should immediately suggest the possibility of transmission to the neonate. Features of the history of the disease and physical examination of skin lesions will often yield the correct diagnosis. For example, a

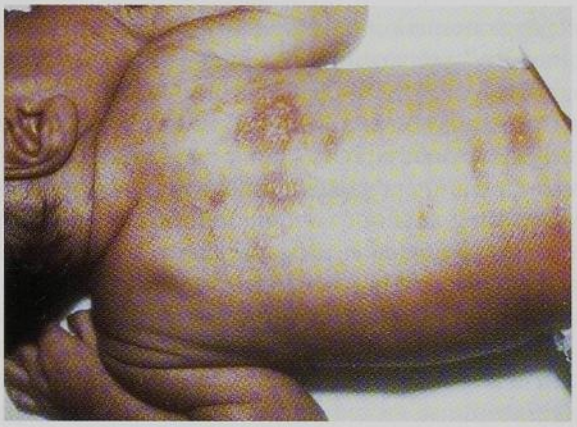

Figure 1
Erythema toxicum neonatorum in a caucasian neonate. (Reproduced with permission from Dr. A.P. Oranje. Aspecten van de kinderdermatologie. De Tijdstroom, Lochem, 1990; page 198)

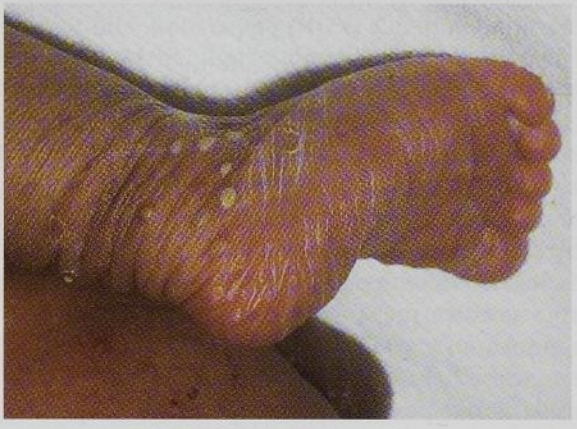

Figure 2
Transient neonatal pustular melanosis in a negroid neonate from Suriname.

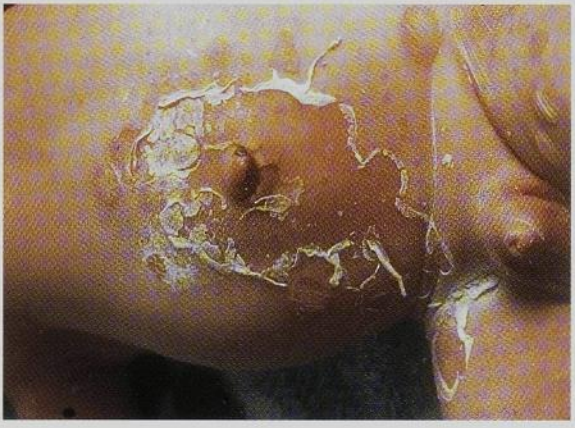

Figure 3
Impetigo bullosa in a caucasian neonate.
(Reproduced with permission from Dr. A.P. Oranje. Aspecten van de kinderdermatologie. De Tijdstroom, Lochem, 1990; page 42)

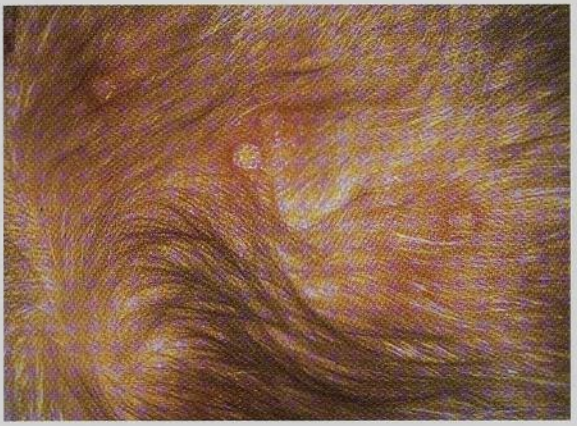

Figure 4
Herpes simplex type 2 in a caucasian neonate.
(Courtesy of Dr. J.H. Sillevis Smitt, dermatologist, AMC, Amsterdam)

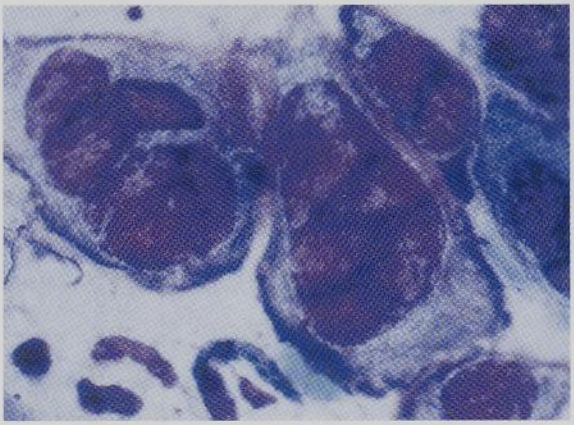

Figure 5
A Tzanck smear of vesicle bases reveals ballooned and multinucleated giant epithelial cells.
(Courtesy of F.H.J.M. van de Noort, cytotechnologist, Department of Pathology, Sint Franciscus Gasthuis, Rotterdam)

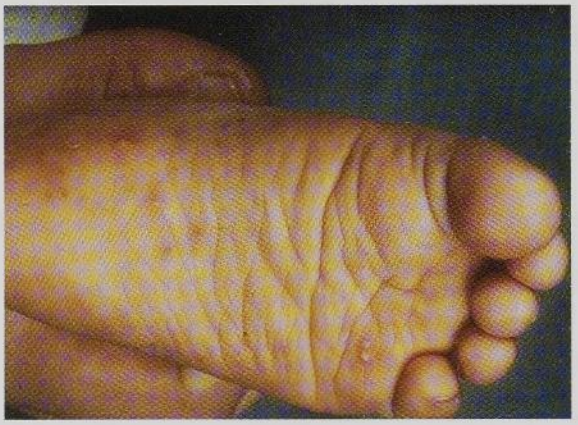

Figure 6
Scabies in a caucasian neonate.

Table 2. *Characteristic data of neonatal pustular disorders*

Disease	Incidence*	Age of onset	Duration **
Erythema toxicum neonatorum	Approximately 1/3 of full-term neonates	24-72 hours	1 week
Infantile acropustulosis	< 1%, possibly increased in blacks and males	Hours after birth to 10 months	2-3 years
Transient neonatal pustular melanosis	5% of all black neonates; <1% in Caucasians	Birth, indicative of intrauterine involvement	Pustules: days Macules: 3 months
Pustular miliaria	Unknown	First weeks of life	Hours to days
Eosinophilic pustular folliculitis (Ofuji's disease)	Unknown reportedeported in adults	From birth; most cases	Years
Congenital self-healing Langerhans cell histiocytosis	Rare	At birth or in the neonatal period	Usually a benign self-limited condition within 4-24 months
Incontinentia pigmenti	Rare	Birth or within the first weeks of life	Linear vesiculopustular lesions evolve into verrucous lesions within a few weeks
Neonatal acne	Unknown	Variable	1 to 3 months
Candidiasis, congenital neonatal	<1%, equal among sexes Approx. 4-5%	Birth-24 hours after the first week of life	2 weeks 2 weeks
Pityrosporum folliculitis	Very rare reported cases in adults	From birth; most	1 to 2 weeks
Scabies	Common, but rare in neonates	2-4 weeks	Responds rapidly to treatment
Impetigo bullosa	<1%	Second day to second week	Approx. 5-10 days
Herpes simplex (HSV)	1/3000 to 1/20.000 births	Majority in the first week life	If left untreated fatal in 80 % of cases survival rate with treatment 50-90%, depending on the degree of initial involvement (disseminated HSV)

* The incidence of most of the above mentioned disorders is not precisely known and shows a strong geographic variation.
** In case of infectious neonatal pustular disorders, average duration after treatment is reported.

history of recurring crops of pustules distributed on the hands and feet should immediately suggest a diagnosis of acropustulosis of infancy. An organized approach to the diagnosis of pustular eruptions in the neonate is outlined in Tables 2 and 3.
The Tzanck smear can be used for detection of an herpetic infection (multinucleated giant cells) as well as noninfectious vesiculopustular eruptions[76]. For example, the Tzanck smear is very useful to identify other cytomorphologic determinants, such as eosinophilic granulocytes in erythema toxicum neonatorum, eosinophilic pustular folliculitis, arthropod bites or incontinentia pigmenti. The Tzanck smear may also be used to detect neutrophilic granulocytes in infantile acropustulosis, or transient neonatal pustular melanosis. Therefore, we advocate the Tzanck smear as the first test to be performed. Secondly, Gram's stain is indicated for observing bacteria.
Good cooperation between the pediatrician and dermatologist is of utmost importance to make a rapid diagnosis and to start appropriate therapy immediately or no treatment at all other than reassurance to the parents in cases of benign transient noninfectious pustular eruptions.

Acknowledgments

The authors are grateful to F.H.J.M. van de Noort (cytotechnologist, Department of Pathology, Sint Franciscus Gasthuis, Rotterdam) for making an excellent colour photograph of a Tzanck smear and Dr. JH Sillevis Smitt (dermatologist, Academic Medical Center, Amsterdam) for providing a beautiful slide of herpes simplex neonatorum.
Financial support for the printing of the color photographs in the article was kindly provided by E. Merck Nederland B.V.

* *Erratum:* Instead of 0,1 to 0.3 ml/kg (dosis mentioned in the original paper)[78-80]

Table 3. *Morphologic, cytologic and histopathologic features of neonatal pustular disorders*

Disease	Clinical picture	Diagnostic test	Characteristic findings
Erythema toxicum neonatorum	Red macules and papules; white to pink pustules, vesicles on the trunk, extremities and face	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Gram smear	Abundant eosinophils, rare neutrophils No bacteria; abundant eosinophils
Infantile acropustulosis	Red papules evolving into pustular and vesicular lesions in one day	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Gram smear Biopsy (in general not necessary)	Early: eosinophils predominate Late: neutrophils predominate No bacteria Intraepidermal vesicles (early): subcorneal pustules (mostly PMNs); mild perivascular infiltrate
Transient neonatal pustular melanosis	Vesicles and pustules desquamate leaving brown macules on the chin, neck, palms, soles	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Gram smear Biopsy (in general not necessary)	Abundant neutrophils; rare eosinophils No bacteria; neutrophils, rare eosinophils Intra- and subcorneal pustule composed almost entirely of neutrophils; rare dermal infiltrate
Pustular miliaria	generalized grouped erythematous papules and pustules with an increase in the intertriginous areas	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Gram stain	Lymphocytes predominate No bacteria
Eosinophilic pustular folliculitis	Crops of papules, vesicles, and pustules that crust, primarily on the scalp with some lesions on the trunk and extremities	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Gram smear	Eosinophils and neutrophils No bacteria
Congenital self healing Langerhans cell histiocytosis	Generalized papules, vesicles, pustules or nodules	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) and Gram s mear Biopsy	Negative for bacteria and fungi In the upper portion of the dermis an infiltrate composed almost entirely of Langerhans cells identified immunohistochemically by markers such as S-100 protein, and CD1a (OKT6), and electron-microscopically by Birbeck granules.

(Table 3. Continued)

Disease	Clinical picture	Diagnostic test	Characteristic findings
Incontinentia pigmenti (rarely pustular)	Linear irregular vesicular and bullous lesions (rarely pustular) over the trunk and the extremities	Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Biopsy	Abundant eosinophils Intraepidermal eosinophilic spongiotic pustules; dermal infiltrate with many eosinophils mixed with lymphocytes in an extrafollicular location
Neonatal acne	Closed comedones predominantly; open comedones, papules and pustules in theface	Gram smear Culture	Bacteria and yeast cells Bacterial: Staphylococcus epidermidis, Propionibacterium acnes Yeast:: *Pityrosporum ovale*
Candidiasis	Pink to red macules and papules evolving into pustules and vesicles	KOH preparation Culture	Pseudohyphae and spores *Candida albicans* isolated
Pityrosporum folliculitis	Follicular papules and sparse pustules on the face and scalp	KOH prepartation Culture	Yeast cells bud monopolarly with a broad base *Pityrosporum ovale* isolated
Scabies	Vesicles, pustules and papules; are burrows on hands, feet trunk, genitalia	KOH or mineral oil	*Sarcoptes scabiei* mites, Preparation eggs, or fecal particles
Impetigo bullosa	vesicles, pustules, bullae on an erythematous base in the diaper area, neck, groin, axilla	Gram smear Gram Tzanck smear (Giemsa, Wright's, or Hemacolor® stain) Bacterial culture	positive cocci in clusters and neutrophils Neutrophils and bacteria *Staphylococcus aureus* isolated
Herpes simplex (HSV)	Grouped or single vesicles on erythematous bases in crops on the skin and mucous membranes Polymerase chain reaction	Tzanck smear Direct immunofluorescence Viral culture	Multinucleated giant cells Positive for HSV Growth of HSV Identification of HSV DNA

HSV, herpes simplex virus; PMNs, polymorphonuclear leucocytes.

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CHAPTER 3

Immunogold electron microscopy in diagnosis of herpes simplex virus and varicella-zoster virus infection

Summary

Electron microscopy can be applied as a rapid method for virus detection, and can be used in validation studies. Immuno-EM can distinguish between VZV, HSV-1 and HSV-2. In this chapter we describe our findings comparing Tzanck smears, viral cultures, and immuno EM to diagnose human alphaherpesvirus infection in clinically suspected cases of HSV and VZV infection.

3.1 The use of colloidal gold immunoelectron microscopy to diagnose varicella-zoster virus (VZV) infections by rapid discrimination between VZV, HSV-1 and HSV-2

Summary

Colloidal gold immunoelectron microscopy was used to diagnose rapidly 53 cases clinically suspected of varicella-zoster virus (VZV) infection and one special case selected from another study on typical herpes simplex virus (HSV) infections. The viruses were identified and subsequently typed within 2.5 h by a direct labelling test for VZV. and within 3.5 h by an indirect labelling test with monoclonal antibodies against HSV type I and type 2.

The protein A purified IgG fraction of human anti-VZV immunoglobulins was adsorbed to colloidal gold particles, and the specificity of the gold-labelled antibodies was tested with several human and animal herpesviruses. Viral envelopes did not crossreact in the direct labelling test. Howevere, an indirect labelling procedure revealed that a small fraction of the anti-VZV antibodies crossreacted with the cores of herpes simplex virus and pseudorabies virus (Aujeszky disease virus).

Virus-infected cellular material taken from typical herpetic lesions was used directly without virus propagation for virus typing. All cases (*N*=54) were analyzed without knowing the clinical description of the results of cytopathologic examination (Tzanck smear) and viral culture. Forty-four cases were identified as VZV; however 5 of the supposed VZV infections were proved to be HSV infections. Although the viral culture of the one HSV case was HSV- and VZV-negative, colloiclal gold labelling identified the case as VZV infection.

In 16 cases virus immunoglobulin complexes were detected by using gold-tagged antibodies against human immunoglobulins. Immunoglobulins on the viral envelopes did not interfere with virus typing by immunogold labelling.

Vreeswijk J [1], Folkers E [2], Wagenaar F [1], Kapsenberg JG [3]

[1] Central Veterinary Institute, Department of Virology, Lelystad, The Netherlands

[2] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands

[1] Central Veterinary Institute, Department of Virology, Lelystad, The Netherlands

[3] National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands

(Published in: J Virol Methods 1988; 22: 255-271)

Introduction

Varicella-zoster virus (VZV). which causes chickenpox and shingles in man, belongs to the family Herpesviridae. This family also includes herpes simplex virus (HSV), cytomegalovirus (CMV). and Epstein-Barr virus (EBV). VZV and HSV are common infectious agents and are usually transmitted by close personal contact. In most cases, local infections of the skin and the mucous membranes are involved, but in severe cases large areas of the skin may be affected, or the infection can spread to the visceral organs and the central nervous system. Effective antiviral therapy requires certainty about the type of infection involved (Kalman et al., 1986; Longson, 1987).

VZV, in contrast to HSV. grows poorly in cell culture. The virus can easily be inactivated during transportation or storage. Therefore, cell cultures must be inoculated as soon as possible after sampling. It is very difficult to combine this strategy with medical praxis. Although the efficiency of viral culture can be increased after short centrifugation of virus infected material on tissue culture monolayers, laboratory diagnosis based on VZV isolation cannot be done within an acceptable time span. Rapier and sufficiently sensitive techniques must be developed. For diagnosis by electron microscopy (EM), transportation and storage conditions are less critical than for viral culture. This study was undertaken to establish whether immuno-EM techniques could replace diagnosis by viral culture and whether they can be used in verification studies during development of other tests.

The present study evaluated the applicability of EM techniques in rapid diagnosis of herpesvirus infection. We studied 54 patients using conventional EM techniques to detect virus particles in material taken directly from lesions. Results of this study were used for the verification of cytodiagnosis (Tzanck test) by light microscopy (Folkers et al., 1988). In the present paper we report on the technical aspects of the immuno-EM technique, the characterization of the diagnostic antibodies used, and the specificity of gold-labelled antibodies. This technique identified virus infection that were not detected by cell cultures, detected immune complexes and identified clinical misinterpretations.

Material and methods

Sample processing

Cellular material was scraped from the bottom of the lesion with the curved side of a vaccinostyle and frozen to -20°C directly after sampling. Some samples were kept at this temperature for several days until transportation was arranged to the EM laboratory. For longer periods (weeks or months), samples still present on the vaccinostyle were stored at -70°C. At the EM laboratory the samples were transferred to a prewetted mortar, and a drop of TE buffer (10 mM Tris, I mM EDTA, pH 7·3) was added together with sterile sand. After homogenization the suspension was diluted with TE buffer to about 400 µl. The sand was collected by low speed centrifugation in a Beckman desk microfuge (type 152, maximum speed) in centrifuge tubes (400 µl) with close fitting snaplids (Beckman No. EET23). The supernatant was transferred to another centrifuge tube, and the content was briefly sonicated in a Branson disrupter (sonifier) equipped with a cuphorn attachment. The sonifier was used with a power setting of 40 W for 2 x 2 s. This suspension is denoted as *suspension 1.*

Suspension 1 was layered on a 70 µl 30% di-potassium tartrate cushion in TE buffer in Beckman polypropylene tubes (No. PAT22) and centrifuged without the snaplids for 30 min at 32000 rpm in a SW 50 rotor (Beckman). The supernatant and the tartrate layer were discarded, and the pellets were suspended in 20 µl TE buffer by brief sonication. The snaplids were applied during the sonication procedure. This suspension is denoted as *suspension* 2.

Reference viruses

VZV (isolate 63-1444), HSV-I (isolate 63-3390), HSV-2 (prototype MS) and EBV were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthovem The Netherlands; CMV (prototype AD169) was a generous gift from Dr. J. v.d. Noordaa, Academic Medical Center (AMC), Amsterdam, The Netherlands.

The Northern Ireland Aujeszky-3 strain (NIA3) (McFerran and Dow, 1975) of swine herpesvirus (pseudorabiesvirus, PRV), the Dutch Lambeek strain of equine herpesvirus serotype 1 (EHV-1. Burrows and Goodridge, 1972), the Australian R19 strain of serotype EHV-4 (Sabine et al., 1981). and the LK strain of serotype EHV2 of equine herpesvirus (Plummer and Waterson, 1963) were used for specificity tests of the diagnostic antibodies. An EHV-3 strain was generously supplied by Dr. J.A. Mumford (Newmarket, U.K.). a bovine herpesvirus 1 (BHV-1) isolate by Dr. A.P.K.M.I. van Nieuwstadt (CDI) and Marek disease virus (strain CV1988) by Dr. G.F. de Boer (CDI).

Virus isolation was carried out according to standard tissue culture techniques using human embryonic lung (HEL) fibroblasts (Dept. of Virology, Sophia Children's Hospital, Rotterdam or Dept. of Virology, GG&GD, Amsterdam). The HSV isolates used in the experimental test system for HSV subtyping with colloidal gold were obtained from the Dept. of Virology, RIVM, Bilthoven. These isolates were subtyped by a virus neutralization assay employing the same monoclonal antibodies that were used in colloidal gold labelling (see below).

Sera and monoclonal antihodies

Anti-varicella serum was obtained from a six-year-old patient with generalized varicella infection complicated with meningitis and cerebellitis. Sera were collected 9 days, 3 weeks and 10 weeks after onset of the disease. The fluorescence titer of anti-VZV and anti-HSV antibodies (IgG and IgM) in the second serum was assessed at the RIVM at Bilthoven. The IgG fraction of this serum was purified by protein A (Pharmacia) affinity chromatography and extensively dialysed against 2 mM borax buffer (pH 7·5).

Rabbit anti-human IgG was obtained from Dakopatts, Denmark and was also purified by affinity chromatography.

Monoclonal antibodies (MCAs) against HSV were prepared by Dr. A.D.M.E. Osterhaus, RIVM. Bilthoven, and Dr. A.J. Scheffer. University of Groningen, The Netherlands. Two MCAs were used, one neutralizing only HSV-l (MCA1, code number 82-13/4-llCl0E5), the other both HSV-1 and HSV-2 (MCA12, code number 82-30/1-20E6). MCAs against human IgG (code number 315-2.2) and IgM (code number 179-1.1) were a generous gift from Dr. J.J. Haaijman, MBL-TNO, Rijswijk, The Netherlands (Haaijman et al., 1994) and are distributed by Nordic Immunological Laboratories, Tilburg, The Netherlands.

Electron microscopy

Negative staining. Suspensions 1 and 2 were examined for the presence of virus particles using 1% phosphotungstic acid (pH 6·8) as a negative stain (PTA stock solution). The virus particles were adsorbed on collodion (Balzers)-coated nickel grids (400-mesh; Veco, Eerbeek, The Netherlands) reinforced with a thin layer of carbon (Balzers). The grids were floated on the sample during 15 min, carbon side down. Negative staining was performed by 2 dips in PTA-Tween (1% PTA, 0.01% Tween 80). Grids were examined in a Philips EM 300 microscope at an accelerating voltage of 80 kV.

Preparation of 18 nm gold particles. Colloidal gold sols were made by reducing chloroauric acid (Merck) with trisodium citrate (Merck) (Geoghegan and Ackerman, 1977). Briefly, 2 ml of a solution of chloroauric acid in water (0.5%) was added to 100 ml of highly purified water (deionized by ion exchange followed by two cycles of distillation) in an Erlenmeyer flask. The flask was used for preparing colloidal gold only and was not treated with detergents or buffers. The solution was heated to boiling and 4 ml of a solution of 1% trisodium citrate in water was added. Boiling was continued under reflux for 30 min. The pH was adjusted to 8·0 with 0.1 M potassium carbonate. The pH measurement was carried out separately in Eppendorf® tubes.

Preparation of gold-IgG complexes. The optimum amount of IgG necessary to stabilize the gold was determined as follows (Geoghegan and Ackerman, 1977): A series of protein solutions with 0.1 ml of increasing concentrations of IgG was prepared in Eppendorf® tubes. Colloidal gold was added to an amount of 0.5 ml. After 5 min 0.1 ml 10% NaCl was added, and the optical density at 580 nm was determined. A color change from red (fully stabilized gold) to violet (flocculated gold) indicated the minimum concentration of protein necessary for optimal coating. Based on these data hatches of 10 ml colloidal gold were coated with IgG. The optimal amount of IgG as deduced from the adsorption isotherm, was diluted with 2 mM borax (pH 7·5) to 2 ml and added to the gold suspension. After 5 min incubation BSA was added to a final concentration of 1%. The gold particles (12 ml) were centrifuged through a 30% w/v sucrose cushion (0.5 ml) in 2 ml tubes in an Eppendorf desk centrifuge for 15 min at maximum speed. They were then suspended in 0.25 ml 1% BSA in PBS per tube and sonicated in a Branson disrupter equipped with a cuphorn attachment for 2 x 2 s with a power setting of 40 W. Suspensions were pooled (3 ml) and the washing procedure was repeated. Thus, 10 ml unstabilized (uncoated) gold sol was concentrated to 3 ml IgG coated gold suspension.

Immunogold labelling procedures. Virus coated grids were prepared as described under negative staining and labelled as follows (Fig. 1). All incubations were done on a drop of solution on Parafilm®. Gold-tagged antibodies were used undiluted.

Direct procedure: incubation with 1% BSA in PBS(10min) followed by goldlabelled antibodies at room temperature for 1.5 h. The grids were extensively washed in PBS buffer, followed by one dip in TE buffer and stained with PTA.

Indirect procedure: grids were incubated with 1% BSA and for 2 h with serial dilutions of the primary antibody (or overnight at 4°C), extensively washed with PBS, incubated with gold-labelled secondary antibody (30 min), washed and stained as above.

Virus typing. VZV detection was carried out in a direct labelling procedure using anti-VZV gold-tagged antibodies. HSV typing was done according to the indirect labelling technique using MCAs against HSV and anti-mouse Ig gold-tagged antibodies in the second labelling

step. The optimal dilution for the MCAs in routine test was 1:400. Antibodies present in viral immunoglobulin complexes were detected with anti-human IgG colloidal gold particles.

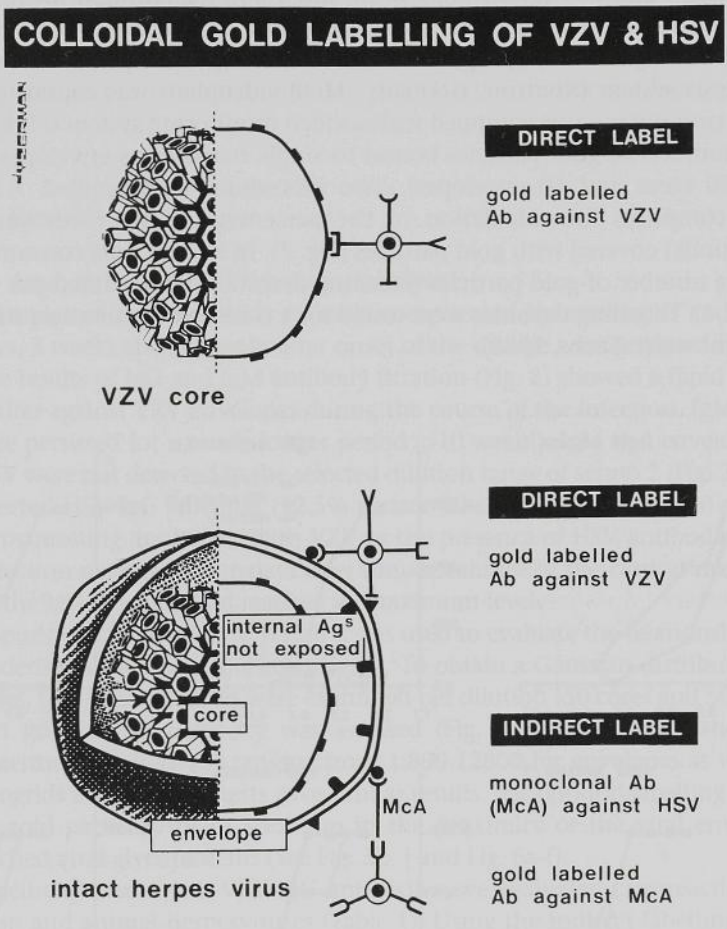


Fig.1. Schematic representation of direct and indirect labelling procedures used in this study.

Serum titrations. The patients' sera were diluted in 2-fold dilutions steps (25-26000) and incubated with VZV-coated grids. Anti-VZV antibody titre was assessed in an indirect labelling assay using anti-human Ig gold-tagged antibodies. IgM and IgG antibody response to VZV was assessed using anti-IgG and anti-IgM MCAs. Optimal concentrations were established at 1:600 for anti-lgG MCA and 1:400 for anti-IgM MCA. Labelling was carried out with anti-mouse Ig gold-tagged antibodies.

Quantitative measurements of gold particle densities on single virus particles. A semiautomatic image analysis system (Kontron, Germany: MOP videoplan) was connected to a Philips EM300 electron microscope equipped with a video monitoring system (Philips LDH 2105). The total numbers of gold particles bound to single herpesvirus envelopes or cores were analysed (50 cores and 50 envelopes). Two procedures were applied. A relatively fast procedure comprises the calculation of the percentage of the virus surface structure (perimeter units) covered with gold particles (Fig. 2). In a more time consuming procedure the absolute number of gold particles (labelling densities) was counted per virus structure (Figs. 3 and 4). Labelling densities were tested for a Gaussian distribution with the Kolmogoroff-Smirnov test (Sachs, 1983).

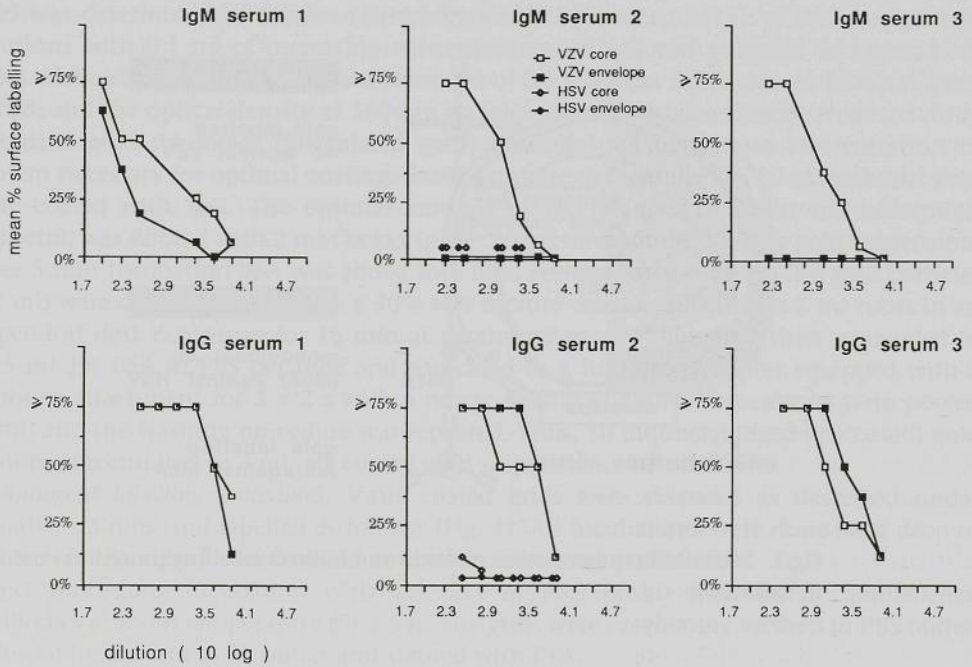


Fig. 2. Characterization of anti-VZV sera used in the production of gold-tagged antihodies. Assessment of IgM and IgG activity by colloidal gold lahelling in 3 different serum samples taken at 9 days (serum 1), 3 weeks (serum 2), and 10 weeks (serum 3) after onsct ol VZV infection. Serum 2 was chosen for the preparation of diagnostie gold conjugates.

Quality control of gold-labelled antibodies. Gold-labelling density data are very important for quality control of gold labels to be used in direct labelling procedures. During this study we prepared more than one batch of the diagnostic antiVZV gold-labelled antibodies. Incidentally, we produced batches of gold-labelled antibodies which showed a mean labelling density of about 20 gold particles per envelope (Fig. 4b). Although we kept all factors for preparation as constant as possible, variations between batches did occur. All further analyses were performed with batches showing mean labelling densities of more than 4() gold particles per viral envelope (Fig. 4a). Batches with lower labelling densities were excluded.

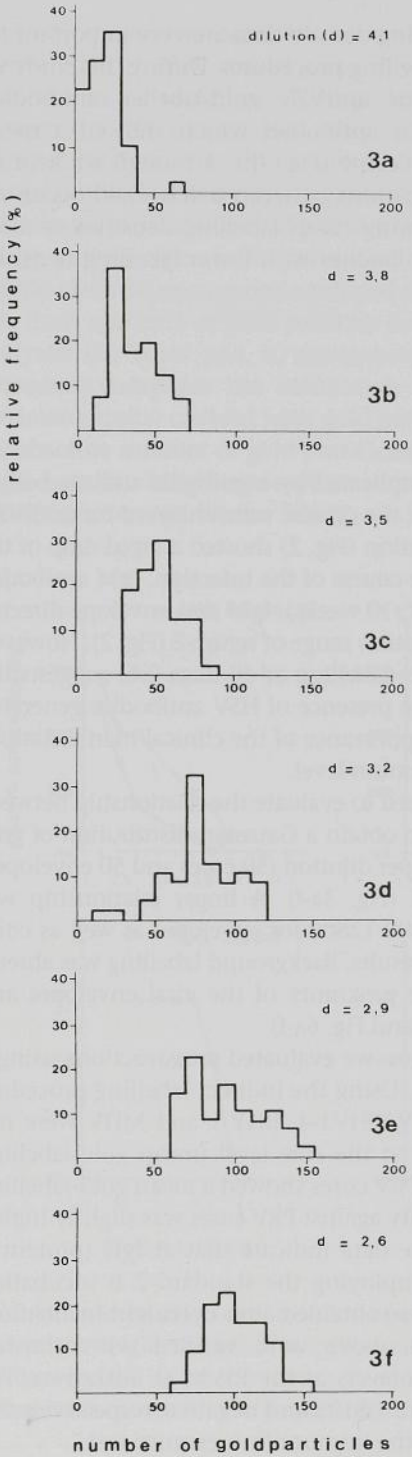
Results

Analysis of anti-VZV immunoglobulins

Serial serum samples of a patient with varicella complicated by meningitis and cerebellitis collected 9 days, 3 weeks and 10 weeks after onset of the disease were analysed for anti-VZV antibodies. The results of IgG and IgM antibody titration (Fig. 2) showed a rapid drop of the IgM antibody titer against VZV envelopes during the course of the infection. IgM antibodies against the core persisted for a much longer period (>10 weeks). IgM and envelope-directed IgG against HSV were not detected in the selected dilution range of serum 2 (Fig. 2). However, a low core-directed HSV IgG labelling (12.5% surface labelling at dilution 2.6) suggests the existence of crossreacting antibodies with VZV or the presence of HSV antibodies generated before VZV infection occurred. Nine days after the appearance of the clinical manifestations of the disease, the IgG labelling had reached its maximum level.

The protein A-purified IgG fraction of serum 2 was used to evaluate the relationship between gold-labelling density and serum dilution (Fig. 3). To obtain a Gaussian distribution of gold particle densities, fifty virus particles were examined per dilution (50 cores and 50 envelopes) and the mean gold-labelling density was assessed (Fig. 3a-f). A linear relationship was observed at intermediate dilutions ranging from 1:800-12800 for envelopes as well as cores (Fig. 3g). Duplogrids and repeated tests gave similar results. Background labelling was absent. Some solitary gold particles might show up in the proximity of the viral envelopes and represent detached viral glycoproteins (See Fig. 3h, j and Fig. 6a-f).

To test the specificity of the anti-VZV IgG antibodies, we evaluated crossreactions using a panel of human and animal herpesviruses (Table 1). Using the indirect labelling procedure crossreactions with envelope antigens of CMV, EBV, EHV1-4, BHV-1 and MDV were not detected. HSV-1 and HSV-2 showed a crossreaction at the core level (mean gold-labelling density of 15 at 1:400 antibody dilution). Similarly, PRV cores showed a mean gold-labelling density of 15 at 1:800 antibody dilution. Thus, activity against PRV cores was slightly higher than activity against HSV cores (one dilution). The data indicate that at IgG (protein A purified) dilution of approximately 1:3000, and employing the standard 2 h incubation period, crossreactions were absent. Similar results were obtained after overnight incubation. The results of immunogold labelling described above were verified with classical immunofluorescence carried out on infected monolayers at the RIVM at Bilthoven. The serum titers of anti-VZV IgG and IgM in serum 2 were 1:8192 and negative, respectively. No reaction was found for anti-HSV IgG and IgM with the immunofluorescence test.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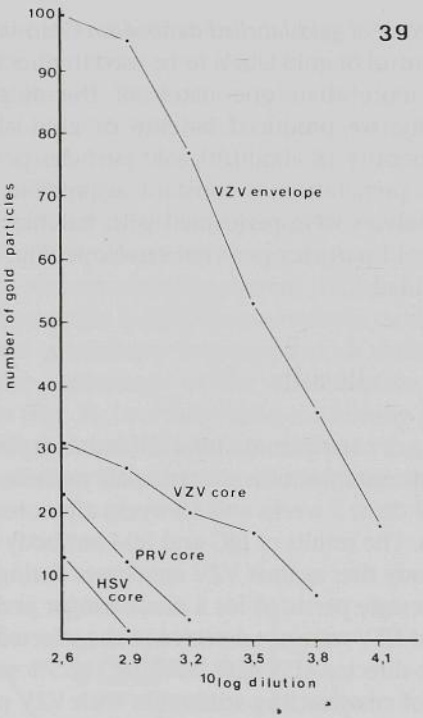
d = 3,8
3b
d = 3,5
3c
d = 3,2
3d
d = 2,9
3e
d = 2,6
3f
number of goldparticles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number of gold particles
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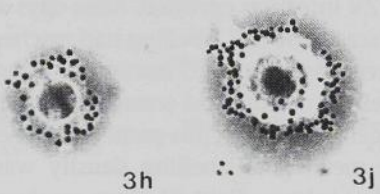
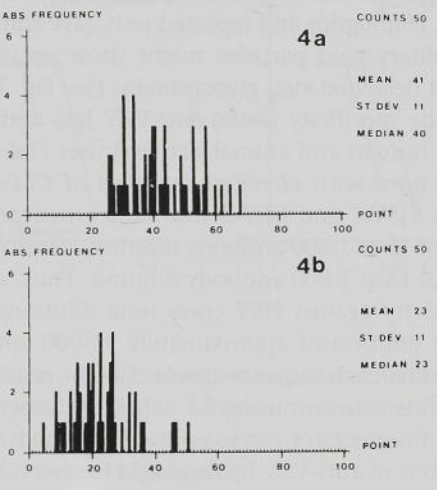
VZV core
PRV core
HSV core
10log dilution


3h
3j


ABS FREQUENCY
4a
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MEDIAN 40
POINT
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4b
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ST DEV 11
MEDIAN 23
POINT

Table 1. *Results of indirect and direct labelling tests using anti-VZV antibodies applied to different herpesviruses*

Virus	Gold-particle counts			
	Indirect test		Direct test	
	Core	Envelope	Core	Envelope
VZV	16 (3.5)	20 (4.1)	15	45
HSV-1	15 (2.6)	–	–	–
HSV-2	15 (2.6)	–	–	–
CMV	–	–	–	–
EBV	ND	ND	–	–
PRV	15 (2.9)	15 (1.7)	–	–
EHV- 1,2.3.4	–	–	–	–
BHV- 1	–	–	–	–
MDV	–	–	–	–

The indirect labelling data indicate the anti-VZV IgG (protein A purified) dilution at which a density of about 10 20 gold particles per virion was obtained (end point, arbitrarily chosen). The direct labelling data give mean labelling densities obtained with the conjugate.
ND not done; ( ) end point IgG dilution, ^{10}log.

◀ Fig. 3. Further characterization of the IgG fraction of serum 2. In an indirect test the gold labelling density of single virus particles was assessed by computer-assisted image analysis. Gold particle count distributions were assessed for all dilution steps (Fig. 3a-f). The plot of mean number of gold particles and serum dilutions is shown in Fig. 3g. Minor crossreactions with PRV and HSV-1 cores were detected. Fig. 3h, j. A typical labelling pattern of a VZV core (Fig. 3h) and VZV envelope (Fig. 3j) obtained after applying the indirect labelling procedure.

◀ Fig. 4. Gold-labelling distribution plots, obtained with two different anti-VZV gold conjugates after computer-assisted image analysis. The same IgG fraction was used for coating, howevere, two different batches of unstabilized gold particles were used.

Specificity of anti-VZV gold-labelled antibodies

Since the indirect procedure takes at least 3.5 h for centrifugation, incubations and examination in the electron microscope, a direct labelling procedure is preferable for rapid diagnosis. However, the most important reason for applying direct labelling is to avoid a reaction of gold-labelled antibodies against human IgG with virus antibody complexes already present in the lesion which might lead to false positive reactions in the indirect test (see below). A concentration step followed by a direct gold-labelling test can be completed in about 2 h. Table 1 illustrates the specificity of gold-labelled anti-VZV antibodies using the panel of different herpesviruses. Again, fifty virus particles were examined to obtain a Gaussian distribution of labelling densities. After adsorption of anti-VZV antibodies to colloidal gold, only a slight PRV crossreaction (mean gold-labelling density 1.3) with the virus cores remained. The crossreaction with HSV cores had disappeared completely.

Analysis of clinical samples

Virus detection. Material obtained from the lesions of 53 patients showing clinical manifestations of VZV infection and from one patient, selected from a similar study based on typical HSV infections, was examined for the presence of virus particles, Both suspension 1 (cell homogenate) and suspension 2 (after ultracentrifugation) were examined. Evaluations of suspension 1 could be obtained about 15 min after homogenization, those of suspension 2, 60 min later.

Table 2 shows that in 49 samples herpesvirus particles could be detected. One of the 5 negative samples showed a positive viral culture for VZV. A *14%* increase of positive samples was obtained by ultracentrifugation of suspension 1. Moreover, EM preparations of suspension 2 were easy to study because of increased virus concentration and the absence of bulk protein. Three samples were not subjected to ultracentrifugation as the concentration of the virus particles was high enough to carry out CG-IEM in a 1:1 dilution of the suspension 1 in TE buffer.

Table 2. *Results of classical EM negative staining applied to rough cell homogenates (suspension 1) and virus suspensions obtained after a concentration (suspension 2) step*

	Herpesvirus detection by EM	
N=54	Suspension 1 (cell homogenate)	Suspension 2 (virus concentrate)
37	+	+
8	–	+
5	–	–*
1	ND	+
3	+	ND

*One sample was positive by virus isolation; ND, not done.

Differentiation of herpesvirus infections. By using anti-VZV gold-labelled antibodies, the herpesvirus particles in 43 samples were identified as VZV (Table 3). In some samples, mostly from patients with varicella infections, the virus concentration was very low. However, none of these preparations led to serious problems in EM interpretation (Fig. 5a). Most of the samples showed labelling densities of 30 to 45 gold particles per envelope. However, some samples showed herpesviruses with lower labelling densities. Since nonspecific background labelling was absent, and HSV viral envelopes did not show labelling, we made a diagnosis on approximately 10 gold particles (arbitrarily chosen) per envelope.

In an attempt to find an explanation for incidental low densities (variations within one sample might also occur), a number of samples was incubated with antihuman Ig gold-tagged antibodies to detect virus antibody complexes (Fig. 5b). Thirteen samples showed antibody-coated virus particles (Table 3). The presence of antibodies did not seriously interfere with diagnosis since these virus particles were still able to bind sufficient anti-VZV gold-tagged antibodies (Fig. 5c).

The diagnosis of one case of clinical varicella, could not be confirmed to CGIEM since virus particles were not detected. Virus isolation succeeded in this case (Table 3). The sensitivity of VZV viral culture is low in comparison with HSV viral culture. This is a well-known problem with VZV cell culture diagnosis, therefore, cell culture data are not considered for further evaluation in this study.

Table 3. *Results of herpesvirus typing by colloidal gold-labelling carried out on 53 patients with clinical signs of VZV and I patient suspected of HSV infection*

N=54	Clinical diagnosis	Gold labelling			TC
		VZV	HSV- 1	IC	
43	VZV	+		+ (13/43)	*
1	HSV	+			–
1	VZV	–	+	–	HSV-1
4	VZV	–	+	+	–
1	VZV	ND			VZV
4	VZV	ND			–

ND, virus particles not detected; *, not relevant (see text); IC, immune complexes; TC, tissue culture typing.

To test the usefulness of the MCAs for the detection of HSV a blind study was carried out with virus isolates obtained from cell cultures and already typed using a neutralization assay (RIVM, Bilthoven). Eight HSV-2 and 9 HSV-1 isolates were analyzed via the indirect gold-labelling procedure (Table 4, Fig. 6). Gold labelling, using MCAs and anti-mouse gold-tagged antibodies, was strictly confined to viral envelopes, i.e. MCA1 to HSV-1 envelopes and MCA12 to both HSV-I and HSV-2 envelopes. No crossreactions with CMV, EBV and

VZV were observed. An HSV-2 diagnosis was scored if a negative reaction with MCA1 and a positive reaction with MCA12 were obtained. The results obtained by gold labelling fully agreed with those of standard neutralization.

Herpesvirus particles were detected by electron microscopy in five out of 54 patients suspected of VZV infection, but the results of virus typing by CG-IEM-VZV were negative (Table 3). Therefore, these patients were suspected of having a HSV infection. After application of indirect gold labelling with anti-HSV MCAs, HSV1 was detected in all five samples. Interestingly, cell culture propagation and HSV subtyping by virus neutralization were positive in one case only (Table 3). A closer examination of the samples with negative virus isolation revealed virus antibody complexes in 4 cases (Fig. 5e). The presence of these antibodies did not interfere with labelling for HSV typing (Fig. 5f). Anti-HSV antibodies were not detected in the cell culture positive sample (Fig. 5d). This suggested that the presence of virus antibody complexes in vivo may interfere with the diagnosis by virus isolation.

Table 4. Colloidal gold-labelling of HSV-I and HSV-2 isolates compared with virus neutralization hy monoclonal antibodies

Sample code	Neutralization		Diagnosis	Gold labelling	
	MCA I	MCA 12		MCA I	MCA 12
82- 3063	+	+	HSV- 1	+	+
3401	+	+	"	+	ND
3919	+	+	"	+	ND
4103	+	+	"	+	ND
4271	+	+	"	+	ND
5830	+	+	"	+	ND
7142	+	+	"	+	ND
7501	+	+	"	+	ND
18188	+	+	"	+	+
82-14865	–	+	HSV-2	–	+
17781	–	+	"	–	+
18295	–	+	"	–	+
22174	–	+	"	–	+
83- 1453	–	+	"	–	+
1769	–	+	"	–	+
3739	–	+	"	–	+
3988	–	+	"	–	+
HSV-1 (63-3390)	+	+		+	+
HSV-2 (MS)	–	+		–	+
CMV, EBV, VZV				–	–

ND not done.

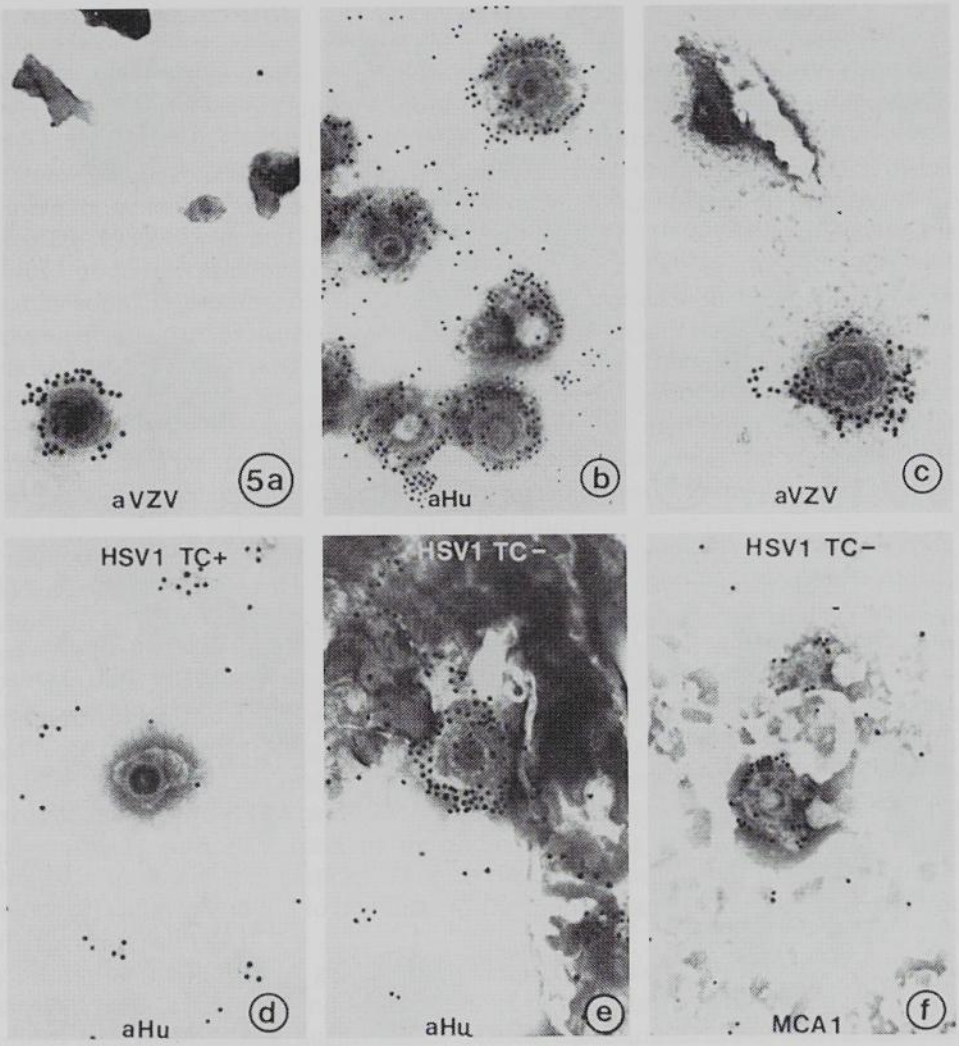

Fig. 5. Colloidal gold immunoelectron microscopy carried out directly on virus particles in tissue homogenates of patients' lesions. The virus shown in a, was the only virus particle detected in this material (a case of varicella). However, with the clear labelling pattern (anti-VZV gold-tagged antibodies) it was still possible to confirm VZV diagnosis. The virus particles shown in b and c were typed as VZV (c, labelled with anti-VZV gold-tagged antibodies). These particles also appeared to have bound a large amount of serum immunoglobulins (b, labelled with anti-human gold-tagged antibodies). Figs. d-f represent HSV-1 virus particles, typed with MCA1 (f). The particle in Fig. e shows bound serum antibodies (labelled with anti-human gold-tagged antibodies). It was not possible to obtain a positive viral culture with this material. In contrast, the particles with positive viral culture shown in d do not showa reaction with anti-human gold-tagged antibodies.

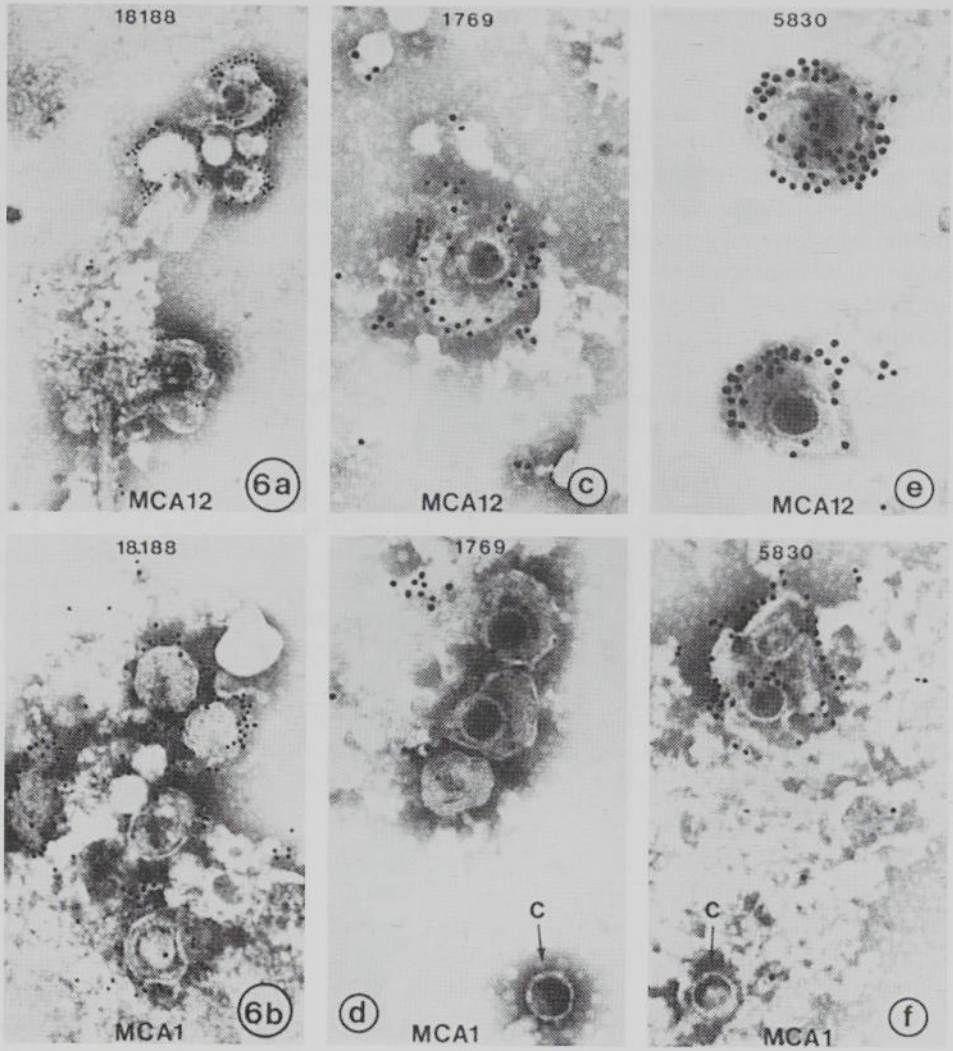

Fig. 6. Analysis of three different HSV isolates after tissue culture propagation in which two monoclonal antibodies were used for differentiation between HSV-1 and HSV-2 (MCA1 and MCA12). MCA1 shows specificity for HSV-1, while MCA12 reacts with both HSV-1 and HSV-2. The isolates 18188 and 5830 were typed as HSV-1 (MCA1 and MCA12 positive). Isolate 1769 showed no reaction with MCA1 and was consequently typed HSV-2.

Discussion

Colloidal gold has been used as an electron-dense marker in several EM studies that focus on detecting viral antigens in thin sections of tissues and cells in tissue, culture (Petrie et al., 1984; Evans and Webb, 1984; Ducatelle et al., 1984; Richardson et al., 1986).
In several studies colloidal gold markers have been combined with classical negative staining for rapid diagnosis of viruses (Stannard et al., 1982; Martin and Palmer, 1983; Na-Sheng Lin, 1984; Hopley and Doane, 1985; Kjeldsberg, 1985, 1986; Beesley and Betts, 1985). Because of its electron opacity, the marker is easily visible in the EM even at low magnification. Depending on the size of the virus particles to be studied, the most convenient gold particle diameter can be selected. In the present study, 18 nm gold particles were chosen for VZV typing. Particles of 18 nm are easily prepared and produce highly stable (>2 years) and reactive conjugates which do not contribute to any background labelling. Moreover, gold-labelled conjugates can be used simply in quantitative evaluations. Smaller particles strongly increase labelling densities but some background labelling sometimes appears. No additional information was obtained by using gold labels smaller than 18 nm. Both indirect and direct labelling procedures established the specificity of the anti-VZV serum used in this study for VZV envelope antigens.
Crossreactions between PRV and EHV-1 are known to occur (Killington et al., 1977). By using indirect CG-IEM we found these reactions occurred exclusively on the viral cores (personal observations). Our results also suggest the existence of common antigens, associated with VZV and PRV. Crossreactivity, known to exist, between VZV and HSV was further substantiated by demonstration of antibodies present in the serum used for preparation of gold-tagged antibodies against VZV which bind to HSV-1 and HSV-2 cores in the indirect labelling test. They did not interfere with differential diagnosis carried out by CG-IEM, since discrimination between HSV and VZV is based on reactivity with the envelope. Antibodies that crossreact with HSV cores in the indirect labelling test were not detected after adsorption to 18 nm gold particles. Thus, in the direct labelling test the specific anti-VZV antibody fraction was found to dominate in the immuno-interactions of the gold particles with the virus particles.
By measuring the number of gold particles bound to viral cores and envelopes, we found that gold particle densities and serial dilutions of the primary antibodies (applied in an indirect labelling test) are quantitatively related. Labelling densities are related to the number of antigenic sites on cellular structures or antigens in cellular compartments. Quantitative evaluations are being done in different fields of cell biology that are mostly confined to ultra-thin section electron microscopy (Posthuma et al., 1986; Kunz et al., 1984). In the present study, however, the analyses were done on negatively stained preparations. The results have implications for different fields in microbiology. such as those concerned with the characterization of sera collected in medical praxis. and in particular for those infections that are still devoid of proper tests.

The antiviral activity of the serum can be designated as gold-labelling titer (dilution). comparable to immunofluorescence and ELISA. Moreover, the antibody binding site can be preciselv located. and in contrast to ELISA techniques, subtracting the background signal is not required. We improved direct and indirect labelling procedures sufficiently to use these tests routinely to diagnose different viruses, to characterize corresponding sera. and to select and characterize monoclonal antibodies.

Another important use of CG-IEM may be to determine IgG and IgM class antibodies and thus to determine the presence of specific antibodies against selected antigens. Furthermore, the class and subclass of virus antibody complexes in vivo can be determined by this technique. We confirmed the presence of anti-VZV IgM and IgG in sera by gold labelling. The drop in envelope-directed IgM during the course of the VZV infection observed with CG-IEM fully agrees with the results obtained with classical techniques. Core-directed IgM inexplicably persisted for more than 10 weeks. Anti-VZV IgM immunofluorescence was absent in serum 2, probably because IgM antibodies were unable to penetrate into the nucleus of the infected cells where cores normally accumulate.

The number of VZV diagnoses made by EM (44 cases) corresponded well with clinical diagnosis. The immuno-EM diagnosis was obtained in only 2.5 h. Because it was possible to detect HSV infections in patients clinically suspected of VZV infection (5 x), we suspected that the opposite was also possible. We confirmed this in one patient who was clinically suspected of HSV infection. In this case, both HSV and VZV viral cultures and CG-IEM with anti-HSV MCAs were negative. Faulty clinical diagnosis, also reported by Kalman et al. (1986), probably more common than is generally acknowledged. The data also suggest that virus-antibody complexes may influence the diagnosis of herpes by viral culture.

The presence study has revealed that immunoelectron microscopy can be used efficiently in rapid diagnosis of VZV. Therefore, we recommend that this technique be used as a standard test in developing other diagnostic techniques for VZV.

Acknowledgements

The authors would like to thank Dr. J.J. Haaijman, Dr. J.G. van Bekkum and Dr. D. van Zaane for critical review of the manuscript. The excellent assistance of Mrs. V. Thatcher (manuscript), Mr. J.M.M. Lepoutre (RIVM) and Mr. T. van Alphen (photographs) is gratefully acknowledged.

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3.2 Improved detection of HSV by electron microscopy in clinical specimens using ultracentrifugation and colloidal gold immunoelectron microscopy: comparison with viral culture and cytodiagnosis

Summary

Three tests were compared to diagnose herpes virus infection: electron microscopy (EM), viral culture (VC) and cytodiagnosis (Tzanck smear). The study comprised 67 patients with skin or mucous membrane lesions suggestive of herpes simplex virus (HSV) infection. The sensitivity of EM increased 25% after virus concentration by ultracentrifugation. Herpes virus infection was confirmed in 55 of the 67 cases by EM or VC or both. EM detected 53 herpes virus-positive lesion samples of which 14 were not detected by VC; only two lesion samples that were herpes virus-positive in VC were not detected by EM. The sensitivities of EM, VC, and Tzanck smear for the group of 55 herpes virus-positive cases were 96%, 75% and 76%, respectively. The specificity of the Tzanck smear was 83% (prevalence 82%). Colloidal gold immuno-EM was used to rapidly type HSV-1, HSV-2 and varicella zoster virus (VZV) present in skin and mucous membrane lesions in less than 4 h. Immuno-EM was able to detect antiviral antibodies on viral envelopes and viral cores in lesion samples with negative VC. Antiviral antibodies do not interfere with typing of herpes viruses by immuno-EM. It is suggested that formation of viral immune complexes and inactivation of virus particles by antibodies may have caused a negative VC. Improved EM is discussed for its applicability to special cases that cannot rely on VC and cytodiagnosis or when rapid diagnosis is required.

Folkers E [1], Vreeswijk J [2], Oranje AP [3], Wagenaar F [2], Duivenvoorden JN [4]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[2] Central Veterinary Institute, Department of Virology, Lelystad, The Netherlands
[3] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University,
[4] Department of Pathological Anatomy (Cytology), Hospital 'de Heel', Zaandam, The Netherlands

(Published in: J Virol Methods 1991; 34: 273-289)

Introduction

Most herpes simplex virus (HSV) infections can be diagnosed on the basis or characteristic clinical signs of the disease. However, because the characteristic vesiculobullous eruptions of HSV can resemble those of other viral and nonviral skin infections, patients can be misdiagnosed (Azon-Masoliver et al., 1990; Bhawan et al., 1984; Halal et al., 1978; Kalman et al., 1986; Oxman, 1986). The facial lesions of HSV and varicella zoster virus (VZV) infections, for example, can be easily confused, probably far more often than is generally acknowledged (Folkers et al., 1989; Kalman et al., 1986).

It is desirable to confirm clinical HSV and VZV diagnosis before treating the infection. The rapidity of obtaining a conclusive diagnosis of these herpes virus infections can be important, especially in immunocompromised patients, for prompt initiation of adequate antiviral therapy to prevent serious complications of the infection. Although viral culture (VC) by the demonstration of characteristic cytopathic effect (CPE) is still the 'gold standard' for the detection of HSV, this method is not completely specific or sensitive (Solomon, 1988). Virus isolation requires at least one day, but the CPE often does not become visible for several days (Gleaves et al., 1990). Because the herpes virus, especially in case of VZV, can be inactivated during transportation or storage, the samples must thus be inoculated as soon as possible, which is usually difficult in non-university hospitals, in out-patient clinics, and in general medical practice.

Colloidal gold immunoelectron microscopy (immuno-EM) was recently introduced as a technique to diagnose rapidly VZV infection (Folkers et al., 1989; Vreeswijk et al., 1988). This test can be used for detection of HSV as well as VZV infection.

The Tzanck smear is rapid, inexpensive, and is still frequently used in office practice. Expertise is required in interpreting virus induced cellular changes in light microscopy. The Tzanck test cannot differentiate between HSV-I, HSV-2 or VZV (Oranje et al., 1986; Solomon, 1986). Besides herpes infections, cytodiagnosis can also be used to detect non-viral agents like Cryptococcus species. Azon-Masoliver et al. (1990) reported that *Cryptococcus* species was demonstrated by cytodiagnosis in a patient suffering from acquired immunodeficiency disease (AIDS) who showed herpes-like skin lesions.

The present study was undertaken to demonstrate that recently developed electron microscopy (EM) techniques can be applied to clinical specimens of skin and mucous membrane lesions for rapid diagnosis of HSV infection.

Material and methods

Specimens

The study was conducted at the out-patient section of the Department of Dermatology and Venereology of Hospital De Heel in Zaandam. Samples were collected from skin, orolabial and genital lesions of 67 patients (46 women and 21 men) with characteristic symptoms of HSV infection. The median age of the population was 35 years, with a range from 2 to 80 years. The herpetic lesions of the skin (n=26) and mucous membranes (n=41) were classified as being maculopapular, vesicular, pustular, ulcerous, or crusted. Thirteen lesions were from genital, 7 from oral, 25 from orolabial, and 22 from other body sites. Of all

genital samples, 4 were collected from skin lesions, 7 from penile and 2 from vulval mucous membrane lesions.

Viral culture

Samples were collected with dry cotton swabs by firmly rubbing the base of the lesion. The swabs were immediately transferred into a transport medium (3 ml Hank's balanced salt solution with 0.5% gelatin, sodium bicarbonate to adjust the pH to 7.2, and antibiotics), and transported within 24 h to the Department of Virology, GG&GD (City Health Services), Amsterdam. If transportation of the specimen could not be done within *24* h, the sample was stored at –80°C within 0.5 h after collection, and then transported afterwards frozen to the laboratory. Virus isolation and virus typing by immunofluorescence were carried out according to standard virological procedures with efforts to cultivate HSV as well as VZV.

Tzanck smear

Samples were generally collected from the same lesion as was used for VC. Scrapings from the edge and base of the lesion were smeared onto a glass slide, air dried, and stained with Hemacolor (Merck) (Folkers et al., 1985). The preparations were examined first by the author (EF) and then double blind by two other investigators (APO and JND). Infected epithelial cells with characteristic nuclear changes were identified by light microscopy (Barr, 1984).

Electron microscopy

Sample processing:

The sample for EM diagnosis was collected from one single lesion, present at the same site as those used for VC and Tzanck smear. Infected cells were scraped from the base and the edges of the lesion with the curved side of a vaccinostyle, fixed with the scarification side in the cap of a plastic sampling tube. After the sample was collected, the cap with the vaccinostyle was placed on an empty tube with a small moistened gauze on the bottom, to prevent freeze drying of the sample. The tube was immediately placed in a refrigerator at 4°C. Within 0.5 h it was moved to a freezer at –25°C. Transportation of the sample to the laboratory of EM was arranged within 2 weeks after sampling. During transportation the temperature was carefully kept below zero. If storage for a longer period was necessary, the samples were frozen at –80°C. Virus suspensions used for EM detection were prepared by two different methods denoted as *collection of viruses from smears*, and *collection of viruses by ultracentrifugation*. Virus collection from smears was used in one experiment that compared the virus particle detection levels of both methods.

Collection of viruses from smears:

Lesion samples were transferred to a glass slide followed by rupture of the tissue by firmly rolling a glass rod over the slide. Viruses were collected in approximately 30 µl (1 drop) distilled water that was spread all over the smear. The virus suspension was carefully removed with a Gilson micro pipette and transferred to Parafilm. Viruses were adsorbed to carbon coated collodionnickel grids for 10 min. The grids were placed on 1% glutaraldehyde in 10 mM Tris-HCI buffer (pH 7·3) for several seconds, washed on two drops of Tris-HCI buffer and stained with 2% PTA (phosphotungstic acid, pH 6·8). Equal parts of the virus suspension and PTA were mixed and some grids were floated on this PTA diluted

virus suspension and dried. Magnifications for screening in the electron microscope (Phillips EM300) were routinely set around 10 000 x.

Collection of viruses by ultracentrifugation:

The lesion sample was removed from the vaccinostyle with 0.4 ml Tris-HCI buffer, transferred to a mortar and homogenized with a minimum amount of ultra-fine sterile quartz sand. The sand was pelleted by low-speed centrifugation. Virus particles, present in the supernatant, were adsorbed to carbon coated collodion-nickel grids for 10 min and fixed and stained as described above.

A virus concentration procedure was carried out to increase further the sensitivity of virus detection (Vreeswijk et al., 1988). The virus suspension obtained after sand homogenization was centrifuged in a Beckman polypropylene tube (n° PAT22) through a 70 µl30% potassium tartrate cushion in a Beckman SW 50 rotor (32 000 rpm, 30 min). The pellet was suspended in 20 µl Tris-HCl buffer and sonicated in closed microtubes by a Branson disrupter with a cuphorn attachment (No. 627-003-020). In an additional experiment this sonicated pellet suspension was clarified by low-speed centrifugation in a desk centrifuge.

Virus particles were adsorbed to carbon coated grids for screening in standard EM, for immuno-EM to detect immune complexes (anti-herpes virus antibodies), and for virus typing (detection of HSV- 1, HSV-2 and VZV) (Vreeswijk et al., 1988).

Solid phase immunoelectron microscopy:

In solid phase immuno-EM (SPIEM) the virus is picked up by a bilayer of protein A and capture antibody absorbed to the EM grid. Nickel grids (400 mesh) supported by a carbon coated collodion film were floated for five minutes on a drop of protein A (25 µg/ml) in PBS. The grids were washed three times with PBS and transferred to a drop of rabbit IgG anti-HSV (National Institute of Public Health and Environmental Protection, RIVM, Bilthoven, the Netherlands), diluted 1:100 in PBS, for 10 min. The optimum concentration of protein A and the optimum serum dilution for HSV trapping were assessed essentially as described by Van Nieuwstadt et al. (1988). Grids were washed with PBS-I % BSA and incubated overnight on a drop of virus suspension. The grids were rinsed with PBS, fixed with 1% glutaraldehyde for a few seconds and stained with PTA.

Immunogold labelling:

Only virus suspensions obtained after ultracentrifugation were used for colloidal gold labelling because soluble proteins present in unpurified suspensions interfere with labelling.

HSV was identified in an indirect labelling test using a monoclonal antibody against a common HSV- I and HSV-2 antigen (RIVM, Bilthoven, the Netherlands) in the first labelling step and gold-tagged rabbit-anti-mouse antibodies in the second labelling step (Fig. 2, obtained from specimen number T5–1745/Table 3). HSV-I and HSV-2 were differentiated in another indirect test; for the antibody used in first step a monoclonal antibody directed against HSV-I (RIVM, Bilthoven, the Netherlands) was used. Virus typing of VZV was carried out with gold-tagged highly specific polyclonal human antibodies against VZV. Identification of antiviral immune complexes was carried out with gold tagged, affinity chromatography purified rabbit anti-human IgG antibodies against IgA, IgM and IgG (Dakopatts A190, Vreeswijk et al., 1988). Virus identification by EM and virus typing by immuno-EM was carried out without prior knowledge of the clinical appearance of the lesion and the results of the other laboratory tests.

Statistical analysis

Sensitivities and Tzanck smear specificity were calculated according to standard methods (Weinstock, 1989). For the difference in sensitivity between two diagnostic tests exact 95% confidence intervals (CI) were calculated (Gardner et al., 1989) and the McNemar test (Siegel, 1956) was applied. McNemar's test was also used to compare the percentages of positive and negative scores obtained from the Tzanck smears by different investigators (EF, APO and JND).

Results

The sensitivities of VC, EM, and Tzanck smear were calculated for the total number of patients with clinically obvious HSV infection (n = 67; Table 2) and for the number of samples that were herpes virus positive confirmed by either VC, EM or both (n= 55; Table 4).

Viral culture

Herpes virus was isolated from 41 of 67 lesion samples obtained from skin or mucous membrane lesions suggestive of HSV infection. (Table 1: all samples). This implies a sensitivity of 61% (95% confidence interval: 48-73%). VC was positive in 58% (15/26) of the skin lesion samples and in 63% of the mucous membrane lesion samples (26/41). Five out of 13 genital (38%), 18 out of 25 labial (72%) and 4 out of 7 oral lesion samples (57%) were VC positive. Of all vesicular, pustular, and ulcerous lesion samples studied, 100%, 63% and 42%, respectively, exhibited CPE in cell culture (Table 1) VC was positive in one single crusted lesion. The single maculopapular lesion showed a negative VC. HSV-1 infection was detected in 34, and HSV-2 in 6 lesion samples. In one patient VC suggested VZV isolation based on typical CPE, reason why HSV typing was omitted; afterwards, HSV-1 was demonstrated by immuno-EM.

Table 1. *Results of viral culture (VC) in several lesion types of 67 patients suspected of HSV infection*

Lesion type	Number of VC positive samples (%)	Virus type	
		HSV-1	HSV-2
Maculopapular (n = 1)	0		
Vesicle (n = 11)	11 (100)	11	
Pustule (n=30)	19 (63)	15*	4
Ulcer (n=24)	10 (42)	8	2
Crusted (n= 1)	1	1	
All samples (n=67)	41 (61)	35	6

*In one patient, herpes infection was confirmed by the typical CPE in VC, suggestive for VZV. Afterwards, HSV-1 typing was done by colloidal gold immuno-EM.

Comparison of classical herpes virus detection by EM and ultracentrifugationenhanced EM

Because this study covers different types of lesions, including ulcerous lesions, the method for sample processing had to be standardized for optimal recovery of virus particles. To find evidence for improvement of herpes virus detection after sand homogenization and ultracentrifugation treatment of the lesion samples, the following experiment was carried out.

Duplo-samples of a vesiculopustular (T5–3625), a pustular (T5–2755) and an ulcerous lesion (T4–3594) with positive TC and positive EM were obtained from 3 different patients and used for comparison of the classical EM technique with the ultracentrifugation-enhanced EM method.

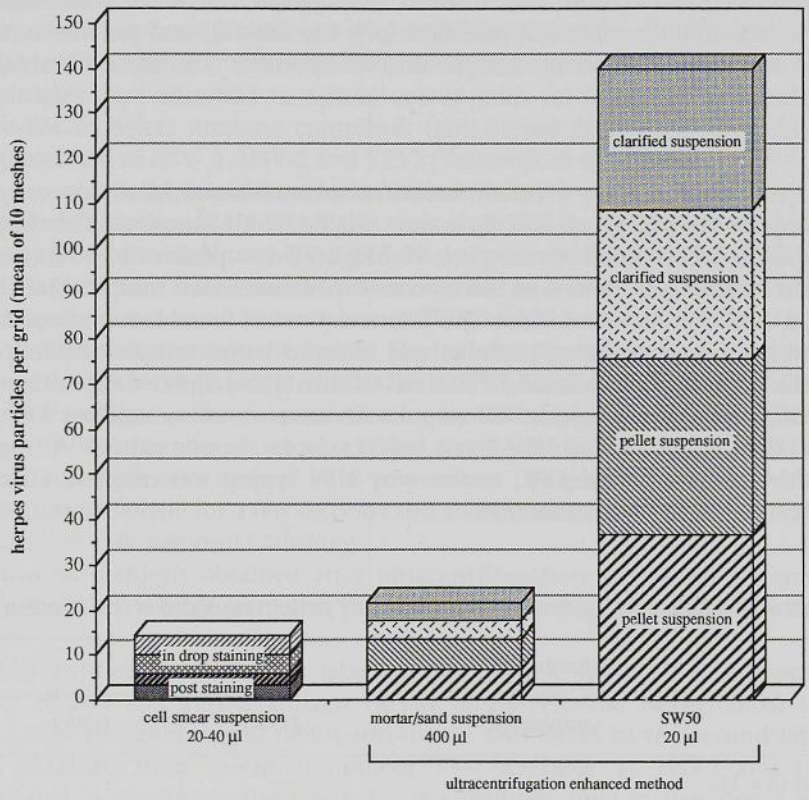

Fig. 1. Herpes virus particle counts obtained with classical direct EM on smears (left stack) and particle counts at 2 different stages of the ultracentrifugation-enhanced method (middle stack and right stack). The 4 layers of each stack represent the mean particle counts obtained with 4 different grids.

The 3 lesion samples were transferred to a glass slide and transformed into a single smear. Three drops of water, for each lesion 1 drop, were used to produce a mixed virus suspension of approximately 60 µl. One third of this suspension (20 µl) was mixed with an equal volume of PTA to 40 µl for herpes virus detection in smears by classical EM. One third was used for adsorption of virus without addition of PTA and fixed and stained afterwards. The third part was transferred to a prewetted mortar with sand. To this sample the ultracentrifugation-enhanced method was applied.
The results of herpes virus detection are shown in Fig. 1. The number of particles detected after ultracentrifugation increased with a factor 10. No difference in detection of virus particles
was observed before and after clarification of the suspension made from the SW50 pellet, nor caused dilution of the virus suspension to 400 µl or use of sand for tissue homogenization a decrease of virus detection in comparison with herpes virus detection in cell smear suspension by classical EM. Experiments in our EM laboratory with different types of herpes viruses (such as equine herpes virus, unpublished data) also confirmed the usefulness of sand during processing of organ tissue samples. Therefore, tissue disruption by sand was used as a standard procedure for EM.

Solid phase immunoelectron microscopy

Solid phase immuno-EM (SPIEM) was applied to some specimens to test its usefullness in combining virus trapping with virus typing by colloidal gold immuno–EM. Virus trapping from either smears or sand homogenates could not be used for routine virus typing by colloidal gold immuno–EM because of high background labeling. However, SPIEM carried out on pellet suspensions obtained after ultracentrifugation showed a better virus/background-labelling ratio, but was still hampered by a higher background labelling, compared with colloidal gold labelling of viruses directly adsorbed to carbon coated collodionnickel grids (compare Fig. 2 and Fig. 3). Therefore, SPIEM was not fully implemented in this study.

Comparison of standard electron and immuno-electron microscopy with viral culture

The sensitivity of EM (36/67) increased approximately 25% (53/67) after virus concentration by ultracentrifugation. A sensitivity of 79% (95% CI: 67% to 88%) was calculated for the 67 patients with clinically overt HSV infection. The results of VC are compared with those of EM in Table 2. Positive VC with positive EM was obtained in 39 lesion samples. Viral culture detected herpes virus infection in 2 lesion samples which could not be confirmed by EM. In contrast, 14 lesion samples were herpes virus positive by EM but showed negative VC. The difference in sensitivity of VC and EM was statistically signifcant (McNemar test: χ^2= 4.65, P_2= 0.03; 95% confidence interval: 6-23% difference in sensitivity).
In 31 of 53 specimens in which herpes virus was demonstrated by EM, herpes virus typing was carried out by immuno-EM (Table 3). The specimen numbers are presented in a way as to create an overview of different typing levels. Since the electron microscope was available for a limited time during this study, the full range of typing tests could not be applied to all lesion samples. One VC (T5-4091) suggested VZV isolation based on the typical CPE (Table

3: column 3, CPE). Therefore, herpes virus typing was omitted but HSV-1 was clearly demonstrated by immuno-EM. In 7 cases, showing negative VC (Table 3: column 3, nd), immuno-EM was positive for HSV. In one sample (T5-1384), with negative VC, immuno-EM demonstrated VZV infection (Table 3: column 9, VZV). This case was included in the calculations for sensitivity of the tests for detecting herpes virus infection.
Incubation with colloidal gold tagged rabbit IgG anti-human IgA, IgM, and IgG antibodies (Table 3: column 5, aHu) to detect antivirus antibody complexes, was done in 25 lesion samples. Antiviral antibody-coated virus particles (labelled viral envelopes and viral cores) were seen in 15 samples, of which 6 correspond to negative VC. In one lesion sample (T5-1010B) only viral cores were labelled with antivirus antibodies but not viral envelopes.
The total number of lesion samples with negative VC and positive EM was 14 (Table 2).

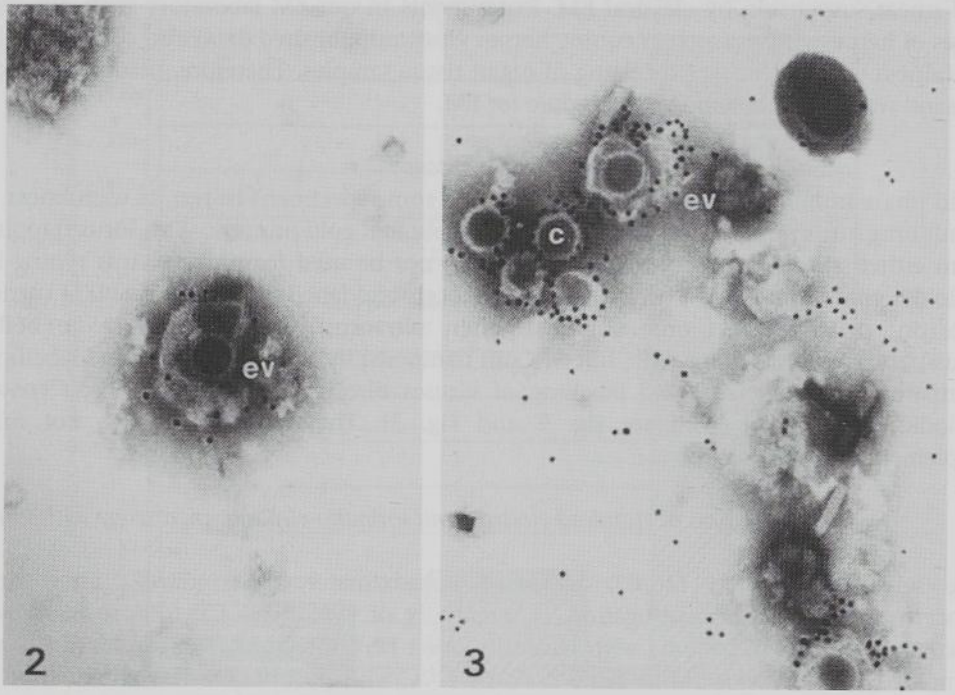

Fig. 2. Immunoelectron microscopy of specimen number T5-1745 (Table 3). Monoclonal antibodies, reactive with common HSV-1 and HSV-2 envelope antigens, were detected with gold labelled anti-mouse antibodies (60 000 x).

Fig. 3. Solid phase immuno-electron microscopy (SPIEM) of specimen number T51745 (Table 3). Virus particles. cores (c) and enveloped virus (ev) were adsorbed to EM grids coated with rabbit anti-HSV antibodies. Trapped virus particles were labelled with anti-HSV-1 monoclonal antibodies, followed by gold labelled anti-mouse antibodies. The efficient antigen trapping, also including viral proteins, is responsible for high background labelling (60 000 x).

Table 2. *Comparison of electron microscopy after virus concentration (EM), viral culture (VC) and Tzanck smear (TS) in 67 patients with clinical symptoms of HSV infection*

Comparison			McNemar	Sensitivity 95% confidence interval
	EM+	EM–	$\chi^2 = 4.65$	6% to 23% difference in sensitivity
VC+	39	2	$P_2 = 0.03$	
VC–	14	12		
	EM+	EM–	$\chi^2 = 4.27$	1% to 20% difference in sensitivity
TS+	41	3	$P_2 = 0.04$	
TS–	12	11		
	VC+	VC–	$\chi^2 = 0.21$	–9% to 17% difference in sensitivity
TS+	33	11	$P_2 > 0.10$	
TS–	8	15		

Eight samples out of these 14 were investigated by immuno–EM and are shown in Table 3 (column 3; nd). In the remaining 6 patients with negative VC, herpes virus infection was demonstrated by EM without further virus typing, which implicates that some of these samples may well contained VZV. A positive Tzanck smear was obtained by all three investigators in 4 of these 6 cases and a negative result in 1 patient; in 1 smear (T5-0004, Table 5) a positive Tzanck smear was obtained by two investigators. The sensitivities of EM and VC were compared for the 55 herpes virus proven lesion samples by either EM, VC or both tests (Table 4).

Tzanck smear

The three investigators EF, APO and JND obtained positive Tzanck smears in 47, 46 and 44 smears, respectively ($n = 67$). We chose the results of JND with the least number of positive scores for statistical calculations and comparisons. This will not overestimate the sensitivity of the Tzanck smear. The sensitivity of the Tzanck smear in the 67 patients with clinically overt HSV lesions is 66% (95% confidence interval: 53-77%). In Table 2 the results of the Tzanck smears are compared with VC and EM. There was disagreement between the Tzanck smear results and VC in 19 cases while for EM disagreement arose in 15 cases. The sensitivities of the Tzanck smear and VC in this study for detecting herpes virus infection were about equal with no signifcant statistical difference (McNemar test: $\chi^2 = 0.21$; $P_2 > 0.10$; 95% confidence interval: –9% to 17%). The difference in sensitivity of Tzanck smear and EM was statistically signifcant (McNemar test: $\chi^2 = 4.27$, $P_2 = 0.04$; 95% confidence interval: 1–20% difference in sensitivity).

Table 3. *Comparison of viral culture, electron microscopy after virus concentration (EM) and immuno-EM at various virus typing levels after application of colloidal gold-tagged antibodies*

Specimen number	Site and type of lesions	Viral culture HSV type	Results of EM (positive/negative)					
			EM	Immuno-EM				
				aHu	aVZV	MCA1	MCA12	Virus type
T4-3537	lip, pustule (m)	nd	+	+	–	+	+	HSV-1
T4-3539	lip, vesicle (m)	1	+	#	#	+	+	HSV-1
T4-3603	face, vesicle (s)	1	+	–	–	+	+	HSV-1
T5-0299	lip, ulcer (m)	nd	+	+	#	+	+	HSV-1
T5-0301	natis, pustule (s)	1	+	#	#	+	+	HSV-1
T5-0302	lip, vesicle (m)	1	+	+	–	+	+	HSV-1
T5-0429	face, crusted (s)	1	+	+	–	+	+	HSV-1
T5-1686	lip, pustule (m)	1	+	+	–	+	+	HSV-1
T5-1745	penis, ulcer (s)	1	+	–	–	+	+	HSV-1
T5-1746	lip, pustule (m)	1	+	+	–	+	+	HSV-1
T5-2755	face, pustule (s)	1	+	+	–	+	+	HSV-1
T5-3186	face, pustule (s)	nd	+	+	–	+	+	HSV-1
T5-3187	lip, ulcer (m)	nd	+	+	–	+	+	HSV-1
T5-4091	face, pustule (s)	CPE	+	+	–	+	+	HSV-1
T4-3629	lip, pustule (m)	1	+	–	–	+	#	HSV-1
T4-3739	lip, vesicle (m)	1	+	–	–	+	#	HSV-1
T5-0163	lip, vesicle (m)	1	+	+	–	+	#	HSV-1
T5-0234	vulva, ulcer (m)	1	+	–	–	+	#	HSV-1
T5-0482	face, pustule (s)	1	+	–	–	+	#	HSV-1
T5-1100	trunk, pustule (s)	nd	+	+	–	+	#	HSV-1
T5-1101	face, pustule (s)	1	+	–	–	+	#	HSV-1
T5-0621	penis, ulcer (m)	2	+	–	–	–	+	HSV-2
T4-3580	lip, pustule (m)	1	+	–	–	#	#	HSV
T5-0398	vulva, pustule (s)	nd	+	+	–	#	+	HSV
T5-0708	lip, pustule (m)	1	+	#	–	#	#	HSV
T5-0709	lip, pustule (m)	1	+	+	–	#	#	HSV
T5-0883	vulva, pustule (s)	nd	+	–	#	#	+	HSV
T5-lOlOA	face, vesicle (s)	1	+	#	–	#	#	HSV
T5-lOlOB	natis, pustule (s)	2	+	e-\c+	–	#	#	HSV
T5-1267	lip, vesicle (m)	1	+	#	–	#	#	HSV
T5-1384	face, ulcer (s)	nd	+	#	+	#	–	VZV

(m) = mucous membrane; (s) = skin; CPE = cytopathological effect; nd = no virus detected; # = not tested; aHu = rabbit IgG anti-human (IgA,IgM,IgG); aVZV = human anti-VZV antibodies; MCA1 = monoclonal antibodies against HSV-1; MCA12 = monoclonal antibodies against HSV-1 and HSV-2; e = viral envelope; c = viral core.

The Tzanck smear sensitivities obtained by investigators EF, APO (dermatovenereologists), and JND (cytotechnologist) for the HSV proven cases were 86% (47/55), 80% (44/55) and 76% (42/55), respectively (data of JND are listed in Table 4: all stages). In 4 patients with no proven herpetic infection by VC or EM (Table 5: T4/3385-T5/2871), the investigators APO and JND obtained 2 positive Tzanck smears in ulcerous mucous membrane lesions. The specificity was 83% (10/12). When the McNemar test was applied, no significant differences were demonstrated between the Tzanck smear results obtained by the three investigators ($P_2 > 0.10$).

Table 4. *Comparison of sensitivities of electron microscopy after virus concentration (EM), viral culture (VC) and Tzanck smear (TS) in detecting herpesvirus infection in 55 lesion samples that were positive in either VC or EM*

Site and stage of lesions		Sensitivity (%)		
		EM	VC	TS*
Mucous membrane				
	oral cavity (n=4)	75	100	50
	lip (n=23)	96	78	87
	genitals (n=6)	100	67	67
	all lesions (n=33)	94	79	79
Skin				
	genitals (n=3)	100	33	67
	other (n= 19)	100	74	74
	all lesions (n=22)	100	68	73
Lesion type				
	vesicule (n = 11)	91	100	91
	pustule (n=27)	100	70	85
	ulcus (n= 16)	94	63	56
	crusted (n= 1)	nr	nr	nr
	all stages (n=55)	96	75	76

*Results obtained by investigator JND; nr, not relevant.

Discussion

Rapid diagnostic techniques for the detection of HSV and VZV are needed for optimal therapeutic management, especially for seriously ill patients. When VC is used for a confrmation test, tissue cultures should be inoculated shortly after collecting lesion samples, but this is generally only possible in university hospitals with laboratories close by. In the present study, because all samples of vesicular lesions were positive in VC (sensitivity 100%; Table 1), we concluded by approximation that the storage and transport conditions of all lesion samples were optimal.

The Tzanck smear is widely used in dermatovenereological practice, because it can give almost instantly information about the disease. Moreover, the Tzanck smear sensitivity and specificity in detecting herpes virus infection compares favorably with those of VC and immuno-fluorescence tests (Solomon, 1988). The Tzanck smear is not specific, which means that it cannot differentiate between HSV and VZV. A second drawback is that the test cannot be applied to detect herpes virus infections in patients with asymptomatic viral shedding.
Several immunological techniques have been developed for detection of HSV antigen. Although these tests are rapid, they are not as reliable as the standard cell culture technique.

Table 5 *Interobserver discrepancies of the Tzanck smear in 67 patients with clinical signs of HSV infection combined with the results of viral culture (VC), and electron microscopy after virus concentration (EM)*

Specimen number	Site and type of the lesion	Results laboratory tests (positive / negative)				
		VC	EM	Tzank smear		
				Observers		
				EF	APO	JND
	Mucous membrane					
T4-3580	lip, pustule	+	+	–	–	+
T4-3665	lip, ulcer	+	+	+	–	–
T5-0163	lip, vesicle	+	+	+	+	–
T5-0299	lip, ulcer	–	+	+	+	–
T5-2855	oral cavity, ulcer	+	+	+	–	+
T4-3385	oral cavity, ulcer	–	–	–	+	–
T4-3723	oral cavity, ulcer	–	–	–	+	–
T5-0970	penis, ulcer	–	–	–	–	+
T5-2871	lip, ulcer	–	–	–	–	+
	Skin					
T4-0301	natis, pustule	+	+	+	+	–
T4-3594	face, ulcer	+	+	+	–	–
T5-0004	natis, pustule	–	+	+	+	–

However, Dascal et al. (1989) introduced an enzyme immunoassay kit that is as sensitive as the viral culture technique. Cao et al. (1989) were able to rapidly detect cutaneous HSV infection with the polymerase chain reaction, but the sample collection in this study necessitates a punch biopsy. We have also tested lesion samples in commercially available ELISA kits, but the results were disappointing, the reason why EM was used as an alternative confirmation technique.

Electron microscopy is expensive and requires considerable experience. It is not available in most diagnostic laboratories, but it can rapidly and specifically diagnose herpetic infection in crude vesicle fluid of lesions (Almeida, 1962). This classical method, still in use by some EM laboratories, is based on detection of particles in suspensions made directly from smears. This technique might work well with lesion samples that largely consist of vesicle fluid and do not need a follow-up with gold labelling techniques. In addition, a large number of lesions are pustular and ulcerous and produce unreliable results with classical EM. The large amount of soluble proteins in such virus suspensions interferes with virus adsorption. The proteins adsorbed to the grid contain a lot of soluble viral antigens that make these grids unsuitable for successive virus labelling. Smith et al. (1962a,b) studied trypsin-chymotrypsin treatment of samples and agar f ltration followed by virus detection in pseudoreplicas (Sharp 1960; Palmer 1975) to increase virus counts. These techniques are not suitable for the routine laboratory.

Ultracentrifugation methods based on the Beckman Airfuge, for more than 12 years in use in our laboratory, had not resulted in acceptable detection levels in other studies. Moreover, this method is hampered by excessive contamination of viruses on the grids with cellular material. Therefore, these grids cannot be used in colloidal gold labelling procedures.

Solid phase immuno-EM (SPIEM) can further enhance the sensitivity of virus detection by EM and is even more sensitive than ELISA (Kohn et al., 1985; Van Nieuwstadt et al., 1988; El-Ghorr et al., 1988). In SPIEM, the virus is picked up by a bilayer of protein A and capture antibody adsorbed to the EM grid (Kohn et al., 1985). The improvement of transmittable gastroenteritis (TGE) virus detection in faeces by SPIEM was at least 100 fold, compared with standard EM (Van Nieuwstadt et al., 1988). El-Ghorr et al. (1988) showed that this EM-adapted 'ELISA technique' can easily be combined with gold labelling techniques. Our studies indicated that this technique can also be used for herpes virus diagnosis (Fig. 3), and the 2 samples that were missed by EM in this study (Table 2) might have been detected in this way. SPIEM certainly will increase herpes virus detection levels. However, SPIEM gold labelling profiles are not consistent because background levels depend largely on the amount of soluble antigen present in a specifc lesion sample. Therefore, it is not advisable to apply SPIEM-gold labelling methods directly to the crude tissue suspension, but labelling profiles will be better when carried out on ultracentrifugationtreated virus suspensions.

Interference of proteins present in the virus suspension with virus adsorption is diminished by dilution of the sample with water or 10 mM Tris buffer. This probably contributed to the high virus particle count observed after diluting the virus suspension 15-20 times to approximately 400 µl. In addition, we found evidence that the use of sand and the effect of short sonication greatly facilitates herpes virus release from cell fragments and improves the detection of equine herpes viruses in the organ tissues of aborted foetuses (unpublished observations). Therefore, we assume that the use of sand facilitates the release of viruses from all types of HSV lesions. Taking into account the above considerations, the improvement of detection by a factor 10–12 after centrifugation through tartrate was no surprise. Moreover, protein-free virus suspensions after ultracentrifugation make all successive gold labelling steps more easy to interpret and will give more reliable and reproducible results.

The immuno-gold labelling data obtained from this study and the data presented in our previous studies confined to VZV (Folkers et al., 1989; Vreeswijk et al., 1988) indicate that EM can diagnose rapidly HSV and VZV infection in skin and mucous membrane lesions, even in cases where VC fails. Therefore, EM must still be considered as an important tool for research and diagnostic laboratories.

Vesicles contain their highest titres of virus within the first 24–48 h (Spruance et al., 1977). In this study the sensitivity of VC for vesicular, pustular and ulcerous lesions (in 55 proven herpetic specimens) was 100%, 70% and 63%, respectively (Table 4). Thus, viral shedding seemed to depend on the stage and duration of the herpetic lesions. However, a relation cannot be found between the lesion type and the presence of human (IgA, IgM, IgG) antiviral immunecomplexes in EM preparations (Table 3), which implies that we cannot explain a decrease of VC sensitivity simply by circulating anti-HSV antibodies, which may have penetrated into the lesions and neutralized the viruses in the final stages of the disease. Since all samples with negative VC, except one, showed viral immune complexes in EM, antibodies may well have played a role in virus inactivation in VC.

In all patients with anti-virus immune complexes, viral cores as well as labelled envelopes were observed. In one patient (Table 3: T5-1010B), however, the viral envelopes were not labelled. In recent EM studies (unpublished observations) we found that anti-viral core antibodies of the IgG class can persist for a long time (several months or even much longer) after anti-viral envelope antibodies have disappeared. Since the patient with specimen number T5-1010 suffered from recurrent infections, a high level of anti-viral core antibodies may have persisted from earlier outbreaks.

In the present study, the overall sensitivity (in 67 clinical specimens) of the Tzanck smear was 66%, and that of VC 61%, with statistically no significant difference when the McNemar test was applied (χ^2=0.21; P_2> 0.10). To further study the difference in sensitivity between Tzanck smear and VC the 95% confidence interval was calculated (Gardner et al., 1989). The difference in sensitivity ranging from –9% to 17% (Table 2) implies that both tests have about equal sensitivities, but due to the limited sample size in this study a difference in sensitivity may be as high as 26%.

Compared to VC, the sensitivity of the Tzanck smear also depends on the stage of the lesion from which the test sample was collected and was 91% in detecting virus infected epithelial cells in vesicular lesions (Table 4). These findings confirmed our earlier results (Oranje et al., 1986; Folkers et al., 1988). However, EM sensitivity did not seem to have a relation with the stage of the lesion (Table 4). Therefore, we conclude that EM was the proper confirmation test for VC and TS in this group of patients. The data presented in Table 5 summarize the TS discrepancies between 3 observers. In 55 smears there was full agreement about the final diagnosis (+/+/+ or –/–/–). The results indicate that reliable sensitivity values can be reached if a second opinion is part of the medical diagnostic strategy.

The present study updates the diagnostic value of electron microscopy, viral culture and Tzanck smear for HSV diagnosis with special attention to the importance of EM as a rapid confirmation technique in special cases where we cannot rely on other laboratory tests. The use of ultracentrifugation-treated virus suspensions is crucial for obtaining reliable results in immuno-electron microscopy. Although new and more sensitive laboratory tests are under way, such as for instance the polymerase chain reaction, and each of them having their own specific drawback, improved EM techniques can still be of practical use for sensitivity and specificity studies.

Acknowledgement

The authors gratefully acknowledge the statistical advice of Dr. H.J.A. Schouten (Department of Medical Informatics and Statistics, University of Limburg, Maastricht).

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3.3 Rapid diagnosis in varicella and herpes zoster: re-evaluation of direct smear (Tzanck test) and electron microscopy including colloidal gold immuno-electron microscopy in comparison with virus isolation

Summary

The Tzanck test and electron microscopy with the technique of colloidal gold labelling in varicella-zoster virus (VZV) infections were compared with virus isolation in 54 patients with clinically suspected varicella or herpes zoster infection. The Tzanck test and direct electron microscopy can determine whether or not an eruption is herpetic but cannot distinguish between herpes simplex virus (HSV) and VZV infection. However, colloidal gold immunoelectron microscopy, using monoclonal antibodies against HSV and anti-VZV IgG, can distinguish between these two herpes viruses. This achieves the same specificity as virus isolation followed by virus neutralization or virus typing using immunofluorescence techniques. The Tzanck test was positive in 91%, virus isolation, under optimal conditions of sampling and transportation, in 80% , direct electron microscopy (negative staining) in 80%, and colloidal gold immuno-electron microscopy after a virus concentration procedure in 95% of the cases. The colloidal gold technique offers a rapid diagnosis in patients with suspected VZV infection.

Folkers E [1], Vreeswijk J [2], Oranje AP [3], Duivenvoorden JN [4]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[2] Central Veterinary Institute, Department of Virology, Laboratory of Electron Microscopy, Lelystad, The Netherlands
[3] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University, Rotterdam, The Netherlands
[4] Department of Pathological Anatomy (Cytology), Hospital 'de Heel', Zaandam, The Netherlands

(Published in: Br J Dermatol 1989; 121: 287-296)

Introduction

In most instances, varicella and herpes zoster can be readily diagnosed. In difficult cases the confirmation of a VZV infection by laboratory tests may be necessary; the commonly applied technique of virus isolation of VZV is specific, but not so good when compared with the isolation of HSV virus. The isolation of VZV is only reliable under optimal conditions of sampling, transportation and storage but needs days and sometimes weeks to confirm.[1] Counter immunoelectrophoresis, immunofluorescence, and ELISA techniques can also differentiate between HSV and VZV infection.[2,3,4] Monoclonal antibodies to VZV antigens are becoming more available, but as yet are not widely used.[5]

A rapid and easy additional procedure is the cytopathological diagnosis, that can support clinical diagnosis of herpetic infection and is based on the Tzanck test. This test is diagnostic for HSV and VZV, but cannot differentiate between the two infections.[6,7] Solomon *et al.*,[1] in their study of the applicability of the Tzanck test in comparison with virus isolation, used the clinical diagnosis of VZV infection as a standard reference, but the vesicular appearance and dermatomal distribution of the herpetic eruption cannot provide a sufficient basis for making a clear differentiation between HSV and VZV infection.[8] Even virus isolation of HSV does not reach 100% sensitivity in typical cases.[9] Therefore, virus isolation cannot be accepted as the only verification standard in detecting or excluding VZV infection. Clearly, there is a need for a rapid supplementary tool for revaluating the Tzanck test in clinical diagnosis of VZV infection. This can be obtained by colloidal gold immuno-electron microscopy, which differentiates between HSV and VZV.[10]

We present the results of a study that illustrates the usefulness and reliability of the Tzanck test with direct electron microscopy (negative staining) and colloidal gold immuno-electron microscopy as compared with virus isolation.

Material and methods

Patients

Samples were collected from skin lesions of 54 patients, during the period from December, 1984 to March, 1986. Eleven of the patients, suspected of having a VZV infection, were attending the out-patient department of Pediatric Dermatology or were hospitalized in the Sophia Children's Hospital Rotterdam (SCH), and 43 were attending the out-patient department of Dermatovenereology or were hospitalized in Hospital 'de Heel' in Zaandam (HdH). The lesions were classified as follows: maculopapular (MP), vesicular (V), pustular (P) and/or crusted (C). The SCH-group and HdH-group together had 11 patients (aged 0–10 years) clinically suspected of having varicella and 43 patients (aged 2–86 years) with symptoms of herpes zoster.

Virus isolation

The vesicular or pustular lesion was opened with a scalpel blade and the fluid collected on a swab which was then transferred immediately to a viral transport medium. In the case of crusted lesions, the crusts were removed and a swab taken from the erosion. With maculopapular lesions, the affected skin was scraped intensively with the edge of the scalpel blade to obtain a superficial erosion and from this a swab was taken.

Swabs obtained from patients of the SCH-group were inoculated in 3 ml transport medium (Dulbecco's modification of Eagle's medium (DMEM) with IO% fetal bovine serum and antibiotics). Within 0.5 h after collection the samples were inoculated into tube cultures of HEL (Human Embryonic Lung) fibroblasts (0.2 ml/tube, 2 tubes/specimen). Virus isolation was attempted at 37°C for a maximum of 2 weeks and cells were scored daily for a cytopathic effect (CPE). Identification of isolated viruses was performed in an immunofluorescence test using monoclonal antibodies against herpes simplex viruses and a polyclonal antiserum against VZV (J. Choufoer-Habova, Department of Virology, SCH). In case of negative results, a second passage was performed.

Swabs obtained from patients of the HdH-group were inoculated in 3 ml transport medium (Hank's balanced salt solution (HBSS) with 0·5%, gelatin, sodium bicarbonate to adjust pH to 7·2, and antibiotics) and transported at room temperature within 24 h to the Department of Virology, GG&GD Amsterdam (Head: Dr R.A. Coutinho). If transportation of the specimen could not be done within 24 h, the sample was inoculated into the transport medium, stored at –80°C within 0·5 h after collection, and then transported afterwards in this frozen condition to the laboratory. The samples were then immediately inoculated into cultures of HEL fibroblasts. The cells were examined for the presence of VZV specific CPE. If no CPE was obtained after 3 weeks of culturing, a second passage was done for another 3 weeks.

Tzanck smear test

Scrapings from the base of the vesicles, pustules, or erosions, were smeared on to a glass slide and air-dried. The material was fixed in methanol and stained within 0·5 min with Hemacolor® (Merck). The slide was dipped five times in methanol, three times in eosin, and three times in thiazine. The slide was then rinsed with water and air-dried and a permanent mounting medium and coverglass applied. The smear was then examined under light microscopy.[11]

Epidermal cells were scored for characteristic nuclear changes as seen in herpetic infections. These nuclear changes included enlargement, multi-nucleation and crowding of the nuclei with nuclear 'moulding'. Different stages of peripheral margination of the nuclear chromatin were seen, and the ground substance of the nucleus was more coarse or showed an opaque appearance (ground glass aspect). In addition, mononuclear, non-giant cell virocytes were recognized on the basis of the nuclear changes described above. Sometimes intranuclear inclusions, surrounded by a prominent halo, were observed.[7,12] All preparations were examined by one of the authors and were then examined 'double-blind' by two others.

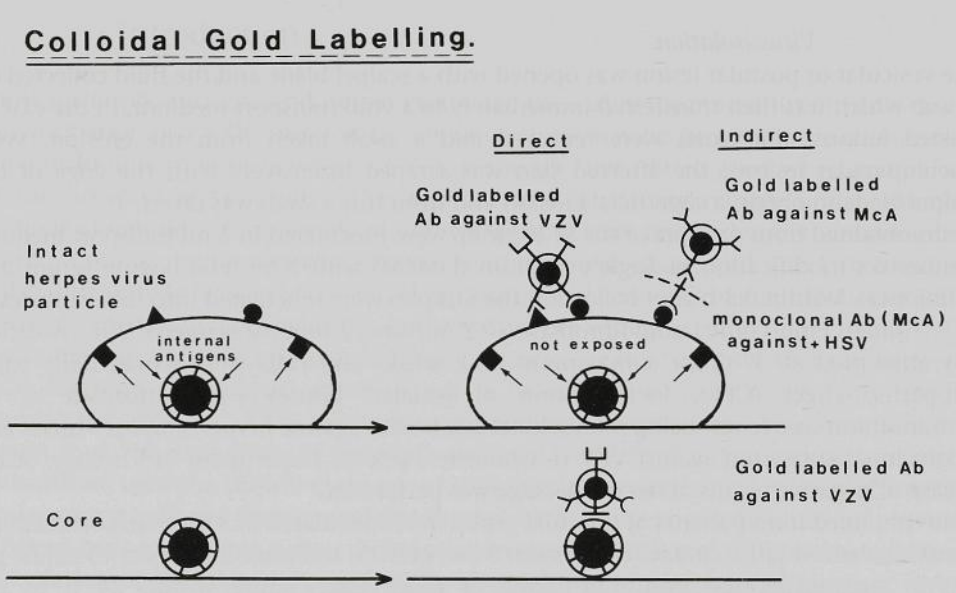

Fig. 1. Schematic representation of colloidal gold labelling steps used in this study. A direct labelling procedure was used for identification of VZV envelopes and cores. An indirect labelling technique was applied to differentiate VZV from HSV.

Electron and immuno-electron microscopy

After obtaining samples for virus isolation and for the Tzanck test, the lesion, or an adjacent one was scraped intensively with the curved side of a vaccinostyle, fixed with the scarification side in the hollow of the cap of a plastic sampling tube. The cap was replaced on the tube which contained a small moistened gauze. Within 0·5 h after sampling, the material was frozen to –25 °C in a normal freezer and transported in frozen condition immediately or within 2 weeks to the laboratory for electron microscopy. At the laboratory, the sample was frozen to –80°C, if storage for a longer period was necessary. Without prior knowledge of the clinical appearance, the results of the Tzanck test or virus isolation, identification of the virus by electron microscopy (EM) and virus typing by colloidal gold immuno-electron microscopy (CG-IEM) was carried out.[10]

The CG-IEM technique can be described briefly as follows. The sample was homogenized in a mortar with sterile sand and diluted with 10 mM Tris-HCl (pH 7·3) to about 500 µl. The sand was pelleted by low-speed centrifugation in a Beckman desk microfuge (maximum speed, 3 s). The virus particles present in the supernatant were adsorbed to the carbon side of collodioncarbon-coated nickel grids (10 min) and stained with 2% phosphotungstic acid (pH 6·8). Virus identification was performed in a Philips EM 300 electron microscope at a voltage of 80 kV. If no virus particles were detected, virus concentration was carried out by centrifugation of the remaining supernatant through a 70 µl 30% potassium tartrate cushion in a Beckman SW 50 rotor (32 000 *g*, 30 min.) The pellet was suspended in 20 µl

10 mM Tris-HCl and sonicated in closed tubes in a cuphorn attachment of a Branson sonifier.[10] Virus adsorption, staining, and virus identification were done as described above. After virus adsorption, the grids were incubated with highly specific gold-tagged, polyclonal antibodies against VZV (showing no cross-reactions with envelopes and cores of HSV, cytomegalovirus, and Epstein-Barr virus) for 1 h at room temperature (Fig.1). The preparation and the specificity of the gold-tagged antibodies have been previously discussed.[10] After incubation, the grids were thoroughly washed with PBS and stained with phosphotungstic acid. The gold particles of the anti-VZV gold conjugate can be seen as electron-dense granules around cores and enveloped particles (Figs 2 and 3). If no reaction with the anti-VZV gold conjugate was seen, HSV typing was carried out using monoclonal antibodies in the first step and gold-tagged, rabbit-anti-mouse antibodies in the second step (Fig. 1).[10]

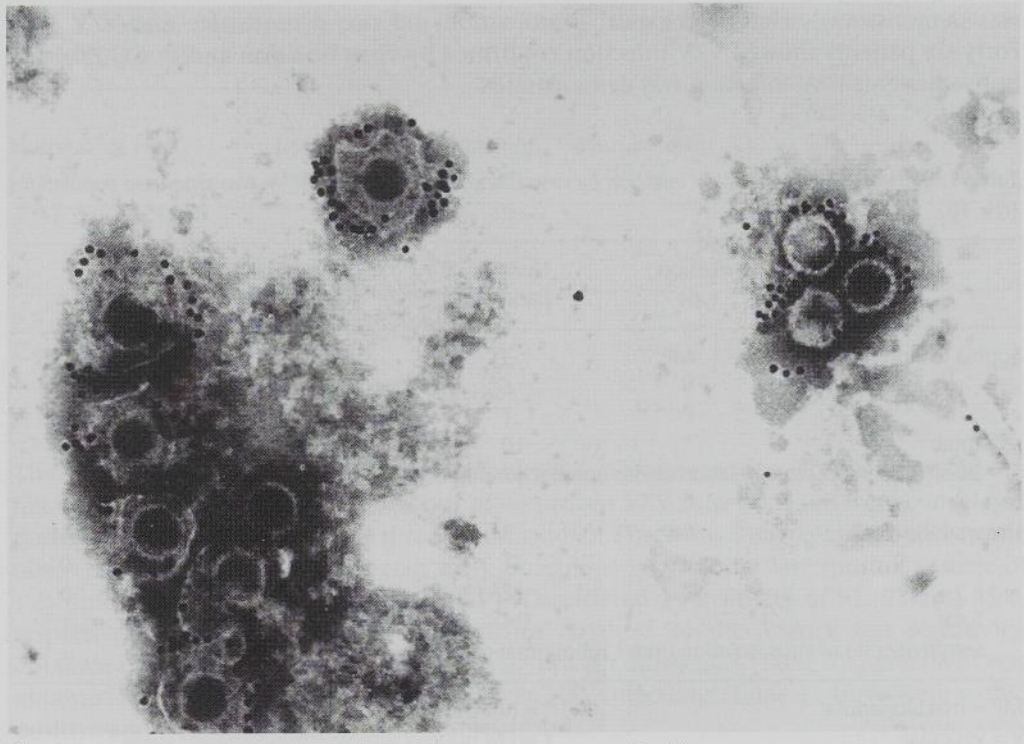

Fig. 2 Fig. 3

Figs. 2 and 3. Gold-tagged polyclonal antibodies against VZV labelled both viral envelopes (Fig. 2) and viral cores (Fig. 3)(x 60 000).

Statistical analysis

McNemar's test[13] was used to compare positive and negative scores obtained from the Tzanck tests as determined by different investigators (EF, APO and JND).

Results

Fifty-four patients were selected on the basis of clinical symptoms of VZV infection. Laboratory tests, however, demonstrated herpes virus infection in 51 patients. The clinical diagnosis 'VZV infection' could not be maintained in three cases. One child with a late stage of varicella had pustules and crusted lesions and the diagnosis has been changed to impetigo. One child had an impetigo with pustules and a localized form of staphylococcal scalded skin syndrome (SSSS). The other patient was suspected as having an early maculopapular stage of VZV infection and on skin biopsy a vasculitis of unknown origin was demonstrated. Here, serological examination did not demonstrate anti-VZV IgM. Forty-six patients showed VZV infection confirmed by virus isolation and/or CG-IEM and in five patients HSV infection was demonstrated.

Table 1. *Patients with proven VZV infection by virus isolation and/or CG–IEM, in two separated populations (n = 46)*

	Lesion type	Number of patients	Virus isolation +	VZV –	CG-IEM +	VZV –
Sophia Children's Hospital	MP	3	2	1	3	0
	V	6	5	1*	5	1*
	P	1	1	0	1	0
Total		10	8	2	9	1
Sensitivity (%) of virus isolation under optimal conditions, 80%						
Hospital 'de Heel'	MP	3	0	3	2	1†
	V	21	4	17	21	0
	P	12	1	11	12	0
Total		36	5	31	35	1
Sensitivity (%) of virus isolation under suboptimal conditions, 14%						

MP = maculopapular.
V = vesicular.
P = pustular.

* = not the same patient.
† = EM positive afterwards in vesicular stage.

Virus isolation

The results of virus isolation obtained in the two investigated populations (SCH and HdHgroup) are shown in Tables 1 and 2. In the SCH-group (n = 11), virus isolation was done under optimal circumstances. VZV infection was confirmed in 10 patients by virus isolation and/or CG-IEM. Virus isolation was positive in eight patients (80%, Table 1). In the HdH-group (n = 43), virus isolation was carried out under suboptimal circumstances (see Methods). Although VZV infection was proven by CG-IEM in 36 patients (including one patient with a positive CG-IEM score afterwards in the vesicular stage of infection), virus could be isolated from no more than five patients (14%, Table 1). In five out of 54 patients with zosteriform herpetic eruptions, HSV was demonstrated in two patients by virus isolation and by CG-IEM; from the other three patients no HSV positive virus isolation could be obtained, but CG-IEM confirmed the presence of HSV particles (Table 2).

Table 2. *Patients, clinically suspected of having VZV infection, with proven HSV infection by virus isolation and/or CG-IEM (n = 5)*

Hospital 'de Heel' (n = 5)	Lesion type	Number of patients	Virus isolation		CG- IEM	
			+	HSV –	VZV	HSV
	P	4	2	2	–	4
	C	1	0	1	–	1

P = pustular.
C = crusted.

Tzanck smear tests

The results of Tzanck smears are listed in Tables 3-5. Agreement between the observers was obtained in 44 Tzanck preparations out of 46 proven VZV infections (42 were considered positive and two negative by all three investigators). There was disagreement between the observers in two cases, involving an early (maculopapular) and a late (pustular) stage of VZV infection. Investigators EF, APO and JND achieved a sensitivity of 91, 93 and 95%, respectively. Using McNemar's test, statistical analysis of the Tzanck test sensitivity, obtained by these investigators, showed no significant differences (Table 3). Agreement was obtained in five Tzanck preparations of proven HSV infections (Table 4): three were scored positive and two negative by all three investigators.

Table 5 summarizes the Tzanck readings of three patients without proven herpetic infection. The observers disagreed in a Tzanck test reading of one child, suspected of having a late stage of varicella with pustules and crusted lesions. Virus isolation and CG-IEM were unable to prove herpetic infection in this case and it was finally diagnosed as impetigo.

Table 3. *Comparison of the Tzanck test scores, obtained by different investigators, in proven VZV infection by virus isolation and/or CG-IEM (n = 46)*

		Number of positive results Tzanck smear			EM	
		Investigators				
Lesion type	Number of patients	EF	APO	JND	Direct	CG-IEM
Maculopapular	6	5	5	6	2	5
Vesicular	27	26	26	26	23	26
Pustular	13	11	12	12	12	13
Total number:	46	42	43	44	37	44
Sensitivity (%)	91*	93*	95*	80	95	

* Using McNemar's test no significant differences were obtained between the Tzanck scores of the investigators ($P > 0.1$).

Table 4. *Comparison of the Tzanck test scores, obtained by different investigators, in patients with a proven HSV infection by virus isolation and/or CG-IEM, clinically suspected of having VZV infection (n = 5)*

		Tzanck test					
Lesion type	Number of patients	Investigator: EF Score: +	−	APO +	−	JND +	−
Pustular	4	3	1	3	1	3	1
Crusted	1		1		1		1

EM and immuno-electron microscopy

In the investigated population ($n = 54$), herpes virus was detected in 40 patients by EM, carried out directly on homogenates of the material obtained from the skin lesions. After a virus concentration step, herpes virus was demonstrated in 49 patients.

The results of EM and CG-IEM in comparison with the Tzanck test are listed in Table 3 and 4. With CG-IEM, varicella-zoster virus was found in 44 cases, and HSV type 1 could be identified in five cases. In one patient with herpes zoster, Tzanck test, virus isolation, and EM examination were negative in the early maculopapular stage; a positive result with CG-IEM was obtained afterwards in the vesicular stage of the infection.

Table 5. *Results of Tzanck tests in patients with clinically VZV infection, without proven herpetic infection (n = 3)*

Diagnosis	Lesion type	Results of Tzanck test obtained by investigator		
		EF	APO	JND
Impetigo	pustular	–	–	+
Impetigo with local SSSS	pustular	–	–	–
Vasculitis	maculopapular	–	–	–

SSSS = staphylococcal scalded skin syndrome.

Discussion

Vesiculobullous eruptions are known to occur during the course of several viral infections, including herpes simplex, herpes zoster, vaccinia (and variola), hand-foot-and-mouth disease and other enteroviral infections. The occurrence of vesicles and vesiculobullous lesions in cytomegalovirus infection has been described,[14,15] and a congenital vesicular eruption due to *Haemophilus influenzae* type B has been observed.[16] Other diseases may also be confused with varicella and herpes zoster.[17,18] However, in most patients a VZV infection can be diagnosed from the typical clinical features.

Disseminated herpes simplex may occasionally resemble varicella. Zoster-like cutaneous manifestations can develop due to HSV infection. Kalman and Laskin[8] reported that 13% of their patients with the clinical diagnosis herpes zoster actually had herpes simplex infections with a facial distribution in four of these six patients. In another report 10%, of patients with clinical impression of herpes zoster, actually suffered from HSV infection as demonstrated by virus isolation.[1] In our study we found that 9% of the patients, clinically suspected of VZV infection, actually had HSV infection. This was demonstrated by virus isolation and CG–IEM. Three of these patients had a facial distribution of their rash and one had involvement of dermatome T12. One child had a generalized varicelliform eruption of HSV infection.

VZV is less tolerant than HSV to transport and storage conditions and the sensitivity of virus isolation in varicella infections at mainly the vesicular stage was 63%. For herpes zoster a sensitivity of 70% in the vesicular stage was obtained and a 40% sensitivity in the pustular stage. In the study of Kalman and Laskin[8] the sensitivity of VZV isolation was 43%. In our previous study,[19] we obtained a sensitivity of virus isolation in varicella of 60% and a sensitivity of virus isolation in five patients with herpes zoster of 100%. In the present study we focused attention on two groups of patients. The first group showed that virus isolation of VZV under optimal conditions could reach a sensitivity as high as 80%. In the second group, representative for the general practitioner, the sensitivity of virus isolation dropped to 14%. Diagnosis on the basis of virus isolation takes several days because the VZV proliferates at a slower rate than HSV. Virus isolation is less suitable for rapid diagnosis.

The Tzanck test has been used for a long time as an aid to confirm the diagnosis of cutaneous and mucosal herpesvirus infections. The typical cytopathological changes in herpesvirusinfected keratinocytes can easily be recognized in early, intact vesicles and pustules. In the very early stage of a herpetic infection the cytological changes can be subtle or equivocal and in the older pustular lesions the necrosis of the infected keratinocytes is obvious and the nuclear details may be unclear. It should be noted that the Tzanck test is not specific for VZV infection but the test can reveal whether or not an eruption is herpetic. Although lesions of VZV show less cell destruction and inflammation in the early stages of infection than those of HSV, these features cannot be used in differential diagnosis.[7,12] In the present study the sensitivity of the Tzanck test was as high as 90%,. Statistical analysis showed no significant differences of Tzanck test sensitivity, obtained by different investigators. The results were largely based on verification with the new immunogold EM technique. A diagnosis of VZV infection can be obtained within 3 h and of HSV infection in 4 h.[10] This technique scored higher than did virus isolation of VZV under optimal conditions. Another advantage of EM is its reduced sensitivity to transport and storage conditions and for this reason, colloidal gold virus typing was used in this study as the standard verification method for the Tzanck test.[10]

Our current results confirm previous conclusions[1] that the Tzanck test is valuable in supporting the clinical diagnosis of VZV infection. It is inexpensive, easy to perform and rapid, but experience and training are required. Proper interpretation of Tzanck tests should not lead to misdiagnoses. Cytopathological re-screening may be an important supplementary diagnostic procedure.[1,19]

Rapid diagnostic techniques in herpetic infections may be necessary for the decision to start antiviral therapy, particularly in cases involving immuno-suppressed or immuno-compromised patients, or in neonatal herpes simplex and neonatal varicella. Since VZV and HSV have varying sensitivity to different antiviral agents, such as acyclovir, early differentiation between these herpes viruses is desirable.[8] In conclusion, this study confirms that the Tzanck test is rapid and reliable in VZV infection, but cannot differentiate between VZV and HSV. Immunofluorescence cytology using monoclonal antibodies, which are now becoming more available, should eliminate this problem in future.[5,20] None the less, the Tzanck test will continue to be important in rapid diagnosis of herpetic skin infections. EM, after a virus concentration step, is another rapid and reliable test in VZV infection. However, the classical EM technique also cannot differentiate between VZV and HSV. CG-IEM provides the possibility for rapid and specific diagnosis in herpetic infections.[10]

Acknowledgements

We thank H.J.A.Schouten (Department of Medical Informatics and Statistics, University of Limburg, Maastricht) for statistical advice, F.Wagenaar (Department of EM, Central Veterinary Institute (CDI), Lelystad) for technical assistance and Prof. J.G.van Bekkum (CDI) and Dr A.L.J.Gielkens (CDI) for their interest in the work.

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3.4 Immunoelectron microscopy for rapid diagnosis of varicella-zoster virus in a complicated case of Human T-Cell Lymphotropic Virus type 1 infection

Summary

Rapid techniques for the detection of herpes simplex virus (HSV) and varicella-zoster virus (VZV) are needed for optimal therapeutic management. VZV infection poses a serious threat, especially to seriously ill patients, for instance, immunocompromised patients. We report a case of human T-cell lymphotropic virus type 1-positive leukemia complicated by atypical multidermatomal herpes zoster. Viral culture and standard serological tests failed to prove VZV infection. Herpesvirus infection was confirmed by cytodiagnosis (Tzanck test). The final diagnosis of VZV was made by immunoelectron microscopy (IEM), which can differentiate between HSV and VZV. Immunoglobulin M antibodies in serum directed against VZV were detected by IEM but not by immunofluorescence. Because IEM was able to identify virus and analyze sera in only 2 h, it is considered a valuable additional tool for the rapid diagnosis of HSV and VZV infections.

Folkers E [1], Vreeswijk J [2], Wagenaar F [2], Kapsenberg JG [3], Hulsebosch HJ [4], Oranje AP [5]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[2] Department of Virology, Central Veterinary Institute, Lelystad, The Netherlands
[3] National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands
[4] Department of Dermatology, University of Amsterdam, Amsterdam, The Netherlands
[5] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University, Rotterdam, The Netherlands

(Published in: J Clin Microbiol 1992; 30: 2487-2491)

Introduction

Most varicella-zoster virus (VZV) infections can be readily diagnosed by characteristic clinical appearance. However, because vesiculobullous eruptions of other viral and nonviral skin infections can resemble those of VZV, the infection can be misdiagnosed.[6] Clinical diagnosis of herpes zoster in immunocompromiscd patients is even more difficult, especially in the early stages of the disease, when only a few herpeticlike vesicles may be present, and these may not have the typical dermatomal localization.[11] In addition, such patients may have hyperkeratotic nodules and crusted verrucous lesions caused by aciclovir-resistant VZV.[1, 8, 9,14] In immunocompromised patients, antiviral treatment of varicella and herpes zoster reduces serious complications of the infection.[15] The rapidity of obtaining a conclusive diagnosis is important for adequate antiviral therapy, because treatment delay diminishes the effectiveness of the antiviral drugs.[2, 3, 15] It is important to differentiate between VZV and herpes simplex virus (HSV) at an early stage of the infection to determine the appropriate dose of the antiviral drug. Laboratory tests are used to confirm the clinical diagnosis. Because the classical viral culture usually requires scveral days, cytologic methods such as the Tzanck test are used to support the clinical diagnosis in an early stage. The Tzanck test cannot distinguish between VZV and HSV infections. Serologic tests can be used to detect rises in VZV antibody titers, but they can only confirm a diagnosis retrospectively.

Virus detection methods, such as the enzyme-linked immunosorbent assay (ELISA) and the polymerase chain reaction, are becoming more sensitive and rapid and can even replace classical diagnostic tests. These methods detect antigen (viral proteins or intact viral particles), immunoglobulin M (IgM)-class antibodies, or nucleic acids. Some tests are unable to give information rapidly (viral culture); others may not be sensitive or specific enough (ELISA) or must be used under very strict laboratory conditions (polymerase chain reaction). We demonstrate in the study described here that immunoelectron microscopy (IEM) can give a rapid (2-h) diagnosis that includes virus detection, virus typing, and study of IgG and IgM antibody levels.

Material and methods

Patient

A 33-year-old Moroccan female immigrant presented with a large necrotic skin ulcer on the left thigh that was surrounded by herpetic pustules (Fig. 1). Zosteriform-localized herpetic pustules were observed on the left upper leg, the right arm, and cranially on the right half of the back, while isolated herpetic vesiculopustules were found scattered all over the trunk. The herpetic eruptions persisted for about 6 weeks, at which time she was admitted to our clinic. The Tzanck test was carried out on smears from pustular lesions on the left thigh to confirm the clinical diagnosis of herpes. VZV infection was diagnosed clinically and was later confirmed by IEM. Aciclovir was administered intravenously for the next 8 days. During therapy the herpes lesions became crusted. Five days after aciclovir medication was stopped, however, the patient developed new papular and, later, vesicular lesions in the area of the older crusted lesions. A relapse of the herpesvirus infection was

demonstrated by the Tzanck test, and VZV was again confirmed by IEM. Acyclovir medication was resumed. During acyclovir medication, vesicular and pustular lesions remained positive for herpesvirus infection, as shown by the Tzanck test. Finally, in about 3 weeks, the lesions became crusted and healed. Virus isolation from the skin ulcer and herpetic lesions was repeatedly unsuccessful. The patient's condition worsened steadily. She developed adult T-cell leukemia and clinical symptoms of AIDS. Serologic examinations (human T-cell lymphotropic virus type 1 enzyme immunoassay kit 307724; Abbott) revealed elevated antibodies against human T-cell lymphotropic virus type 1, but tests for human immunodeficiency virus (HIV) were negative. Despite intensive antileukemia treatment, the patient died within 2 months after the onset of the leukemia. Disseminated herpes zoster is frequently associated with the presence of a malignancy or underlying immune deficiency.[1] Herpes zoster occurs seven times more frequently in HIV-infected individuals than in noninfected people and appears early in HIV-induced immunodeficiency.[7] In our patient, the first sign that suggested acquired immune deficiency was a multidermatomal herpes zoster infection with a large necrotic ulcer on the left thigh. This ulcer finally healed after prolonged acyclovir medication.

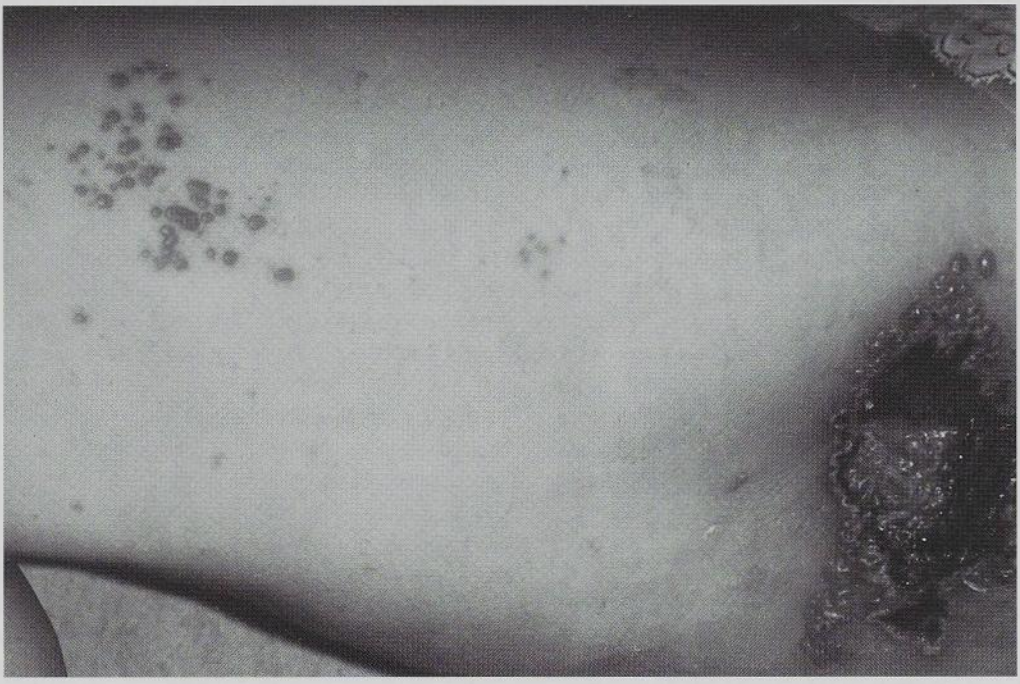

Fig. 1. Necrotic skin ulcer and herpetic pustules on the left leg of the case patient on the day of admission.

Standard virological methods

The Tzanck test was the first laboratory test that diagnosed a clinical herpesvirus infection in our patient and was applied as described before.[4-6,10] Immunofluorescence tests (IFTs) performed on smears of lesions can distinguish between VZV and HSV, but studies that make use of IFT are scarce. Some investigators have used VZV culture as a reference test for determining the sensitivity of these IFTs.[11, 12] The isolation of VZV usually takes days, if the virus multiplies at all, and therefore is inappropriate for sensitivity studies and rapid diagnosis. Although cytodiagnosis by immunofluorescence can be very specific, sensitivity probably is influenced by unpredictable factors caused by the stage of the lesion. Therefore, IFT was not used to detect virus in smears in our study. Herpesvirus isolation and virus typing by immunofluorescence were carried out by standard virological procedures. Recent VZV and HSV infection could not be proven serologically by complement fixation tests (Table 1). IgG and IgM antibody titers were assessed by IFT performed on acetone-methanolfixed HSV- and VZV-infected monolayers. No specific IgM antibodies against VZV were detected by IFT (Table 1).

Electron microscopy

The use of colloidal gold IEM techniques for the diagnosis of VZV and HSV infections has previously been described in detail.[5, 6, 16] Virus particles were incubated with highly specific gold-tagged human antibodies directed against VZV. Monoclonal antibodies against HSV and gold-tagged rabbit antibodies directed against mouse Ig were used to detect HSV and to distinguish between HSV types 1 and 2. The patient's antibodies, which were bound in vivo to the virus particles that were present in the lesions, were detected with gold-tagged rabbit IgG antibodies directed against human Igs. The antibody class was further characterized with monoclonal antibodies directed against IgG and IgM. These monoclonal antibodies were also used to assess the VZV and HSV antibody titer in the serum of the patient. The reference virus strains, test sera, monoclonal antibodies, and colloidal gold conjugates used were those described previously.[16] Two reference serum samples were included in this study (Table 1); one was from a patient with herpes zoster, and one was from a patient with varicella. Serum samples from two healthy people (two of the authors of this report) with no clinical signs of VZV or HSV infection during a period of at least 20 years before testing were used as negative control sera. Pseudorabies virus, a herpesvirus that causes Aujeszky's disease in pigs, was tested by IEM in order to exclude nonspecific reactions.

Results and discussion

The complications observed in our patient required an immediate diagnosis of herpes infection and differentiation between HSV and VZV in order to give the proper acyclovir dosage. The dosage administered for VZV infection is two times the dosage administcred for HSV infection. Virus particles obtained from skin lesions (about 6 weeks after the first clinical signs of VZV infection) were all trapped in virus-antibody complexes (Fig. 2a). Immunogold labeling identified the antibodies as IgG. Virus typing, which was still possible on antibody-coated virus particles[16], revealed the presence of VZV.

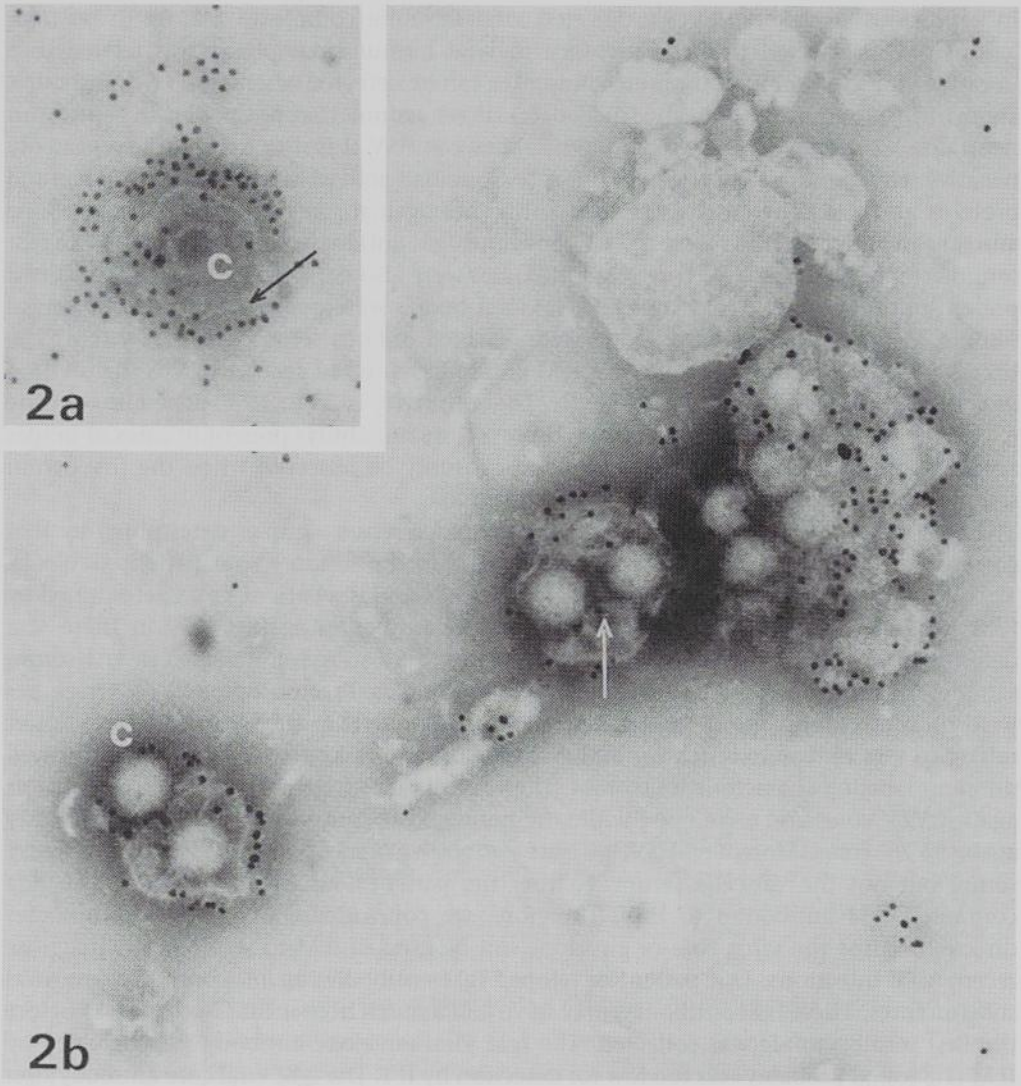

Fig. 2. IEM. (a) VZV particle obtained from the patient's lesion labeled with 18-nm-diameter colloidal gold particles (black dots) coupled to rabbit anti-human antibodies. The gold particles detect patient's antibodies bound to the viral envelope in vivo. c, viral core; arrow, viral envelope with antibody layer. (b) VZV test virus from infected tissue culture cells was first incubated with a dilution of the patient's serum and then with monoclonal antibodies directed against human IgM. The monoclonal antibodies were detected with 18-nm-diameter gold particles coupled to anti-mouse antibodies. c, viral core; arrow, virus particle with two cores. Magnifications, x65,000.

In a previous study on HSV we detected viral immune complexes mainly in pustular lesions.[6] We suspected that the presence of viral immune complexes was related to a negative viral culture. Viral immune complexes can be detected when anti-VZV antibodies appear in the serum (unpublished IEM data). If we assume that antibodies in serum can neutralize VZV in vivo more efficiently than they can HSV, this may explain the repeatedly negative viral cultures in the present case.Colloidal gold IEM detects viral structural proteins and can differentiate between antibodies against viral core and viral envelope antigens in patient serum (Table 1). VZV envelope IgM antibodies were detected in the first serum sample (Fig. 2b). VZV core IgM antibodies were also detected by IEM and remained present in the serum 4 weeks later. HSV IgM antibodies were not detected in either serum sample. VZV and HSV IgG antibodies were detected in both serum samples by IEM. The titers showed little change. Testing for IgM antibodies directed against VZV is the method of choice in the serodiagnosis of varicella. The sensitivity is 27 to 70%, depending on the technique used.[13] These IgM antibodies, however, are not always present in cases of herpes zoster. A rise in titer of VZV IgG antibodies can only be observed when the first serum sample is collected at an early stage of the infection.

The antibody titers against VZV of the two reference serum samples determined by IEM corresponded well to the titers of VZV antibodies in the serum of our patient (Table 1). The titers of VZV IgG antibodies in the reference sera and the patient's sera determined by IEM were in the same range as the titers of IgG antibodies against VZV in these sera determined by IFT. IgM antibodies directed against HSV were not detected in any serum sample, including that from our patient. IgM antibodies directed against VZV envelopes were not detected in the herpes zoster reference serum. The varicella and herpes zoster reference sera that were tested for IgM and IgG antibodies against VZV and HSV showed different labeling characteristics by IEM. The varicella serum had high IgM antibody titers against VZV cores and envelopes, while the herpes zoster serum had IgM antibodies only against VZV cores. Moreover, HSV IgGclass antibodies were detected in the herpes zoster serum but not the varicella serum. Neither the patient's serum nor the reference sera contained IgM antibodies to HSV. Therefore, we concluded that IgM-class antibodies directed against the virus core or envelope can be used in IEM to serologically diagnose recent VZV infections. Our patient developed IgM antibodies against both of these viral substructures. These IgM antibodies may have had a much higher titer in the weeks before the first serum sample was collected. The IgM viral envelope antibody titer detected by IEM (1.8 $\log_{10}$) probably was too low for detection by IFT. The IgM viral core antibody titer (3.6 $\log_{10}$) also was not detected by IFT, although acetone methanol fixation of the test cells allowed the penetration of IgM molecules into the nucleus of the infected cells, where viral cores normally accumulate. The reason for the discrepancy between the IEM and IFT results is not clear.

It may be that a different subset of IgM-class antibodies which cannot be detected by IFT are detected by IEM, and so we can explain why, in the serum of the patient, an IgM anti-VZV titer was not demonstrated by IFT.

High IgG and IgM antibody levels against cytomegalovirus (CMV) were found by IEM. CMV IgM antibodies were not detected in the second serum sample or in human reference sera of VZV-infected patients. Therefore, we assume that in this case, the IgM antibodies against CMV were induced by a coexisting CMV infection, which is common in immuno-

TABLE 1. Serodiagnosis of herpes virus infection in two serum samples from the patient and two reference serum samples

Patient or viral infection (patient age)	Time (wk) after onset of disease	Test virus[a]	Serum Antibody titer in serum determined by[b]:						
			CFT	IFT (log_{10})		IEM (log_{10})			
				IgM	IgG	IgM		IgG	
						Core	Envelope	Core	Envelope
Case (33 yr)	6	VZV	1:16	–	3.0	3.6	1.8	4.0	3.5
		HSV	1:16	–	3.6	–	–	3.8	3.2
		CMV				2.3	2.0	>4.0	3.5
		EBV				–	–	>2.6	2.0
		EBV-CA		–	2.0				
		EBV-NA			+				
		EBV-EA			–				
		PRV				–	–	–	–
Case (33 yr)	10	VZV	1:32	–	3.6	2.0	–	3.5	3.2
		HSV	1:8	–	3.6	–	–	3.5	3.2
		CMV				–	–	>4.0	3.2
		EBV				–	–	>2.6	2.4
Varicella (50 wk)	2	VZV		>2.1	>4	4.4	3.5	3.5	3.5
		HSV		–	–	–	–	–	–
		CMV				–	–	–	–
Herpes zoster (22 yr)	1	VZV		2.7	4.8	3	–	3	4
		HSV		–	4.2	–	–	4	3.8
		CMV				–	–	–	–
Control 1 (43 yr)		VZV				–	–	–	–
		HSV				–	–	–	–
Control 2 (45 yr)		VZV				–	–	–	1.7
		HSV				–	–	–	–

[a] CMV, cytomegalovirus; EBV, Epstein-Barr virus; EBV-CA, Epstein-Barr virus capsid antigen; EBV-NA, Epstein-Barr virus nuclear antigen; EBV-EA, Epstein-Barr virus early antigen; HSV, herpes simplex virus; VC, viral culture; PRV, pseudorabies virus; VZV, varicella-zoster virus.
[b] CFT, complement fixation test; IEM, immunoelectron microscopy; IFT, immunofluorescence test; –, no titer.

compromised patients. Low-level antibody titers against Epstein-Barr virus were detected by IEM. Epstein-Barr virus antibodies were also found by IFT.

Virus typing and diagnosis of viral infection in serum by IEM were completed in about 2 h after sampling and were conclusive for the diagnosis of VZV infection. No other diagnostic technique was as rapid and specific as the IEM. Moreover, the virus was typed and the antibody titer in serum was assessed in one experimental setup. Many hospitals, especially university hospitals, that now use the electron microscope (EM) for pathologic examinations can also use it for rapid virus diagnosis. Because viruses are killed by glutaraldehyde before the EM grids are examined, there is no danger of EM laboratory workers being exposed to infectious viruses.

Acknowledgements

We thank M. Bakker and M. Tersmette, Laboratory of Human Retrovirology, Academic Medical Center, University of Amsterdam, and J. M. M. Lepoutre, National Institute of Public Health and Environmental Protection, Bilthoven. We also thank V. Thatcher and J. G. van Bekkum, Central Veterinary Institute, Lelystad, for critical review of the manuscript.

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Addendum to chapter 3

A simple storage system for EM grids. Quality management in complicated immuno-EM studies for research and routine diagnosis

Introduction

Newly developed electron microscopy (EM) methods for virus research and routine virus diagnosis comprise trapping of viral antigens on antibody coated grids (solid-phase immunosorbent EM, SPIEM)[1, 2] and colloidal gold labeling (colloidal gold immuno-EM, CGIEM)[3]. Immuno-EM can offer high sensitivity (SPIEM) and high specificity (CGIEM) in specially designed validation studies for new laboratory tests and for rapid diagnosis of virus samples, even in cases when other methods are not currently available.[4, 5] SPIEM and CGIEM also can be applied in combination on one single grid which permits catching of the antigen and characterization of the antigen by immunolabelling to take place in one session.[6] In this way coronaviruses or their characteristic peplomers could be extracted from faeces and tested for the presence of the characteristic protein using monoclonal antibodies.[5]

Folkers E[1], Vreeswijk J[2]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands
[2] Central Veterinary Institute, Department of Virology, Laboratory of Electron Microscopy, Lelystad, The Netherlands

During the studies carried out for this thesis a quality management problem arose during administration and storage of large amount of EM grids. Often 30-50 grids (including duplo grids) were obtained in typical experiments, e.g. the titration of sera on different herpes viruses, testing of catching antibodies, gold conjugates, and complement bound to viral immune complexes. Typical experiments carried out on patients lesion samples comprised: direct EM (testing of skin samples for virus), direct labeling with VZV or HSV gold conjugate, test for bound antibodies (total IgX) and assessment of the antibody subtype.

The standard grid boxes used in the EM laboratory did not meet our requirements for proper management of the grids because:

- grids can easily be placed or returned to wrong locations inside the boxes,
- there is considerable danger of loosing grids during uncontrolled or forced opening of the gridboxes,
- grids can be easily lost or damaged during bringing the grid in- or outside the box,
- grid documentation must be related to box- and location numbers unrelated to the patients' numbering system,
- grids cannot be labeled in a way that can give instant overview based on notations – describing experimental conditions or results made during the EM session.

Our storage system is based on using large petriedishes. Although the petriedishes take more place to store, it solved the above-mentioned quality problems.

Material and methods

A round-cut fine filter paper was placed on the bottom of the dish using Scotch double-sided adhesive 9 transfer tape (924) and the Scotch tape stripper ATG752. Small reinforcement rings (Avery, 1 cm diameter) were placed to the filter paper. Grids were forced with a small part of the outer ring between the filter paper and the reinforcement ring (Fig. 1). In the middle of the reinforcement ring round labels in different colors were placed, each colour referring to different groups of agents prepared for diagnosis. On the colour label a short notation of the type or the date of the experiment was written while on the filter paper additional information about the grids, the experiment (such as patient number, scheme of dilution steps, settings of the EM, results of diagnosis, etc.) was noted down. This facilitates rapid searches, especially when the grids must be reexamined by other persons. Colour pictures were taken from all petriedishes to keep track of the individual grids.

Discussion

EM experiments and grids can be traced and localized in a minimum of time by screening the colour photographs. Since all grids stick firmly to the dish we never faced problems anymore with unidentified grids or in the worst case, falling on the floor accidently. Also, this way of storage avoids codes such as complicated gridbox numbers that can often lead to confusions.

1a

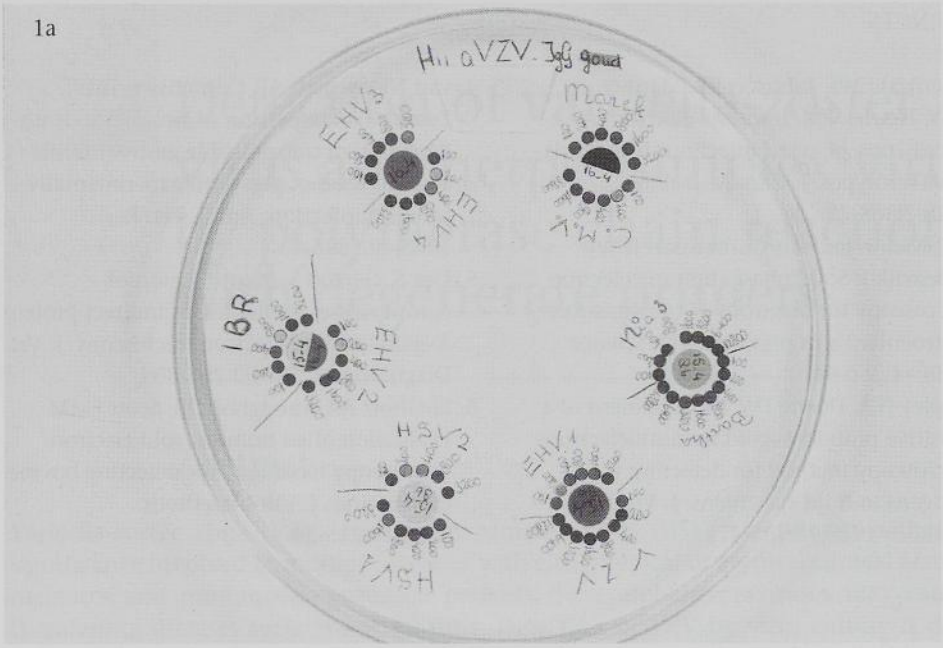
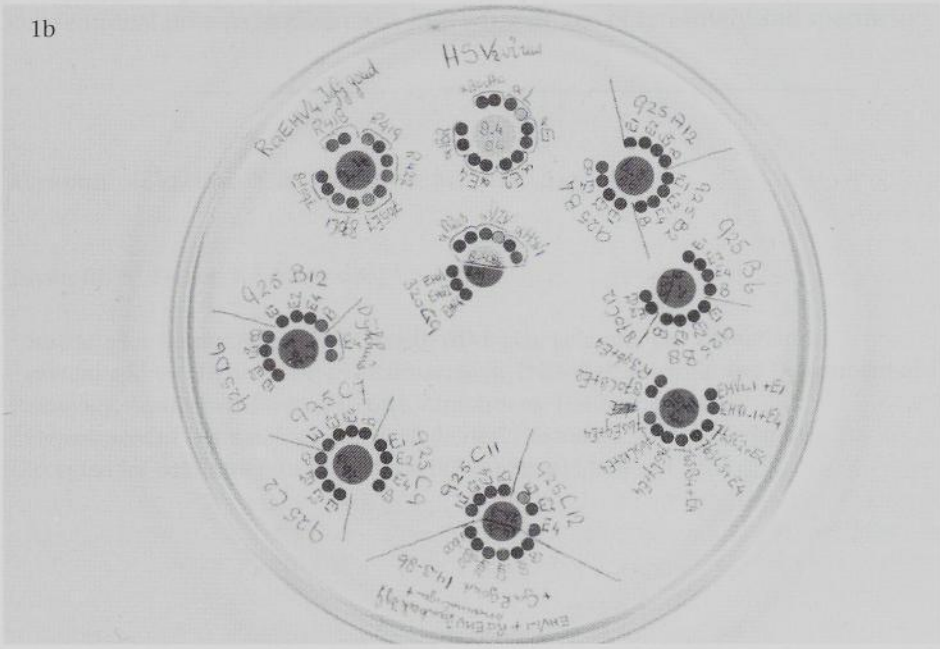


Fig. 1a. and Fig. 1b. Large petriedishes with grids forced with a small part of the outer ring between the filter paper and reinforcement rings.

1b



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CHAPTER 4

Detection of varicella-zoster virus and herpes simplex virus by polymerase chain reaction with degenerate primers

Abstract

Varicella-zoster virus (VZV) and herpes simplex virus (HSV) are human pathogens of significance involved in multiple diseases with either typical or atypical clinical features. In neonates and immunocompromised patients these alphaherpesviruses may cause life-threatening diseases such as encephalitis. Detection of VZV by virus culture is difficult. Polymerase chain reaction (PCR) is quicker and more sensitive and applicable in most clinical microbiological laboratories. Using degenerate primers, glycoprotein B DNA was amplified from all alphaherpesvirus field strains present in clinical samples. The amplification of glycoprotein B allowed virus typing of VZV, HSV-1 and HSV-2 using restriction enzyme digestion of the PCR products. Degenerate primers can replace conventional primers in diagnostic PCR without loss of sensitivity and specificity.

Keywords: VZV; HSV; PCR; degenerate primers; diagnosis

Jacobs JJL [1,2], Folkers E [3], Vreeswijk J [1]

[1] Institute for Animal Science and Health (ID-DLO), Lelystad, The Netherlands
[2] present address: Department of Pharmacology, TNO-PML, Rijswijk, and Department of Pathology, Academical Medical Centre, Amsterdam, The Netherlands
[3] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands

(Accepted for publication in the Journal of Virological Methods)

1. Introduction

The three human alphaherpesviruses, varicella-zoster virus (VZV) and herpes simplex virus (HSV) types 1 and 2, can produce a wide range of mucocutaneous manifestations. The severity of the disease depends on the entry site of the virus, the kind of infection (primary or recurrent), and the patient's immune state (healthy, elderly or immunocompromised). Clinical diagnosis is mostly based on the presence of the characteristic vesicular eruptions, but these might also resemble other viral and nonviral skin infections (Azon-Masoliver et al., 1990; Bhawan et al., 1984; Cohen, 1994; Halal et al., 1978; Kalman and Laskin, 1986). Immunocompromised patients may have atypical clinical signs of HSV and VZV infection such as mucosa-associated inflammatory pseudotumors, generalized papular eruptions, long-standing ulceration, hyperkeratotic nodules and crusted verrucous lesions caused by acyclovir-resistant viruses (Beasley et al., 1997; Cohen and Grossman, 1989; Hoppenjans et al., 1990; Husak et al., 1998; LeBoit et al., 1992; Rawlinson et al., 1989; Smith et al., 1991). The Tzanck smear, direct immunofluorescence tests, and virus culture are the tests most used to confirm the clinical diagnosis of human alphaherpesvirus infections (Coffin and Hodinka, 1995; Cohen, 1994). Confirmation of the clinical diagnosis of VZV, HSV-1 and HSV-2 infection by virus culture may be difficult, especially that for VZV. Immunoelectron microscopy (IEM), another tool to detect herpesviruses, is only available in specialized microbiological laboratories (Folkers et al., 1991; Vreeswijk et al., 1988). Polymerase chain reaction (PCR) might than be an attractive method for use in most routine microbiological laboratories, because it can be applied to all developmental stages of herpetic lesions, and to atypical mucocutaneous manifestations in an immunocompromised population. Moreover, results can be obtained within one day, whereas virus culture may take weeks (Kido et al., 1991; Nahass et al., 1995a; Nikkels et al., 1998). In this study we investigated the detection of human alphaherpesvirus and to discriminate between VZV, HSV type 1 and 2, using PCR with degenerate primers. We chose to amplify a gene that is essential for virus replication. It has been shown that several herpes genes can be deleted without affecting virus replication in tissue culture (Haarr and Skulstad, 1994; Nishiyama, 1996; Subak-Sharpe and Dargan, 1998). Loss of these genes could occur during viral replication in the field. Therefore, a diagnosis based on nonessential genes might overlook such mutant viruses.

Our preference for glycoprotein B (gB) was because: (1) this gene is indispensable for virus culture; (2) it is the major immunologic virus determinant; and (3) it is the major virus type determinant (Haarr and Skulstad, 1994; Nishiyama, 1996; Subak-Sharpe and Dargan, 1998). PCR can be used to amplify alphaherpesvirus gB DNA sequences. Type-specific restriction-enzyme digestion profiles of the PCR products can be used to discriminate between HSV-1, HSV-2 and VZV. Analysis of alphaherpesvirus gB DNA sequences (Bzik et al., 1984; Davison and Scott, 1986; Stuve et al., 1987) for conventional primer sites was unsuccessful. However, we were able to select several homologous stretches of approximately 7 amino acids long in alignments of the gBs of HSVs and VZV.

A degenerate primer can be designed to encompass all the possible DNA sequences coding for the selected amino acid sequence. For example, a hypothetical DNA stretch of 7 alanines will result in a degeneracy of $4^7 = 16{,}384$ primer variants. Degenerate primers have the property to bind to virus field strains harboring silent nucleotide variations.

Degenerate primers based on conserved amino acids have been used for cloning new genes (Compton, 1990; Lee and Caskey, 1990). However, they have rarely been use for diagnostic purposes. Furthermore, the primers most used were less than 50-fold degenerative. Primers with 512-fold and 8,192-fold degeneration were used for the detection of the Human Papilloma Virus (HPV) DNA (Snijders et al., 1991). These primers were only degenerate for known nucleic acid variation. We successfully used highly degenerate primers for reverse transcriptase PCR (RT–PCR) detection of Foot-and-Mouth Disease virus (FMDV) RNA (Jacobs et al., personal observations). In this report, we describe a PCR for VZV, HSV–1 and HSV–2 with degenerate primers. Discrimination between the PCR products of VZV, HSV–1 and HSV–2 was carried out with restriction-enzyme digestions.

2. Materials and Methods

2.1. Specimens

Selection of the patients was based on proven human alphaherpesvirus infection by virus culture or detection by IEM. PCR was carried out without prior knowledge of the virus type detected in the specimens, except for the cultured viruses used.

2.1.1. Virus samples stored in dry form at –70°C

Three samples were collected from the herpetic lesions on each patient. The samples consisted of cellular material, which was scraped from the edge and base of the lesion with the curved side of a vaccinostyle. One sample was used for virus culture, one was preserved for IEM (Folkers et al., 1989; Folkers et al., 1991; Vreeswijk et al., 1988) and the third sample was kept in dry storage at –70°C until this study was started. Specimens from one patient were stored dry at –70°C for less than two weeks (Table 4, < 2 wks). PCR was carried out on the dry-stored samples from 15 patients with proven HSV, and from 13 patients with proven VZV (Tables 4 and 5).

2.1.2. Virus samples stored in buffer at –70°C

Swab specimens from skin, orolabial, and genital herpetic lesions were placed in Hank's balanced salt solution (HBSS) and transported to the virus culture laboratory. Inoculation of cell cultures was done immediately upon arrival at the laboratory. Samples of proven HSV (n=6) and VZV (n=8) infection were stored in HBSS at –70°C for up to two years, until used in this study (Tables 4 and 5).

2.1.3. Nonviral control specimens stored in dry form at –70°C

Samples were obtained from nonviral vesiculo-bullous lesions of four patients, attending the outpatient section of the Department of Dermatology and Venereology of Hospital De Heel in Zaandam. One patient was suffering from *pompholyx*, one from *prurigo bullosa*, one from bacterial infected *dermatomycosis*, and one patient from facial bullous *erysipelas*. Scrapings from the edge and base of the lesions were made using the curved side of a vaccinostyle, and stored at –70°C for 9 years. Uninfected HeLa cells obtained from the laboratory of the department of Virology of the Municipal Health Service (GG&GD) of Amsterdam, stored at –70°C, were used as reference control specimens in the PCR.

2.1.4. Virus culture samples stored at –70°C

The investigated HSV–1 and HSV–2 virus culture samples (Table 5) were assigned to the following two groups according to the duration of storage: (1) less than two years (< 2 yrs); or (2) at least nine years (> 9 yrs) of storage. Other viruses used in this study were varicella-zoster virus (VZV), cytomegalovirus (CMV) and Epstein-Barr virus (EBV). Laboratory cultured viruses were generous gifts from Dr. G.J.J. van Doornum of the GG&GD Amsterdam, Dr. P.M.E. Wertheim-van Dillen of the Academic Medical Center (AMC) Amsterdam, and Dr. J.G. Kapsenberg of the National Institute of Public Health and Environmental Protection (RIVM) Bilthoven. Titrated HSV–1 and HSV–2 cultures ($TCID_{50}$) were generous gifts from Dr. A.M. van Loon of the Academic Hospital Utrecht (AZU) Utrecht, the Netherlands.

2.2. Procedures

2.2.1. DNA extraction

Prior to DNA extraction, dry tissue samples were dissolved in 200 µl TE in a microcentrifuge tube (0.5 ml) to obtain a wet form. The wetted tissue and HBSS samples (swabs) were freeze-thawed twice in liquid nitrogen and subsequently heated in a water-bath to 37°C. Samples were treated with SDS (final concentration 0.5%) and proteinase K (final concentration 100 µg in 250 µl), mixed, and incubated for 45 minutes at 37°C. Subsequently a phenol – chloroform – isoamyl-alcohol extraction (25 : 24 : 1) in phenol extraction buffer (10 mM Tris, 100 mM NaCl, 0.5% SDS, 5 mM EDTA; pH 9.0) was carried out, followed by precipitation in two volumes of ethanol (Sambrook et al., 1989). After ethanol precipitation the extracted DNA was washed with 70% ethanol, air dried for 10 minutes and dissolved in 50 µl aqua dest. PCR was either carried out directly with freshly isolated DNA or, later on with DNA that had been stored at –20°C prior to use.

2.2.2. DNA amplification

DNA amplification was carried out in 0.5 ml microcentrifuge tubes (Biozyme). Each tube of 50 µl solution contained: 1x GeneAmp®, 10x PCR buffer II {10 mM Tris-HCl, pH 8.3 (at 25°C; 50 mM KCl} (Perkin Elmer), 2.5 mM $MgCl_2$ (Perkin Elmer), 4x 0.2 mM dNTPs (Pharmacia, Biotechnology), 2x 50 nM primers (Table 1), 0.625 U Taq polymerase (Perkin Elmer), and 2 µl extracted DNA solution. The solution was covered with two drops of mineral oil. Amplification was carried out in a Perkin Elmer DNA Thermal Cycler 480. The following program was started: 2 min. 'hot start' at 94°C, 40 cycles (each 30 sec. 94°C, 30 sec. 60°C and 2 min. 72°C), followed by a final elongation step of 7 min. at 72°C. Procedures to minimize PCR product carry-over were applied as recommended (Kwok, 1990).

2.2.3. Enzymatic digestions of the PCR products

A 7.5 µl aliquot of each PCR product was incubated for one hr in a 10 µl reaction volume containing 3 U *Sma* I, 3 U *Sca* I or 6 U *Sal* I according to the manufacturer's recommendation (New England Biolabs).

2.2.4. DNA alignments

The following sequences were used in alignment studies. GenBank accession numbers for HSV–1: K01760 (Bzik et al., 1984); D10879, K02720, K03541, M14164, M21629, S65444, S74390, U49121, X14112. For HSV–2: U12175 (Stuve et al., 1987); AF021340, M14923, M15118, M24771, U12172, U12173, U12174, Z86099. For VZV: P09257 (Davison and Scott, 1986).

2.2.5 Tilted gel electrophoresis and markers

Two µl loading buffer was added to 10 µl PCR product or 10 µl of restriction enzyme digestion product. Samples were loaded on a 1.5 % agarose gel in TBE containing ethidium bromide. During electrophoresis the gel was tilted at an angle of approximately 4° (You and Sun, 1995). This procedure allowed a better separation of dsDNA sized between 100 bp and 1 kb. The digestion products of plasmid DNA of pGEM®-3 with *Hinf* I, *Rsa* I, and *Sin* I were used as size marker (see Fig. 2). The DNA fragments were photographed with UV illumination.

3. Results

3.1 Primers

For this study we selected two homologous amino-acid stretches, approximately 700 bp apart and located in the the human alphaherpesvirus glycoprotein B (gB) gene, to develop degenerate PCR primers (Fig 1 and Table 1). The PCR product generated by these primers contained sufficient virus-specific DNA sequences for virus typing with restriction enzymes. The PCR product comprised of an upstream primer HB4 and a downstream primer HB6. PCR with this primer set generated products that can be discriminated by restriction enzymes. As the primers do not align with human beta and gammaherpesviruses, we expected that they will only bind to alphaherpesvirus DNA and not produce amplification products of the human beta- and gammaherpesviruses. Screening of these primers in GenBank did not reveal alignments that could lead to other undesired, nonspecific PCR products. The primers were tested in three modifications (A, C, and D) with different degrees of degeneracy. The degenerative positions were encoded by standard molecular biological one–letter codes: N, Y, R, W, S, D, or H (Table 1a, and 1b).

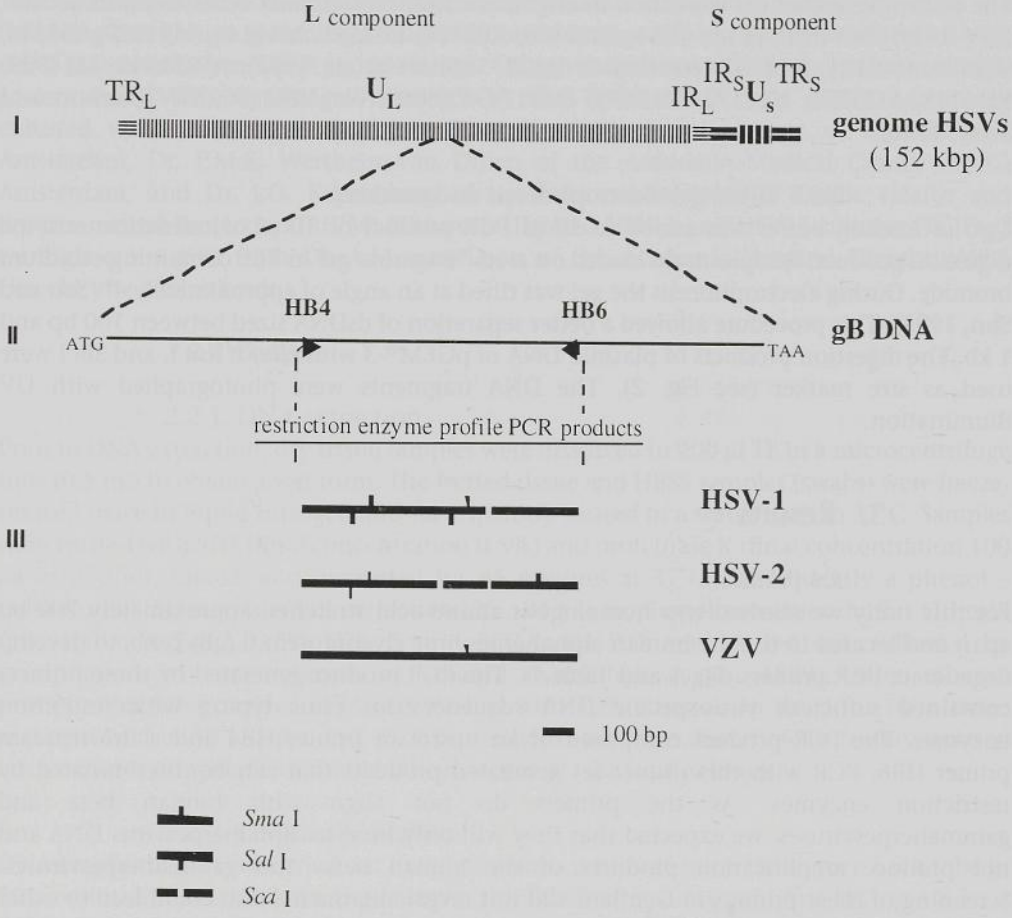

Fig. 1. Setup for the detection of herpes viral DNA.
Level I: The HSV genome with terminal repeats (TR), inverted repeats (IR), and the long (UL) and short (US) coding regions. VZV (125 kb) and HSV (152kb) genomes differ in the size of the coding and repeat regions. Level II: Magnification of gB, and the primer locations of HB4 and HB6. Level III: PCR products of HSV-1, HSV-2, and VZV, generated by HB4–HB6 primer combinations, and the restriction-enzyme locations of Sma I, Sal I and Sca I within the PCR products.

Table 1a *Alignments of primer variants with human herpes viral DNA*

Primer HB4 template (5′ – 3′)		Degeneracy*		
HB4A	AY TGY ATH RTN GAR GAR GTN GAN GC**	3,072		
HB4D	AY TGY ATH GTN GAR GAR GTN GAN GC	1,536		
HB4C	AC TGC ATC GTC GAG GAG GTG GAC GC	1		
		Number of mismatches		
Viral template DNA (+) strand (5′ – 3′)		HB4A	HB4D	HB4C
HSV-1	AC TGC ATC GTC GAG GAG GTG GAC GC	0	0	0
HSV-2	AC TGC ATC GTC GAG GAG GTG GAC GC	0	0	0
VZV	AT TGC ATC ATT GAG GAA GTT GAA GC	0	1	6

No good alignment with CMV, HHV-6, HHV-7, EBV, and HHV-8

Primer HB6 template (5′ – 3′)		Degeneracy*		
HB6A	TGD ATR TGR TCR TAN GTR AAY TGN AG	1,536		
HB6D	TGD ATR TGR TTR TAN GTR AAY TGN AG	1,536		
HB6C	TGT ATG TGG TTR TAC GTA AAC TGC AG	2		
		Number of mismatches		
Viral template DNA (-) strand (5′ – 3′)		HB6A	HB6D	HB6C
HSV-1	TGA ATG TGG TTA TAC GTA AAC TGC AG	1	0	0
HSV-2	TGA ATG TGG TTG TAC GTA AAC TGC AG	1	0	0
VZV	TGA ATG TGG TCA TAT GTA AAC TGG AG	0	1	3

No good alignment with CMV, HHV-6, HHV-7, EBV, and HHV-8

* Degeneracy is the number of variants present in a primer

** D= A,G,T S=C,G
H= A,C,T W=A,T
N= A,C,G,T Y=C,T
R= A,G

Table 1b. *Display of* A–C *base mispairing (primer sets with HB4A & HB6A) and* G–T *base mispairing (primer sets with HB4D & HB6D)*

```
VZV(+)   5′–ATTGCATCATTGAGGAAGTTGAAGC... ... CTCCAGTTTACATATGACCACATTCA–3′ VZV(+)
                              <———— 3′-GANGTYAARTGNATRCTRGTRTADGT–5′ HB6A
                              <———— 3′-GANGTYAARTGNATRTTRGTRTADGT–5′ HB6D
                 ..................... ... CTCCAGTTTACATATAACCACATTCA–3′ HSV(+)
VZV(-)   3′–TAACGTAGTAACTCCTTCAACTTCG–5′... .....
HB4A     5′–AYTGYATHRTNGARGARGTNGANGC–3′ ————>
HB4D     5′–AYTGYATHGTNGARGARGTNGANGC–3′ ————>
HSV(-)   3′–TAACGTAGCAACTCCTTCAACTTCG–5′... .....
```

(A–C and G–T mispairings are underlined)

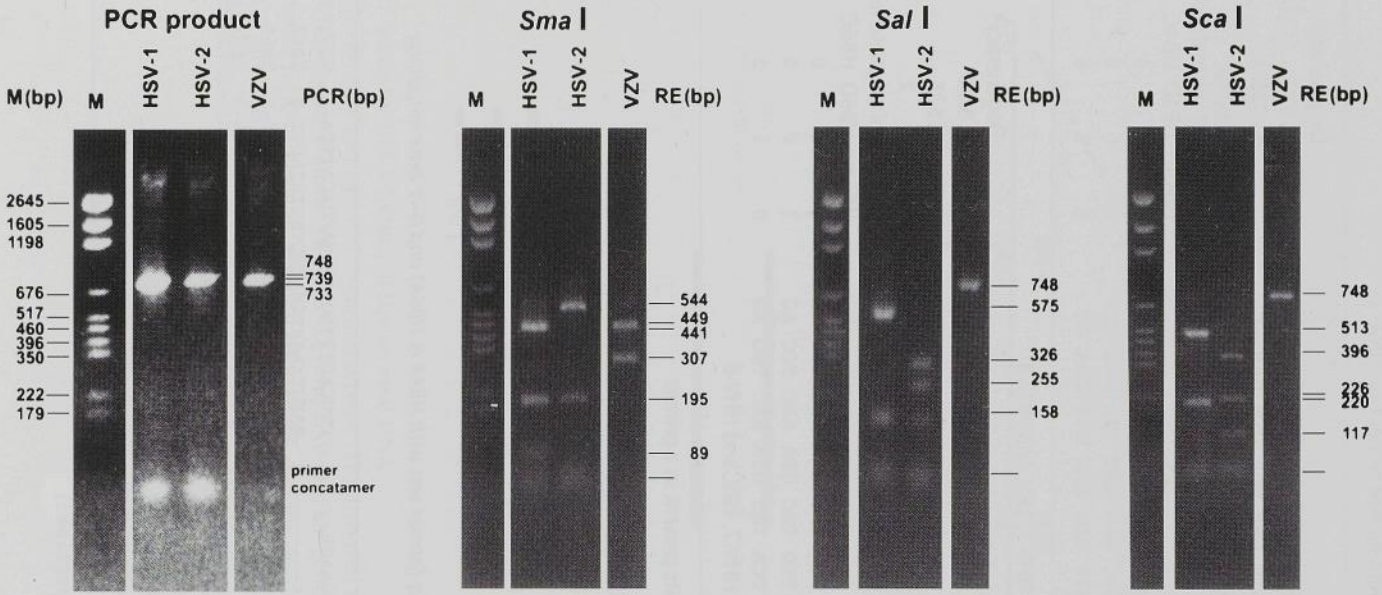

Fig. 2. PCR products of HSV-1, HSV-2, and VZV obtained with degenerated primer set IV. Restriction digestion profiles obtained with *Sma* I, *Sal* I, and *Sca* I. Lane on the left shows the pGEM®-3 markers (M).

3.2 Detection of viral DNA using cultured viruses

The primer sets I - IV were used to amplify VZV DNA (Table 2). Only two of these (set III and set IV) allowed the amplification of HSV-1 and HSV-2 DNA.

Table 2. *Results of PCR carried out with different primer combinations for the detection of human herpes viral DNA*

	degenerate primer sets				conventional primer
	I	II	III	IV	V
	HB4A-HB6A	HB4D-HB6A	HB4A-HB6D	HB4D-HB6D	HB4C-HB6C
Cultured virus					
HSV-1	–	–	+	+	+
HSV-2	–	–	+	+	+
VZV	+	+	+	+	–
CMV	–	–	–	–	–
EBV	–	–	–	–	–
Controls (1-5)*	–	–	–	–	–

* Controls: 4 clinical specimens (*pompholyx, prurigo bullosa*, bacterial infected *dermatomycosis*, facial *erysipelas*), and uninfected HeLa cells. Virus cultures of HHV-6, -7 and -8 were not available for testing.

3.3 Sensitivity

In sensitivity studies of the PCR with degenerate primers, culture samples with a know virus concentration were diluted in uninfected HeLa cells. The sensitivity of the PCR to HSV-1 was 4.2 log $TCID_{50}$, obtained with both degenerate and conventional primers. The titrated sample of HSV-2 contained 3.2 log $TCID_{50}$ virus particles, 10-fold lower than the detection limit of HSV-1. This titre was not detected in our PCR. Two PCRs, one with conventional primers and one with degenerate primers (set IV), were applied to all HSV samples and their dilutions. PCRs with conventional or degenerate primers gave identical results, indicating that the use of degenerate primers does not affect PCR sensitivity. VZV could not be amplified using our conventional primers for HSV. The sensitivity of the PCR detection of VZV is not known because no titrated virus sample for VZV detection was available. Primer set I and IV gave identical results for VZV.

3.4 Restriction-enzyme digestion profiles of the PCR products

The expected products of PCR carried out with the HB4-HB6 primer sets and restriction-enzyme digestion profiles are listed in Table 3. The results are shown in Fig. 2.

Table 3. *Expected products of PCR carried out with HB4-HB6 primer sets and restriction-enzyme digestion profiles of the products*

Virus	PCR product Size (in bp) *Sma* I	Products of restriction-enzyme digestion Size (in bp) *Sca* I	*Sal* I	
HSV-1	733	449	513	575
		195	220	158
		89		
HSV-2	739	544	396	326
		195	226	255
			117	158
VZV	748	441	748	748
		307		

3.5 Detection of viral DNA using clinical specimens

Similar results were obtained with PCR primer set I and with PCR primer set IV when both tests were applied to all VZV samples (n=24), samples stored dry plus those stored in HBSS. PCR on all VZV samples stored in a dry form at –70°C (n=13) produced positive results (Table 4), while on VZV samples stored in HBSS at –70°C (n=8) PCR with the same primer sets detected viral DNA in only five of the samples. The storage of VZV samples in HBSS varied from two weeks to two years, which was much shorter than the dry storage of samples. One specimen was stored both in dry and in wet form. The PCR detected VZV DNA in the sample stored in dry form, but not in the sample stored in HBSS. PCR with primer set IV was carried out with HSV-clinical samples (n=21) and HSV–laboratory cultures (n=16) (Table 5). Five out of six PCR results obtained with HSV–laboratory cultures that were stored for less than two years had positive scores. However, the PCR was negative (n=10) when HSV–laboratory cultures were stored for 9 years. PCR detected HSV DNA in one of the HSV-clinical samples that had been stored in dry form for nine years (n=15). HSV DNA could be detected in only one out of six clinical samples, stored in HBSS for less than eight weeks (Table 5). Fig. 3 shows some clinical samples that were analysed by PCR, and characterized for herpesvirus type by restriction-enzyme digestion profile (*Sma* I).

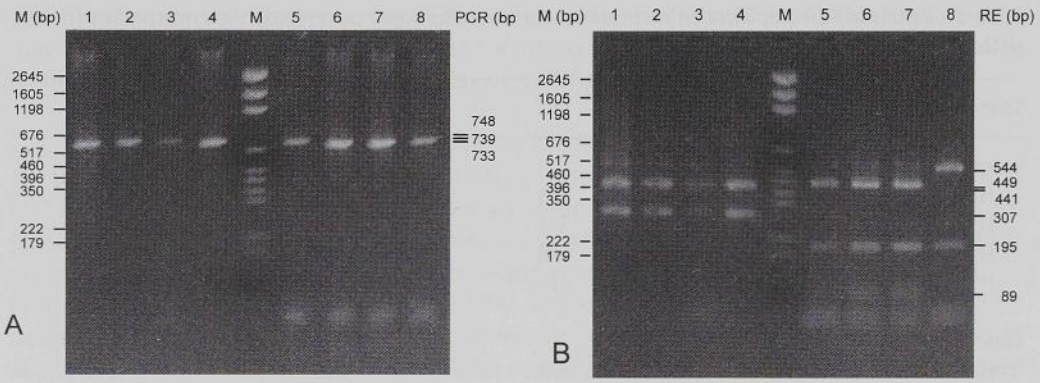

Fig. 3. Detection and identification of human alphaherpesviruses in clinical specimens. Panel A: Agarose-gel electrophoresis of PCR products. PCR was carried out with primer set IV. Virus strains: VZV (lanes 1-4); HSV-1 (lanes 5-7); HSV-2 (lane 8). Sample 7 was a cultured and titrated HSV-1 specimen with a concentration of 4.2log$TCID_{50}$. Lane M: DNA-ladder size marker (pGEM®-3 DNA digested with *Hinf* I, *Rsa* I, and *Sin* I). Panel B: Agarose-gel electrophoresis of *Sma* I digests of the PCR products shown in panel A.

Table 4. *Results of PCR with degenerate primers followed by restriction-enzyme virus typing carried out on samples from patients with VZV infection*

Disease	Lesion type	Storage	Tzanck smear‡	Virus culture‡	IEM ‡	PCR$
Varicella	vesicular	dry>9 yrs	1/1	0/1	1/1	1/1
Varicella	pustular	dry>9 yrs	1/1	0/1	1/1	1/1
Herpes zoster	vesicular	dry>9 yrs	3/3	1/3	3/3	3/3
Herpes zoster	pustular	dry>9 yrs	7/7	1/5	6/6	7/7
Herpes zoster*	pustular	dry <2wks	1/1	nd	nd	1/1
Herpes zoster	pustular	HBSS<2yrs	2/2	7/7	nd	5/7
Herpes zoster*	pustular	HBSS<2wks	1/1	1/1	nd	0/1

‡: done before storage
$: PCR results with primer set I were similar to those with primer set IV
*: same patient
nd: not done

Table 5. *Results of PCR amplification and subsequent diagnosis based on restriction-enzyme profiles (HSV-1, HSV-2) in relation to storage conditions*

Specimen	Storage	VC‡	IEM‡	PCR$
Laboratory	VC -70°C			
HSV–1	< 2 yrs	3/3	nd	3/3
HSV–2	< 2 yrs	3/3	nd	2/3
HSV–1	> 9 yrs	8/8	nd	0/8
HSV–2	> 9 yrs	2/2	nd	0/2
Clinical	HBSS -70°C			
HSV–1	< 8 wks	3/3	nd	0/3
HSV–2	< 8 wks	3/3	nd	1/3
Clinical	dry -70°C			
HSV–1	> 9 yrs	4/4	3/3	1/4
HSV–2	> 9 yrs	6/11	5/5	0/11

HBSS: Hank's balanced salt solution
IEM: Immunoelectron microscopy
VC: Virus culture
nd: not done
$: PCR results with primer set V were similar to those with primer set IV
‡ : done before storag

4. Discussion

There are several methods available for the detection of VZV. Virus culture, which is a sensitive technique for the detection of HSV is less useful for detection of VZV. VZV is prone to substantial inactivation during sampling and transportation (Echevarria et al., 1997; Folkers et al., 1989; Nahass et al., 1992). Having previously shown that IEM is a highly sensitive technique for the detection and diagnosis of VZV (Folkers et al., 1989; Vreeswijk et al., 1988), we used IEM to validate our newly developed diagnostic PCR with degenerate primers. Recent PCR results obtained with VZV samples, which were dry- stored for nine years (Table 4), were equal to the IEM results obtained nine years ago with the same samples (Folkers et al., 1989).

4.1 Sampling

PCR detected VZV DNA in all dry stored VZV samples, but only in 63% HBSS samples (Table 4). This indicates that PCRs carried out on dry-stored samples have a better chance of obtaining positive results than those carried out on HBSS–stored samples ($p < 0.01$, in accordance to the 2 x 2 Contingency Table (Mead and Curnow, 1990)). This is in agreement with results obtained by Nahass and coworkers (1995) who reached high sensitivity of detection in dry-stored specimens (Nahass et al., 1995a; Nahass et al., 1995b).Long–term storage at –70°C (> 9 years) appeared to be detrimental to HSV

detection, while VZV detection by PCR seemed to be unaffected. A possible explanation may be that the VZV particle mainly resides intracellularly (Cohen and Straus, 1996) and, is therefore protected against extracellular degrading enzymes.

4.2 Restriction enzyme typing of the PCR products

To simplify the practical procedure in our study, restriction–enzyme digestions were carried out directly in the PCR mixtures. Although some restriction enzyme activities are very sensitive to differences in salt concentrationsü the activity of the selected restriction enzymes, *Sma* I, *Sca* I, *Sal* I, are fairly robust and partial digestions were rarely observed.
From a theoretical point of view, subtle DNA sequence variations between different virus isolates may be expected as a result of errors made by the viral DNA polymerase (Abbotts et al., 1987). These DNA variations may alter restriction–enzyme recognition sites, thereby prohibiting enzymatic digestion. Thus, certain virus strains may exhibit different restriction-enzyme digestion profiles compared to other strains within the same virus type. The same holds for creation of a new restriction-enzyme site by mutation. In both cases the other two restriction-enzyme digestion patterns will remain unaltered, so that discrimination between the alphaherpesviruses remains possible.
A variation was found only at the *Sal* I recognition site on comparing the sequences of ten different HSV-1 strains. HSV–1 strain F was among two out of ten HSV–1 strains (GenBank accession numbers M14164 and M21629) with the *Sal* I sequence GTCGAC changed into GTTGAC, which is not recognized by *Sal* I. The full–length PCR product (733 bp) of these HSV-1 strains, therefore, will not be cleaved by *Sal* I, and thus remain similar to that of VZV (748 bp). The restriction-enzyme digestion patterns of *Sma* I and *Sca* I would be unaltered. Based on these profiles, typing of the variant strains as HSV–1 remains feasible. Similarly, on comparing nine HSV–2 strains, a variation was only found at the *Sma* I site. In the HSV–2 strains MMA and CAM4B (GenBank accession numbers U12172 and U12174), the *Sma* I site was changed from CCCGGG into CCCAGG, which is not recognized by *Sma* I. The full-length PCR products (739 bp) of these two HSV–2 strains, therefore, will not be digested by *Sma* I, and thus remain similar to that of VZV (748 bp), which lacks a *Sma* I site. The restriction–enzyme profiles of *Sal* I and *Sca* I would be unaltered. Therefore, typing of these variant strains as HSV-2 will still be possible. In this study, we encountered no aberrant restriction-enzyme digestion profiles.

4.3 PCR with degenerate primers

Conventional primers are commonly used in diagnostic PCR. Sites for conventional primers are selected at conserved nucleotide stretches of virus types. In general, fully conserved nucleotide stretches can be found in highly conserved genes. PCR amplification products of highly conserved genes are not likely to contain many subtype-specific nucleotide sequences. Thus virus-type discrimination between PCR products obtained with conventional primers can be difficult. Another approach is the use of degenerate primers. Selection of sites for degenerate primers is not done on conserved nucleotide stretches, but on conserved amino acid stretches, which may be important for maintaining the protein structure or function. Consequently, a PCR with degenerate primers selected from conserved amino acid stretches, will enable amplification of less conserved nucleotide sequences. These sequences are likely to bear virus type-specific nucleotide stretches, which

may be used for discrimination by different methods: hybridization with a type-specific probe, type-specific nested PCR, nested multiplex PCR, direct sequencing of the PCR product, and restriction-enzyme digestions.
The degenerate HB6D primer was effective in the amplification of HSV DNA but primer HB6A was not (Table 2). A single mispairing between HSV DNA with the HB6A primer might have been responsible for the failure to detect HSV-1 and HSV-2 DNA with primer sets I and II (HB4A - HB6A, and HB4D - HB6A, respectively). The mismatches of the HB6A primer with HSV-1 and HSV-2 DNAs comprised A–C base mispairings. No stabilizing interaction can be expected from A–C mismatches (Crick, 1966). Both primers HB4D and HB6D had a G–T mismatch with VZV DNA. However, in case of G–T mismatching, two stabilizing hydrogen bonds can occur similar to those present in A–T bonds (Crick, 1966). This is probably the reason why VZV DNA amplification was able to proceed with the primer combination IV. In PCR with conventional primers, a single base mispairing is not detrimental to amplification, unless located at the 3' primed site, where it can interfere with strand elongation (Barnes, 1994). In case of the HB6A primer, the A–C mismatch was located at a distant position (16^{th} nucleotide) from the 3' elongation site (underlined in Table 1b). Using conventional primers, the location of this A–C mismatch is apparently far enough from the primer elongation site to allow PCR amplification. PCR with degenerate primers seems to be more dependent on correct primer–template pairings. This decreased tolerance to mispairing was also found in RT–PCRs with degenerate primers for FMDV (Jacobs et al., unpublished results). Decreased tolerance by 2 to 3 mismatches in PCR with degenerate primers prevents amplification of virus type-unrelated sequences. Theoretically, the decreased tolerance will compensate the loss of primer specificity introduced by a degeneracy of several thousands (Jacobs et al., unpublished results). In practice, the specificity of degenerate primers may be underestimated, as these primers are designed to 'tolerate' silent mutations, but discriminate more strongly against unrelated sequences.

5. Conclusion

Contrary to conventional primers, degenerate primers enable PCR amplification of less conserved nucleotide sequences, like gB DNA. Glycoprotein B can be used for virus-type discrimination. Type-specific restriction-enzyme patterns of the PCR products were used to discriminate between the human alphaherpesviruses VZV, HSV-1, and HSV-2. This is a fast and reliable method, which can be introduced in a diagnostic laboratory with a high throughput of samples. Sensitivity of PCR with degenerate primers may be increased using nested PCR or using hybridization of the PCR product with a labeled internal oligonucleotide probe. This study has demonstrated that degenerate primers can be effectively used in diagnostic PCR without loss of specificity and sensitivity.

Acknowledgements

We are grateful to Sylvia Bruisten (Department of Virology of the Municipal Health Service (GG&GD), Amsterdam) for her critical reading of the manuscript.

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CHAPTER 5

Immune response to varicella-zoster virus infection *in vivo*: immunoglobulins, complement and cellular infiltrates

Abstract

Herpesviruses, like varicella-zoster virus (VZV) use different strategies to escape host immune-effector mechanisms. Systemic immune responses were monitored by serological techniques employing fluorescent antibody to membrane antigen (FAMA) and immunoelectron microscopy (IEM). Cellular infiltrates in herpetic skin lesions were analyzed for different leukocytic populations in correlation with the presence of virus, immunoglobulins, virus-immune complexes and complement factors. Between the primary (varicella) and the reactivated infection (herpes zoster) differences in interactions between the immune system and VZV were noticed. Early in varicella, T lymphocytes infiltrate lesions solely; in herpes zoster and late varicella, innate leukocytes appear in addition to T lymphocytes. In varicella, virus immune complexes (VICs) emerge, when serum antibodies can be detected. In herpes zoster VZV hides, possibly intracellular, from immunoglobulins, but when the alternative complement pathway is activated, VZV is released and VICs are formed. Activation of immune effector mechanisms against VZV and differences in the immune response in varicella versus herpes zoster are discussed. It is suggested that type-2 mediated responses, which stimulate antibody production, are the predominant effector mechanisms in varicella. In herpes zoster, after reactivation, VZV evades neutralization by antibodies. Therefore, memory type-1 mediated cytotoxic-immune responses are expected to be more important for virus eradication in herpes zoster.

Folkers E [1], Jacobs JJL [2], Oranje AP [3], ten Kate FJW [4], Vreeswijk J [5]

[1] Department of Dermatology, Hospital 'de Heel', Zaandam, The Netherlands

[2] Institute for Animal Science and Health (ID-DLO), Lelystad, The Netherlands
present address: Dept. Pharmacology, TNO-PML, Rijswijk, and dept. Pathology, Academical Medical Centre, Amsterdam, The Netherlands

[3] Department of Dermatology and Venereology (Pediatric Dermatology), Erasmus University, Rotterdam, The Netherlands

[4] Department of Phatological Anatomy, University Hospital Rotterdam, and Erasmus University, Rotterdam, The Netherlands
present address: Dept. of Pathology, Academical Medical Centre,Amsterdam, The Netherlands

[5] Central Veterinary Institute, Department of Virology, Lelystad, The Netherlands

(Manuscript in preparation)

1. Introduction

The human body employs different strategies to respond to a virus infection, either innate or antigen specific. Innate responses can be humoral, such as the complement system, or cellular, such as responses mediated by macrophages, granulocytes and natural killer (NK) cells. Antigen-specific responses can also be humoral, e.g. immunoglobulins, and cellular, e.g. mediated by cytotoxic or helper T cells. Antigen-specific immune responses will mature upon infection and become stronger and more effective at reinfection or reactivation. Antigen-specific responses will also recruit the innate immune system in order to rapidly eliminate a virus [1].

In order to persist, a virus must evade the host defence immune response. Viruses have evolved many different strategies to avoid eradication by the immune system. RNA and retroviruses alter their antigens to escape antibody [2, 3] and/or cytotoxic T lymphocyte (CTL) responses [4]. In contrast to RNA viruses, DNA viruses, like herpes, do not have high mutation rates that would enable antigen changes fast enough to escape immune effector mechanisms[5, 6]. The immune system uses both innate and antigen specific responses to eliminate viruses, like VZV [7, 8]. The immune system has multiple approaches to eliminate viruses, effective escape of viruses from the immune response involves disarming of multiple effector mechanisms.

Two different reasons exist for the interest in the interactions between VZV and the immune system. Firstly, the severity of VZV infection seems related to the patient's immune status. Varicella (chickenpox) results mostly in a mild infection, but can be more severe in older, otherwise healthy individuals. Neonates, pregnant women, and immunocompromized patients belong to high-risk patients who can develop a progressive, life-threatening disease after a primary VZV infection. Beside a primary infection, also reactivation of the latent virus may occure, resulting in herpes zoster (shingles). Patient at risk for herpes zoster are both otherwise healthy, older individuals and immunocompromized patients. In immunocompromized patients, disseminated herpes zoster is likely to occur [9-12]. Secondly, two different strategies of immunotherapy for VZV are employed, vaccination to generate T-cell responses and immunoglobulin prophylaxis. VZV vaccination leads to T-lymphocyte mediated immune effector mechanisms [13, 14] and reduces the frequency and severity of herpes zoster and its complications [15]. Treatment of varicella infection by serum immunoglobulin prophylactic is indicated for patients at high risk for complications, after they have been exposed to VZV [16-18].

VZV antibody titers rapidly increase during herpes zoster infection [19, 20]. IgG antibodies in serum, in concert with the cellular immune response can inactivate VZV efficiently *in vivo*. This is supported by detection *in vivo* of virus-immune complexes (VICs) by thin section immunoelectron microscopy (IEM) [21]. These VICs can be related to a negative virus culture [22]. Small amounts of antibodies and polymorphonuclear leukocytes contribute to *in vitro* inactivation of cell-associated VZV [23, 24].

The present study was undertaken to resolve both cellular and humoral immune-effector mechanisms that may contribute to the local eradication of VZV infections. Different leukocyte populations, complement factors, immunoglobulins, presence of virus and VICs were studied in herpetic skin lesions.

2. Materials and Methods

2.1 Patients

2.1.1 Sera and lesion samples from adults

The study comprised five, otherwise healthy adults, who attended during the period 1985 – 1987 the out-patient department of Dermatovenereology of Hospital de Heel. Two patients had varicella (case V-1 and V-2) and 3 herpes zoster (cases Z-1, Z-2 and Z-3). All 5 case histories mentioned earlier suffering from labial herpes. Based on the absence of labial and/or oral herpetic lesions during the examinations related to this study, dual infection with herpes simplex virus (HSV) was excluded. The patients were followed during a period of two weeks after onset of the disease. Blood samples, lesion scrapings, and biopsies were taken at regular intervals for serology, IEM, and immunohistology. The samples were taken within 2 to 5 days after the onset of the skin lesions. Additional sampling was done at intervals of 2 to 4 days after taking the first sample.

Lesion smears, skin biopsies, and blood samples were jointly taken during the first and the subsequent clinical consultations. Varicella lesions usually develop in crops. Therefore, all developmental stages can be present in involved skin areas. During the patient's first consultation, chickenpox lesions at the same stage of development were marked with a water-resistant marker pen. Some of these lesions were used for sampling, the others, present in the marked area, were left for follow-up sampling. In classical herpes zoster clusters of herpetic lesions emerge unilaterally over one to three sensory nerve dermatomes. The longest time-existing herpes zoster lesions were selected for sampling.

A negative control serum sample (C-1, table 1), from a healthy adult without clinical VZV and HSV infection for at least 20 years was included in this study [25].

2.1.2 Sera from children

For ethical reasons taking biopsies from children for experimental research is not allowed. Sera were examined from three hospitalized, varicella infected children, 18 weeks, 50 weeks, and 6 years old, respectively (V-3, V-4, V-5, table 1). Their case history did not mention earlier HSV infection. Patient V-4 was described before [25].

2.2 Serological testing

Sera were investigated for presence of anti-VZV antibodies (IgM and IgG) by IEM (22) and fluorescent antibody to membrane antigen (FAMA) carried out on infected monolayer cells [26]. Rheumatoid factor antibody can interfere with IgM detection [27]. All sera were found negative for rheumatoid factor antibodies. Serum titers by IEM were calculated from sigmoid plots (dilutions versus gold particle counts per virus particle) as described before [22].

2.3 Virus-immune complexes

Lesion scrapings were processed for virus typing and detection of VICs by IEM [28, 29]. Gold-tagged affinity chromatography purified rabbit anti-human immunoglobulin antibodies (Dacopatts A190) were used to label VICs. Monoclonal antibodies (Nordic) were used for detection of IgG and IgM in VICs [22].

2.4 Immunohistology

For immunohistology, skin biopsies were cut in serial cryostat sections. Immunofluorescence (IF) was applied to detect VZV antigens, immunoglobulin types (IgM, IgG and IgA) and complement factors (B1a, C1q, total complement). VZV antigens were detected with monoclonal antibodies obtained from Sanbio. All other antibodies were polyclonal and obtained from CLB (Amsterdam).

Immunohistochemistry (IHC) was done as an indirect immunoperoxidase staining with monoclonal antibodies (Becton Dickinson) for $CD3^+$ T lymphocytes (Leu-4), $CD4^+$ T-helper cells (Leu-3), $CD8^+$ CTLs (Leu-2), $CD1a^+$ epidermal Langerhans cells (Leu-6), and $CD22^+$ B lymphocytes (Leu-14). Quantitative measurements of immunohistochemical labeled leukocytes were carried out with a semiautomatic image analysis system (Kontron, Germany). Images of sections were projected through a side tube on an electronic (x-y) tablet. Leukocyte counts were expressed as the number of labeled cells per 100 infiltrating leukocytes.

3. Results

The results of serology using IEM and FAMA are listed in table 1. Patients with varicella simultaneously developed anti-VZV IgM antibodies directed against virus cores and envelopes (Table 1, patients V-1 and V-2). During the development of an IgG titer, however, the appearance of IgG antibodies directed against envelopes preceded IgG antibodies directed against virus cores (Table 1, patients V-1 and V-2). In one patient with herpes zoster (Z-1) IgG directed against virus envelopes also preceded IgG directed against virus cores (Table 1).

Cross-reactive antibodies between HSV and VZV may contribute to a false positive outcome of routine diagnostic serology. The common antigens of VZV and HSV seem widely distributed in the virion [30, 31]. Cross-reacting antigens of HSV and VZV, not associated with the virus particle, have been identified [32]. Cross reactions between VZV and HSV nucleocapsid proteins have been demonstrated [33, 34]. Cross reactivity of IgG antibodies directed against HSV-1 and VZV gB (major immunogenic protein) exists, most frequently observed in HSV-seropositive patients with primary VZV infection [35]. A titer increase of mainly IgM against HSV was found in patient V-1, which is indicative for a cross reaction between VZV and HSV (Table 1). Preexisting titers of anti-HSV serum IgG in patients with herpes zoster (cases Z-1, Z-2 and Z-3) remained unchanged during a raise of anti-VZV serum IgG antibodies (Table 1). Also no increase in anti-HSV core IgM could be found in patient V-2 (Table 1).

Table 1. *IgM and IgG anti–VZV and anti–HSV serum-antibody titers (log 10) detected by FAMA and immuno-EM in patients with VZV infection*

patient	diagnosis	age	type lesion	days	anti-VZV IgM FAMA	anti-VZV IgM IEM core	anti-VZV IgM IEM env.	anti-VZV IgG FAMA	anti-VZV IgG IEM core	anti-VZV IgG IEM env.	anti-HSV IgM FAMA	anti-HSV IgM IEM core	anti-HSV IgM IEM env.	anti-HSV IgG FAMA	anti-HSV IgG IEM core	anti-HSV IgG IEM env.
V-1	varicella	21 yr	vesicle	2	–	–	–	–	–	–	–	–	–	–	–	–
V-1	varicella	21 yr	pustule	5	3.9	3.5	3.2	3.6	–	–	2.1	2.9	–	–	–	–
V-1	varicella	21 yr	pustule	7	3.9	3.5	3.2	4.2	–	3.2	3	3.2	2.6	–	2.9	–
V-2	varicella	30 yr	vesicle	3	2.4	2.9	2.9	3	–	–	–	2.6	–	–	–	–
V-2	varicella	30 yr	pustule	5	nd	3.2	3.2	nd	–	2.9	nd	2.9	–	nd	–	–
V-2	varicella	30 yr	pustule	9	3.6	3.5	3.6	3.6	3.2	3.2	–	3.2	–	–	–	–
Z-1	herpes zoster	22 yr	vesicle	2	–	2.6	–	3	–	3.5	–	–	–	3.6	3.5	3.5
Z-1	herpes zoster	22 yr	pustule	4	–	2.6	–	4.2	–	3.8	–	–	–	3.9	3.5	3.5
Z-1	herpes zoster	22 yr	pustule	6	2.7	2.9	–	4.8	2.9	4.1	–	–	–	4.2	4.1	3.8
Z-2	herpes zoster	54 yr	vesicle	3	–	–	–	3	2.9	2.9	–	–	–	4.2	3.8	3.8
Z-2	herpes zoster	54 yr	pustule	5	2.7	–	–	3.6	3.2	4.1	–	–	–	4.2	3.8	3.8
Z-2	herpes zoster	54 yr	pustule	8	2.7	–	–	4.8	5	5	–	–	–	4.2	3.8	3.5
Z-3	herpes zoster	61 yr	pustule	5	2.4	–	–	4.2	2.9	3.5	–	–	–	3.9	3.8	3.8
Z-3	herpes zoster	61 yr	pustule	7	3.3	–	2.3	4.8	3.2	4.1	–	–	–	3.8	3.8	3.8
V-3	varicella	18 wk	nr	12	2.1	3.8	3.8	3.9	3.5	4.1	–	2	–	3	3.5	3.5
V-4	varicella	50 wk	nr	12	2.1	4.4	3.5	3.9	3.5	3.5	–	–	–	–	–	–
V-5	varicella	6 yr	nr	21	–	3.8	–	3.9	3.8	3.8	–	–	–	–	2.9	–
C-1	healthy	43 yr	na	na	–	–	–	–	–	–	–	–	–	–	–	–

Env. = virus envelope; nr = not relevant; na = not applicable; nd = not done

Table 2. *The activated complement system and the detection of VICs in VZV-skin lesions*

		IEM						IF	
		anti-VZV Ig		anti-VZV IgM		anti-VZV IgG		complement alt.	clas.
patient	days	serum env.	lesion VICs	serum env.	lesion VICs	serum env.	lesion VICs	lesion B1a	C1q
V–1	2	–	–	–	–	–	–	–	–
V–1	5	+	+	+	+	–	–	–	–
V–1	7	+	+	+	±	+	+	+	–
V–2	3	+	+	+	+	–	–	–	–
V–2	5	+	+	+	+	+	–	–	–
V–2	9	+	+	+	+	+	+	+	+
Z–1	2	+	–	–	–	+	–	–	–
Z–1	4	+	–	–	–	+	–	–	+
Z–1	6	+	+	–	–	+	+	+	+
Z–2	3	+	–	–	–	+	–	–	+
Z–2	5	+	+	–	–	+	+	+	+
Z–2	8	+	+	–	–	+	+	+	+
Z–3	5	+	–	–	–	+	–	–	+
Z–3	7	+	+	+	±	+	+	+	+

+ = present; ± = very little of labeled enveoped virus; – = absent; env. = virus envelope; VICs = virus immune complexes; alt. = alternative complement pathway; clas.= classical complement pathway; Ig = all human immunoglobulins as detected with polyclonal rabbit anti human serum

The IgG antibodies found in 18-weeks old patient V-3 (Table 1), most likely represent maternal antibodies, due to genital HSV infection in its mother.
In varicella patients V-1 and V-2 IgM and IgG serum titers were similar in FAMA. The IEM showed IgM titers before IgG could be detected against VZV (Table 1). These results indicate that these adults indeed suffered from primary VZV infection. In secondary infection, herpes zoster, as well as late in varicella (after day 12) IgG titers were more than 1.5 log over IgM titers (Table 1, cases Z-1, Z-2, Z-3, and V-3, V-4,V-5). The higher IgG titers compared to IgM titers is in accordance with the presence of a memory B lymphocyte response in herpes zoster.

In table 2 serology is compared with the detection of virus immune complexes (VICs) and complement. Serum Igs against the viral core were present in the same samples, albeit some differences existed in Ig isotype, i.e. IgM or IgG (Table 1). In all lesions VZV antigen was detected by IEM and IF (data not shown). Serum anti-VZV immunoglobulins were present in all patients except in V-1 at day 2 (Table 2). Immunohistology showed that total IgM, IgG and IgA were also present in lesions (data not shown). We reasoned that if non specific immunoglobulins (Igs) were present in lesions and serum contains anti-VZV Igs, anti-VZV Igs should also be present in the lesions. In varicella patients, VICs were always detected in lesions when anti-VZV Igs could be detected in serum (patients V-1 and V-2,

Table 2). The decrease of IgM-labelled VICs in patients V-1 and Z-3 at day 7 can be explained by competition between IgM and affinity-matured IgG [1]. In contrast to varicella, in the early samples from herpes-zoster patients (Z-1, Z-2 and Z-3) no VICs were detected (Table 2, column anti-VZV Ig). The intralesional presence of B1a, a marker of the alternative complement pathway, remarkably corresponds with the IgG containing VICs (Table 2). In contrast, the detection of C1q, a marker of the classical complement pathway (known to be activated by immune complexes) did not show any relation with VICs.
In table 3 cellular infiltrates of lesions are compared with the presence of VICs. $CD1a^+$ Langerhans cells and CD22+ B cells were not detected in lesions (data not shown). Intact epidermis approximate to the lesion appeared to be depleted of Langerhans cells (data not shown). The number of non-lymphocyte infiltrating cells was calculated either as the number of cells not stained with CD3, or not stained with CD4 and CD8 (Table 3). Remarkably, early in varicella (day 2 or 3) the cells present in the lesion are almost exclusively T lymphocytes (85 - 90 %). Later in varicella, as well as early in herpes zoster, 30 - 50% of infiltrating cells were monomyeloid leukocytes (Table 3). Early in varicella and herpes zoster infection (day 2 or 3) the ratio $CD4^+$ and $CD8^+$ T lymphocytes below 0.2 or above 2.0. From day 4 on the ratio between T cell populations approach 1.0.

Table 3. *The influx of inflammatory cells in VZV skin lesions (% labeled leukocytes) compared with the presence of VICs*

	IHC								IEM
patient code	$CD3^+$ day	$CD4^+$ T cells	$CD8^+$ T-helper	ratio CTL	$CD3^-$ $CD4^+/CD8^+$	$CD4^-$ 8^- non-T	mean non-T	Ig non-T	VICs
V-1	2	88	62	31	2.0	12	7	10	–
V-1	5	77	26	34	0.8	23	40	32	+
V-1	7	65	20	43	0.5	35	37	36	+
V-2	3	83	14	76	0.2	17	10	14	+
V-2	5	55	26	29	0.9	45	45	45	+
V-2	9	59	28	31	0.9	41	41	41	+
Z-1	2	57	10	55	0.2	43	35	39	–
Z-1	4	60	23	24	1.0	40	53	47	–
Z-1	6	57	23	29	0.8	43	48	46	+
Z-2	3	75	40	19	2.1	25	41	33	–
Z-2	5	66	33	28	1.2	34	39	37	+
Z-2	8	68	33	21	1.6	32	46	39	+
Z-3	5	74	22	28	0.8	26	50	38	–
Z-3	7	69	26	25	1.0	31	49	40	+

% = number of intralesional peroxidase positive cells per 100 leukocytes; + = present; – = absent;
Ig = all human immunoglobulins as detected by IEM with polyclonal rabbit anti human serum

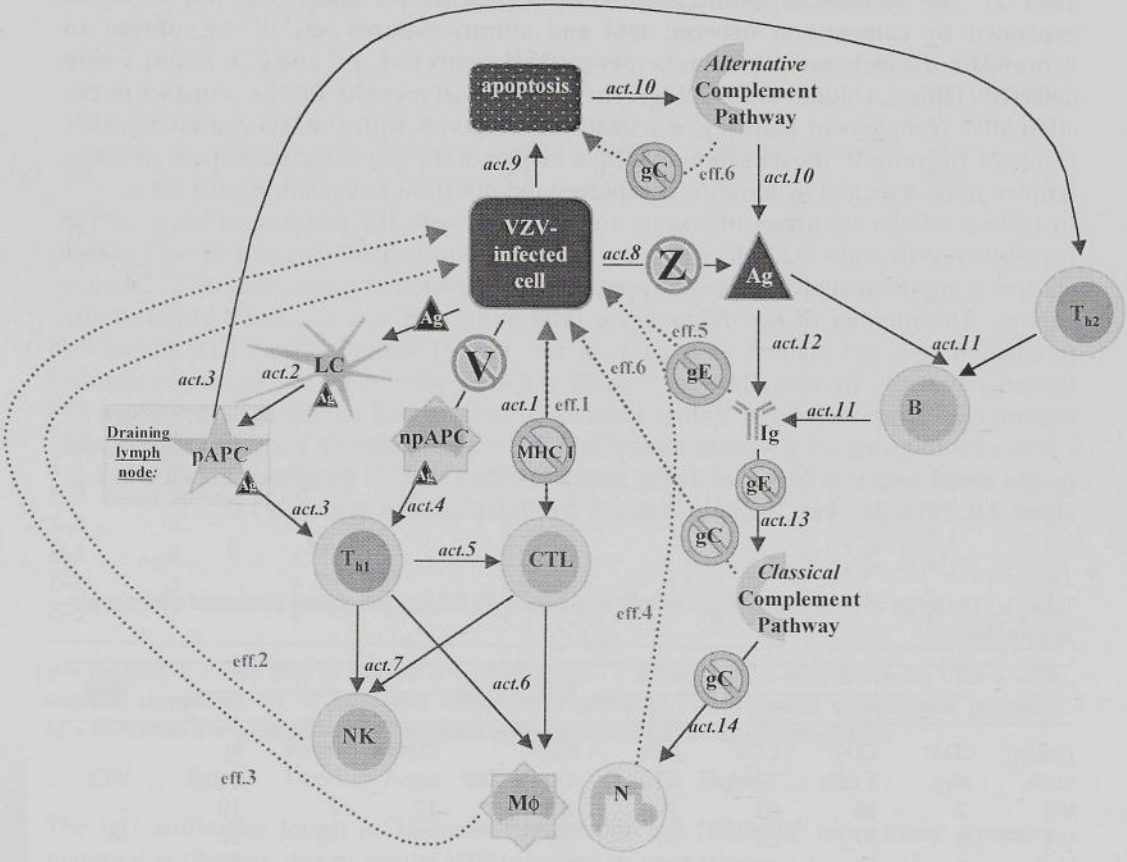
apoptosis
act.10
Alternative Complement Pathway
act.9
gC
eff.6
act.10
VZV-infected cell
act.8
Z
Ag
Ag
V
eff.5
act.12
act.11
T h2
act.3
act.2
LC
eff.6
gE
act.1
eff.1
act.11
B
Draining lymph node:
pAPC
npAPC
MHC I
Ig
act.3
act.4
gE
act.13
gC
T h1
act.5
CTL
Classical Complement Pathway
eff.4
eff.2
act.7
act.6
gC
NK
act.14
eff.3
Mϕ
N

Fig. 1. Mechanisms of immune evasion by varicella-zoster virus

Legends:

→ Response activating pathways (*act.*):

1 infected cells present VZV antigens to CTLs in MHC-I context;
2 antigen uptake by LCs, and maturation of LCs to professional APCs;
3 pAPCs present VZV antigens to T_h lymphocytes in MHC-II context;
4 in memory immune response (herpes zoster), non-professional APCs present VZV antigens to $T_h$lymphocytes in MHC-II context;
5 activation of CTLs;
6 activation of macrophages;
7 activation of NK cells;
8 only in varicella, virus antigens are liberated from infected cells;
9 infected cells become apoptotic;
10 lysis of apoptotic cells by the alternative complement pathway results in liberated antigens;
11 VZV antigens together with T_{h2} cell stimulate B lymphocytes to produce antibodies;
12 free VZV particles forms VICs with immunoglobulins;
13 VICs stimulate the classical complement pathway;
14 the classical complement pathway activates neutrophils.

⇢ Immune effector mechanisms (eff.):

1 CTLs kill infected cells by recognizing VZV antigens in CTLs in MHC-I context;
2 NK cells recognize absence of MHC I on (infected) cells;
3 activated macrophages become cytotoxic;
4 neutrophils are cytotoxic in combination with immunoglobulins;
5 neutralizing immunoglobulins eliminate VZV;
6 activated complement pathways lyse infected cells.

Tools of VZV to block immune effector mechanisms:

V = only in varicella
Z = only in herpes zoster
gC = gC functions as a complement receptor
gE = gE functions as an Fc_g receptor
MHC-I = down regulation of MHC-I

Ag = antigen
APC = antigen-presenting cell
pAPC = professional APC
npAPC = non-professional APC
B = B lymphocyte
CTL = cytotoxic T lymphocytes
LC = Langerhans cell
N = neutrophilic granulocyte
NK = natural killer cell
Mf = macrophage
T_h = T-helper lymphocyte

4. Discussion

VZV enters the body through the respiratory mucous membranes, spreads via regional lymph nodes into the blood, resulting in a first viremic phase. During a second viremic phase, infected peripheral blood mononuclear cells permit transportation of VZV to the skin. The incubation period before the appearance of skin lesions is about 2 weeks. After arrival at the skin VZV may spread to fibroblasts and keratinocytes. In the cutaneous sites of virus replication local immune responses will develop to eradicate the virus. The virus is presumed to move from the skin along neural pathways to sensory ganglia cells of the dorsal root to establish latency. In herpes zoster, VZV is reactivated, and spreads along the neural pathways into the skin, resulting in a second clinical manifest infection (9). Data in literature focusing on advanced immune evasion strategies of human herpesviruses are reviewed below, and combined with the results of our study to work out a hypothesis about the interactions between VZV and the immune system (Fig. 1):

- HSV-gene ICP47 encodes a inhibitor to block transport of (virus) peptides in to the endoplasmatic reticulum by the peptide transporter (TAP). By this mechanism, HSV avoids formation of the MHC-I peptide complex and thus antigen presentation and recognition by CTLs [36, 37]. In addition, HSV-infected cells appear to be resistant against CTL-induced apoptosis [38]. Like HSV, VZV is reported to induce MHC-I down regulation [39], which would enable escape from CTLs.
- Natural killer (NK) cells are cytotoxic to cells that lack MHC I. NK cells recognize their target cells and are cytotoxic, unless the NK killer-inhibitory receptor (KIR) binds to MHC I of the target cell [40]. HSV-infected cells avoid NK cytotoxicity, although MHC I is down regulated [41]. Cytomegalovirus, a betaherpesvirus, has been reported to code for an MHC-I analogue, which is recognized by KIRs and thereby inhibits NK-mediated cytotoxicity [42, 43]. Whether a similar escape mechanism exists for VZV remains to be tested.
- A third mechanism of immune escape is mediated by glycoprotein C (gC), which is conserved between the alphaherpesviruses. HSV-1 and HSV-2 gC have been shown to inhibit complement-mediated cell lysis by binding to activated forms of C3 [44-46]; VZV gC remains to be tested for this activity.
- A fourth immune-escape mechanism is described for the cooperation of two other glycoproteins, gE and gI. HSV gE and gI form a heterodimer which functions as an IgG (Fcγ) receptor [47]; VZV gE is capable to function as Fcγ receptor as well [48].

The immune escape mechanisms of alphaherpesviruses, may delay the immune effector response. We have shown *in vivo* that, T lymphocytes are the first cells recruited in varicella (Table 3). Only memory T lymphocytes are recruited in tissue [49], and almost all activated $CD8^+$ T lymphocytes are antigen specific [50, 51]. Antigen-stimulated T cells, secrete chemokines which attract innate immune cells, like macrophages and neutrophil granulocytes. In herpes zoster we found that the innate immune cells were already present at day 2 or 3 of the memory immune response (Table 3, Z-1 and Z-2). This agrees with the general immunologic concept that antigen-specific memory responses occur faster and stronger than primary immune responses [1]. Early in herpes zoster, in vesicular lesions, clustering of inflammatory cells around infected keratinocytes is absent (no rosette

formation of leukocytes). Later, in pustular lesions, the appearance of rosettes consisting of granulocytes and small lymphocytes around lytic keratinocytes, is prominent [52], and indicates that immune effector mechanisms are activated.

Immune complexes can activate the classical complement pathway [1]. In herpes zoster we noticed that the classical complement pathway was activated at day 3 - 4 of the clinical disease, while in varicella, C1q was not deposited until day 9 (Table 2). We detected VICs in varicella earlier than in herpes zoster. Thus C1q deposition did not correlate with VICs. Memory T cells may initiate an immune response, thereby inducing acute phase proteins, which can activate the classical complement pathway [53, 54]. Enhanced T-lymphocyte responses would then explain our finding that C1q deposition occurs earlier in herpes zoster than in varicella.

In varicella, the intralesional presence of VZV and antibodies resulted in VICs. This indicates that VZV during a primary infection is accessible to immunoglobulins. In herpes zoster, however, virus particles and serum antibodies were found but VICs were not always formed (Table 2). Apparently, in herpes zoster, the virus hides from antibodies, e.g. by keeping its antigens intracellular. Early in herpes zoster, in vesicular lesions, clustering of inflammatory cells around infected keratinocytes is absent (no leukocytes rosette formation of leukocytes). Later, in pustular lesions, the appearance of rosettes consisting of granulocytes and small lymphocytes around lytic keratinocytes, is prominent, and indicates that immune effector mechanisms are activated [52]. A putative explanation for the absence of VICs could be a decrease in production of viral mRNAs and proteins as has been reported in chronic VZV lesions [55, 56].

A decrease of VZV mRNA and (glyco)proteins could explain the absence of VICs due to reduced virus particle formation. However, decreased VZV-particle production in herpes zoster contradicts with our IHC and IEM detection of VZV, suggesting another mechanism is responsible for the absences of VICs. Such a mechanism could be intracellular hiding of VZV particles. Some glycoproteins of clinical isolates of HSV were not processed and remained intracellular, while in laboratory adapted HSV-strains glycoprotein processing occurred without delay [57]. The intracellular arrest of glycoproteins could be a tool for alphaherpesviruses to hide from antibody-mediated immune responses. If our hypothesis is true and the viral antigens remain intracellular, cell lysis is required to form VICs with IgM or IgG. Generally, virus infection may render cells apoptotic. Apoptotic cells diminish membrane expression of complement-regulatory molecules and in this way apoptotic (infected) cells become susceptible for complement-mediated cell lysis by the alternative pathway [58, 59]. Moreover, T lymphocytes [60], macrophages [61] and neutrophils [62], have been shown to produce properdin, a positive regulator of the alternative pathway of complement activation [63]. The requirement for complement of the alternative pathway during the process of lysis of virus-infected cells has been demonstrated in *in vitro* studies [64, 65]. We found that the alternative pathway was activated, as indicated by B1a deposition, when VICs occurred in herpes zoster (Table 2). This suggests that the alternative complement pathway is able to release VZV antigens from apoptotic cells.

After discussing the effector mechanisms above, we will now briefly review the activation of immune cells that generates these mechanisms. In healthy children with mild varicella, T-cell proliferation to VZV antigens occurs in 3 days after onset of clinical disease[66, 67]. In varicella, VZV-activated T-helper lymphocytes stimulate B lymphocytes [68], but stimulate

also the generation of CTLs [69]. Both higher T-cell proliferation and lower interferon-γ production were associated with reduced varicella lesion numbers[66, 70]. In line with our finding of B-lymphocyte mediated effector mechanisms in varicella, T-helper lymphocytes are either unpolarized type 0 or a weakly to polarized to a type 2 response.
In conclusion, differences in type 2 (antibody) and type 1 (cellular) mediated effector mechanism could be found between varicella and herpes zoster, respectively. In varicella, anti-VZV antibodies form VICs. Antibody-mediated responses, like antibody-dependent cytotoxicity, may thus be important effectors. These results could explain the beneficial effects of immune prophylaxis with antibodies after first contact with VZV and early in varicella. In herpes zoster, VZV avoids recognition by antibodies and the T-helper 2 response may thus loose most of its virus neutralizing potency. Enhanced, memory cellular cytotoxic responses of the T-helper 1 type, may be important for the immune control of herpes zoster. The latter has been shown before by the association of VZV reactivation (herpes zoster) with decreased CTL activity [71].
We do not know if other viruses that cause persistent, chronic or reactivated infections, use a similar mechanism to evade neutralization by antibodies. Examples of such viruses are HSV, beta- and gammaherpesviruses and human immunodeficiency virus (HIV). It might be useful to investigate antibodies, virus particles and the forming of VICs in other infections as well. VICs can be examined by the IEM method, as we did, as well as by a sandwich ELISA, using e.g. a capturing antibody for the virus and a detecting antibody against the immunoglobulins that form VICs.

Acknowledgments

The authors would like to thank Johan van Lier (Department of Pathological Anatomy, University Hospital Rotterdam, and Erasmus University, Rotterdam) for performing the immunohistochemical studies. Frans Wagenaar (Department of Virology, ID-DLO, Lelystad) is acknowledged for his technical assistance with IEM. We are grateful to professor Mohammed R. Daha (Leiden University Medical Center, Department of Nephrology) for critically reading the manuscript.

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CHAPTER 6 Summary & Samenvatting

Chapter 1: General introduction

The herpes viruses (*Herpesviridae*) belong to a family of double-stranded DNA viruses. The herpes viruses are found in eukaryotes, organisms consisting of cells whose nucleus can divide and is surrounded by a nucleus membrane. Due to the fact that every animal species has its own herpes family, it is presumed, that the virus is of very old origin and that through millions of years it has been able to adapt itself to life on earth.
The family of herpes viruses are subdivided into three subfamilies.
Alphaherpesviruses are rapidly growing viruses which finally destroy the cells in which they grow and, once present in the body, have a permanent latent existence.
Betaherpesviruses grow slower than the alpaherpesviruses, causing swelling of the infected cells and slower destruction. Gammaherpes viruses multiply in lymphocytes causing transformation into malignant cells.
At present, eight herpes viruses are known to infect humans. Three of them are alphaherpesviruses: herpes simplex virus 1 (HSV-1) which causes a cold sore on the lips, herpes simplex virus 2 (HSV-2), which is responsible for the sexually transmitted condition *herpes genitalis* which causes recurrent painful eruptions on the genitals and in the region of the anus, and the varicella-zoster virus (VZV) which persists latently in the body of everyone who has suffered from chickenpox and which can manifest itself later as shingles. The cytomegalovirus, which is a betaherpes virus, causes serious general illness in patients with a low resistance such as patients suffering from AIDS and patients who have undergone an organ transplant. The Epstein-Barr virus is a gammaherpes virus and causes, amongst other conditions, glandular fever. Additionally, there are two herpes viruses which are simply called number 6 and 7, which cause fever and skin eruptions. Herpes virus 8, which has recently been discovered, is said to be related to certain skin tumors with AIDS patients, the Kaposi sarcoom. Although, at present, eight human herpes viruses are known, the dermatovenereologist is mostly confronted with the symptoms which are caused by the alphaherpesviruses.
The diagnosis chickenpox (varicella), shingles (herpes zoster) and herpes (herpes simplex) is generally made on the grounds of clinical evidence. But other viral and non-viral skin conditions, resulting in blebs and blisters or pustules, sometimes strongly resemble the herpetiform eruptions of the skin and bordering mucous membranes which are caused by the human alphaherpesviruses. Especially with complaints in the genital and anal region, the dermato-venereologist sometimes has to consult a laboratory to be able to make a correct diagnosis.

The herpesvirus sometimes manifests itself in the affected parts of patients with eczema. Seeing as eczema is usually treated by medication which suppresses the defence mechanism, the wrong treatment can easily be given leading to proliferation of the virus and an increasingly sick patient.
It is of crucial importance to a small group of people that the diagnosis is made as quickly as possible.They often have a defect immunity system through illnesses such as AIDS or leukaemia or through defence repressive medication following a transplant. Additional laboratory research may therefore be necessary to confirm the clinical diagnosis. Herpes can also have fatal consequences for infants whose immunity system is still in the early stages of development. The first vesicles on the baby`s skin could be the first symptoms of a life threatening situation. In the case of a herpes infection, the treatment must begin at the earliest possible stage to prevent a serious outcome.
Until the eighties there were no quick laboratory methods available to diagnose a herpes infection and the Doctor was entirely dependent on clinical evidence . Nowadays, the diagnosis is still made in this fashion but certain cases give rise to reservations.
Laboratories make frequent use of the immunofluorescent test in which labelled antibodies, which attach themselves to the herpesvirus, are used. Due to the fact that these antibodies light up once exposed to the light of a special microscope, it is possible to establish the presence of the herpesvirus.This test lasts at least two hours. Seeing as the test is not fully reliable the virus also has to be cultivated. In the case of a herpes simplex the virologist receives the results within two days and is also informed if the pathogen is resistant to certain medication. The cultivation of the varicella-zoster virus is far more difficult and lasts much longer, often too long for the daily dermatovenereologists practise.
The Tzanck test is a simple, cheap and quick test with use of the lightmicroscope which gives the dermatovenereologist the results within a few minutes. The Tzanck test can easily be performed during the surgery hours. This test is only the first step to the final diagnosis, seeing as it can not determine the type of alphaherpes virus which one is dealing with.
Viral culture is an excellent method for academic hospitals to gain an accurate diagnosis but most of the local hospitals in the Netherlands do not have the facilities to do so. They send their herpes viral material to a central virological laboratory in the area. Alphaherpesviruses, in particular the varicella-zoster virus, are extremely vunerable and can be easily impaired during transport. This makes the culture of herpesviruses less reliable for the local hospitals.
If lightmicroscopic research and viralculture can not supply a final diagnosis, more advanced research, e.g. with the help of immunoelectron microscopy or molecule-diagnostic DNA-augmenting techniques such as the polymerase-chain-reaction (PCR), is needed.

Chapter 2: Tzanck smear and viral culture in diagnosis of herpes simplex virus and varicella-zoster virus infection

In *chapter 2.1* the Tzanck smear is presented as a very easy and rapid test for the detection of a herpetic infection in skin lesions. For the Tzanck smear, a scalpel is used to carefully scrape material from the base of a fresh vesicle or pustule, and the scraping is smeared on a

slide and air dried. After drying, the material is fixed in methanol and stained preferentially with a rapid staining based on the immersion procedure, because the staining can be done within one minute.
In *chapter 2.2* the results of our study, conducted in a pediatric clinic, focussed on herpetic and non-herpetic vesicular and bullous skin disorders, indicated the high sensitivity (> 80%) and specificity (>90%) of the Tzanck smear.
Comparable results were obtained with herpetic skin lesions in our study dealing with genital herpes, as described in *chapter 2.3*. In vesicular skin and mucous membrane lesions a Tzanck smear specificity of 100% was obtained. In women with genital ulcerous mucous membrane lesions the Tzanck smear sensitivity drops to 44%, that makes cytodiagnosis not suitable for a routine screening test to exclude genital herpetic infection. On the other hand positive Tzanck smear results are very reliable in confirming the clinical diagnosis of herpetic infection.
In *chapter 2.4* a systematic diagnostic approach of pustular eruptions in the neonate is proposed. The need for investigating every neonate with pustules for an infectious disease is emphasized. The Tzanck smear, the Gram stain and a potassium hydroxide preparation are important quick diagnostic tests. The Tzanck smear is a very easy, rapid and sensitive test for detection of a herpetic infection as well as noninfectious pustular eruptions. Therefore, the Tzanck smear should be the first test to be performed. Moreover, a Gram stain and potassium hydroxide preparation should be performed in cases of neonatal pustular disorders to detect bacterial and fungal infections. The goal of this diagnostic approach is to spare a healthy neonate, with a benign transient condition, an invasive evaluation for sepsis, potentially harmful antibiotic therapy, and prolonged hospitalization with its own inherent morbidity.

Chapter 3: Immunogold electron microscopy in diagnosis of herpes simplex virus and varicella-zoster virus infection

In many hospitals in Holland an adequate laboratory facility for viral culture is not available. Specimens must be sent to specialized virological laboratories elsewhere, and therefore the processing of viral specimens will be delayed. Especially VZV can loose infectivity very rapidly when kept in transport medium for longer periods. Even under optimal laboratory conditions, VZV isolation is substantially less sensitive than those for HSV. Electron microscopy can be applied as a rapid method for virus diagnosis, and can be used in validation studies.
In *chapter3.1* colloidal gold immunoelectron microscopy for rapid diagnosis of VZV infections by discrimination between VZV, HSV-1 and HSV-2, is described in detail. *Chapter3.2* describes improved methods for detection of HSV by electron microscopy in clinical specimens. A virus concentration procedure by ultracentrifugation is essential to increase the sensitivity of herpes virus detection by electron microscopy. The usefulness of solid phase immunoelectron microscopy (SPIEM) to enhance the sensitivity of herpes virus detection was also investigated. Virus and viral proteins were picked up by a bilayer of protein A and capture antibody absorbed to the EM grid. In comparison with colloidal gold labelling of viruses directly adsorbed to carbon coated collodion-nickel grids

simultaneously trapping of these viral proteins causes high background labeling. SPIEM-gold labelling methods for herpes virus detection in crude tissue suspension are therefore not advisable. The results of immuno-EM were compared with those obtained by virus culture and cytodiagnosis. In this study the sensitivity of virus culture for vesicular, pustular and ulcerous lesions was 100%, 70% and 63%, respectively. The Tzanck smear sensitivity for the same lesion types was 91%, 85% and 56%, respectively. However, EM sensitivity did not seem to have a relation with the stage of the lesion: 91%, 100% and 94%, respectively. The use of ultracentrifugation-treated virus suspensions is crucial for obtaining reliable results in immuno-EM as a confirmation test in HSV-diagnosis.
Chapter3.3 outlines the sensitivity and specificity of the Tzanck preparation in diagnosing herpes virus/VZV infection in comparison with viral culture and electron microscopy. VZV is less tolerant than HSV to transport and storage conditions. Furthermore, diagnosis on the basis of virus isolation takes several days because the VZV proliferates at a slower rate than HSV. Virus isolation is therefore less suitable for rapid diagnosis of VZV infection. Virus isolation of VZV under optimal conditions reached a sensitivity of 80%. In circumstances representative for the general practitioner and medical specialists in hospitals without facilities for virus culturing the sensitivity of VZV isolation dropped dramatically to 14%. The sensitivity of the Tzanck test in this study was as high as 90%. The Tzanck test cannot differentiate between VZV and HSV, but is valuable in supporting the clinical diagnosis of VZV infection. Direct electron microscopy (negative staining) reached a sensitivity of 80%, comparable to the results of virus isolation under optimal conditions. The sensitivity of colloidal gold immuno-electron microscopy after a virus concentration procedure with ultracentrifugation was 95%. The classical EM technique cannot differentiate between VZV and HSV. CG-IEM provides the possibility for rapid and specific diagnosis in herpetic infections.
Chapter 3.4 reports on a case of human T-cell lymphotropic virus type 1-positive leukemia complicated by atypical multidermatomal herpes zoster. Where standard tests failed, cytodiagnosis (Tzanck test) and immuno-electron microscopy unmistakably have proven VZV infection.
In *Addendum to Chapter 3,* an easy method of storage of EM-grids, applied to this study, is described. The conventional grid storage boxes were replaced by large petri dishes. In this way, numerous grids of multiple EM experiments can be traced and localized in a minimum of time.

Chapter 4: Detection of varicella-zoster virus and herpes simplex virus by polymerase chain reaction with degenerated primers

As already mentioned, diagnosis of VZV by cell culture is difficult, because virus culture facilities are not present in most of the non-academic hospitals. Electron microscopy is only available in specialized laboratories. The polymerase chain reaction (PCR) may be an attractive method for routine microbiological laboratories. It can be applied in all developmental stages of herpetic lesions. The results of PCR can be obtained within days, whereas viral culture may take weeks.
In *chapter 4,* the results of our study with PCR based on degenerative primers to confirm

herpes virus infection were described. Theoretically, the use of degenerative primers may give a higher sensitivity in case of detecting (mutant) herpes viruses. The sensitivity of virus detection by PCR depends upon the virus sampling procedure: dry sampling allows more sensitive detection than wet sampling in Hank's balanced salt solution (HBSS, used in clinical practice to conserve virus for culture). Our PCR results strongly suggest that dry specimens are more suitable for (storage prior to) PCR detection than HBSS.

Chapter 5: Immune response to varicella-zoster virus infection in vivo: immunoglobulins, complement and cellular infiltrates

Host defence against viral infection is complex and includes multiple innate, or nonspecific, as well as antigen specific mechanisms. *Chapter 5* focuses on the detection of antiviral antibodies, complement activation, and cellular immune response by VZV infection. This chapter reviews the various processes by which VZV evades the host defence immune response. Between the primary (varicella) and the reactivated infection (herpes zoster) differences in immune system-virus interactions were noticed. Early in varicella, T lymphocytes infiltrate lesions solely; in herpes zoster and late varicella, innate leukocytes appear in addition to T lymphocytes. In varicella, virus immune complexes emerge, when serum antibodies can be detected. In herpes zoster VZV hides, possibly intracellular, from immunoglobulins, but when the alternative complement pathway is activated, VZV is released and VICs are formed.

In conclusion, differences in type 2 (antibody) and type 1 (cellular) mediated effector mechanism were found between varicella and herpes zoster, respectively. In varicella, anti-VZV antibodies form VICs. Antibody-mediated responses, like antibody-dependent cytotoxicity, may be important effectors. These results could explain the beneficial effects of immune prophylaxis with antibodies after first contact with VZV and early in varicella. In herpes zoster, VZV avoids recognition by antibodies and the T-helper 2 response may loose most of its virus neutralizing potency. Enhanced, memory cellular cytotoxic responses of the T-helper 1 type, may be important for the immune control of herpes zoster.

Hoofdstuk 1: Algemene inleiding

De herpesvirussen (*Herpesviridae)* behoren tot een familie van dubbelstrengs DNA-virussen. De herpesvirussen komen voor bij eukaryoten, organismen die bestaan uit cellen die een celkern hebben die zich kan delen en die omgeven wordt door een kernmembraan. Omdat vrijwel iedere diersoort zijn eigen herpesfamilie heeft bestaat het vermoeden dat het om een heel oud virus gaat, dat zich door miljoenen jaren heen aan het leven op aarde heeft aangepast.
De familie van herpesvirussen wordt onderverdeeld in drie subfamilies. Alphaherpesvirussen zijn snel groeiende virussen die uiteindelijk de cellen waarin ze groeien stuk maken en de eigenschap hebben om, eenmaal in het lichaam aanwezig, er voor altijd sluimerend in aanwezig te blijven. Betaherpesvirussen groeien langzamer dan de alphaherpesvirussen, waardoor de geïnfecteerde cellen kunnen opzwellen en uiteindelijk veel later stuk gaan. Gammaherpesvirussen vermenigvuldigen zich in lymphocyten en waardoor deze in kwaadaardige cellen kunnen veranderen.
Bij de mens zijn momenteel acht herpesvirusen bekend. Daaronder zijn drie alphaherpesvirussen: herpes simplex-virus 1 (HSV-1) dat de *koortslip* veroorzaakt, herpes simplex-virus 2 (HSV-2), verantwoordelijk voor de seksueel overdraagbare aandoening *herpes genitalis* dat steeds terugkerende pijnlijke zweertjes aan de geslachtsdelen en in de anaalstreek veroorzaakt, en het varicella-zoster virus (VZV) dat bij iedereen die ooit de*waterpokken* kreeg sluimerend aanwezig blijft en later in de vorm van *gordelroos* opnieuw de kop kan opsteken. Verder komt het cytomegalovirus voor, een betaherpesvirus, dat bijvoorbeeld bij mensen met een verlaagde afweer, zoals na orgaantransplantatie of bij AIDS, ernstige algehele ziekteverschijnselen kan veroorzaken. Het Epstein-Barr virus is een gammaherpesvirus en veroorzaakt onder meer de ziekte van Pfeiffer. Dan zijn er nog de herpesvirussen die simpelweg nummer 6 en 7 worden genoemd en die koorts en huiduitslag kunnen veroorzaken. Het recent ontdekte herpesvirus 8 wordt in verband gebracht met bepaalde huidtumortjes bij AIDS, het Kaposi sarcoom. Hoewel er momenteel acht humane herpesvirussen bekend zijn, wordt de dermatovenereoloog het meeste met de verschijnselen die door de alphaherpesvirussen veroorzaakt worden, geconfronteerd.
De diagnose waterpokken (varicella), gordelroos (herpes zoster) en herpes (herpes simplex) wordt gewoonlijk gesteld op grond van het klinische beeld.
Maar andere virale en niet virale huidaandoeningen, die gepaard kunnen gaan met blaasjes, blaartjes en pustels lijken soms sterk op door de humane alphaherpesvirussen veroorzaakte herpetiforme erupties van de huid en aangrenzende slijmvliezen. Met name bij afwijkingen in de genitaal- en anaalstreek moet de dermatovenereoloog soms een beroep doen op een laboratorium om de juiste diagnose te kunnen stellen. Bij mensen met eczeem maakt het herpesvirus soms van de gelegenheid gebruik om zich op de getroffen plekken te manifesteren, zodat de diagnose gemakkelijk gemist kan worden. Dat kan leiden tot een verkeerde behandeling, aangezien eczeem meestal wordt bestreden met middelen die de afweer onderdrukken. Daardoor krijgt herpes vrij spel en wordt de patiënt alleen maar zieker.
Bij een kleine groep mensen is het van levensbelang om snel te bepalen of er sprake is van herpes. Zij hebben vaak een minder goed werkend immuunsysteem door ziekten als aids of leukemie of door afweer onderdrukkende medicijnen na een transplantatie. Aanvullend laboratoriumonderzoek kan daarom nodig zijn om de klinisch gestelde diagnose te

bevestigen. Herpes kan eveneens fatale gevolgen hebben voor zuigelingen, van wie het immuunsysteem zich nog niet volledig heeft ontwikkeld. Als de eerste blaasjes op de babyhuid verschijnen, kan er al sprake zijn van een levensbedreigende aandoening. In het geval van herpes moet de behandeling zo vroeg mogelijk gestart worden om een ernstige afloop te voorkomen.
Tot in de jaren tachtig waren er geen snelle laboratoriummethoden beschikbaar om een herpesinfectie vast te stellen en kon een arts alleen afgaan op de klinische verschijnselen. Tegenwoordig wordt de diagnose vaak nog steeds op die manier gesteld, maar in bepaalde gevallen blijft er twijfel bestaan.
In het laboratorium wordt vaak gebruik gemaakt van de immunofluorescentietest, waarbij gemerkte antistoffen worden gebruikt die zich alleen aan herpesvirussen hechten. Doordat deze antistoffen oplichten bij het licht van een speciale microscoop, is het mogelijk vast te stellen of er sprake is van herpes. Deze test duurt op zijn minst een paar uur. Omdat deze test toch niet altijd volledig betrouwbaar is moet het virus ook worden gekweekt. In het geval van herpes simplex heeft de viroloog meestal binnen twee dagen de uitslag en weet hij meteen of de ziekteverwekker resistent is tegen bepaalde medicijnen. Het kweken van het varicella-zoster virus is veel moeilijker en duurt bovendien ook veel langer. Deze testen zijn in de dagelijkse dermatovenereologische praktijk meestal niet snel genoeg.
De Tzanck-test is een eenvoudige, goedkope en snelle test met behulp van het lichtmicroscoop, waarmee de dermatovenereoloog al binnen enkele minuten de uitslag kan hebben. De Tzanck-test kan daarom gemakkelijk tijdens het spreekuur worden gedaan. Deze test is een eerste stap in de uiteindelijke diagnose, want de Tzanck-test kan geen uitsluitsel geven over het type alphaherpesvirus waarmee men te maken heeft.
Viruskweken zijn voor academische ziekenhuizen een prima oplossing voor preciese diagnostiek, maar de meeste perifere ziekenhuizen in Nederland beschikken niet over de faciliteiten om dat zelf te doen. Zij sturen hun herpesvirusmateriaal naar een centraal virologisch laboratorium in de buurt. Alphaherpesvirussen, maar vooral het varicella-zoster virus, zijn erg kwetsbaar en kunnen tijdens het transport kapot gaan. Dat maakt de herpesviruskweek voor de perifere ziekenhuizen minder betrouwbaar.
In het geval dat lichtmicroscopisch onderzoek en viruskweek geen definitieve diagnose kunnen geven, is meer geavanceerd onderzoek met behulp van bijvoorbeeld (immuno-)electronen microscopie of moleculair-diagnostische DNA-vermeerderingstechnieken zoals de polymerase-chain-reactie (PCR) gewenst.

Hoofdstuk 2: De Tzanck-test en de viruskweek bij de diagnose van herpes simplex-virus en varicella-zoster virus infectie

In *hoofdstuk 2.1* wordt de Tzanck-test gepresenteerd. Het is een eenvoudige en snelle test om een herpesinfectie te kunnen vaststellen bij huidafwijkingen die gepaard gaan met blaasjes en pustels. Met behulp van, bijvoorbeeld, een vaccinostyle wordt materiaal geschraapt van de bodem en de rand van een blaasje of pustel. Dit materiaal wordt uitgestreken op een objectglas en aan de lucht gedroogd.Vervolgens wordt het uitstrijkje gefixeerd in methanol en bij voorkeur gekleurd volgens een snelle kleurmethode, die ongeveer een minuut duurt.

In *hoofdstuk 2.2* worden de resultaten gepresenteerd van een vergelijkend onderzoek naar de resultaten van de Tzanck-test en de herpesviruskweek. Dit onderzoek werd verricht bij patiënten in het voormalige Sophia Kinderziekenhuis te Rotterdam. Deze patiënten hadden huidafwijkingen die gepaard gingen met blaasjes, blaartjes en pustels. Ten opzichte van de viruskweek had de Tzanck-test een gevoeligheid van meer dan 80% en een specificiteit van meer dan 90%.
Vergelijkbare resultaten worden verkregen bij herpes genitalis in het geval van herpeslesies op de huid. Dit onderzoek wordt besproken in *hoofdstuk 2.3* . Hier had de Tzanck-test bij herpesblaasjes op huid en slijmvliezen een specificiteit van 100%. Bij vrouwen met genitale slijmvlieswondjes daalde de gevoeligheid van de Tzanck-test naar 44%. Deze methode is daarom in het geval van genitale herpes niet geschikt als screeningsmethode in alle stadia van herpeslesies. Daarentegen is een positieve Tzanck-test betrouwbaar ter bevestiging van de klinische diagnose herpesvirusinfectie.
In *hoofdstuk 2.4* wordt een voorstel gedaan om te komen tot een systematische diagnostische aanpak in het geval van pustuleuze afwijkingen bij neonaten. Met nadruk wordt erop gewezen dat niet uitsluitend op het klinische beeld een diagnose mag worden gesteld, maar dat deze met behulp van aanvullend onderzoek dient te worden bevestigd. Daarbij zijn de Tzanck-test, het Gram-preparaat en het directe KOH-preparaat belangrijke diagnostische hulpmiddelen. De Tzanck-test is een erg eenvoudige, snelle en gevoelige test bij het vaststellen van een herpesinfectie van de huid en hij kan ook gebruikt worden bij het stellen van de diagnose bij niet-infectieuze pustuleuze aandoeningen zoals bij het erythema toxicum neonatorum. Een Gram-preparaat en het directe KOH-preparaat van pustels kunnen gebruikt worden bij het vast stellen van een bacteriële of een schimmelinfectie. Het uiteindelijke doel van deze schematische diagnostische aanpak is, om de uiteindelijk gezonde neonaat, die achteraf blijkt een onschuldige voorbijgaande huiduitslag te hebben gehad, invasief onderzoek ter uitsluiting van sepsis, behandeling met potentieel schadelijke antibiotica en een langdurig verblijf in het ziekenhuis, met alle gevaren van dien, te besparen.

Hoofdstuk 3: Electronenmicroscopie met behulp van immuno-goud-labeling bij de diagnose van herpes simplex-virus en varicella-zoster virus infectie

De meeste ziekenhuizen in Nederland beschikken niet over de mogelijkheid om herpesviruskweken zelf te verrichten. Het te kweken materiaal moet daarom naar daarin gespecialiseerde microbiologische laboratoria worden verstuurd. Vooral het varicella-zoster virus is erg kwetsbaar voor transport en kan daardoor zijn vermogen verliezen om in viruskweek zich te vermenigvuldigen. Zelfs onder optimale laboratoriumomstandigheden is het varicella-zoster virus minder goed instaat zich te vermenigvuldigen in de viruskweek dan het herpes simplexvirus. Electronenmicroscopisch (EM-) onderzoek, dat slechts in enkele laboratoria beschikbaar is, kan als een snelle diagnostische techniek worden gebruikt en is overigens ook geschikt om de uitkomst van andere diagnostische tests te valideren.
In *hoofdstuk 3.1* wordt gedetailleerd beschreven hoe EM-onderzoek met behulp van

immuno-goud labeling (immuno-EM) kan worden toegepast als snelle diagnostische test, waarbij tevens onderscheid gemaakt kan worden tussen VZV, HSV-1 en HSV-2.
Hoofdstuk 3.2 beschrijft verbeterde methoden bij de detectie met behulp van de electronenmicroscoop van het herpes simplex-virus in van patiënten afkomstig materiaal. Een virus-concentratieprocedure door middel van ultracentrifuge is essentieel om de detectiegevoeligheid van het EM-onderzoek te verhogen. De bruikbaarheid van de SPIEM-methode ('solid phase immunoelectron microscopy') om te komen tot een verhoogde detectiegevoeligheid voor het herpes simplex-virus werd tevens onderzocht. Daarbij worden het virus en virale eiwitten gevangen in een dubbellaag van proteïne A en het antigeen bindend antilichaam, die gebonden is aan een vaste plastic drager, zoals bijvoorbeeld het collodion-vlies over een metalen EM-grid. In vergelijking met de immuno-goud labelings methode, waarbij virussen direct worden geadsorbeerd aan een met een koolstoflaag bedekt EM-grid, veroorzaakt de SPIEM-methode een hoge achtergrond labeling door de tegelijkertijd mee ingevangen virale proteinen van niet meer intacte virusdeeltjes. Het gebruik van de SPIEM-methode voor de detectie van herpesvirussen in niet-voorbehandeld patiëntenmateriaal wordt daarom afgeraden. Verder worden in deze studie de resultaten van de immuno-EM-techniek vergeleken met die van de viruskweek en de Tzanck-test. In deze studie bleek de gevoeligheid van de viruskweek van herpes blaasjes, pustels en wondjes respectievelijk 100%, 70% en 63% te zijn. De gevoeligheid van de Tzanck-test bij deze lesies was respectievelijk 91%, 85% en 56%. De gevoeligheid van de in deze studie toegepaste EM-techniek leek niet te worden beïnvloed door het type herpes lesie en was respectievelijk 91%, 100% en 94%. Het gebruik van met ultracentrifuge voorbehandelde herpes simplex-virussuspensies is van cruciaal belang voor het verkrijgen van betrouwbare resultaten bij het gebruik van immuno-EM.
In *hoofdstuk 3.3* wordt de gevoeligheid en de specificiteit van de Tzanck-test bij het diagnostiseren van varicella-zoster virus infecties vergeleken met die van de viruskweek en het EM-onderzoek. VZV is kwetsbaarder dan HSV bij transport en in opslag. Omdat VZV bovendien langzamer groeit dan HSV is de viruskweek in dit geval niet geschikt voor sneldiagnostiek. Zelfs onder optimale condities werd in ons onderzoek voor VZV slechts een gevoeligheid van de viruskweek verkregen van 80%. In een situatie die representatief is voor de perifere dermato-venereologische praktijk bleek er een dramatisch verschil te bestaan met de viruskweek onder optimale omstandigheden; er werd slechts een gevoeligheid verkregen van 14%.
Daarentegen werd in deze studie met de Tzanck-test een gevoeligheid bereikt van meer dan 90%. De Tzanck-test kan geen onderscheid maken tussen VZV en HSV, maar is desondanks waardevol voor de ondersteuning van de klinische diagnose bij VZV-infecties.
Bij EM met gebruikmaking van de negatief-kleuringstechniek werd een gevoeligheid bereikt van 80%, een vergelijkbaar resultaat ten opzichte van wat bereikt werd met de viruskweek onder optimale omstandigheden. Na toepassing van ultra-centrifugatie bedroeg de gevoeligheid van immuno-EM met gebruikmaking van de colloïdaal-goudkleuringstechniek 95%. Met de klassieke EM-techniek met behulp van negatief-kleuring kan geen onderscheid worden gemaakt tussen VZV en HSV. Dit is wel mogelijk met immuno-EM en deze methode is dan ook geschikt voor sneldiagnostiek bij humane alphaherpesvirusinfecties.
In *hoofdstuk 3.4* wordt een patiënt beschreven met leukemie veroorzaakt door het humane

T-cel-lymphotrope virus type 1 (HTLV-1) en herpes zoster, verspreid over meerdere dermatomen. Met behulp van de viruskweek kon de diagnose VZV-infectie niet worden bevestigd. De Tzanck-test ondersteunde de klinische diagnose wel en met immuno-EM kon VZV worden aangetoond.
In het *addendum bij hoofdstuk 3* wordt een gemakkelijke opslagmethode voor EM-grids beschreven, zoals die door ons bij EM-onderzoek werd gebruikt. De conventionele opslagdoosjes voor EM-grids werden vervangen door grote petri-schalen. Met de door ons beschreven opslagmethode kunnen wij de EM-grids, ondanks de grote hoeveelheden, in een minimum van tijd gemakkelijk terugvinden.

Hoofdstuk 4: Het aantonen van het varicella-zoster virus en herpes simplex-virus door middel van een polymerase kettingreactie (PCR) met degeneratieve primers

Zoals al is opgemerkt, kan het moeilijk zijn om VZV aan te tonen door middel van de viruskweek, omdat de meeste niet-academische ziekenhuizen daarvoor de faciliteiten niet hebben. Electronenmicroscopie kan alleen worden toegepast in daarin gespecialiseerde laboratoria. De PCR kan als een aantrekkelijke routinemethode worden beschouwd voor diagnostiek in microbiologische laboratoria. Deze methode is toepasbaar in elk stadium van een herpeslesie. De resultaten van deze PCR zijn binnen twee dagen te verkrijgen, terwijl de viruskweek op zijn minst meerdere dagen in beslag neemt.
In dit hoofdstuk wordt beschreven wat het resultaat is van ons onderzoek naar PCR gebaseerd op *degeneratieve primers* om alphaherpesvirusinfecties vast te stellen. De gevoeligheid van deze PCR is afhankelijk van de bewaarprocedure van specimina van het virus. Het blijkt dat droge virusmonsters meer geschikt zijn - en dat droog bewaren beter is - voor de diagnostiek met PCR dan bewaren in vochtige media zoals die volgens Hank (Hanks Balanced Salt Solution, HBSS).

Hoofdstuk 5: Immuun respons op varicella-zoster virusinfectie in vivo : immunoglobulinen, complement en cellulair infiltraat

De afweer tegen een virusinfectie is complex en bestaat zowel uit aangeboren afweermechanismen, als uit niet-specifiek en specifiek tegen het antigeen gerichte verdedigingsmechanismen. Hoofdstuk 5 geeft in het bijzonder aandacht aan het aantonen van virale antilichamen, activatie van het complementsysteem en de cellulaire immuunrespons bij infectie met VZV. Dit hoofdstuk geeft een overzicht over de literatuur met betrekking tot de verschillende processen waarmee VZV reageert op het afweersysteem van de gastheer. Er werden verschillen opgemerkt in de interactie van VZV met het immuunsysteem tussen een primaire infectie (varicella, waterpokken) en een latere, lokale opvlamming (herpes zoster, gordelroos). In het beginstadium van varicella bestaat het cellulaire infiltraat vrijwel uitsluitend uit T lymphocyten; bij herpes zoster en in een laat stadium van varicella bestaat het infiltraat naast T lymphocyten ook uit leukocyten. In varicella kunnen met immuno-EM virale immuuncomplexen (VICs) worden aangetoond

wanneer in het serum antilichamen tegen het virus kunnen worden aangetoond. In het geval van herpes zoster lijkt het erop dat VZV zich als het ware (waarschijnlijk intracellulair) verstopt voor de virale antilichamen. Pas wanneer het alternatieve complement-systeem wordt geactiveerd worden er VICs gevormd. Samengevat kan gesteld worden dat er verschillen bestaan tussen varicella en herpes zoster in de type 2 (antilichaam-gemediëerde) en type 1 (cellulair gemediëerde) effector mechanismen. In varicella vormen anti-VZV-antilichamen VICs, waarbij antilichaam-gemediëerde immuunrespons, zoals antilichaam-afhankelijke cytotoxiciteit, mogelijk een belangrijk rol speelt. Dit zou kunnen verklaren waarom immuunglobulinen-prophylaxe na een eerste contact met VZV en in een vroeg stadium van varicella effectief is. In het geval van herpes zoster lijkt het erop dat VZV herkenning door antilichamen tegengaat, waardoor virusneutralisatie door de T-helper-2-respons grotendeels achterwege blijft. Een verhoging van de cytotoxische cellulaire 'memory-response' van het T-helper-1-type kan een belangrijke rol spelen in de immunologische afweer bij herpes zoster.

CHAPTER 7

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About the author

The author of this thesis, Elzo Folkers, was born on March 15th 1948 in Willemstad, Curaçao.
From 1960 to 1966 he attended high school in Ede (Gymnasium ß).
He obtained his medical degree at the medical faculty of the Free University of Amsterdam in March 1975. From April 1975 until April 1979 he was trained as a specialist in Dermato-venereology at the Free University Hospital, Amsterdam, the Netherlands.
In April 1979 he joined the team of medical specialists as a Dermato-venereologist in Hospital "de Heel", Zaandam, the Netherlands.

Curriculum vitae en dankwoord

Elzo Folkers werd op 15 maart 1948 te Willemstad, Curaçao, geboren. Hij doorliep de middelbare school (Gymnasium ß) te Ede.
Van september 1966 tot en met maart 1975 studeerde hij medicijnen aan de Vrije Universiteit te Amsterdam.
Daarna volgde hij vanaf 1 April 1975 een specialisatie dermatologie/venereologie op de afdeling Dermatologie en Venereologie van het Academisch Ziekenhuis der Vrije Universiteit (hoofd: prof. dr. E. van Dijk). Op 1 april 1979 vond registratie als huidarts plaats.
Sindsdien is hij als dermato-venereoloog werkzaam in Stichting Ziekenhuis "de Heel" te Zaandam.

In de eerste jaren van mijn medische opleiding werkte ik als verpleeghulp op de afdeling dermatologie in het Academisch Ziekenhuis van de Vrije Universiteit. Daar kwam ik in kontakt met mijn latere opleiders, professor dr. E. Van Dijk, die daar toen als chef de policlinique dermatologie werkzaam was en professor dr. G.L. Kalsbeek†, chef de clinique. In die tijd heb ik het *collegedictaat Dermatologie* uitgegeven, dat door (toen nog) dr.E. van Dijk werd gecorrigeerd. En zo is het begonnen. Omdat ik er belangstelling voor had, werd ik door hen dikwijls bij bijzondere patiënten gehaald en zij maakten mij enthousiast voor het specialisme dermatologie. Toen de opleiding dermatologie aan het AZVU eenmaal een feit was, kreeg ik daar de gelegenheid om mij verder als huidarts te specialiseren. Het was dr.G.L.Kalsbeek† die mij leerde om de Tzanck-test te gebruiken als ondersteuning voor de klinisch gestelde diagnose herpesvirusinfectie.
Tijdens mijn opleiding tot huidarts raakte ik bevriend met Han Vreeswijk, die toen bezig was met zijn promotieonderzoek over het molluscum contagiosum virus.
Mijn opleiding werd afgerond en ik ging werken als dermatoloog in het pas opgerichte fusie-ziekenhuis "de Heel" in samenwerkingsverband met collega dr.Theo G. Nelemans, die mij verder leerde hoe te werken in een perifere dermatologische praktijk.
Steeds meer raakte ik als perifeer werkzaam dermatoloog overtuigd van de waarde van de Tzanck-test bij het stellen van de diagnose bij die huidziekten, die gepaard gaan met blaasjes, blaartjes en pustels. Op een congres, ik weet niet meer precies wanneer, maar het

is waarschijnlijk tijdens een nascholingscursus geweest, ontmoette ik mijn co-promotor dr. Arnold P. Oranje. Beiden raakten we verder in de ban van de cytologische snel-diagnostiek bij humane alphaherpesvirusinfecties en we schreven een klinische les over de Tzanck-test voor het Nederlands Tijdschrift voor Geneeskunde. In die tijd belde Han Vreeswijk, die toen verbonden was aan de afdeling electronenmicroscopie van het voormalige CDI (het Centraal Diergeneeskundig Instituut te Lelystad) mij op met de vraag of ik humaan herpesvirusmateriaal zou kunnen leveren aan zijn laboratorium. Hij was een soort "deus ex machina": hij beschikte over de sleutel om de Tzanck-test verder te valideren in die gevallen waarin de herpesviruskweek verstek liet gaan. En zo is het verder gegaan. Jan N. Duivenvoorden, werkzaam als cytologisch hoofdanalist op de afdeling Pathologie van mijn ziekenhuis, werd ook door ons enthousiasme "besmet". Het uiteindelijke resultaat van dit alles werd in de voorafgaande bladzijden door ons als team beschreven. Alléén is zoiets onmogelijk!
Nog iemand raakte in de loop der tijd "geïnfecteerd", Marinus C.G.van Praag (zie hfdstk 2.4), die ik voor het eerst in Athene op de Acropolis echt leerde kennen. En door reorganisaties binnen het voormalige CDI kwam ik, door de veranderde werkzaamheden van mijn uiteindelijke co-promotor dr. J. Vreeswijk, in contact met de mogelijkheden van de PCR-diagnostiek. En zo kwam uiteindelijk ook John J.L. Jacobs in "ons" herpes-team verzeild.
Ook nu is dit verhaal nog niet compleet, zal de misschien geïnteresserde lezer opmerken. Waarom een promotie aan de Universiteit van Amsterdam? Dat is de laatste schakel in deze geschiedenis. Ziekenhuis de Heel had een affiliatiecontract met deze universiteit en collega Nelemans en ik waren bij de eersten in de periferie, die co-assistenten dermatologie van de UvA onder onze hoede kregen. De afgelopen jaren werden de contacten met de vakgroep dermatologie van het AMC steeds hechter en uiteindelijk gebeurde het onvermijdelijke: prof dr. Jan D. Bos werd mijn promotor.
Mijn kinderen Elle en Anne wil ik bedanken voor het geduld dat ze de afgelopen jaren met mij hebben gehad, mijn echtgenote Elsa voor incidentele kritische opmerkingen, niet alleen op het terrein van de Neerlandistiek.Ook mijn huidige collegae Armand J. Landwehr en dr.Victor Bakels wil ik in mijn dankwoord niet onvermeld laten, evenals de polikliniekassistentes en de (niet alleen) voor mij langst werkende secretaresse Corrie Holleman-Rovers. Tenslotte rest mij, als "last but not least", ook de collegae huisartsen uit de regio Zaanstad, de collegae specialisten uit mijn ziekenhuis en de patiënten die betrokken waren bij het onderzoek te bedanken voor hun medewerking.

Tenslotte

Zo zou ik kunnen doorgaan tot ik ophoud

Daar is veel voor te zeggen, niets daarna.

Rutger Kopland,
uit: Tot het ons loslaat (1977).