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Varicella immunity and vaccination

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Summary

Varicella-zoster virus (VZV) is an air-borne, extremely contagious herpesvirus. It causes chickenpox, which usually is a benign illness in childhood. Adults, pregnant women and immunocompromised patients often get a severe disease with complications. After infection the virus stays latent in sensory ganglia. It can be reactivated and cause shingles if the cell-mediated immunity (CMI) deteriorates. In the western world more than 90% of adults have had chickenpox. VZV vaccines have been introduced in many countries. They have been included in the childhood vaccination programme in e.g. the USA and Germany but not in Sweden due to lack of knowledge on many aspects of long-term effects.

More detailed information on the nature of the VZV-specific immune response is needed. To provide sufficient and reliable information the studies must be performed at a large scale. Such studies have been difficult since the methods for determination of antibody mediated immunity to VZV are not well standardised and the conventional lymphoproliferation assays for CMI are labour intensive.

We therefore developed a Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood (FASCIA) for the detection of CMI against VZV. The results were more reproducible than with the conventional method. The sensitivity and specificity in relation to the VZV serostatus increased with immunophenotyping by cell-surface staining of the proliferating cells. FASCIA is easy to handle, can be performed at a large scale and is suitable for large scale studies on specific CMI.

Serology and FASCIA with immunophenotyping were used to compare the VZV-specific CMI response before and after immunization against VZV in VZV seronegative, adult persons and in controls with past chickenpox. One year after vaccination the humoral and the CMI responses were lower in the vaccinees than in persons with past infection. In a study to determine the feasibility of co-administration of a VZV vaccine and the measles, mumps, rubella (MMR) vaccine booster at 12 years of age, 5/17 VZV seronegative pupils did not seroconvert after one VZV vaccine. After a second dose, 3/5 that could be examined seroconverted. VZV CMI was measurable in one of them before the second dose, and in 3/3 after vaccination. The VZV vaccine did not affect the MMR-response, but the age group should be recommended 2 doses of VZV vaccine. In the study, the seroprevalence for VZV was calculated to be 97% among the 12 years-old, which was higher than expected. We therefore evaluated if there has been a change in seroprevalence to VZV during the past 30 years, since the changes in social conditions and life-style in Sweden may have affected the spread of the viruses. The seroprevalence for the air-borne VZV had increased dramatically from 50% to 98%, and we suggest that it may be due to a change in child care pattern. The seroprevalence for HSV and EBV that infect by close contact had not changed dramatically, but there seemed to have been an unexplainable increase in the CMV sero prevalence.

Original papers and manuscript

The thesis is based on the following papers and manuscript, which will be referred to in the text by their Roman numerals:

- I** Svahn A, Linde A, Thorstensson R, Karlen K, Andersson L, Gaines H. Development and evaluation of a flow-cytometric assay of specific cell-mediated immune response in activated whole blood for the detection of cell-mediated immunity against varicella-zoster virus. *J Immunol Methods* 2003;277(1-2):17-25.

- II** Parment PA, Svahn A, Ruden U, Brakenhielm G, Storsaeter J, Akesson L, Linde A. Immunogenicity and reactogenicity of a single dose of live attenuated varicella vaccine and a booster dose of measles-mumps-rubella vaccine given concomitantly at 12 years of age. *Scand J Infect Dis* 2003;35(10):736-42.

- III** Svahn, A, Berggren J, Parke A, Storsaeter J, Thorstensson R, Linde A. Changes in seroprevalence to four herpesviruses over 30 years in Swedish children aged 9-12 years. *J Clin Virol* 2006;37(2):118-23.

- IV** Svahn A, Linde A, Andersson L, Pauksens K, Dahl H, Gaines H. Humoral and cell-mediated varicella-zoster virus response in vaccinated and naturally infected adults in Sweden. Submitted.

Abbreviations

AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphatic leukemia
C	Cytosine
CMI	Cell-mediated immunity
CMV	Cytomegalovirus
CNS	Central nervous system
CRS	Congenital rubella syndrome
DNA	Deoxyribonucleic acid
EA	Early antigen
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
G	Guanosine
HHV-6	Human herpes virus 6
HHV-7	Human herpes virus 7
HHV-8	Human herpes virus 8
HIV	Human immunodeficiency virus
HLA	Human leucocyte antigen
HZ	Herpes zoster
IFA	Immunofluorescence assay
IIF	Indirect immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
MMR	Measles-Mumps-Rubella
MMRV	Measles-Mumps-Rubella-Varicella
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pfu	Plaque forming units
PHN	Postherpetic neuralgia
VCA	Viral capsid antigen
VZV	Varicella-zoster virus

Human herpes viruses

Introduction

The herpesviruses are large, enveloped DNA-viruses and have been found in many different animal species. The herpesviruses share the following features (Roizman et al 2001).

- Double stranded linear DNA with a length varying from 120 to 250 kilo-base pairs (kbp)
- Icosahedral capsid about 100-110 nm in diameter containing 162 capsomeres
- The size of the virion varies from 120 to 300 nm
- Synthesis of viral DNAs and capsid assembly occurs in the nucleus. Capsids are enveloped as they transit through the nuclear membrane
- Tegument consisting of amorphous, sometimes asymmetric material between the capsid and the envelope
- An envelope containing viral glycoprotein spikes on its surface
- Remain latent in their natural host after infection. Latent genomes retain the capacity to replicate and cause disease upon reactivation.
- The size of the virion varies from 120 to 300 nm

About 130 herpesviruses have been identified. So far eight of them have been isolated from humans. The family *Herpesviridae* was divided in the subfamilies alfa-, beta-, and gamma *herpesvirinae* on the basis of biological properties before the DNA sequences of the individual members of the family were known.

- *Alfaherpesvirinae* have a variable host range, relatively short reproductive cycle, rapid spread in culture, efficient destruction of infected cells, and capacity to establish latent infection primarily but not exclusively in sensory ganglia.
- *Betaherpesvirinae* have a restricted host range, long reproductive cycle and infection progresses slowly in culture. The infected cells frequently become enlarged (cytomegalia) and establish latency in different cell types.
- *Gammaherpesvirinae* have a limited host range, replicate in lymphoblastoid cells in vitro with B- or T-lymphocytes as the main targets, Latency is often found in lymphoid tissues (Roitzman et al 2001).

Table 1 The human herpesviruses family

Subfamily Herpesvirinae	Genus	Virus	Designation
Alfa	Herpes simplex virus	Herpes simplex virus 1	Human herpesvirus 1
		Herpes simplex virus 2	Human herpesvirus 2
	Varicellovirus	Varicella-zoster virus	Human herpesvirus 3
Beta	Cytomegalovirus	Cytomegalovirus	Human herpesvirus 5
	Roseolovirus	Human herpesvirus 6 A	
		Human herpesvirus 6 B	
		Human herpesvirus 7	
Gamma	Lymphocryptovirus	Epstein-Barr virus	Human herpesvirus 4
	Rhadinovirus	Kaposi's sarcoma-associated herpesvirus	Human herpesvirus 8

Varicella-zoster virus

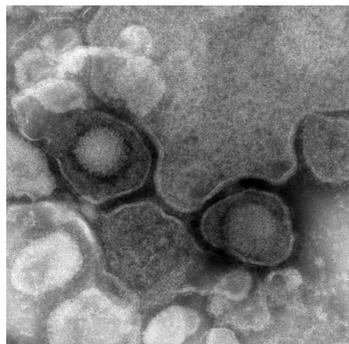


Photo: Kjell-Olof Hedlund, SMI

History

Why chickenpox is called chickenpox is not clear but shingles is suggested to derive from the Medieval Latin word “cingulus”, a girdle. Zoster is probably from the classic Greek, where a warrior used a zoster – a-belt-like binding – to secure his armor (Weller TH 2000). Similarities between varicella and smallpox initially made it difficult to differentiate between the diseases. Varicella was first described as separate clinical disease by Heberden the older in 1767 (Heberden W 1767). Transmission of vesicular fluid samples from children with chickenpox to healthy children caused chickenpox and demonstrated that varicella was caused by an infectious agent (Steiner 1875). Histopatologic similarities between varicella and zoster were also described (Tyzzer EE 1906). The occurrence of varicella in children after exposure to herpes zoster made von Bókay in 1909 suggest that there was a relationship between the diseases, but it was not until 1943 that zoster was suggested to be a reactivation of latent varicella virus (Garland J 1943). It is still difficult to cultivate VZV and it was first in 1953 that the trials were successful (Weller 1953). The virus was called varicella-zoster virus on the basis of epidemiological and laboratory evidence that the virus causes the diseases (Weller and Witton 1958) and molecular characterization also showed that isolates from varicella and zoster were identical (Straus et al 1984). The complete DNA sequence of varicella-zoster virus was reported in 1986 (Davison and Scott 1986).

Structure, genome and replication

The lipid envelope is 180-200 nm in diameter with glycoprotein spikes. The VZV genome consists of a linear, double-stranded DNA of approximately 125 kbp with an average G+C content of 46%. The genome of the Dumas strain was sequenced in 1986. A unique long region (UL) of 105 kbp is flanked by terminal repeat long (TRL), internal repeat long (IRL) and a unique short region (US) flanked by terminal repeat short (TRS) and internal repeat short (IRS) regions. There are about 69 open reading frames (ORFs). The genome consists of 5 regions with direct sequence repeats termed R1 to R5 (Davison AJ Scott JE 1986). There are similarities between the HSV-1 genome and VZV (Davison AJ, Wilkie NM 1983).

The replication of the VZV genome is thought to proceed by a rolling-circle mechanism (Stow ND, Davison AJ 1986). It has been difficult to study VZV due to its narrow host range and the low titres of cell-free virus that are obtained *in vitro*. The VZV attaches to the host cell surface which is achieved by interaction of the virus with heparan sulphate constituents of proteoglycans on the host cell surface (Quinlivan and Breuer 2005). This interaction is mediated by gB, gH and gI molecules embedded in the outer envelope of the virus. Mannos-6-phosphate residues on the viral glycoprotein ectodomains then bind to mannos-6-phosphate on the host cell surface and trigger penetration and fusion. Viral tegument proteins are released into the host cell cytoplasm which migrates to and enter the host cell nucleus. Fusion of naked viral nucleocapsids with the outer nuclear membrane releases the viral DNA genome into the nucleus where it circularises (Quinlivan and Breuer 2005).

VZV proteins located in the viral tegument may be released and transported with the nucleocapsid to the nucleus where they may serve to initiate transcription of viral genes. VZV IE messenger RNAs (mRNAs) are transcribed, transported to the cytoplasm, translated and the proteins are transported back to the nucleus. VZV early mRNAs may then be transcribed in the nucleus, translated in the cytoplasm, and transported back to the nucleus and facilitate viral DNA replication. VZV late mRNAs are transcribed, and translated in the cytoplasm. Capsid proteins are transported back to the nucleus for assembly of capsids. Newly replicated VZV DNA is then packaged into capsids and transported out of the nucleus. The capsids are enveloped and carried to the cytoplasmic membrane where the virions are released. There are discussions regarding the mode of nucleocapsid egress from the nucleus, the site of envelopment and the pathway leading to release of particles from the effected cell (Gershon AA et al 1994, Harson and Grose C 1995, Quinlivan et al 2005).

The seven known VZV glycoproteins are gB (ORF31), gC (ORF14), gE (ORF68), gH (ORF37), gI (ORF67), gK (ORF5), and gL (ORF60) (Kinchington PR and Cohen JI 2000). All but gK and gL are targets for neutralizing antibodies. VZV gE is one of the most immunogenic proteins of VZV and the main target for both the humoral and cellular immune response (Kinchington PR and Cohen JI 2000). VZV contains sequences for glykoproteins gM and gN but it has not been established if the are glycosylated (Yamagishi et al 2007). Glykoprotein gM is also mentioned as the putative gM (Quinlivan et al 2005).

Table 2 Some important open reading frames of VZV and functions of the proteins encoded

ORF	Function	Reference
28	DNA polymerase	Mar et al 1978
36	Thymidine kinase	Sawyer et al 1986
40	Major nucleocapsid protein, detectable in purified virions and nucleocapsids.	Vafai et al 1990, Kinchington et al 1992
47	Protein kinase, important for virus replication in lymphocytes	Ng et al 1994
62	Transactivator, tegument protein, major target for the CMI response to VZV	Kinchington et al 1992
66	Protein kinase	Kinchington et al 2000

Table 3 VZV glycoproteins and some of their functions

ORF	Glykoprotein	Function	Reference
31	gB*	Virus entry and cell spread Accumulates in the Golgi of infected cells	Heineman et al 2004
14	gC	Suggested important for virion production	Storlie et al 2006
68	gE*	Major glycoprotein essential for VZV replication Important for cell-to-cell spread, envelopment and possibly entry One of the most immunogenic proteins of VZV target for cellular and humoral immune response	Berarducci et al 2006, 2007 Moffat et al 2004 Mo et al 2003
37	gH*	Acts as a fusogen that facilitates cell-to-cell spread of virus gL is needed for maturation and membrane expression	Pasička et al 2004 Duus et al 1996
67	gI	Neurotropism Co-localizes with gE on the surface of VZV infected cells	Zerbonie et al 2007 Yao et al 1993
5	gK	Participates in membrane fusion and cytoplasmic virion morphogenesis Half-life shorter than gB, gE, gH Indispensable for viral replication	Hall et al 2007 Mo C et al 1999
60	gL	Expressed alone or with gH accumulates in cytoplasm not on the surface of transfected cells contains an endoplasmatic reticulum-target sequence	Duus et al 1995, 1996

* major glycoproteins

Only one serotype of VZV has been recognized. Sequencing of VZV isolates has identified some minor differences between the OKA vaccine strain and wild-type virus which can be used distinguishing between them by restriction fragment-length polymorphism (LaRussa et al 1998) or PCR (Parker et al 2006) but strain variation is not thought to affect immunity to new strains after past infection. Four major subtypes of wild VZV have been identified by single nucleotide polymorphisms in ORFs 1, 21, 37, 60, and 62. The OKA vaccine strain can be differentiated from the wild VZV by SNPs in ORF62 (Parker et al 2006).

VZV is susceptible to disinfection, degradation by physical and chemical treatments such heating to 60°C, prolonged storage of -10°C or above, extreme pH or ultrasonic disruption.

Pathogenesis

Primary infection

The Fenner model for mousepox is suggested as a model for the pathogenesis of the primary varicella infection (Grose 1981). The transmission of VZV is by air-borne water droplets or by close contact. During the incubation phase the virus is inoculated in the respiratory mucosa and spread to regional lymph nodes where replication may take place. A primary viremia is thought to transport the virus to liver, spleen and other cells of the reticuloendothelial system. A second viremia a few days before after the onset of rash by cell-has been detected by PBMC cultivation, PCR. This cell-associated viremia transports the virus to skin and mucous membranes sites but the mechanisms are not clear. The virus may exit the capillaries while within lymphocytes and enter the epidermal layer (Harson and Grose 1995). Replication in epidermal cells causes the rash. Virus is also released into respiratory secretion (Sawyer et al 1992). Cellular tropism of VZV involves T-lymphocytes, skin cells and cells of dorsal root ganglia but also lungs, liver, central nervous system and other organs during disseminated infection. It has recently been shown that memory T-cells probably start transporting the virus to the skin immediately after viral replication begins in the respiratory mucosa (Ku et al 2004, Quinlivan et al 2005).

Latency and reactivation

The virus has been found to stay latent in dorsal root ganglia (Kennedy et al 1999), trigeminal ganglia (Mahalingam et al 1993), geniculate ganglia (Furuta et al 1992) and olfactory bulbs (Liedtke et al 1993). It is suggested that the virus is spread haematogenously to sensory ganglia or by retrograde axonal transport from infected epidermal and dermal tissues. Latent virus has been showed to reside in neurons (Hyman et al 1983) and nonneuronal cells (Meier et al 1993). The mechanisms by which VZV establishes and maintains latency, avoids the immune system or reactivates are still not known. A decrease in cell-mediated immunity has showed to be of importance for the reactivation (Saibara et al 1993, Park et al 2004).

Epidemiology

VZV is an exclusive human pathogen and distributed world-wide. Chickenpox is usually contracted early in childhood. Unfortunately, the assays for seropositivity analyses vary in sensitivity (Gershon and Hambleton 2004) and even when the same assay is used in different laboratories and with the same panel of samples, the evaluation of whether low reactivity should be regarded as sign of past infection or background reactivity varies (de Ory et al 2006). Therefore, studies of seroprevalence are not exactly comparable, but they give a good indication on major differences between regions. In the USA and the rest of Western world the seroprevalence for adults is > 90 % (Marin et al 2007, Sengupta et al 2007). In Europe the percentage of VZV seronegative varies between 1.2 % in the Netherlands to 18.3 % in Italy for the age group 10 -14 years (Nardone et al 2007). However, in tropical countries the seroprevalence has been reported to be only about 40-80% among adults (Garnett et al 1993, Ooi et al 1992). A comparison of seroprevalences among health care workers from different regions working in Saudi-Arabia revealed that the people from the Middle East had the lowest prevalence (46%). Since the same method was used for all participants in the study, the reliability of the found differences is high (Almuneff et al 2003).

A seroprevalence of 42 % in the rural tropical areas and 96% in the urban parts has been reported (Mandal et al 1998). The reason for the differences is not known, but probably the density of the population plays a major role for the spread. In Sweden about 100 persons are hospitalized for varicella and 1-6 die every year (Socialstyrelsens EpiCs database). In the USA, before the introduction of the VZV vaccine in 1995 the overall mortality rate was reported in 1970-1994 to 0.4 deaths per million population, and during 1988-1995 the hospitalization rate was 2.3 -6.0 per 100 000 population. In 2005 the hospitalization rate decreased to 0.8 per 100 000 population and the overall mortality rate to 0.14 per million population during 1999-2001 (Marin et al 2007).

Herpes zoster is caused by reactivation of latent VZV and is associated with a decrease in cell-mediated immunity. The risk of herpes zoster increases with old age and occurs at an annual rate of about 3.2 -11.8 per 1000 persons 60 years or older (Donahue et al 1995, Helgason et al 1996, Yawn et al 2007). Herpes zoster is rare in children and a rate of 0.23 to 0.74 per 1000 children has been reported (Marin et al 2007). There is an increased risk of zoster patients with suppressed cell-mediated immunity such as transplanted, HIV infected, and haematological malignancies to develop herpes zoster (Miller et al 2007, McNulty et al 1997, Rusthoven JJ 1994). The hospitalization rate was reported to 4.4 hospitalisation per 100 000 population for herpes zoster in England and Wales (Brisson et al 2003). In Sweden about 500 persons are hospitalized and about 50 die yearly (Socialstyrelsen EpiCs database).

VZV seronegative individuals may contract varicella from persons with herpes zoster. Exposure to exogenous varicella seems to boost the immune system and protects individuals with latent varicella-zoster virus infection from herpes zoster (Thomas et al 2002).

Clinical feature

Varicella

Primary VZV infection, chickenpox, is often acquired in childhood and is then a quite harmless disease in healthy individuals. When adolescents and adults contract varicella, they are at risk of more severe symptoms and complications. Infected individuals are contagious for 24 to 48 hours before the onset of varicella. The incubation period is about 2-3 weeks. Prodromal symptoms like fever, headache, malaise, anorexia, and abdominal pain are followed by a maculopapular exanthema. It begins on the scalp, face or trunk and then spreads to the arms and legs. The cutaneous lesions soon become clear, fluid-filled vesicles with an erythema margin. New lesions appear and vesicles in different stages can be seen during a week. When the vesicles dry, they form crusts. Pruritus can be severe. Vesicles may also develop on mucous membranes such as oropharynx, conjunctiva, trachea, rectum and vagina. Sometimes the symptoms are very mild with no fever and few blisters and the varicella diagnosis may then not be considered. About 60-80% of those who do not know or believe that they have not had chickenpox are VZV seropositive when examined for VZV serostatus (Boulianne et al 2001, Harel et al 2001).

Complications such as secondary bacterial infections of skin lesions, hepatitis, thrombocytopenia, encephalitis or cerebellar ataxi may occur (Zeibold et al 2001). Aspirin is shall not be given children with varicella as it may contribute to the development of Reye's syndrome of fatty degeneration of the liver and encephalopathy (Hurwitz et al 1987). Pneumonia is common in adults (Frangidis et al 2004).

Herpes zoster

After the primary infection VZV stays latent in the sensory ganglia. Herpes zoster is a consequence of reactivation of VZV. The lesions are localized on one side and within the dermatomal distribution of one or more sensory nerves. The symptoms are vesicles, pruritus, neuropathic pain, and hypersensitivity (Yawn et al 2007). The thoracic, cranial and lumbosacral dermatoms are most commonly involved. In healthy individuals, symptoms heal within about 2 weeks but can persist for 4-6 weeks. Neurological pain without vesicles “zoster sine herpette” may also occur (Gilden et al 1992). Reactivation from the geniculate ganglia causes herpes zoster oticus and facial nerve palsy, referred to as the Ramsey Hunt syndrome (Grose et al 2002, Furuta et al 2005). Facial nerve palsy without cutaneous symptoms has also been reported (Furuta et al 2005).

The most common complication of herpes zoster is the severe chronic pain, postherpetic neuralgia (PHN) (Jung et al 2004). PHN is defined as pain >90 days after development of cutaneous lesions. Suggested risk factors for PHN are old age, female sex, presence of prodrome, extensive rash and severe acute pain (Jung et al 2004). Zoster ophthalmicus is associated with conjunctivitis, keratitis, anterior uveitis, and iridocyclitis and may affect the vision on the eye (Liesegang 2004).

Encephalitis may occur, and is now recognized to be a vasculopathy that affects large or small vessels. Large-vessel arterial disease (granulomatous arteritis) occurs predominantly in immunocompetent persons while small-vessel mediated encephalitis is found in immunodeficient patients (Studahl et al 1998, Gilden et al 2002).

Risk groups for severe infection*Pregnant women*

VZV seronegative pregnant women are at risk to contract serious varicella and the most common complication is pneumonitis (Katz et al 1995). However, in Sweden only one hospital admission and no death was found during a 10 years period in pregnant women with pneumonitis (Nilsson and Ortqvist 1996). Spontaneous abortion has not been reported to be over represented in pregnant women with varicella (Sauerbrei et al 2007). Maternal chickenpox before week 20 has been reported to cause congenital anomalies in about 1-2% (Enders et al 1994, Prober et al 1990). Skin lesions with dermatomal distribution, neurological defects, and limb hypoplasia are some of the symptoms of the congenital varicella syndrome (Sauerbrei et al 2007). If the mother develops chickenpox about 4 or 5 days before or 2 days after delivery the newborn child is at serious risk for life-threatening neonatal chickenpox since the virus but no maternal IgG antibodies has been transferred. Herpes zoster of the pregnant woman is not considered to be a risk during the perinatal period for the child since maternal VZV IgG antibodies have been transferred to the foetus (Sauerbrei et al 2007).

Immunocompromised patients

Immunocompromised children are at risk for prolonged varicella with increased numbers of cutaneous lesions, dissemination and complications such as pneumonia, hepatitis, encephalitis and disseminated intravascular coagulopathy (Balfour HH Jr 1988). Prednisone therapy during the VZV incubation period significantly increases the risk for developing severe varicella infection in children with acute lymphatic leukemia (ALL) (Hill et al 2005). HIV infected children are reported to be more severely ill than healthy children but less than those

with leucemia. However, the HIV infected children more often developed herpes zoster (Gershon et al 1997).

The severity of herpes zoster and risk of complications are increased in immunocompromised patients. HIV patients have an increased risk for herpes zoster when the CD4+ lymphocyte count decreases (Chaisson et al 1991). HIV and AIDS patients may develop cutaneous and visceral dissemination, as well as recurrent or chronic herpes zoster. Other complications are retinal necrosis, PHN, and encephalitis (Vafai et al 2001). About 25% of patients with Hodgkin's disease developed herpes zoster 150 days after bone marrow transplantation (Christiansen NP et al 1991).

Treatment and prophylaxis

Antiviral chemotherapy

Acyclovir and Valaciclovir

Acyclovir (9- $\{2$ -hydroxyethoxymethyl $\}$ guanine) belongs to the nucleoside analogues and is a synthetic acyclic purine analog. Conversion by virus-encoded thymidine kinase to its monophosphate derivative occurs mainly in virus infected cells (Fyfe et al 1978). Di- and triphosphorylation is catalyzed by cellular enzymes and result in acyclovir-triphosphate concentrations 40 to 100 times higher in HSV and VZV infected cells than in uninfected cells. Acyclovir triphosphate competes with the deoxyguanosine triphosphate as a substrate for viral DNA polymerase (Derse et al 1981), and thereby the viral DNA synthesis is inhibited. Acyclovir triphosphate lacks the 3' hydroxyl group required for DNA chain elongation and viral DNA synthesis is terminated (Furman et al 1984). Little incorporation of acyclovir into cellular DNA occurs as the viral polymerase has more affinity for acyclovir triphosphate than does cellular DNA polymerase (Collins and Bauer 1979). The active compound of valaciclovir is acyclovir is cleaved to acyclovir by valine hydrolase. Acyclovir penetrates into most body compartments including the brain. Acyclovir is available in topical, oral and intravenous preparations. It penetrates in most body tissues including the brain. Valaciclovir is only available in tablets and has a better oral bioavailability. Treatment of VZV demands a higher dose than treatment of HSV.

Famciclovir

Famciclovir also belongs to the guanine nucleosid family and is the prodrug of penciclovir. Famciclovir has an excellent oral bioavailability which penciclovir does not have. The antiviral spectrum is similar to that of acyclovir (Sutton et Kern 1993). Penciclovir is phosphorylated the same way as acyclovir. Penciclovir-triphosphate has a longer intracellular half-life than acyclovir-triphosphate in HSV-1, HSV-2, and VZV infected cells after drug removal. There is cross resistance between acyclovir and penciclovir. Penciclovir is only available in topical form and famciclovir as tablets.

Foscarnet

Foscarnet is a pyrophosphate analogue of phosphonoacetic acid (PAA) and acts directly on the virus DNA polymerase which is inhibited by blocking of the pyrophosphate binding site. The formation of the 3'-5' phosphodiester bond between the primer and the substrate is inhibited and the chain elongation is thereby prevented. It can be used when VZV or HSV-2 are resistant to acyclovir and penciclovir (Chatis et al 1989). Foscarnet is administered by the intravenous route.

Ganciclovir

Ganciclovir also belongs to the nucleoside analogue group and is only approved for treatment of CMV infections. However, it has effect also on VZV and HSV when CMV infection is treated. Ganciclovir can be administered by the intravenous route as well as orally.

Side effects

Acyclovir and penciclovir are usually well tolerated and have low toxicity as they are metabolized mainly in the infected cells. Nausea and headache have been reported (Tyring et al 2002), as well as severe symptoms from the central nervous system (Feldman S et al 1988). Foscarnet in too high doses may be nephrotoxic. Ganciclovir has haematological toxicity and neutropeni, anemia, and thrombocytopenia have been reported

Resistance

Immunocompromised patients, especially AIDS patients, may have to be treated with antiviral drugs for long time and then resistance can occur. This is more common for HSV and CMV than for VZV.

Passive antibody prophylaxis

VZV hyper immunoglobulins are antibody preparations containing VZV IgG antibodies made from high-titre human serum. It should be administrated within 48 to 96 hours after exposure, and then may totally/partially protect against symptomatic/severe infections. It is recommended as post exposure prophylactics to individuals at high risk for severe or fatal varicella such as newborn infants exposed for maternal varicella, immunocompromised, seronegative children and pregnant women (Miron et al 2007). It does, however, not eliminate the possibility that varicella will occur. After onset of illness it does not alter the severity of varicella or herpes zoster.

Treatment

Varicella is not recommended for treatment of healthy children in Sweden unless the symptoms are severe. Persons >18 years and immunocompromised children and adults are recommended treatment (Läkemedelsverket 4:2005) and it should be started within 24 hours from onset of symptoms but may start after that time if the symptoms are severe. Acyclovir has documented effect on varicella.

Treatment of herpes zoster is recommended for persons >50 years and immunocompromised children and adults. Herpes zoster may be treated with acyclovir, valaciclovir or famciclovir. Treatment should start as soon as possible and preferably within 72 hours. If the pregnant woman has a serious chickenpox with complications, treatment is recommended at any time during pregnancy with intravenous acyclovir. If the symptoms are milder oral treatment can be performed before week 35 but the known effects on the foetus of the high doses of acyclovir that has to be used against VZV is limited. Treatment is recommended after week 35. The new born baby should be treated with varicella-zoster virus immunoglobulin and acyclovir if the mother has chickenpox 1 week before or after delivery-

Vaccine against varicella-zoster virus

A live attenuated VZV vaccine was developed in Japan in the 1970s (Takahashi et al 1974) and is the first human herpes vaccine. The VZV strain (OKA) was isolated from a 3 years old healthy boy with chickenpox, and attenuated by repeated passage in various cell-lines. This strain has since then been used in the vaccine production from Merck & Co Inc, GlaxoSmithKline, Pasteur Merieux Connaught, and Birken. In the USA the Varivax® (Merck) is used and Varilrix® by (GlaxoSmithKline) dominates in Sweden and most of Europe.

The OKA vaccines from different companies have been considered to be “more equal than different” concerning effect and side effects (Rentier B, Gershon AA 2004, Hambleton S, Gershon AA 2005, Chiu SS, Lau YL 2005). They can vary in some ways, such as passage numbers, dose of virus, stabilizers and temperature for storage. Varilrix® is lyophilized and one dose (0.5 mL) contains $\geq 10^{3.3}$ plaque forming units (PFU), and may be stored in 2 °C to 8 °C. Varivax® is also lyophilized and one dose (0.5 mL) contains at least 1350 PFU, and should be stored in -15 °C or colder.

In Sweden, the vaccines are recommended for VZV seronegative individuals at risk for severe varicella such as adolescents, adults, and different risk groups. Contradictions are pregnancy, severe immune deficiency and allergic to components in the vaccine. One dose has so far been recommended below the age of 13 years, and two doses thereafter with 1-2 months intervals between them. Two doses are also recommended to patients with immune deficiency disorders if vaccination is not a contradiction. The induced VZV immunity response is comment in the immune response part of the thesis.

The vaccines are considered to be safe with few side effects. It is approved for use in many countries and included in the childhood vaccination programme in Japan and Chorea since the 80ies, the USA since 1996 (Marin et al 2007) and in Germany since 2004 (Sengupta et al 2007). Varilrix® and Varivax® are licensed for VZV seronegative individuals from the age of 9 months but are not included in the Swedish childhood vaccination programme. The vaccine virus becomes latent like wild-type virus, and there is a risk of the virus to cause zoster but the symptom may be milder. Since chickenpox gives protection from zoster, the incidence of zoster may rise markedly in persons with previous natural infection, if the circulation of VZV is strongly reduced. A temporary, sharp increase of zoster has been modelled (Wagenfeil et al 2004), and this prediction has to be taken into consideration when the vaccine is introduced into the childhood vaccination programme. Today there is a zoster vaccine, Zostavax®, which reduces the number and severity of zoster cases when administered to persons above (Oxman et al 2005). Possibly it could be used to prevent the increase of zoster cases after general vaccination. Recommendations for administering varicella vaccine to individuals with underlying immunodeficiency disorders have been published (Sartori 2004 review, Miron et al 2007).

Post-exposure prophylaxis

Varicella vaccine can also be given post prophylactic to immunocompetent persons if given within three days from the known exposure. It may also have effect given four or five days after exposure. It should not be in combination with antiviral chemotherapy as this can reduce the effect of the vaccine (Läkemedelsverket 4:2005).

MMRV and Zoster vaccine

A combination vaccine of MMR and varicella was registered by Merck in 2005 and by GSK in 2006 (Vesikari et al 2007, Hammerschmidt et al 2007). In the USA a live zoster vaccine Zostavax® (Merck) has been registered for individuals >60 years to booster the varicella immunity in order to prevent herpes zoster or severe PHN (Betts RF 2007, Marin et al 2007). One dose (0.65 mL) of the zoster vaccine contains at least 19 400 pfu.

Breakthrough varicella

Breakthrough varicella is infection of wild-type VZV occurring >42 days after VZV immunization. It has been reported in an increased frequency in the USA the last years. Outbreaks have occurred despite vaccination at day-care centres and schools (Miron D et al 2005, Lopez et al 2006, Galil et al 2002). The vaccinees in general had milder disease than unvaccinated (Miron et al 2005, Vazquez et al 2004). Low age at vaccination and vaccination >2 years before exposure have been identified risk factors for breakthrough infection. Two doses have also proved to elicit more effective protections also in children (Kuter B et al 2004) and are now recommended in the USA, with the first dose between the age 12-15 months and a second at 4-6 years. The second dose can be given earlier, provided that the interval between the first and second dose is >3 months. Individuals who only had one dose should receive a second dose (Miron et al 2007). In adults breakthrough varicella after two does has been reported to 9% during en observations period of 8 weeks to 11.8 years (mean 3.3 years) (Ampofo et al 2002).

Morbidity, hospitalization and mortality

The effect of VZV vaccination in US has been monitored for different regions. Since the introduction of the childhood vaccine in the USA the varicella cases as well as hospitalization and mortality have decreased dramatically. With a VZV vaccination coverage of 74-84% in children aged 19-35 months the reported cases of varicella had declined 71-84% in 2000 (Marin et al 2007). In 2005 two areas had vaccine coverage of 90% and a decline of varicella cases of 91%. The incidence declined most in children but there was a decline in all age groups, indication herd immunity. In 2005 the hospitalization rate decreased to 0.8 per 100 000 population and the overall mortality rate to 0.14 per million population during 1999-2001. The age-adjusted morality rate decreased with 66% from 1994 to 2001 (Miron et al 2007). There are no consistent trends in herpes zoster incidence in the USA since the introduction of the vaccine, but there has not been such a close monitoring of zoster as of chickenpox. It has been concluded in the US that the risk for reactivation of VZV after immunization need to be studied further (Miron et al 2007).

Side effects

The most common side effects of VZV vaccination are fever and varicella lesions. The lesions are less contagious than those in natural infection and household contacts have been reported to have contracted mild chickenpox or asymptomatic seroconversion (Gershon LaRussa 1992). Herpes zoster after vaccination has also been reported both in healthy children (Plotkin et al 1989) as well as and in children with leukaemia (Hardy et al 1991).

Titres

Titres for protection after vaccination are discussed and also if they should be measured and how (Sharrar et al 2000, Gershon Hamblton 2004). By the not commercial gpELISA a titre of ≥ 5 has been considered to be protective but breakthrough infections have occurred (Li et al 2002). By the reference method FAMA a titre of $>1/4$ has been considered to be protective but this method cannot be performed in large scale and is mainly used for research purposes (Gershon et al 1994). Presence of FAMA antibody in sera does not predict immunity in all circumstances (Gershon AA et al 1984, LaRussa et al 1985).

Immune response

Primary wilde-type varicella-zoster infection

The innate immune system starts to work within a few hours after infection and does not require previous exposure to antigen and do not develop memory. Natural killer (NK) cells cytotoxicity, IFN α , and granulysins are involved in the control of the spread of VZV. Granulysin is a cytolytic protein made by NK cells and CD8+ T-lymphocytes and blocks the replication of VZV and triggers apoptosis of infected cells (Hata et al 1999). IFN α is made by dendritic cells, monocytes and NK cells and inhibits VZV replication in vitro. IFN α is detected in serum during acute varicella but in lower levels in adults than in children (Arvin et al 1986, Wallace et al 1994). NK T-cells are suggested to contribute in the host defence against varicella-zoster virus (Levy O et al 2003). However, the innate responses are not enough to prevent symptoms of illness in most individuals with chickenpox.

VZV specific antibodies can usually be detected at low concentration within 3 days after the onset of lesions, with IgM appearing first. VZV-antibodies of the IgG, IgM and IgA classes are directed against the glycoproteins of VZV as well as against the nucleocapsid and tegument (Hambleton et al 2005). They and can mediate viral neutralization directly or in the presence of complements and mediate lysis of infected cells by antibody-dependent cell mediated cytotoxicity (ADCC) (Ihara et al 1984). IgM antibodies appear during acute varicella and decline within a few months. IgG are also produced and they persist after the acute disease and may mediate protection against re-infection. IgG antibodies appear at the latest five days after infections, and persist after the disease over long time.

Passively given IgG antibodies protects against infection and indicates that presence of a sufficient amount of IgG antibodies protect against re-infection (Arvin AM 1996, Miron et al 2007). IgA antibodies have been found in the nasopharyngeal secretion and serum during the first week of illness and have been shown to increase during the following 3 to 4 weeks and measured up to 60 days after onset of symptoms (Wittek et al 1983). Children with congenital T-cell disorders or AIDS are more likely to develop disseminated varicella infection than those with agammaglobulinemia suggesting that specific CMI responses are more valuable than VZV-antibodies for the protection against dissemination of VZV and development of severe disease (Arvin 1996, Cohen et al 1999). Detection of high IgG or IgM titres early during infection in previously healthy children does not predict mild infection and immunocompromised children may develop progressive varicella infection in spite of adequate production of VZV antibodies (Arvin et al 1986).

Cell-mediated immune response

VZV-specific T-lymphocytes can be detected 24-72 hours after onset of varicella. Intact cellular immunity seems to be important for terminating cell-associated viremia, as well as for limiting the virus replication at cutaneous sites. Individuals who display demonstrable antigen-specific T lymphocyte response within 24-72 hrs period are more likely to have a mild primary infection (Asano et al 1985, Arvin et al 1986). Immunodeficient, susceptible individuals with delayed or no acquisition of VZV-specific T-Cells are at risk for disseminated disease (Arvin AM et al 1992).

Both MHC class I restricted CD8⁺ T-lymphocytes and MHC class II restricted CD4⁺ T-lymphocytes are sensitized to VZV antigens during the primary VZV infection. CD4⁺ T-lymphocytes mediate the early T-lymphocyte proliferative response to VZV antigen by release of the Th1 type cytokines such as IL-2, IL12, and IFN- γ (Bergen et al 1991, Ito et al 1991;1992, Zhang et al 1994, Jenkins et al 1998). IFN- γ was less frequently in individuals <19 years and >55 years than in the age group 20-25 years. There was no difference in the frequency of IL-4 productions between the age groups (Zhang Y et al 1994). T-lymphocytes recognize VZV glycoproteins, the major tegument protein, and transactivator IE62 (Arvin 1996). An increased cell-surface expression of the Human Leucocyte antigen (HLA-DR) antigen was shown on circulating lymphocytes from subjects with acute VZV infection and IFN- α as well as IFN- γ was produced (Arvin et al 1986). The expression of the activation markers HLA-DR and IL-2 was significantly increased on PBMCs from seropositive individuals when stimulated with VZV antigen for 6 days and this was observed in both CD4⁺ and CD8⁺ T-lymphocytes (Ito et al 1992). The VZV-specific T-cell response after primary infection and vaccination is reported to be dominated by CD4⁺ cells (Jones et al 2006).

Herpes zoster

As has been mentioned, a decrease in the cellular immunity, above all of cytotoxic T-cell activity, precludes herpes zoster (Saibara et al 1993, Park et al 2004). Reactivation of VZV is not associated with decreased titres of VZV IgG antibodies (Weigle KA et al 1984). A 2.6 time increase in VZV IgG titres after herpes zoster has been reported as well as an increase in IgM and IgA titres (Opstelten W 2007). An increase in antibody response for 2 years was found in individuals having had herpes zoster (Hayward et al 1991).

With increasing age there is a decrease in CMI and an increased risk for herpes zoster. Also immunocompromised patients have an increased risk. Low or absent CMI response to VZV represents an essential but perhaps not in itself, sufficient reason for reactivation (Abendroth A et al 2001). Many individuals do not develop herpes zoster in spite of old age or immunosuppressive treatment so other not yet known factors are likely to contribute to the susceptibility associated with reactivation. When reactivation occurs there is an immediate increase of the T-lymphocyte response. The frequency of VZV specific T-lymphocytes were higher in elderly persons who had herpes zoster infection than in age-matched controls, and the high level persisted for at least 2 years (Hayward et al 1991). The enhanced CMI and humoral immunity may explain why second episodes of herpes zoster are rare. The rate of later development of herpes zoster was 70% in HIV-infected children who had low levels CD4⁺ lymphocytes detected at the time of development of varicella (Gershon AA et al 1997).

Varicella-zoster virus vaccination

VZV vaccination elicits both humoral and CMI response. Neutralizing antibodies and antibodies to gB and gE in titres were elicited in titres equivalent both in natural infected and vaccinated (Haumont et al 1997). In another study vaccinated children displayed 1/10 of the titres of natural infected (LaRussa et al 1990). Antibodies have been measured by gpELISA after 9 years in children who received one or two doses of vaccine. Two doses induced a better protection than one dose against breakthrough infection. Herpes zoster occurred in 2 cases in the one dose group vaccine (Kuter et al 2004). Three months after immunization 100 % of children had measurable IgG antibodies, 97.7% after one year and 93.3% five years later. In adults a seroconversion rate of 100 % was record three months and one year after immunization, and after 5 years 94.4% were positive (Zerboni et al 1998).

Adolescents and adults had a seroconversion rate of 82% after one dose and 94% after 2 doses (Gershon et al 1988). In a review by Hambleton and Gerson seroconversion rates in children were reported to be 86-99% after one dose and 99-100% after two doses. In adolescents 79% seroconverted after one dose. After one dose 75% of adults and adolescents seroconverted and 99% after the second dose (Hambleton S Gershon AA 2005). Loss of antibodies has been reported to 35% 1 month to 7 years (mean 1.2 years) after vaccination (Ampfo et al 2002). Another study reports a loss of 31% after 1-11 years (Saiman et al 2001). Thus, the studies of effect of vaccination render quite varying results and are difficult to evaluate due to variations in vaccinations schedule and methods for determination of immunity.

Cell-mediated immune response

CMI was measurable in 89.7% of children after one year and in 86.7% after 5 years. In adults 94.1% had measurable CMI one and 5 years after immunization (Zerbone et al 1998). In comparison of CMI among children receiving one dose to two doses the CMI response was significantly higher among the children that received two doses. The CMI measured after 2-12 weeks was higher than after one year (Nader et al 1995). In this study a diminished CMI response in adults vaccinees were accompanied by lower concentrations of IgG antibodies specific for VZV antigen one year after immunization (Nader et al 1995).

In children receiving one dose and adults receiving two doses the T cell proliferative response were equal 8 months after immunization (Jenkins D et al 1998). By 15 months the T cell proliferative response to VZV antigen in children was nearly twice that measured in vaccinated adults although the children had only been given one dose. During the first 8 months the mean SI was not significantly different in vaccinated adults compared to naturally infected adults but after 15 months the mean SI in vaccinees was significantly lower than those with past naturally infection (Jenkins et al 1999). The IFN γ production was equal in vaccinees and those with natural infection after 15 months though there was a lower response in the vaccinees at 8 months. The mean IFN- γ response in adults and children was not statistically different nor when the response was induced by vaccination neither following natural infection. The IL-10 response increased immediately after immunization and decreased to baseline after 8-15 months both in vaccinated adults and children, and in naturally infected. IL-12 increased initially and decreased after 8 to 15 months. The kinetic of the IL12 production were similar for vaccinated adults and naturally infected (Jenkins D et al 1998).

In vaccinated children aged 12-28 months both humoral and CMI were detected 6 weeks after immunization. HLA DR expression on CD4+ and CD8+ cells as well as production of IFN γ and IL10 were detected (Habermehl et al 1999). Proliferative response against several viral proteins has been demonstrated (Diaz et al 1989, Sharp et al 1992, Watson et al 1990). Results from some of these studies are summarized in table 3. In conclusion, it seems that the responses after vaccination and persons with past chickenpox, the responses are of similar quality, but that the vaccine-induced responses are deteriorating more rapidly than immunity after infection.

Immunocompromised patients

In bone marrow transplanted children 8 of 9 seroconverted after one dose VZV vaccine, and the last one after a second dose. Six of them had detectable antibodies after 2 years (Sauerbrei et al 1997). The incidence of herpes zoster in vaccinated leukemic children was 2% and 15% in leukemic children with past history of varicella. The vaccinees that developed herpes zoster had lower CMI than those with past history (Hardy I et al 1991).

HIV-infected children with CD4+ T-cell percentage of $\geq 15\%$ and a CD4+ T cell count of ≥ 200 cell/microL did benefit from VZV vaccination and 79% developed antibodies/and or CMI 2 months after 2 doses and 83% remained responders after one year (Levin et al 2006).

Table 4 Seroconversion rate and CMI, shown by demonstrable response or by mean for SI, in children and adult vaccinees in some studies (n d = not done).

Reference	No of doses	Sample size	Age group	Timing of test	Seroconversion rate (%)	CMI % or SI
Zerboni et al 1998	1 or 2	60	Children	3 months	100%	N d
				1 year	97.7%	89.7%
				5 years	93.3%	86.7%
Kuter et al 2004	1	657	Children	1 year	100%	
	1	400		5 years	100%	
	1	277		9 years	99.6%	
	2	588		1 years	99.8%	
	2	376		5 years	100%	
	2	237		9 years	99.6%	
Jenkins et al 1999	1	13	Children	Before		SI 1.5
				21 d		SI 8.8
				8 months		SI 5.5
				15 months		SI 11.5
Nader et al 1995	1 or 2	90	Children 2-12 years	6 weeks	100%	n d
				1 year	100 %	97%
Watson B et al 1995	1 or 2	151	Children 1-12 years (mean 4.5)	6 weeks	100%	n d
				1 year		93%
Zerboni et al 1998	2	18	23-43 years	3 months	100%	
				1 year	100%	94.1%
				5 years	94.4%	94.1%
Jenkins et al 1999	2	23	adults	Before		SI 1.5
				21 d		SI 11
				8 months		SI 6.9
				15 months		SI 6.3
Nader et al 1995	2	39	adults	6 weeks	100%	
				1 year	94%	94%

SI = stimulation index

Herpes simplex virus, human cytomegalovirus, and Epstein-Barr virus

Herpes simplex virus

HSV was the first herpesvirus to be discovered. The virus is transmitted by direct or indirect contact from lesions, or shed by infected, asymptomatic persons. The virus must come in contact with mucosal surfaces or abraded skin for infection to be initiated. The viral replication takes place at the site of infection. Nerve endings are infected, and both HSV-1 and HSV-2 are transported by retrograde movement to the nuclei of sensory ganglia (Stevens JG, Cook ML 1971). Humans are so far the sole reservoir for transmission. The mouth and the lips are the most common sites of primary HSV-1 infection with symptoms as blisters, aphthae, gingivitis, and stomatogingivitis herpeticus with high fever. Skin or any organ can also be affected.

Primary HSV-1 infection usually occurs in young children and is often asymptomatic (Whitley RJ 2002). Parent-to-child transmission has been suggested as 30% of children by the age of 30 months had detectable antibodies to HSV (Tunback P et al 2007). Changes of sexual behaviour have made genital infections caused by HSV-1 more common (Lowhagen GB et al 2002, Challenor et al 2007). HSV-2 is associated with genital infections, and usually sexually transmitted but orolabial infections by HSV-2 has also become more common (Lowhagen GB et al 2002, Wald A et al 2004). Only 12% of Swedish adults were reported to be seronegative for both HSV-1 and HSV-2 in the 1990s (Jonsson MK et al 2006). In the USA about 58% of adults were reported to be seropositive for HSV-1 (Xu et al 2006).

Recurrent HSV labialis and HSV genitalis are common. Reactivation of the viruses may occur after local or systemic stimuli such as emotional and physical stress, fever, ultraviolet light or immune suppression. Other manifestations of infection are keratoconjunctivitis, encephalitis or meningitis. HSV-1 is the most frequent cause of encephalitis (Skoldenberg B 1996). Recurrent meningitis is often caused by HSV-2 (Bergstrom T et al 1990).

Immunocompromised patients have an increased risk for severe HSV infection, especially transplant recipients (Miller GG, Dummer JS 2007). Congenital infection is extremely uncommon but may cause malformations of the foetus and when infection during partus the new-born may develop herpes disseminatus neonatorum (Kropp RY et al 2006). HSV-1 infection is more common in childhood in poor countries and lower socioeconomic classes, whereas in developed countries and with high socioeconomic status the primary infection may be delayed until adolescence or adulthood. In Europe the seroprevalence varied between countries and HSV-1 was reported to 52% in Finland to 82% in Bulgaria. For HSV-2 the seroprevalence was 4% in England and Wales and 24% in Bulgaria (Pebody et al 2004).

Human cytomegalovirus

CMV infects via saliva, urine, breast milk, semen, cervical secretions, tears and blood. The key reservoirs for CMV latent infection are bone marrow and peripheral blood (Dankner WM et al 1990, Taylor-Wiedeman J et al 1991, Ibanez CE et al 1991). The primary infection is often asymptomatic in immunocompetent persons but mononucleosis-like symptoms may occur. Transmission from mother to foetus is common and plays an important role in maintaining CMV infection in the population. CMV may be spread from the mother to the baby transplacental, intrapartum, and by human milk. Young infants often shed virus for years and provide a source of transmission to other children and adults. Congenital CMV infection may cause mental retardation, hepatosplenomegaly, hearing problems, haematologic abnormalities, and retinal lesions (Malm et al 2007).

CMV is one of the most common and harmful pathogens in immunocompromised patients. The most severe infections are seen in AIDS patients with low CD4 counts and in transplanted patients. The seroprevalence is higher and CMV is acquired earlier in life in poor countries and in low socioeconomic groups. Serology tests to determine vulnerability for primary infection and for diagnosis of ongoing infections are of value for organ and blood donors, pregnant women and in patients with mononucleosis. They are less useful in immunocompromised patients. Molecular methods are being used more and more frequently for diagnosis, especially since immunocompromised patients often do not mount an adequate antibody response.

Epstein-Barr virus

The virus enters B-lymphocytes which are the only carriers of CD21, the complement receptor which is also the receptor for EBV. In the B-cells, latency is established. Production of progeny viruses occurs in epithelial cells, mainly in the oropharynx but also genitally. EBV is transmitted mainly by oral secretions, but also sexually (Higgins CD et al 2007, Thomas R et al 2006). The virus is frequently reactivated and shed by secretions, such as saliva. Virus has also been detected genitally (Higgins et al 2007, Thomas et al 2006) and in the lungs (Ho et al 2006) from seropositive individuals which support the view that once the virus has colonized the B-lymphoid system, reactivation from latency can occur at any mucosal site with a B-cell infiltrate. Most primary infections occur in childhood and are asymptomatic. Early infection may be protective against the development of an allergic phenotype (Nilsson C et al 2005). Probably parent-to-child transmission by the oral route is the most common way of transmission.

When adolescents and young adults are infected, about half of them get mononucleosis (kissing disease), a disease characterised by symptoms such as high fever, tonsillitis, lymphadenopathy, atypical lymphocytosis and involvement of the liver and spleen. Encephalitis, myelitis and Guillain-Barré, severe generalized, lethal infection and spleen rupture are rare complications. The malignancies Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease are associated with EBV infections (Rezk et al 2007). Transplant recipients are at risk for post-transplant lymphoproliferative disease caused by EBV (Peterson MR et al 2006).

Heterophile antibodies can be detected by quick tests but the assay lacks in sensitivity and specificity, especially in small children. Specific EBV serology is the most reliable diagnostic tool, and is traditionally based on detection of IgM and IgG antibodies to viral capsid antigen (VCA) and IgG to EBV nuclear antigens (EBNA). At onset of symptoms IgM and IgG VCA can be detected. VCA IgM disappears after convalescence and EBNA antibodies appear after 1-6 months, and persist throughout a person's lifetime together with VCA IgG. About 82% of Swedish 16 years old girls were reported seropositive (Andersson-Ellström A et al 1995).

Table 3 Methods used for the diagnosis of herpesvirus infections

Method	HSV	CMV	EBV	VZV
Isolation	xx	xx*		x
Antigen detection	xx	x	x	xx
Serology IgG/IgM	x	xx	xx	xx
Molecular	xx	xx	xx	xx

*is being replaced by molecular methods for diagnostic purpose

xx commonly used

x less used routinely, mainly selected material or in research

Measles, mumps, and rubella viruses

Measles virus

Measles virus belongs to the paramyxoviridae family and is an RNA virus. It is spread by aerosol and the respiratory route. It causes a severe childhood disease. After an incubation period of 10-14 days fever, coryza cough and conjunctivitis occur for a couple of days and are followed by maculopapular rash. Inside of the cheek white lesions (the Koplik's spots) are seen early during infection. Encephalitis, pneumonitis and meningitis, and other bacterial complications due to the immunosuppressive effect of the virus, are severe complications. No antiviral treatment is available but immune serum can be administered and provide passive immunity. Infection results in long-lasting immunity in immuno-competent persons.

Mumps virus

Mumps virus belongs to the paramyxoviridae family and is an RNA virus. It is spread by saliva, and causes another of common childhood diseases, mumps, and infectious parotitis. The incubation period is about 2-3 weeks. The symptoms are fever and salivary gland swelling, particularly of the parotid glands. Complications such as meningitis, meningoencephalitis, encephalitis, and pancreatitis can occur. Mumps used to be the most common cause of meningitis of childhood before the vaccination era (Bjorvatn et al 1978). Another complication is mumps orchitis, which may occur upon infection after puberty, and may cause sterility if both testes are infected. Re-infection can occur. No treatment is available. Infection usually results in varying degree of immunity in immuno-competent persons.

Rubella virus

Rubella virus belongs to the Toga viridae family and is an RNA virus. It is endemic worldwide, outside areas where it is included in the childhood vaccination programme. The incubation period is about 2-3 weeks and it appears to be spread by aerosol. Often the symptoms are mild and subclinical. Clinical symptoms include maculopapular rash, lymphadenopathy, conjunctivitis, sore throat and arthralgia. Complications such as meningitis, thrombocytopenia and arthritis may occur. Rubella is very teratogenic, and infection before week 20 of a pregnancy causes serious malformations such as cataract, deafness, congenital heart failure and mental retardation. Congenital rubella syndrome (CRS) is unusual since the introduction of the MMR vaccine. However, in countries without vaccination the incidence of CRS is 0.6-2.2 per 1000 live births which is in comparison with figures before the pre-vaccination era in industrialized countries (Cutts et al 1997). No antiviral treatment is available but hyper-immune serum can be administered. Infection results in long-lasting immunity in immuno-competent persons.

Table 5 Methods used for diagnosis of measles, mumps and rubella

Method	Measles virus	Mumps virus	Rubella virus
Isolation	x	x	x
Serology IgG/IgM	xx	xx	xx
Molecular	xx	xx	xx

The Mumps-Measles-Rubella vaccine (MMR)

Since 1982 two doses of the live attenuated MMR vaccine are included in the childhood vaccination programme in Sweden. The doses have been given at the age of 18 months and 12 years. For children born 2002 and later the second dose from the year 2007 is given at the age of 6-8 years (SOSFS 2006:22 M). The coverage of the vaccination is >90% in Sweden. Every 10th years there is a serological surveillance study performed in Sweden, in which the immunity to MMR in different age groups has been investigated. In the latest study, reported in 1997 (Olin et al 2004), the immunity to measles was found to be satisfactory but the antibodies against mumps virus and rubella virus had declined at the age of 8-9 years and 10-11 years before the second dose was given. Therefore it was decided to give the 2nd dose earlier. Since the introduction of the MMR vaccine, the incidence of the diseases has decreased dramatically in Sweden but small outbreaks of measles and mumps have been reported. However, these diseases cause serious problems in other parts of the world where there are no general vaccination programmes.

Aims of the study

- To establish a flow-cytometric method that can be used at a large-scale for evaluation of the cell-mediated immune responses against varicella-zoster virus
- To use this method for measurement of cell-mediated immune response against varicella-zoster virus before and after varicella-zoster vaccination and natural infection
- To evaluate the humoral response against varicella-zoster virus before and after vaccination and natural infection
- To investigate the seroprevalence of varicella-zoster virus antibody in Swedish children during thirty years

Studies in this thesis

Methods used in the studies

VZV Serology

Paper I and IV

An in-house IgG enzyme-linked immunosorbent assay (ELISA) in microplates with the wells coated with nucleocapsid antigens from VZV (Sundqvist and Wahren 1982) was used. The sera were examined in ten-fold dilutions from 1/100. An end-point titre of <100 was considered negative (no immunity), ≥ 500 was considered positive (immune). Titres 100-499 were considered equivocal (uncertain immunity). In case of equivocal or negative results in ELISA, indirect immunofluorescence assay (IIF) was performed to determine the definite VZV serostatus (Grandien et al 1976). For the IgG IIF VZV-infected fibroblasts fixed on galls slides were incubated with serum diluted one in ten and thereafter with fluorescein-labelled anti-IgG antibodies. The preparations were examined in a fluorescence microscope at magnification 1/40, and the assay was interpreted as positive if there was a distinct fluorescence in the cytoplasm of infected cells. Only nuclear fluorescence was regarded non-specific. The inverted value of dilution 1/2 was given as the titre. A titre of <2 was interpreted as no previous exposure to VZV, ≤ 2 possible exposure but no certain immunity, and >2 was regarded sign of immunity. The assay has been in use since 1976, and before the introduction of ELISA, a titre of >2 was regarded as a sign of immunity. Since it has been described that very low titres in the reference method FAMA are not protective (Gershon AA et al., 1994) and we have experienced at least one severe chickenpox infection in a person with detectable antibodies by IIF but VZV ELISA IgG <100 , our definitions today are: A titre of <2 indicates no previous exposure to VZV, ≤ 2 indicates likely previous exposure, without certain immunity, and >2 indicates previous exposure and in combination with detectable antibodies in the IgG ELISA (≥ 100) regarded as a sign of immunity.

VZV IgM: An in-house VZV IgM capture ELISA with peroxidase-labelled nucleocapsid antigen was used as previously described (Sundqvist V.-A 1982).

Paper II

Since it should be possible to correlate the results to other VZV-vaccination studies performed by GlaxoSmithKline (GSK), methods recommended by GSK and employed in other studies were used.

For screening of the whole material, Enzygnost VZV IgG ELISA (Beringwerke, Germany) was performed according to the recommendations from the manufacturer. An optical density (OD) of ≥ 0.2 should be regarded as diagnostic for the presence of VZV antibodies, 0.1-0.2 should be regarded as equivocal and the assay should be repeated. >0.1 should be regarded as negative. The ELISA results were also expressed as arbitrary units (AU/ml) according to the manufacturer, and these results were used for statistical comparison of antibody levels in different groups.

Equivocal results were re-examined both in ELISA and IIF. IgG Virgo immunofluorescence (IF) KIT (Hemagen Diagnostics, Maryland, USA) was performed according to the recommendations from the manufacturer. To increase the sensitivity of the IF, the serum samples were examined in dilutions of 1/2, 1/4 according to recommendations from the laboratory at GSKBio, with vast experience of the assay. A typical cytoplasmatic fluorescence in a dilution of 1/4 was regarded as a sign of the presence of specific antibodies in the Virgo assay.

To verify the sensitivity and specificity of the Virgo examination, a panel of 14 reference samples including samples positive by the Virgo assay but negative in the ELISA, were sent to Professor Anne Gershon (Columbia University, New York, USA) who examined them by a reference method, fluorescent antibody membrane assay (FAMA). This is an indirect immunofluorescence antibody test, using unfixed VZV-infected cells (Williams et al., 1974). The conformational structure of VZV glycoprotein antigens on the surface of infected cells is probably altered by fixation, which leads to decreased sensitivity and specificity of antibody binding. Since FAMA is extremely labour intensive, demands long experience for reproducible results and requires working with live virus it is performed at very few laboratories in the world. According to professor Gershon, a titre >1:4 is highly correlated (96%) with protection from varicella after close exposure to VZV in healthy vaccinees or after natural infection (Gershon et al., 1994) Parallel examinations on the 14 samples were also performed with our in-house IIF. In 13714 samples the results correlated, but one of the 14 sera that were negative in our IIF had detectable antibodies at dilution ½ in the FAMA and Virgo assays. The adult person who had donated the serum fell ill with a severe chickenpox two years after the study, further illustrating the importance carefully interpreting of the protective effect of very low IIF- or FAMA titres.

Cell-mediated immunity

Paper I and IV

Flow-cytometry

The flow-cytometry identifies individual cells and other particles as they pass in a stream through a laser beam. Each cell then scatters the laser light, which gives information about the size (forward scatter light, FSC) and granularity of the cell (side scatter light, SSC). If the cell has been labelled with a fluorescent reagent (fluorochrome) it emits fluorescence. The scattered and fluorescent light is collected by optics, transferred through specific filters to photodetectors and further digitalised and analysed by a computer.

The VZV nucleocapsid antigen (Sundqvist et al., 1982) used for the IgG ELISA was also used for cultures for activating specific cell-responses. The final concentrations of nucleocapsid antigens including CMV antigen (Sundqvist et al., 1983) in whole-blood and PBMC cultures; 3.2 µg/mL and 5.0 µg/mL, respectively, had been determined prior to the study. The control antigen was made from human lung cells used for cultivation of the viruses. Staphylococcal enterotoxin A (SEA) (10 ng/mL), Staphylococcal enterotoxin B (SEB) (5 ng/mL) (Sigma Aldrich Co., Stockholm, Sweden), and Phytohaemagglutinin (PHA) (Murex Diagnostics, Dartford, England) were used for control of cell proliferation.

FASCIA

The blood was diluted 1/10 in RPMI 1640 (Gibco/BRL Ltd, UK), supplemented with 10 000 IU/ml penicillin (Gibco/BRL), 10 000 µg/mL streptomycin (Gibco/BRL), and 2 mM L-glutamine (Gibco/BRL) [RPMI medium]. Four hundred fifty microliters of the diluted blood and 50µL of antigen or medium only were added to 12 x 75 mm polystyrene round-bottom tubes with caps (Falcon 2058, Becton Dickinson Labware, NJ) and incubated for 6-7 days in a humidified atmosphere at +37 °C with 5 % CO₂ in air. Negative (medium only) and positive (SEA+B) control cultures were run in triplicate and duplicate, respectively. For these mean values were calculated and given as results, whereas specific stimulations were run in one tube only.

After incubation, the tubes were centrifuged at 300 x g and the supernatants were removed and kept at -70°C until required for analysis of cytokine concentrations. The pellets were stained with the following monoclonal antibodies: anti-CD3-PerCP, anti-CD4-APC, anti-CD45RO-PE, and anti-HLA-DR-FITC [Becton Dickinson Immunocytometry Systems (BDIS), Stockholm, Sweden] for 10-15 minutes in room temperature. A lysing solution (1.0 mL ammonium chloride; Orthomune Lysing Reagent, Ortho Diagnostics, Stockholm, Sweden) was added for 5-10 minutes at room temperature, followed by centrifugation, removal of the supernatants, additional lysing if needed, washing with phosphate buffered saline (PBS), and resuspension in 250 – 500 μL PBS with 2 % paraformaldehyde. The samples were stored in the dark at 4°C until acquired on a FACSCalibur (BDIS) using CellQuest software (BDIS) within 4 hours. The instrument was set for four-colour analysis using FACSComp software (BDIS) in conjuncture with CaliBRITE (BDIS), and 10,000 events were acquired from each tube and saved as list mode data for analysis, mainly using a previously described method (Gaines et al 1996, Gaines et al 2000) with modifications.

PBMC culture and detection of DNA synthesis (paper I, II and IV)

The conventional proliferation assay was conducted as previously described (Jordan and Merigan, 1974, Ljungman et al., 1985, Wang et al., 1999). Briefly, mononuclear cells were purified by Ficoll-Hypaque gradient centrifugation and 1.5×10^5 cells per well cultivated for 6-7 days in medium with human AB+ serum in flat-bottomed microwell plates with or without antigens (VZV, CMV, PHA or SEA + B) 50 μL ^3H -thymidine were added to each well during the last 4 hours of cultures, followed by harvesting onto glass fiber pads and measurement of incorporated ^3H -thymidine by a β -cell counter. The amount of incorporated ^3H -thymidine was measured in a β -cell counter (LKB Wallac, Turku, Finland) as counts per minute (cpm). The results were expressed as stimulation indices (SI), and net stimulation (net S). [SI= A/B (> 2 positive), net cpm= A-B (> 1000 positive). A= mean cpm from VZV antigen stimulated cultures, B= mean cpm control antigen stimulated cultures. Both criteria should be fulfilled to verify presence of cell-mediated immunity CMI against VZV.

Cytokine detection (paper I)

The culture supernatants were analysed in duplicate for concentrations of IFN- γ , IL-5, and IL-10 by in-house ELISAs, using monoclonal antibody pairs from Mabtech (IFN- γ ; Nacka Sweden) or Nordic Biosite AB (IL-5 and IL-10; Täby, Sweden). ELISA protocols recommended by the manufacturers were used with slight modifications. The detection limit was 20 pg/ml for IFN- γ and IL-5, and 15 pg/ml for IL-10; the net results were calculated as levels in cultures in the presence of activators minus levels in cultures with medium only.

Serology for other viruses:

Paper II

Measles virus: indirect in-house IgG ELISA with sucrose gradient purified measles virus (Lec) virions cultivated on MA-104 cells as antigen. The results were correlated to an international standard serum (NIBS, UK). The cut-off value was set to 0.12 IU/mL.

Mumps virus: indirect in-house IgG ELISA with antigen prepared from embryonated hen's eggs inoculated with mumps virus (strain SBL 1/67 Enders type). An international reference serum was arbitrarily given the value 100 units/mL (AU/mL). After comparison with a neutralization test, serum samples with ELISA results of < 8 AU/mL were considered negative. A protective antibody level cannot be given for mumps since reinfection may occur.

Rubella virus: indirect in-house IgG ELISA with antigen supplied by Orion Diagnostica (Finland) was used. The results were correlated to an international standard serum (NIBS, UK). The cut-off value for immunity was set to 10 IU/mL.

Paper III

HSV: IgG type-common in-house IgG ELISA (Sundqvist et Wahren 1982)

VZV: in-house IgG ELISA, nucleocapsid antigen (Sundqvist et Wahren B 1982)

CMV: in-house IgG ELISA, nucleocapsid antigen (Sundqvist et Wahren 1981)

Serum dilutions 10^{-2} and 10^{-3} were used both for HSV, CMV and VZV. The cut off value for positive result was optical density (OD) ≥ 0.2 in dilution 10^{-2} . The 10^{-3} dilution was included to give better specificity by enabling monitoring of appropriate decrease in absorbance with dilution. No antibody quantification was performed.

EBV: in-house indirect IgG immunofluorescence assay with the viral capsid antigen (VCA) expressing P3HR1 cell-line as antigen (Linde et al., 1987) The serum dilutions used were 1/20 and 1/80. A characteristic staining reaction in serum dilution $\geq 1/20$ was considered positive. The 1/80 dilution was included to ensure specificity in case of non-specific fluorescence of 1/20. The specimens were investigated for seropositivity or seronegativity.

Paper III

Measurement of Total IgG.

To evaluate the quality of long-term stored samples, total IgG concentrations were measured in 20 randomly selected samples from each cohort. The analysis was performed with Turbox Immunoglobulin G Assay (Orion Diagnostica, Trosa, Sweden) for quantitative nephelometric determination of IgG in human serum or plasma with Turbox/Turbox plus analyser, in accordance with the instructions of the manufacturer. The assay is a liquid-phase immunoprecipitation assay with nephelometric end-point detection. The results are expressed as concentration units (g/L). The measurement range is 4-30 g/L.

Paper IV

VZV PCR was used for the confirmation of the VZV infection (Puchhammer -Stöckl et al 1991).

Paper I

Background

Detailed information on the nature of VZV-specific immune response following natural infection, reactivation and VZV immunization is needed. To obtain this measurement of both the humoral and the CMI response is necessary. The studies must be performed on a large scale to provide sufficient and reliable information, since individual variation may be vast. However, the conventional lymphoproliferation assay for CMI detecting thymidine incorporation in peripheral blood mononuclear cells (PBMCs) (Ljungman et al 1985, Wang et al 1999) is technically complex, labour-intensive, time-consuming, employs radioactive material, about 10 mL blood is needed, and is difficult to perform on a large scale (Diaz et al 1989, Sharp et al 1992, Watson et al 1990). A more convenient flow-cytometric method for measurement of CMI in activated whole-blood was therefore developed and evaluated.

Material

Fifty-two healthy children aged 1-14 years were enrolled in the study. Four VZV IgG positive, healthy laboratory workers were bled once a week during three consecutive weeks and cultures were run in five replicates to investigate the intra-assay and inter-assay variation of FASCIA in comparison with the conventional lymphoproliferation assay. Three ml of peripheral blood was collected in sodium heparin from the children and 10 ml from the laboratory workers, as the conventional assay demands more blood. Antibody detection was performed on plasma stored at -70 °C until analysis.

Results and discussion

Thirty-seven children were VZV seropositive and 15 VZV seronegative. Two groups were established for evaluation of the sensitivity and specificity of the VZV specific CMI response. The seropositive group was expected to display VZV specific CMI and the seronegative to be lacking CMI response.

The FASCIA results were expressed as stimulation index (SI), net stimulation (net S), and percentage stimulation (%S). SI was calculated as results in VZV cultures / results in medium only cultures; net S as results in VZV cultures - results in medium only cultures; and % S as $100 \times \text{net S} / (\text{results in PHA cultures} - \text{results in medium only cultures})$. Net S and %S provided more reproducible results in terms of both intra-assay and inter-assay variation and were also more useful as the background was very low or absent, and zero cannot be employed for calculations of ratios. Hence %S was selected for the study. The conventional lymphoproliferation assay displayed a higher variation than the FASCIA.

Measurement of CMI can be performed by identification of the lymphocytes and lymphoblasts on there scatter profile. More accurate results can be achieved by immunophenotyping and the cellular cultures were stained with CD3, CD4, CD45RO and HLA-Dr. In total 16 different combinations of parameters were evaluated and the combination that provided the most accurate results was selected. Irrespectively of the number of parameters a median VZV-specific CMI of about 10% %S was recorded in seropositive subjects and a majority of the seronegatives displayed 0.00%. The sensitivity and specificity were calculated with the presumption that seropositive subject should display VZV-specific CMI and seronegative should not. Two or three cell-surface markers for activation appeared to provide more accurate results than none, one, or four markers. A sensitivity of 95-97 % and a specificity of 93-100 % were calculated for different combinations and 0.00 -1.00 % treshold cut-off levels. IFN- γ was detected in 78% of the cultures from the seropositive children and in none of the cultures from the seronegative. Ten of the 28 IFN- γ positive cultures also contained other cytokines: IL-10 was only detected in 8 cases, IL-5 only in one, and one had both IL-10 and IL-5. This is in concordance with findings of Trannoy et al 2000 that VZV-specific memory cells generally produce IFN- γ suggesting a Th-1 profile.

FASCIA is easy to work with. Less blood is needed than for convetional anlysis which is valuable when children are examined. The cells are kept in an environment more similar to that in vivo than when PBMCs are utilized. Possible enrichment or depletion of cell subsets and pre-activation of cells during the preparation, resulting in an increased background and a decreased sensitivity is avoided. FASCIA can be used both for single samples and in a large scale for determination of CMI after vaccination and possible also for the evaluation of VZV reactivation resulting in shingles.

Paper II

Background

General childhood VZV vaccination has not been introduced in Sweden, and vaccination of VZV-seronegative pupils at 12 years of age, together with the MMR booster, could be a cost-effective way to avoid the severe VZV in teen-agers and adults. This study aimed to determine the feasibility of co-administration of a VZV vaccine and the MMR booster at 12 years of age.

Material

The study was performed during two school years (1998-99, 1999-2000) in the southern parts of Stockholm. In the first year, guardians of 1231 pupils were asked about the child's history of chickenpox. The majority, 1041 children, had a history of chickenpox and 190 were history negative. Of the history negative 62 were vaccinated. Further history negative pupils were included the following year, and in total 100 were admitted to the study. In total 99 were vaccinated, 98 could be evaluated for safety, and 89 were examined for immunogenicity as 11 children dropped out. As control, 201 history positive children were recruited. Seventeen of them dropped out and in total 199 were evaluated for safety, and 184 for immunogenicity. The vaccines used were VZV vaccine Varilrix® (GSK), and the MMR vaccine MMR II® (Aventis Pasteur, MSD). Serum samples were collected before the vaccination and about 8 weeks after. The history positive pupils received only the MMR vaccine. The history negative pupils received the MMR vaccine and the VZV vaccine at the same occasion but in different arms.

Results and discussion

In total 17/89 in the history negative group were also VZV seronegative before VZV immunization. Twelve of them seroconverted after 1 dose, and 5 did not mount any antibodies. Among the VZV history-negative but VZV seropositive children, the VZV IgG titres before vaccination were significantly lower than in the VZV history positive group. After VZV immunization the VZV titres in the history negative group increased and became higher than in the history positive group that only received the MMR vaccine. The MMR response was comparable in those that had received only MMR and in those that had got also the VZV vaccine. Anti-mumps seropositive rates were higher in the history negative groups than in the history positive group both before and after MMR vaccination. The predictive value of self-reported VZV infection for positive serology was shown to be 100 % (ELISA) and 99.5 % (IF) compared to serology for subjects with a history of chickenpox infection. However, in the history negative group, 80.9 % were found to be VZV seropositive for antibodies against varicella by ELISA and IF before vaccination.

Five initially VZV seronegative pupils did not have measurable VZV Ig G antibodies after the first varicella vaccination. Four accepted a second dose after about one year. One child had moved and could not be contacted for revaccination. VZV IgG antibodies and antigen-specific lymphocyte proliferation were investigated before and after the second dose. From one child, only a blood sample before the second dose could be obtained since he then refused further sampling. He had no detectable VZV IgG antibodies or CMI before the second dose. Before and 4-12 weeks after the second dose blood samples were drawn from the other 3 children. All three were seronegative before the second dose but one had measurable CMI. That child had also measurable CMI after vaccination and seroconverted. The other two had no measurable CMI before the second dose but seroconverted and developed detectable VZV-specific CMI after the second dose of VZV vaccine.

Concordant results concerning VZV serostatus were obtained in 97 % of the samples at first examination. After re-examination 13/15 samples were equivocal by ELISA but positive in titre 1/8 by IF. One sample was negative by ELISA but displayed a titre of 1/4 by ELISA. These samples were considered as seropositive as IF was reference method. One sample was positive in ELISA by displayed a titre of 1/2 by IF and considered seronegative.

There were few adverse and effects reported. Redness of injection site was the most frequently reported. The unsolicited symptoms were low in both groups (<10%). No serious adverse effects were reported.

In an unpublished study from 1992 two hundred 12 years old children in Stockholm the seroprevalence was 90 %. In this study it was calculated to be about 97 %. Day care attendance and indoor living may have contributed to an increase in seroprevalence. The immune response to the MMR vaccine was not affected by the concomitant administration of VZV vaccine but whether MMR affects the response to the VZV vaccine requires further studies. All children had received one dose of MMR before the age of 11 years, but measurable antibodies before the dose at 12 years were lacking in 6.6% for measles and in 26.4% for rubella. In a measles outbreak in Stockholm 49/52 cases were unvaccinated and 3 had only received one dose of measles vaccine (Parment PA, Insulander M 2000). This emphasizes the need for a second dose of MMR. The rate of mumps seropositivity found in any study depends on a great extent on inter-methods variations between assays employed by different laboratories. Before MMR, 66 % of the samples were positive and 83 % after vaccination, measured with by in-house ELISA and in-house neutralization test. The sera were retested at Merck's laboratory with the vaccine strain used as antigen in ELISA. Then only 4 % of the sera were demonstrated to lack antibodies before immunization and all were seropositive after the second dose.

Lack of history of chickenpox cannot be used as the only criterion for VZV vaccination, since it would result in costly over-vaccination of 4/5 of history negatives in Sweden. VZV serology before vaccination would probably be cost-effective. Two doses of VZV vaccine should be recommended also to 12 years old children, since only 12/17 seroconverted after the first dose. The feasibility of vaccinating VZV seronegative children within the existing Swedish school health-care system still needs further studies comparing VZV vaccine alone and concomitantly with MMR. Also, the possibility to vaccinate together with MMR at 12 years of age has now disappeared since the MMR boost in 2007 was moved to 6-8 years.

Paper III

Background

In our paper II the antibody seroprevalence for 12 years old children was approximately 97% which was higher than expected. We therefore wanted to investigate if the had been a change in prevalence of antibodies during 30 years. The serostatus to EBV, CMV and HSV were included for comparison, since these viruses are generally spread during direct contact, whereas VZV is generally transmitted by air-borne droplets. This was to get information on whether the mode of spread might have affected the change, and since it was of general interest since the social conditions and life-style in Sweden may have affected the spread of these herpesviruses. Age at primary infections may affect the severity of outcome of all these infections. The age group 9-12 years was examined since most of the pre-pubertal, non-sexual transfer of herpesviruses is likely to have occurred by that age.

Material

A total of 1185 serum samples from children aged 9-12 years in 1967-68, 1977-78, or 1997, respectively, were available in a serum bank at SMI. The sera were considered to be representative for each age group. Only the year of birth and area of residence were known for each child. The materials were regarded as representative for the whole of Sweden (WS), except for the 152 sera collected 1977-78 from children living in the Stockholm area (Sto). Of the samples from 1967-68, and 1977-78, 22% (152/696) had deteriorated. For WS 1967-68 308/396 samples, Sto 1977-78 152/216 and for WS 1977-78 all 84 available samples were examined. Samples collected in 1997 were randomly selected from a larger collection in order to match the number of samples from the other years. The sera had been stored in -20 °C and freeze-thawed 2-4 times. The sera from 1977-78 were stored diluted in virus cultivation media and the dilutions used for analysis were adjusted accordingly.

Results and discussion

The seropositivity for VZV was 50% in 1967-68, and 74-82% in 1977-78, and 98% in 1997. The corresponding figures were 31%, 53%, 50%, and 58% for CMV, 35%, 35%, 32% and 38% for HSV, and 64% in 1967-68 and in 1977-78 (both cohorts), and 62% in 1997 for EBV. The rate of seropositivity to VZV from the whole of Sweden had risen significantly from 50% in 1967-68 to 98% in 1997. The HSV and EBV seroprevalence were relatively stable. The seroprevalence for CMV had increased from 31% to 50% between 1967-68 and 1977-78. This increase was not significant but a further increase to 58% made the difference between 1967-68 and 1997 significant ($p < 0.001$). This p -value is for the difference between 1967-68 and 1997 and 1977-78 is not taken into account.

The quality of the old sera was a major issue. In order to investigate the quality of the samples examination of the total IgG content was performed. No correlation between the total IgG content and the specific OD values in the specific ELISAs was found. The p -values were >0.05 for the virus combinations and also for each virus separately. This indicates that the quality of the samples was sufficient in terms of their immunoglobulin content to be used to study antibody prevalence. The median values for the IgG content differed. It was usually higher and more varied in the older samples, indicating desiccation of some samples. Higher seroprevalence might be expected in those samples, but was not found. The dilution of the samples for cohort 1977-78 appears not to have affected the result. However, the difference in seroprevalence with time for the different viruses increases the plausibility that the results are not due to deterioration of IgG in the old samples. Intact specific binding of the IgG of the old samples can, however, not be guaranteed.

In our study (paper II) the seroprevalence was 97% in 12 years old children which corresponds to the figures in this study. For 11-12 years old Swedish children born 1949 a seroprevalence of 70% have been reported which could indicate some deterioration of the old samples in our study (Strom 1980). However, it is not clear how the antibodies were measured in that report. The seroprevalence of 50% correlates to findings in 8 years old children in the UK in the 1980s (Kangro et al 1994). In Finland a seropositivity of 83% was reported in 8 years old children born 1989 and 1990 (Aarnisalo et al 2003) but our assays may have a slight higher sensitivity (de Ory et al 2006). In England and Wales the seroprevalence was 80-85% in 9-12 years old children in sera from 1996 (Vyse et al 2004). In several studies from different countries $>90\%$ seropositivity at the ages 10-14 years has been described (Muench et al 1986, Salleras et al 2001, Wunzler et al 2001).

Higher incidence of chickenpox among children attending day care centres has been reported and that school and preschool patterns probably play an important role of the spread of VZV (Jorm and Capon 1994, Hurwitz et al 1997, MacDonald et al 1997, Brisson et al 2001). Today about 80% of Swedish children attend day care today from the age of one year compared to 10% in the 1960s (National Agency for Education Report no 236, 2003, Landgren Möller, 2005). The VZV vaccine was introduced 1994 and has been used very little and VZV-vaccination can thus not have affected the results of this study.

A CMV seropositivity of 51% was reported in the age group 10-19 years in Swedish sera collected 1973-1982 (Ahlfors 1984). A seroprevalence of 50-58% in the age group 4-12 years is reported from other parts of the world (Kangro et al 1994, Shen et al 1992). Changes in CMV epidemiology may be associated with changes in breast-feeding and childcare, and transmission at day-care centres is described (Adler 1988, Stagno and Cloud 1994, Kashiwagi et al 2001). Since CMV is transmitted by breast milk, cervical secretion and congenitally the seroprevalence of the mother affect the status in the children. Decrease in breastfeeding from 1930s to 1970s could explain the low figures for children born 1955-58 but the increase in the cohort born 1967-68 is contradictory (Statistics Health and Diseases Breast-Feeding, Children Born 1998, 2000). The increase in promiscuity during the 1960s may have affected the seroprevalence of the mothers but such data is not available. The reasons for changes in CMV seroprevalence are multifactorial and the exact reasons are difficult to identify.

The seroprevalence for HSV was relatively stable. In a recent Swedish study it was 31% in the age group 0-19 years for HSV-1 which is matching our figures (Tunback et al 2003). In 14-15 years old girls in 1972 the corresponding figure was 23% (Christenson et al 1992). In Belgium, England and the Netherlands the seroprevalence for HSV-1 was 25-35%, and in Finland, 15-20% seropositives were found in children 5-9 years old using samples collected in 1989-2000 (Peabody et al 2004). A decline in the seroprevalence for HSV-1 from 34% in 1986-87 to 24% in 1994-95 for the age group 10-14 years was reported in England and Wales (Vyse et al 2000). We did not find such a decline in our study. The outcome of infections late in life may be affected by delayed primary infection. The decrease in childhood HSV infection may have contributed to the increase in genital HSV-1 infection in England and Wales (Vyse et al 2000).

The seroprevalence for EBV was relatively stable. It was reported in a recent study to 52% in Swedish children aged 4 years (Sidorchuk et al 2003), and in the UK to 61% in the age group 4-12 years (Kangro et al 1994). In England and Wales the corresponding figure was 54% in children aged 10-14 years in 1994. A strong association between HSV-1 and EBV seropositivity was reported and most likely explained by the similarities in the mode of transmission (Morris et al 2002).

The strength in this study is that sera from different age-matched cohorts were examined at the same time and with modern methods. A possible weakness is that the samples may have deteriorated somewhat during storage and that cohort 1977-78 not was selected in the same manner as the others. However, three conclusions seem justified; HSV and EBV that infect by close contact have not changed dramatically, but it seems like there has been an increase in CMV prevalence. CMV epidemiology is complex and the reason for this increase is difficult to identify. However, a dramatic increase in prevalence of the air borne transmitted VZV have occurred, probably as an effect of change in child care patterns.

Paper IV

Background

VZV seronegative adults are at risk for severe chickenpox and VZV immunization is recommended, but the long term efficiency has not been well studied in adults. In this study humoral IgG response was also examined using ELISA and IIF. FASCIA with immunophenotyping against CD3 and CD4 was used to evaluate the VZV-specific CMI response before and after immunization with the VZV vaccine Varilrix® in VZV seronegative adults and in controls with past chickenpox.

Materials

Eighteen VZV seronegatives out of around 4000 questioned for VZV history were enrolled in the study. Seven were lost to follow up after the first or second dose. Eleven were monitored up to one year after vaccination. Eleven individuals with past chickenpox history were included as controls. Samples were drawn before the first dose, about 6-8 weeks after the second and around one year after the first dose. One vaccinee contracted a breakthrough varicella 2 ½ years after immunization and from this subject, samples were drawn before and after vaccination, during the disease and 2 years after the disease. The CMI results were calculated as %S as presented in paper I.

Results and discussion

The 11 naturally infected subjects had significantly higher IgG titres ($p < 0.05$) than the vaccinees after one year with a median (range) IgG antibody titre by ELISA of 4300 (900-26000) compared with 210 (<100-680) in the vaccinees. The ELISA IgG titre was >100 in 3/11 tested, and >500 in 4/11. The IIF antibodies were >2 in 9 of 11 vaccinees tested by IIF one year after vaccination. The two groups also differed in CMI response showing a median (range) %S of 7.0 (0.8-29.8) in the controls and %S of 1.7 (0.0-16.4) in the vaccinees. The difference nearly reached significance ($p < 0.053$).

The woman with breakthrough infection had low humoral response before and after the second dose. The CMI before and after the first vaccination were 1.3, and 2.1 %S respectively, and decreased to 0.43 % S after the second dose. She was lost to follow-up and a sample after one year was thus not obtained. She contracted breakthrough infection after 2 ½ year and 2 days after onset of cutaneous lesions. The IgG titre was 1 000 and the % S was 5.99. No IgM antibodies were detected. The VZV diagnosis was confirmed by PCR analysis of a sample obtained from one of the lesions. Two years later the ELISA IgG titres were 6 000 and the %S was 64.34.

VZV seronegative adults are rare in Sweden and it was difficult to get a large study group despite extensive screening for negative histories. The problem of vaccination of adults and monitoring policies for health care workers (HCW) may therefore seem minute, but a case of VZV in one HCW (or pregnant woman) may cause considerable damage. It is worrisome that one year after using two doses of vaccine both the humoral and CMI responses in the vaccinees were inferior to what was found in the natural infected individuals, and that 1/11 individuals got a breakthrough infections, Similar results have been reported also in another study (Gershon AA, Steinberg SP, 1990).

Four of eleven vaccinated persons in our study would be regarded at risk of chickenpox upon exposure according to our revised criteria for immunity. The protective significance of the level of the CMI responses recorded is difficult to determine but it does not seem to be of high magnitude or long duration in the vaccinees in comparison to persons with past infection. The

woman with breakthrough infection had ELISA IgG titre and %S in the same levels as the naturally infected controls 2 years after her breakthrough disease which indicates that she did not have any general immune defect.

In our study, 36% lacked specific antibodies one year after immunization. Loss of antibodies in 31-35% of vaccinees has been reported in other studies (Saisman L et al 2001, Ampofo K et al 2002). Seroconversion in 100 % of subjects 6 weeks after the second dose, loss of antibodies in 4% after one year, decreasing titres and one breakthrough infection were reported by Burgess et al 1999. Seroconversion achieved in 100% three months and one year after vaccination, and 94.4% measurable antibodies after 5 years have also been reported (Zerboni et al 1998). In the same study, 94.1% had specific CMI after one and 5 years. One explanation for the difference in the results may be the vaccine used. Most studies have used the Varivax vaccine, which is stored frozen. In our paper II only 71% of 12 years old children seroconverted after one dose Varilrix®. However, it has been suggested that the different vaccines from the OKA strain should be considered more equal than different until more data is available (Lay YL et al, 2002, Lay YL et al 2004, Rentier B, Gershon AA 2005, Hambleton S, Gershon AA 2005, Chius SS, Lau YL 2005).

It has been suggested that in case of loss of detectable antibodies, CMI may offer protection against infection (Ampofo S et al 2002, Siaman et al 2001). Loss of antibodies in spite of measurable VZV specific CD4 response has been reported (Seward et al 2004). Measurement of both specific antibodies and CMI has therefore been recommended (Ampofo et al 2002, Numbe et al 1985). However, this study does not support that there may be a strong CMI despite absent antibodies. In our study only 64% of adult HCW vaccinated twice displayed what we regard as protective antibody titres one year after immunization. In addition, the antibody and CMI responses were lower than in naturally infected subjects, and one vaccinee had a break through infection. Since such infections are usually milder than in unvaccinated, vaccination may still be recommended in those at risk for severe infection. According to our results we suggest that vaccinated HCW should be examined for serostatus if exposed for varicella. If the vaccinee has uncertain or seronegative serostatus we suggest that the individual should be treated no different than an unvaccinated with the same results.

In conclusion, the low immunity one year after vaccination, and the contrasting results concerning VZV-vaccination of adults require further studies, including comparisons of the effect of different vaccines, and examination of cell-mediated immunity.

Conclusions

- Flow-cytometric Assay of Specific Cell-mediated Immune response in Activated whole blood (FASCIA) has been developed and established for use at a large-scale. FASCIA has many advantages compared to the conventional lymphocyte proliferation assay and some of them are:
 - Less blood is needed e.g. 0.5 mL compared to 10 mL
 - Much less labour-intensive – can be conveniently performed at a large scale as well as for single samples
 - No radioactive material is handled
 - Cells are kept in an environment more similar to that in vivo
 - Less inter-assay and intra-assay variation
 - High sensitivity and specificity
- FASCIA has showed to be a useful method for measurement of VZV cell-mediated immune response in children, in vaccinated adults before and after immunization and in naturally infected adults and can be further developed and evaluated also for other microbial pathogens.
- Humoral response in adults before and after varicella-zoster virus vaccination and in naturally infected has been evaluated. The response one year after VZV immunization was significantly lower than in naturally infected.
- Children by the age of 12 years should be recommended 2 doses of VZV vaccine since only about 30% seroconverted after one dose.
- VZV vaccine does not alter the MMR response.
- The seroprevalence in 9-12 years old children has increased dramatically to VZV, but not to other herpesviruses like HSV, CMV and EBV in Sweden over recent decades. Changed patterns of early childcare may have contributed to the change.

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