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DIAGNOSIS IN ASEPTIC MENINGITIS AND IMMUNE RESPONSE IN HERPES SIMPLEX VIRUS INFECTIONS

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Stockholm 2009

Published by Karolinska Institutet. Printed by Eprint.

ISBN 978-91-7409-447-3

ABSTRACT

Herpes simplex virus type 1 and 2 are ubiquitous and appear often asymptomatic but some individuals suffer from recurrent infections and the causes are largely unknown. In this thesis I present results from evaluations of diagnostic and etiologic studies of methods used for the detection of acute aseptic meningitis (AAM) and in HSV-2 meningitis in particular. The second part we investigated the innate and/or adaptive immune response in patients affected by HSV-1 and 2 in primary genital infection as well as recurrent HSV-2 meningitis compared to recurrent genital infection or seropositive blood donors.

The first paper addresses the need for efficient and sensitive diagnostic methods in the management of virus infections in the central nervous system. The objective was to evaluate a real-time PCR for the detection of HSV-2 and VZV DNA from cerebrospinal fluid samples in clinically well characterized patients with HSV-2 meningitis and AAM of unknown origin. The sensitivity of real-time PCR was found to be 87% (33/38) in primary and 70% (19/27) in recurrent HSV-2 meningitis. VZV was detected in 2 of 45 samples (4.4%). The quantitative real-time PCR was also compared with the nested qualitative PCR and found to identify more cases in the recurrent meningitis group.

The second paper addresses the etiology of AAM and the diagnostic efficiency in an adult population in Stockholm, using a limited first-line combination of microbiological assays. PCR assays for HSV- DNA and enterovirus (EV) RNA in the CSF as well as ELISA for IgM to tick-borne encephalitis virus (TBEV) in serum were performed. A viral diagnosis was obtained in 255 of the 419 cases (62%) with these routinely performed assays. Thus, consistent use of CSF-PCR for EV and HSV and TBEV serology established a diagnosis in the majority of AAM patients.

The third paper addresses the immune response in patients experiencing a first episode genital HSV infection. In a prospective clinical study the cell-mediated immune response (CMI) was measured and the cytokine profile identified and followed during one year. In patients with primary HSV infection CMI responses declined over time, whereas patients with non-primary HSV infection displayed stable CMI during the follow up year. For patients with primary HSV-2 infection, levels of HSV-specific IL-10 and IL-4 responses at first visit were significantly inversely correlated with number of recurrences during the subsequent year.

The fourth paper addresses HSV-specific immune response in patients affected by recurrent meningitis caused by HSV-2. During asymptomatic periods, these patients expressed elevated T-cell blasting and cytokine responses against HSV-antigens an also an increased expression of TLR 3 and 9 on dendritic cells as well as increased TLR induced interferon responses in comparison with patients with recurrent genital HSV-2 infection and asymptomatic seropositive HSV-2 individuals. Thus, we did not find that recurrent HSV-2 meningitis was due to deficiencies in adaptive or innate immune functions.

LIST OF PUBLICATIONS

Papers included in this thesis:

I. Elisabeth Franzen-Röhl, Annika Tieveljung-Lindell, Lena Grillner and Elisabeth Aurelius
Increased Detection Rate in Diagnosis of Herpes Simplex Virus type 2 Meningitis by Real –Time PCR Using Cerebrospinal Fluid Samples
Journal of Clinical Microbiology, Aug. 2007, p. 2516-2520.

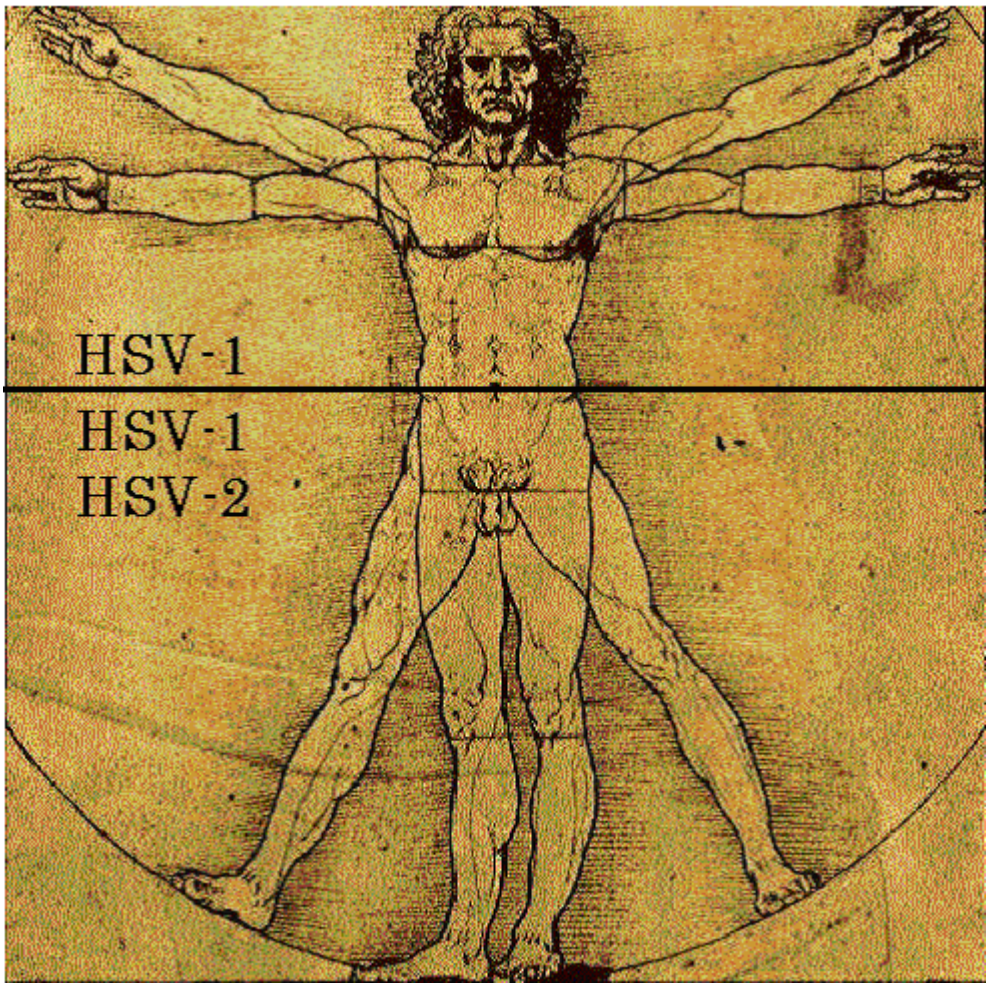
II. Elisabeth Franzen-Röhl, Kenny Larsson, Eva Skoog, Annika Tiveljung-Lindell, Lena Grillner, Elisabeth Aurelius& Martin Glimåker
High diagnostic yield by CSF-PCR for entero- and herpes simplex viruses and TBEV serology in adults with acute aseptic meningitis in Stockholm
Scandinavian Journal of Infectious diseases, 2008, 1-8, First article!

III. Elisabeth Franzen-Röhl, Danika Schepis, Fredrik Atterfeldt, Kristna Franck, Arne Vikström, Jan-Åke Liljeqvist, Tomas Bergström, Elisabeth Aurelius, Klas Kärre, Louise Berg, Hans Gaines
Herpes Simplex Specific T cell Responses in Primary Infection Correlate Inversely with Frequency of Subsequent Recurrences
Manuscript

IV. Elisabeth Franzen-Röhl*, Danika Schepis*, Maria Lagrelius, Kristina Franck, Petra Jones, Jan-Åke Liljeqvist, Tomas Bergström, Elisabeth Aurelius, Klas Kärre, Louise Berg& Hans Gaines&
Increased Cell Mediated Immune Responses in Patients with Recurrent Herpes Simplex virus Type 2 Meningitis
Manuscript

*this authors contributed equally to the study
& this authors contributed equally to the study
The papers will be referred to by their Roman numerals.

To Daniel, Samuel, Gabriel, Maria and Isac


HSV-1
HSV-1
HSV-2

Our destination is to pass beyond all things, to leave everything, to thrive towards the End and in the End discover our Beginning, our constant new Beginning without an End.
By Thomas Merton

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LIST OF ABBREVIATIONS

AAM	Acute aseptic meningitis
APC	Antigen-presenting-cell
BCR	B-cell receptor
CMI	Cell-mediated immune response
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
DC	Dendritic cells
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EV	Enteroviruses
FACS	Fluorescence activated cell sorter
HSE	Herpes simplex encephalitis
HSM	Herpes simplex meningitis
HSV	Herpes simplex virus
HSV-1	Herpes simplex type 1
HSV-2	Herpes simplex type 2
IFNg	Interferon gamma
IL	Interleukin
IP-10	Interferon gamma inducible protein 10
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PRRs	Pattern-recognition receptors
RG	Recurrent genitalis
RM	Recurrent meningitis
RT-PCR	Real-time polymerase-chain reaction
TBEV	Tick-borne encephalitis virus
TCR	T-cell receptor
Th	T helper cell type
TLR	Toll-like receptor
TNF-a	Tumor necrosis factor factor a
VZV	Varicella-Zoster virus

INTRODUCTION

Primarily some background aspects of aseptic meningitis will be discussed followed by a more thorough presentation of infection with the herpes simplex viruses.

Acute aseptic meningitis (AAM) is the most common type of infection of the central nervous system (CNS). AAM and mild meningoencephalitis affects approximately 10-20/100 000 inhabitants per year in Sweden. The vast majority of AAM and mild meningoencephalitis cases are caused by viruses. In many cases the etiology has remained unknown. About twenty years ago a specific pathogen was found in less than 25% due to inadequate testing, limitations of the diagnostic methods and unawareness of the clinical spectrum. The development of new serological and molecular methods has considerably increased the possibilities to establish etiological diagnosis in viral CNS infections. Some viral agents causing AAM are considered to be of greater importance in our country: enterovirus (EV), tick-borne encephalitis virus (TBEV) and herpes simplex viruses type 2(HSV-2) and varicella zoster virus (VZV).

MENINGITIS

Enterovirus meningitis

Epidemiology, virology: Enterovirus comprises a major subgroup of Picornaviridae and includes the polio, coxsackie A, coxsackie B, echoviruses and simply enteroviruses.. The genome consists of a single stranded RNA. Enterovirus (EV) is the major cause of AAM in adults in Scandinavia, similar to the western world (Sköldenberg 1972, Glimåker 1990, Gunter1997, Koskiniemi 2001). The fecal-oral route spreads the virus. In temperate climate EV infections are far more common during the late summer and early fall than during the rest of the year.

Clinical picture: Meningitis due to enteroviruses may be associated with a biphasic onset of symptoms with a first phase with upper respiratory tract symptoms, muscular tenderness, a mild rash, and conjunctivitis and, in small children diarrhea followed by the second meningitis phase. The meningitis is usually a self-limiting disease and only in rare cases complicated by a myelitis or severe encephalitis. Since poliomyelitis is an EV disease and since outbreaks with other more neuropahogenic EV strains appear it is important to type EV in cases with neurological symptoms for polio surveillance and to detect emerging neuropathogenic EV infections.

Diagnostics: EV may be isolated from CSF, throat and fecal samples (Sköldenberg 1972, Chonmaitree 1982). Virus isolation creates possibilities for EV sub-typing. The IgM assays may allow relatively rapid report of probable EV infection but the specificities of these tests are incomplete. Detection of IgG antibodies by IgG-EIA is more specific and is used for confirmation of the diagnosis (Glimåker1992). PCR for the detection of EV-RNA can be applied for both CSF and stool samples. This method is specific and more sensitive than viral isolation (Glimåker 1993). CSF-PCR is the method of choice for EV meningitis. Isolation and RNA detection from feces may be supportive of an EV meningitis diagnosis but not confirmative as EV may be present many months after an acute infection.

Tick-borne encephalitis/meningitis

Epidemiology, virology: Tick-borne encephalitis virus (TBEV) is a member of the genus Flavivirus, family Flaviviridae. Flaviviruses are icosahedral enveloped 50 nm viruses with RNA genome. TBEV is transmitted by Ixodes spp ticks in a vast area from Western Europe to the eastern coast of Japan. More than 10 000 cases of this disease arise every year world-wide (Kunz 2003). In Europe, 3000 cases are treated in hospitals and/or reported each year with increasing numbers during the past decade. TBEV meningitis occurs with a seasonal variation from April through November in Sweden (Haglund 1996, Gunther 1997). A total of 224 cases were reported in 2008 in Sweden, out of which 119 were diagnosed in the Stockholm region (Smittskydd Stockholm April 2009). Climate change and leisure habits expose people more often to ticks and have contributed to increasing numbers of cases and extended geographical spread.

Clinical picture: Typical is the biphasic clinical presentation affecting 72-80% of the patients. In the first viremic phase the dominant symptoms are fever, fatigue, malaise and headache. After a symptom free interval neurological symptoms appear; most often meningitis but the clinical spectrum ranges from mild meningitis to severe encephalitis with or without myelitis and spinal paralysis. A "post-TBEV syndrome" with mainly cognitive CNS dysfunctions may require a rehabilitation period of many years (Haglund 1996, Gunther1997, Mickiene 2002).

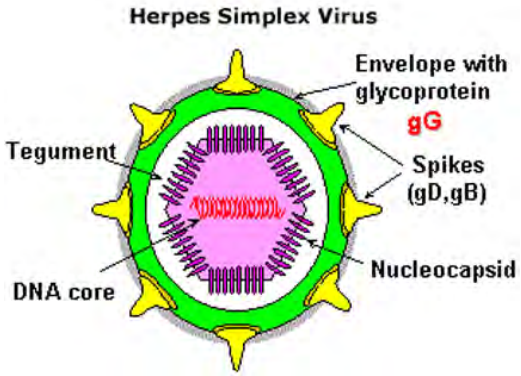
Diagnostics: TBEV may be diagnosed by findings of TBEV specific IgM in serum by capture ELISA technique. This assay is specific and sensitive in the meningoencephalitic phase of the disease (Haglund, 1996, 2003, Mickiene 2002). Demonstration of TBEV-RNA in CSF is only reported in some few cases and not used routinely. Assays for demonstration of intrathecal antibody synthesis are available and may be used for the discrimination of acute disease in previously vaccinated patients (Gunther 1997).

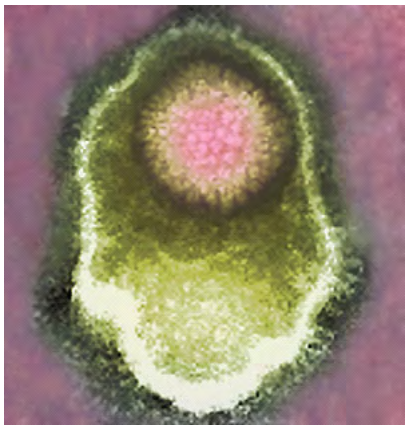
Varicella zoster virus meningitis

Epidemiology, virology: In recent years increased attention has been paid to CNS infections by VZV, which often occur without a zoster rash (Bergström 1996, Echevarria 1997, Studahl 2000, Koskinemi 2002, Kupila 2006). VZV is a double-stranded DNA virus belonging to the same family alphaherpesviridae as HSV-1 and 2.
Clinical picture: VZV may cause a broad spectrum of neurological symptoms, ranging from mild meningitis (1/3), encephalitis and cerebrovascular syndromes. After the primary infection VZV stays latent in the sensory ganglia. The majority of CNS complications among adults are due to reactivation.
Diagnostics: Isolation of VZV in CSF is possible but has lately been replaced by detection of VZV DNA by PCR because it is rapid and more sensitive than isolation. VZV may be demonstrated by immunofluorescens in vesicles, supportive of theVZV meningitis diagnosis. In primary infection IgM antibodies may be detected in serum and also an IgG titre but both may be lacking in reactivated infection. When VZV is not detectable in CSF, VZV specific inrathecal antibody production can be demonstrated (ELISA). The sensitivity of VZV PCR in CSF and demonstration of intrathecal antibody production in VZV meningitis is not systematically evaluated.

HSV-1 AND HSV-2

The herpes simplex virus





VIRION

HSV-1 and HSV-2 together with VZV comprise the alpha herpes viruses, in the herpesviridea family of large DNA viruses, which are known to infect many different animals. They have a comparably rapid replication cycle and persist primarily in sensory ganglia.
The herpes simplex virion consists of largely collinear double-stranded DNA packaged in icosahedral protein nucleocapsids (100-110 nm in diameter). Although the subtypes are genetically highly homologous they are known to differ in location of some viral proteins.
The capsid consists of 162 capsomeres and is surrounded by the tightly adherent membrane identified as the tegument (Roizman 1971). An envelope, derived from cellular membrane surrounds the capsid and tegument. Embedded in the envelope are the viral glycoproteins which appear as protrusions or spikes (Roizman B 1990). Two biologic properties of HSV that directly influence human disease are neurotropism and latency. Neurovirulence refers to the affinity with which HSV is drawn to and propagated in neuronal tissue, which can result in profound disease with severe neurological sequele, as in the case with HSE and neonatal HSV CNS disease. Sites on the herpes simplex genome that mediate this propensity for neurovirulence have a.o. been suggested to be in the thymidine kinas (TK) gene.

HSV Envelope glycoproteins

The glycoproteins mediate attachment of the virus to cells and elicit host response to the virus (Spear 2000). Protruding envelope glycoproteins are of several morphological types, and these spikes tend to be randomly clustered, which could be of possible functional importance during viral entry (Bergström 2006). Viral surface glycoproteins mediate attachment and penetration of HSV into cells and provoke host immune responses. Eleven glycoproteins of HSV have been identified (gB, gC, gD, gE, gH, gG, gI, gJ, gK, gL and gM), with a twelfth being predicted (gN). Glycoprotein D is the most

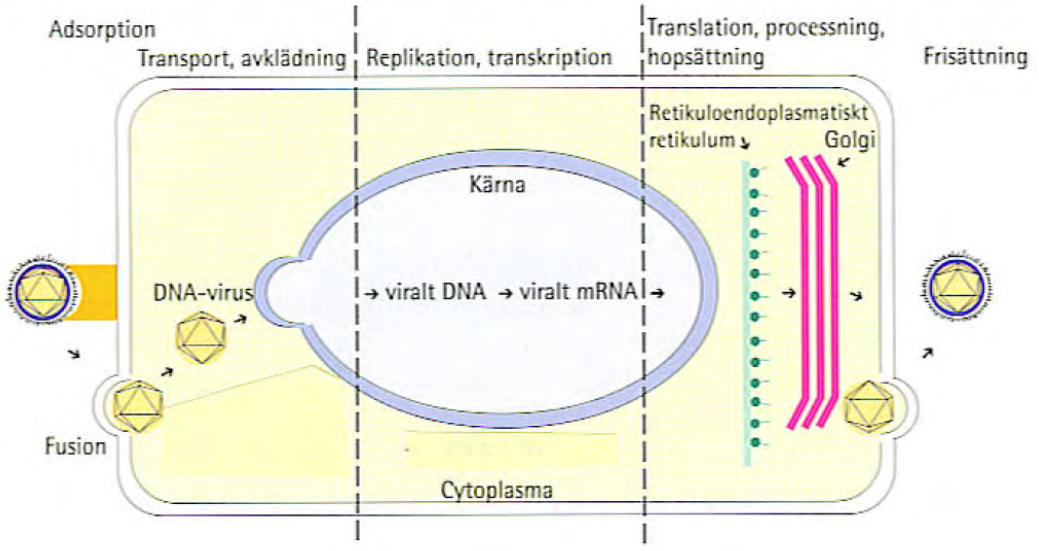
potent inducer of neutralizing antibodies and appears to be related to viral entry into a cell; gB is required for infectivity. Glycoprotein G (gG) provides antigenic specificity to HSV-2 and therefore results in an antibody response that allows for the distinction between HSV-1 (gG-1) and HSV-2 (gG-2). These glycoproteins are dominating targets for human B-and T-cell mediated immune response as well as mediators of immune evasion.

Differences in pathogenicity between HSV-1 and HSV-2

The differentiation of HSV into two types in the 1960s permitted association with site of involvement and with certain forms of herpetic diseases. Beyond the neonatal age, the great majority of oro-labial, facial and ocular infections as well as the more severe infections such as acute fatal encephalitis were associated with HSV-1. During the same 2 decades, HSV-2 was noted to be the major type isolated from the genital and ano-rectal sites and found in the cerebrospinal fluid in association with primary genital or anal infections. Recent changes in epidemiological patterns at e.g. in Australia and parts of Europe may question the concept of type selective viral oral/genital tropism as there are indications that HSV-1 is increasingly detected in primary genital herpes (Christie 1997, Löwhagen 2002, Scoular 2002, Tran 2003). However, the following conditions strongly argue for differences in HSV biology and pathogenesis in human infection:

1) uncommon isolation of HSV-2 from classical oro-labial region and of HSV-1 from cervix;
2) less common reactivation of genital HSV-1 and facial HSV-2;
3) differences in CNS complications in form of HSV-1 causing encephalitis and HSV-2 meningitis and
4) profound differences (HSV-2>HSV-1 in virulence during neonatal CNS infections

(Lafferty 1987, Bergström 2006).



HSV entry and replication

To initiate infection the virus must attach to cell surface receptors, fuse its envelope to the plasma membrane, and allow the de-enveloped capsid to be transported to the nucleus of the cell where transcription, replication of viral DNA and assembly take place. The HSV nucleocapsids spread to the cell somas by retrograde axonal flow probably utilizing the cellular skeleton of microtubules. Cell to cell spread is a crucial

fact of HSV pathogenesis, both during formation of vesicular lesions and the spread within the nervous system. Whether HSV uses different receptors for entry, cell-to-cell spread and egress is a question that remains to be fully elucidated.

In the cell the nucleocapsids release their linear DNA content into the nucleus then transcription occurs after circularization of the DNA. After synthesis and assembly in the nucleus, nucleocapsids are temporarily enveloped by inner nuclear membrane into the perinuclear space through budding. This enveloped particle is de-enveloped while passing through the outer nuclear membrane. In the trans-Golgi network, secondary envelopment by permanent membrane containing surface projections occurs and the mature virions are transported in Golgi derived vesicles to the cell surface where mature extracellular viruses are formed (Granzov 2001).

HSV persistency/latency/reactivation

An important characteristic of all members of the alpha-herpes virus group is the ability to establish latency in neuronal ganglia from where it can reactivate. Following primary infection, usually in the oral or genital mucosa, the virus is transported by retrograde flow along axons that connect the point of entry into the body to the nuclei of sensory neurons. Viral multiplication occurs in a small number of sensory neurons and the viral genome then remains in a latent state in the dorsal root ganglia for the life of the host. This event allows the virus to establish a latent infection, which in fact, is a life-long co-existence of the neuron-hidden virus and human host (Cook Stevens 1973, Kristensson 1971) HSV can then periodically reactivate under a variety of stimuli of environmental stress (Scott 1997). The HSV reactivation relies on an anterograde transmission of viral material in neuronal axons back to muccocutaneous epithelial/ epidermal tissue where the recurrent lytic or unapparent infection takes place. According to modern understanding of HSV infection, the virus reactivation can continuously occur at low levels throughout the life of infected individuals (Koelle & Wald, 2000).

It has been demonstrated that HSV-1 predominately resides in the trigeminal ganglia whereas HSV-2 generally resides latently in the sacral ganglia (Baringer 1973, Croen 1991). After reactivation, HSV is believed to migrate along nerves towards the skin or mucosa causing peripheral lesions or asymptomatic shedding. In some cases the virus is thought to be transported towards the meninges where it may cause an inflammatory response. Genital lesions may precede the meningitis or may appear independently or in extra-genital sites, the latter may indicate spread of virus to other ganglia even if some cases may be explained by autoinoculation. Spread within the CNS may be an early event indicated by focal neurological signs accompanying primary meningitis (Bergström 1990). The mechanisms by which HSV establishes latency are being investigated intensely but remain incompletely understood at this time (Sasadusz 1994). An HSV reactivation leading to clinical symptoms is termed recurrence. In many instances viral reactivation does not lead to overt clinical symptoms, as shown by findings of asymptomatic shedding in genital herpes (Wald 2000). How often viral reactivation leads to migration to the meninges is not known nor is it known whether reactivation necessarily leads to clinical meningitis. Viral reactivation may pass unnoticed but may also be the cause of recurrent limited attacks of headache without other findings of meningitis either clinically or in the CSF (Bergström 1990).

Epidemiology HSV meningitis

Epidemiology: Herpes simplex virus (HSV) is ubiquitous. There are two subtypes, herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2). The seroprevalence of HSV-1 is about 80% and HSV-2 20-30% in adult population in Sweden (Forsgren 1994, Löwhagen 1990). Female gender, more advanced age, and lower socioeconomic status are associated with higher HSV-2 seroprevalence (Johnson RE 1989, Löwhagen 1990, Corey 1993, Forsgren 1994, Buxbaum 2003). Although the subtypes are genetically highly homologous, they give rise to different clinical pictures. HSV-1 and HSV-2 usually cause mucocutaneous lesions. HSV-1 is traditionally linked to oro-labial herpes and HSV-2 to genital mucocutaneous lesions. Lately the percentage of genital herpes caused by HSV-1 is increasing. Both types may also induce a wide range of neurological symptoms. HSV-1 in rare cases causes severe focal encephalitis (HSE). An estimated incidence of HSE is between two and four persons per million per year (Sköldenberg 1991, Whitley 1995), where as HSV-2 aetiology accounts for approximately 5 % of these cases (Aurelius 1993).
The most common manifestation of HSV-2 neurologically among immunocompetent adults is aseptic meningitis, sometimes with transient mild to moderate encephalitis and /or myelitis and radiculitis. The frequency of aseptic meningitis due to HSV-2 has probably been underestimated and undetermined due to previous limitations in diagnostic methods. Our knowledge of the extent of meningitis in HSV-2 infection is incomplete but meningitis is known to be a frequent complication to primary genital infection (Corey 1983). Symptoms suggestive of aseptic meningitis were found in a population with primary episode of genital HSV-2 in 36% of the women and 13% of the men. There is a female predominance in HSV-2 meningitis, with a female-to male ratio 2:1-6:1 (Corey 1983, Bergström 1990, Read 1999, Aurelius 2002) and in a study from U.K. on CSF samples, the relative incidence of HSV-2 was high and HSV-2 was the virus most frequently detected among adult young women with aseptic meningitis (Read 1999). Furthermore, neurological HSV-2 disease carries a risk of recurrences. There is no data available on the incidence of HSV-1 meningitis in immunocompetent adults.

CLINICAL PICTURE OF HSV INFECTIONS

Mucocutaneous infection

The typical manifestation of viral disease is mucocutaneous lesions known as cold sores or fever blisters (HSV-1) or genital ulcers (HSV-2). At the cellular level, both HSV-1 and HSV-2 infect the cells of stratified squamous epithelium that fashion either mucosa or epidermis at the mucocutaneous borders found in oral and genital regions. In general, HSV infections are clinically more severe when they occur as primary infections

The most common clinical manifestation of primary infection with HSV 1 is acute *herpetic gingivostomatitis* which occurs most frequently in small children (one to three years of age). The diagnosis is indicated by the age of the patient together with the characteristic extensive vesiculo-ulcerative lesions of the buccal mucosa, gingival, pharynx and tongue accompanied by fever, pain and malaise. The lesions tend to heal within two to three weeks. However, many infections are subclinical.

The majority of primary Herpes simplex eye infection occurs in adolescents and adults (Dawson 1976, Darougar S 1985). The primary HSV-1 infection in the eye consists of *keratoconjuctivitis* which is either unilateral or bilateral and may be accompanied by vesicles on the eyelids and surrounding skin.

Genital herpes: Constitutional symptoms consisting of headache, malaise, and myalgia, often precede the onset of genital symptoms. Local itching and soreness are quickly followed by pain at the site of lesions and inflamed inguinal lymph nodes, usually bilaterally. Many individuals also experience neuralgic pain or tingling paraesthesiae in the sacral dermatomes. The symptoms of first episodes tend to be worse in women who often have more severe constitutional symptoms and dysuria. Lesions in women are more widespread and affect the labia majora, the labia minora, the introitus, the cervix, the perineum, the perianal areas and buttocks. Excessive mucopurulent vaginal discharge occurs when there is associated herpetic cervicitis. New crops of lesions can appear during the first 10 days of the illness. Thus papules, vesicles and ulcers may coexist. Lesions on males are most common on the penis especially on the glans penis. Proctitis on homosexual men presents with perianal pain and rectal discharge. In untreated episodes, viral shedding continues for a median duration of 11 days, and the total duration of the illness may last for three to four weeks.

Asymptomatic herpes: However, the majority of infected persons are unaware that they have acquired genital HSV infection and over 50% of transmission occurs without symptoms. Of HSV-2 seropositive persons, only 50% have undiagnosed but clinically detectable disease, and 25% are truly asymptomatic (Corey 1983, Kinghorn 1993, Langenberg 1999).

The incubation period between transmission and symptom onset is generally short; between 1 and 7 days. Based on the patient history, clinical episodes can be subdivided in to first and recurrent episodes.

The first episode may be a true primary infection with either HSV-1 or HSV-2 in a patient with no prior serological evidence of HSV infection.

It may also be a non-primary first episode where prior evidence of infection by HSV-1 or HSV-2 is found in the serum of a patient with a first clinical episode genital herpes.

As earlier mentioned an increasing frequency of genital herpes caused by HSV-1 has followed possibly with more wide spread orogenital sexual habits. Although the clinical picture cannot be discerned, HSV-1 genitally tends to cause fewer recurrences than HSV-2 (Benedetti 1999). This is analogous to HSV-2 in the orofacial area where it is believed to recur far less frequently than in the genital area.

HSV-2 meningitis

Clinical picture: Meningitis due to HSV-2 has some characteristics that distinguish it from meningitis of other origins: 1) the association with muccocutaneous herpetic lesions and 2) associated additional neurological symptoms, along with 3) the appearance of recurrent disease.

Headache develops during 2-3 days, together with varying degrees of other signs and symptoms of meningeal irritation such as neck stiffness, photophobia, nausea and vomiting. Fever is common but not an obligatory finding. In most cases, the acute symptoms of primary meningitis resolve spontaneously within about a week, although sometimes after a protracted illness. Genital lesions may precede meningitis with an overall interval of about a week. Primary, as well as recurrent meningitis may however appear without muccocutaneous symptoms, which is now known to be the most frequent presentation (Sköldenberg 1975, Hevron 1977, Bergström 1990, Schlesinger 1995, Tedder 1994, Sasadeusz 1994). The fact that the lesions are missing in many cases might well be explained by the fact that asymptomatic genital HSV-2 infections are common. Cerebrospinal fluid typically shows a mild to moderate predominately monocytic pleocytosis with an average of around 400 leukocytes x 10^6 in primary meningitis, slightly increased protein, a CSF serum glucose ratio more than 0.5 and normal lactate. Hypoglycorrhea has been reported in a number of cases and occasionally a slightly increased lactate concentration can be found (Chin 1996, Brenton 1980, Bachmeyer 1996, Jensenius 1998). Neurasthenic symptoms such as mild headache, labiality, concentration disabilities and fatigue may last for several weeks (Sköldenberg 1975, Aurelius 2002).

Pathogenesis of meningitis:

The factors that are of decisive importance for symptomatic neurological HSV-2 disease in otherwise immunocompetent individual are not clear. The reason for the much more frequent neurological complications in women than in men also is not clear. It has been suggested that this may be due to the fact that females possess larger total surface of infection from where the viral load is forwarded to a larger number of neuronal ganglia (Corey 1983, Sasadeusz 1994). Further, a prior HSV-1 infection indicated by a pre-existing humoral immune response to HSV-1 might be a marker of protection and, vice versa, a lack of HSV antibodies constitutes a risk factor for developing neurological disease induced by HSV-2 infection. HSV-1 specific seroprevalence in a group of young adult women with HSV-2 induced meningitis was significantly lower than in a matched control group and than was expected from seroepidemiological studies in the same geographic area (Aurelius 2006). Neuropathogenic and neuro invasive characteristics of viral subtypes may be involved (Bergström 1990).

Recurrent herpetic meningitis

Neurological HSV-2 disease carries a risk of multiple recurrences. Considerable neurological morbidity may follow with recurrent episodes of meningitis and myeloradiculitis and also a wide variety of less distinct neurological symptoms. Attacks of headache without overt signs of meningitis and without pleocytosis have been reported after herpes meningitis.

In recurrent meningitis, as in recurrent genital herpes, the subsequent episodes vary in intensity but tend to come with milder clinical and laboratory symptoms and are of shorter duration than primary attack (Bergström 1990, Bachmeyer 1996). Recurrent attacks of meningitis may appear more or less frequently and after a shorter or longer period of time without symptoms. They may occur once or twice and up to 20 times or more. The symptom-free interval may vary from months to several years and even decades. Whether a shorter intervals and higher frequency of recurrences are correlated with the severity of clinical symptoms in primary infection remains unclear. In many instances viral reactivation may not lead to overt clinical symptoms, as shown by findings of asymptomatic shedding in genital herpes (Wald 2000). This may be explained by partial protection by the specific immune defense. Virus reactivation may pass unnoticed but may for instance also be the cause of recurrent limited attacks of headache without other findings of meningitis, either clinically or in the CSF (Bergström 1990, Aurelius 2002). Stimuli to reactivation of latent HSV-infection are most probably relevant also for recurrences of neurological disease, for instance, recurrent attacks of meningitis are reported to follow periods of psychological stress as well as lower back trauma.

Mollaret´s syndrome

A syndrome that is characterized by repeated episodes of aseptic meningitis, separated by symptom free intervals, and by distinctive CSF cytology, was described by a French neurologist named Mollaret in 1944. Mollaret´s cells were demonstrated in the CSF by Picard et al. (1993), in 2 out of 3 patients with recurrent meningitis of HSV-2 origin. Most reported cases of recurrent meningitis of verified HSV-2 origin which are often termed Mollaret meningitis, have a clinical course in-line with the one stated in Mollaret´s syndrome, however the characteristic CSF cytology is either not found or, more commonly, not sought. It seems reasonable that the diagnosis of Mollaret´s meningitis should be made only after other recognized causes of recurrent lymphocytic meningitis have been excluded.

HSV in immune compromised

Immunosuppressed patients are at risk of developing infections because of immune system impairment. Infections by viruses are of special significance for the immunity involving the cytotoxic T cells. A deficit of T-cell mediated immunity is encountered in a number of conditions dominated by immune suppression, including transplanted and oncology patients, as well as patients with other congenital or acquired immune deficiencies like the human immunodeficiency virus (HIV). Primary and recurrent oro-labial disease represents the most common manifestation of HSV infection following transplantation and accounts for 85% of HSV related disease in the early post-transplant setting (Meyers JD, 1985). Clinical manifestations of muccocutaneous HSV disease in transplant patients do not significantly differ from those of immunocompetent hosts. Herpetic lesions may involve lips, tongue, oral mucosa, and pharynx and perioral skin. Anogenital herpes accounts for 10-15 % of all HSV-related diseases in the transplanted patient. However, reactivation of HSV can occur quickly after prophylaxis is stopped (Urban-Ispizua A. 2002). Compared to HIV negative individuals, HIV infected persons show a more prolonged disease and viral shedding and higher rate of recurrences. In patients with advanced infection and very low number of CD4-positive cells, sever muccocutaneous lesions are observed together with involvement of visceral organs, such as the gastrointestinal tract, lung, eye and central nervous system CNS.

Classical clinical pictures of encephalitis or meningitis of HSV origin do not seem to be more frequently appearing in immune compromised patients like HIV infected. In patients with severely impaired immunity the better recognized clinical picture is subacute focal encephalitis or ventricular-encephalitis (Vago 1996, Cinque 1998). More recently, cases presenting with clinical features of meningitis or meningo-encephalitis and mainly by HSV-2 have been reported (Mommeja-Martin H 2003).

DIAGNOSIS OF HSV INFECTION

The four corner-stones in viral diagnosis, namely viral isolation, genome detection by PCR, antigen detection and serology, are all applicable for establishing etiological diagnosis in HSV infection.

Diagnosis of muccocutaneous HSV infection

Viral isolation from muccocutaneous herpetic lesions has traditionally been applied. Culture on GM (green monkey kidney) cells has been followed by typing with HSV-type specific monoclonal antibodies. Isolation is the only method giving viable virus and may still be of value e.g. when resistance to antivirals is suspected or in diagnosis of atypical lesions**.**

Direct demonstration of antigen from herpetic lesions is a rapid and rather reliable method that functions well also in non-sophisticated circumstances.

Detection of HSV DNA after amplification by PCR from muccocutaneous lesions has now replaced isolation and antigen detection in routine diagnosis, mainly because it is more sensitive and also renders a more rapid result.

Serology: Until recently most available antibody tests were type-common, i.e. could not separate the two HSV-types, but assays to type-specific antigens are now available. In Western blotting, where the patient serum is reacted with HSV proteins that have been separated by electrophoresis, the pattern of antibody binding bands is type-specific. IgG seroconversion to type-specific HSV antigens in the HSV-1 and HSV-2 glycoproteins G (gG1 and gG2) demonstrated by ELISA may verify the diagnosis in primary infection. These methods are less labor-intensive and now used in routine diagnosis (Ashley R.1999). Seroconversion to HSV-2 may take about 3 weeks but seldom 4-5 weeks or even later. Significantly rises in serum titers are usually not observed in recurrent HSV-infection. Demonstration of IgM may be used as a support for a recent infection and may also be detected in reactivation but is not a consistent finding.

Diagnosis of HSV meningitis
Isolation of HSV in the CSF may be done and has been reported in several cases in primary meningitis of both types. With few exceptions attempts to isolate HSV in the CSF in recurrent meningitis is unsuccessful and have been documented in only a single case with HSV-1. In a large series, HSV-2 was recovered from the CSF in 21 of 27 patients with a primary infection, but from none of 10 with recurrent disease (Bergström 1990)

Detection of HSV DNA after amplification by PCR in the CSF seems to be very sensitive but is thought to be less sensitive in recurrent meningitis. Since the introduction, the PCR methods have been developed further and different methods for quantitation of the viral copies are established. These methods are easily adapted for simultaneous analyses to detect other presumptive agents and they are rapid, yielding a result in hours. The viral load may be put in relation to the clinical course and may be used for monitoring effect of antiviral therapy. In the first paper of this thesis the sensitivity and specificity of a quantitative real-time (RT) PCR is estimated in CSF samples from a large and clinically well characterized material from HSV-meningitis patients and patients with AAM of unknown origin, and compared to the to the previously used qualitative HSV-PCR.

Viral culture or demonstration of viral antigen or genome from genital or lumbosacral lesions preceding or during the meningitis attack may employed to support the meningitis diagnosis, although it does not rule out that peripheral activation could be a concurrent phenomenon and thus be of no relevance to the etiology of the meningitis.

Serology In the absence of peripheral lesions, serum and CSF antibodies have been used to support the diagnosis. In recurrent meningitis the HSV-2 diagnosis may be supported by the finding of HSV-2 antibodies in the acute serum, indicating a pre-existing HSV-2 infection and thus a prerequisite for reactivation.

The intrathecal antibody response in HSV meningitis has not been thoroughly studied since successive lumbar punctures are not frequently performed for practical and ethical reasons. Unlike HSV- encephalitis kinetics of the intrathecal antibody response in HSV meningitis are not fully known. An intrathecal response is probably not detectable in the very initial phase of the attack. HSV-2 immuno-blotting analysis of

serum and CSF may be useful to demonstrate an intrathecal antibody response (Bergström 1990, Monteyne 1997). The method may help in the diagnosis of recurrent meningitis, where the intrathecal response seems to be enhanced with increasing numbers of episodes. In complicated cases where an etiological diagnosis is needed, a later lumbar puncture and CSF analyzed together with serum for the detection of specific intrathecal antibody synthesis may be of value.

TREATMENT IN HSV INFECTION

The replication of the viral DNA-genome is performed by virus-coding enzymes. Most antiviral substances that are used are targeted at these replication enzymes. Aciclovir (ACV) and penciclovir (PCV)are guanosin analogues. Both are mono-phosphorylated by the viral thymidine kinase (TK) and further phosphorylated to ACV- or PCV-triphosphate via cellular kinas. ACV-triphosphate competitively inhibits viral DNA-polymerase and after incorporation in the viral DNA chain the synthesis will be terminated. PCV will be incorporated as well and cause decreased replicatition and often stops synthesis. The two virus specific steps result in metabolisation restricted to virus-infected cells and thus a low grade of toxicity. Enhanced bioavailability is achieved by the prodrugs valaciclovir and famciclovir which are converted to aciclovir and famciclovir respectively during the passage through the liver.

The alternate drug foscarnet is a pyrophosphate analogue and does not need any intracellular metabolism but blocks the pyrophosphate-binding site directly. The nucleotide analogue cidofovir does only need to be phosphorylated by cellular kinases to get an active substrate for the viral DNA polymerase. Both drugs are hampered by toxic side effects, especially nephrotoxic, and are in HSV infection used only in cases with severe disease and resistant virus.

Currently, the antiviral drugs acyclovir, valaciclovir, famciclovir and topical formulations of penciclovir represent the standard therapy of herpes simplex virus infections.

Treatment HSV meningitis

In genital herpes antiviral therapy with acyclovir was shown to be effective both in the short term treatment of primary episodes (Wald 1994) and in the suppression of recurrences by continuous prophylactic medication (Goldberg 1993).

Acute meningitis is a self-limiting disease that heals without specific antiviral treatment in immunocompetent individuals. Treatment of HSV induced meningitis with acyclovir has been documented since the beginning of the 1980´s but there has not yet been a controlled study. A number of cases are reported where aciclovir was thought capable of shortening the duration of symptoms. (Levy 1984, Bergström 1990, Schlesinger 1995, Cohen 1994). It is not known whether the long-term course is affected compared with untreated individuals. Antiviral treatment in acute episodes of recurrent meningitis is sparsely documented. Probable effect of intermittent or suppressive treatment with aciclovir in recurrent meningitis is reported. Patients with previous frequent meningitis attacks were free of recurrences during continuous aciclovir suppression (Bergström 1990, Berger 1991).

In primary meningitis antiviral intervention seems justified in light of the long duration of symptoms, in analogy with primary muccocutaneous disease (Wald 1994). In cases with severe symptoms intravenous aciclovir (5mg/kg x 3) may be used, otherwise

peroral therapy with Valaciclovir 1g x3 should ensure CSF concentrations in the range required for anti-HSV-effect (Lycke 2003). In recurrent meningitis a decision to avoid medication may be arrived at in some patients or antiviral may be instituted promptly, thereby increasing the potential clinical effect. Patients with frequent attacks would rather benefit from prophylactic treatment with aciclovir, valaciclovir or famciclovir. It may be helpful to go thorough the patient history in order to identify patterns in the course of disease including precipitating factors and periods at risk. A successful alternative may then be intermittent suppressive prophylaxis when an attack is expected.

HSV Resistance

Already in the 1980´s the first report on resistance to acyclovir was presented (Coen DM 1980). Naturally are 0.01 % of HSV-1 and 0.1-0.9 % of HSV-2 populations are resistant because of changed TK. The majority o of resistance conferring mutations (95%) clusters in the thymidine kinas (TK, UL23) and a minority (< 5%) of the mutations is found in the HSV DNA polymerase gene (DNA pol, UL30). The mutation frequency of HSV-2 is somewhat higher compared to HSV serotype 1. The majority of the TK negative strains are less virulent. Resistance correlates inversely with the immune status of the host, severe clinical manifestation, and start and duration of treatment; basically it correlates with the viral load that is selected for resistant virus in the presence of drugs. In summary resistant strains may comprise a therapeutically clinical problem among immune compromised patients whereas treatment of immune competent individuals is considered effective, safe and carrying a very small risk of developing resistance.

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IMMUNOLOGY

Like all living creatures on this planet we are surrounded by a large number of bacteria, viruses and other microorganisms. This coexistence of pathogens with their host is a sophisticated act of balance. Our first line of defense against invaders consists of physical barriers produced by the epithelial tissues. The skin is generally thought of as the main barrier but more important is the large area covered with thick mucous membranes that line up our digestive, respiratory and reproductive tracts. This protective membrane consists of a chemical shield of antibacterial peptides, lyzozymes, gastric acid or sebum. Additionally, the mucosa produces a constant secretion of fluids that subsequently clears pathogens through mucilliary transport. This action helps to prevent the pathogens from reaching the epithelial layer. However, viruses can cross these barriers and enter human cells, where they manage to take control over the host cell's machinery to produce many more copies of the virus. These newly made viruses burst out of the infected cells and go on to infect other cells in the neighborhood thus multiplying exponentially.

THE IMMUNE SYSTEM

The immune system protects us from invading pathogens. Detailed insight into the interaction of pathogens with this system will lead to better understanding and help to develop efficient protective strategies. The immune system can roughly be divided into the innate and the adaptive immune systems, with the innate system readily activated to response within minutes, while efficient adaptive immune responses take days. The adaptive is further divided into a humoral and cell-mediated response. The traditional view of innate and adaptive immunity as two separated entities is increasingly being replaced by a more integrated picture. General examples of the new integrated interpretation include that secretion of cytokines that have biological effects on both innate and adaptive cells and that the binding of innate Fc receptors to targets opsonised with antibodies derived from adaptive responses.

The innate immune system. Any invader that breaches the physical barrier of skin or mucosa is greeted by cells of the innate immune system such as macrophages and neutrophils. Twenty years ago the innate system was considered unspecific but this has been revaluated and specificity is found in the detection of microbial infection by pattern recognition receptors, such as the Toll like receptors (TLRs) (Janeway 1989). TLRs are now thought to be one key component of the innate system, which detects microbial infection and subsequently triggers antimicrobial host defence responses. They activate multiple steps in the inflammatory reactions that help eliminate the invading pathogens and coordinate systemic defences (Iwasaki 2004). This will be further discussed below.

The adaptive immune system is a defence system with a memory function profile that can adapt and protect us against almost any invader. The main actors of this system are different T and B-cells ready to eliminate intruders with the use of their antigen-specific cell surface receptors, i.e. T-cell receptors and B-cell receptors. Immunity can be induced when there is a history of recognition by the host so called a memory immune response.

When meeting an antigen for the first time through an infection or vaccination, the adaptive immune response will respond within approximately a week with B-cell production of antibodies that can specifically bind the antigen. This is called a primary immune response and results from the activation of naive B cells. The type and amount of antibodies produced vary according to the type of antigen, involvement of T-cells, prior history of antigen exposure and anatomic site of infection. Primary responses are characterized by relatively slow kinetics and small magnitude compared with responses after a second or subsequent exposure. A typical picture is the initial rise of serum levels of IgM antibodies and later of all antibody types but primarily IgG. This stage will last for a couple of weeks and then decline to reach slightly higher antibody levels as prior to the exposure to the antigen. The next encounter with same antigen will give rise to a much quicker and stronger second immune response than the first one. This time the IgM level will reach same peak level but IgG will react faster and more powerful and with antibodies of a higher affinity due to the process of B-cell maturation called affinity maturation.
Recently scientists have discovered a subpopulation of natural killer cells that can mediate long-lived antigen-specific adaptive recall responses independently of B and T-cells (O'Leary 2006). These findings reveal properties of NK cells that were previously attributed only to cells of the adaptive immune system (Sun 2009). Therefore, the innate and adaptive immune systems do not operate independently but instead cooperate to produce a more efficient total response than either alone and are decisive in the way the memory function is developed and established. Cells in the innate system are of major importance to attract and activate T-cells. This is true for granulocytes, dendritic cells; secreting chemotaxis, inducing chemokines or NK cells (Kiessling 1975) secreting important cytokines to activate the adaptive immune system.

THE INNATE IMMUNE SYSTEM

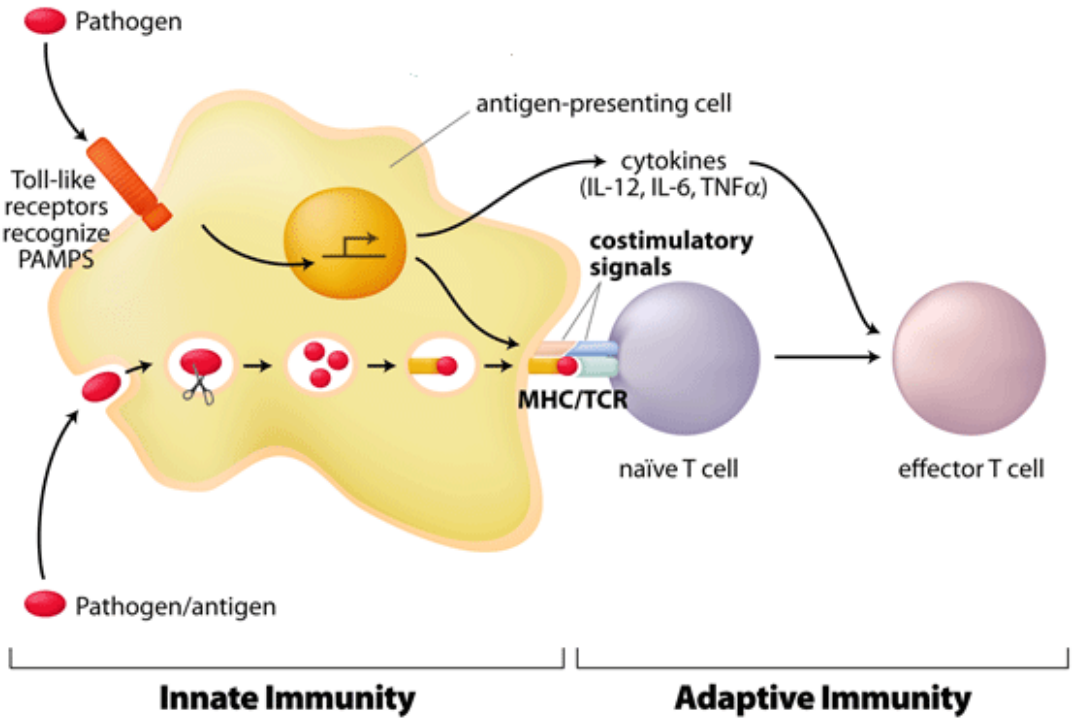
This system is specialized for the control of invading pathogens during the primary phase of an infection. The response is generated within hours after encounter of pathogen and do not require prior antigen exposure. The cellular constituents of the innate immune system include granulocytes, dendritic cells (DCs), monocytes and macrophages and the natural killer cells (NK).

The complement system is composed of about twenty different proteins that work together to destroy invaders and to signal other immune participants. The primary role of the complement system is to facilitate opsonisation and subsequent elimination of microbes by binding to them in different ways; the classical pathway of antibody-antigen complexes; the alternative pathway, which is antibody-independent and works by binding of the complement C3b to microbial surfaces or the lectin pathway which is initiated by mannan-binding lectins (acute phase protein) binding to carbohydrates on bacterial surfaces. Besides opsoning microbes, activated complement form a membrane attack complex that effectively lyses microbes. In addition cleavages from activated complement are efficient inflammatory mediators.

The most important phagocyte is the **neutrophil granulocytes** which accounts for 70% of all white blood cells in circulation. In contrast to macrophages, neutrophils are not

antigen presenting cells but professional killers ready to phagocytose microorganisms on demand. They are extremely important in our line of defence against many bacteria and are quickly recruited by signal substances from the site of infection. Three types of molecules must be expressed before neutrophils leave the bloodstream and become active. The activation is induced by macrophages at the site of infection that secrete IL-1 and TNF upon recognition of pathogens, i.e. via TLR ligation. IL-1 and TNF than activate endothelial cells, which will start expressing adhesion molecules important for extravasations of leukocytes.

M**onocytes and macrophages** are large blood cells that also are able to eliminate microorganisms by phagocytosis. Monocytes are circulating precursors of macrophages by entering the inflamed tissues they mature to macrophages. Macrophages and dendrite cells can stimulate naïve T-cells by presenting peptides bound to MHZ molecules T-cells. Conversely, T-cells, and also NK cells, can stimulate the activity of macrophages and dendritic cells by secreting the cytokine IFNγ. As result, with more IFNγ around, more macrophages can be primed to engulf microbes more efficiently, and to present antigens. Many specialized macrophages are resident in tissues such as Kupfer cells in the liver or macrophage like cells of the brain. Macrophages are long lived and can also be carriers of intracellular microorganisms. Many microorganisms have developed systems to avoid the macrophage killing functions and instead establish persistent intracellular infections.



Toll-like receptors (TLRs) on macrophages and dendritic cells have recently emerged as a key component of the innate system. They detect microbial structures or patterns and trigger antimicrobial host defence responses. This discovery implies that the innate

system also has some degree of specificity in its response; different TLR bind different microbial structures. The targets of pattern recognition, sometimes called pathogen associated molecular patterns (PAMPs) are detected by pattern recognition receptors (PRRs) that signal the presence of infection to the host. The TLR family is the best-characterized class of PRRs in mammalian species. TLRs 1, 4, 5 and 6 seem to specialize mainly in the recognition of unique bacterial products. In contrast TLRs 3, 7, 8 and 9, specialized on viral detection and recognize mainly microbial nucleic acids, such as double-stranded RNA and unmethylated DNA. The biological importance of any given combination of TLR expressed on a cell, different ligand specificity and signaling specific for each individual TLR is largely unknown (Iwasaki 2004).
Scientists have found that TLR 3 and 9 are essential in the immune defence against herpes viruses (Casrouge 2006). A recent report by Kurt Jones et al. 2004 reveals that TLR2s sense HSV-1 and trigger the inflammatory cytokine response. Additionally, the role it plays in alerting the innate response has pathological consequences (by contributing to an inflammatory process) rather than protective consequences (Morrison 2004).
Although originally described as receptors for molecular patterns found in bacterial and fungal pathogens, TLR recognition of viral products has more recently been appreciated.
Ten human TLRs have now been identified, along with natural or synthetic ligands for nine of these (Table 1).

Table 1. The Toll-like receptor (TLR) family and its ligands

TLR molecule	Ligands (organisms of origin)
TLR1	Triacylated lipopeptides (bacteria) Soluble factors (bacteria)
TLR2	Envelope protein (virus) Lipopeptide(virus) Lipoprotein (bacteria) Peptidoglycan (bacteria) Atypical LPS (bacteria) Glycolipids (spirochetes) Zymosan (yeast)
TLR3	Double-stranded RNA (virus)
TLR 4	LPS (Gram-negative bacteria) Envelope protein (virus)
TLR 5	Flagellin (bacteria)
TLR 6	Diacylated lipopeptides (bacteria)
TLR 7	Single stranded RNA(virus)
TLR 8	Single –stranded RNA (virus) (in humans)
TLR 9	Unmetylated CpG DNA(virus, bacteria) Chromatin-IgG complexes (host)
TLR 10	unknown

Dendritic cells, macrophages and B-cells are antigen presenting cells (APC) i.e. cells that display peptide fragments of protein antigens in association with MHC molecules on the cell surface. They activate antigen-specific T-cells. Professional APCs express class II constitutively, while non-professional APCs such as fibroblasts and epithelial cells have an inducible expression of MHC class II. In addition to displaying peptide-MHC complexes, APCs must also express co-stimulatory molecules, such as B7.1 and B7.2 in order to activate naïve T-cells optimally. T-cells use their receptors (TCR) to view this presented antigen, and while this is considered the first signal, the signal induced via B7-1/B7.2 ligation to CD28 on the T-cell is termed the second signal. Naïve T-cells require both signal one and two for activation, while previously activated T-cells can be activated by the first signal alone. Once a naïve helper T-cell has been activated by this two-key system it proliferates to build up a clone composed of many helper T-cells that recognize the same antigen.

Dendritic cells (DCs) are present in lymphoid organs, the epithelia of the skin, the gastrointestinal and respiratory tracts and in most parenchymal organs. DCs can arise from both lymphoid and myeloid precursors and can be grouped into conventional DCs and plasmacytoid DCs (pDCs). Theses are extreme producers of IFN-α which is a cytokine with pronounced antiviral effects. DCs are often referred to as sentinels of the immune system, representing a crucial link between innate and adaptive immunity. One function of DCs is to capture microbial protein antigens and to transport the antigen to draining lymph nodes. It is the ability to "travel when stimulated" that makes the DC so central in initiating adaptive immunoreactions. By the time the DC reaches a lymph node it expresses the cell surface proteins necessary to activate naive T-cells - high levels of class I and class II MHC molecules and plenty of B7 proteins. The first DCs described were Langerhans cells that are found just below the skin and occupy as much as 25% of the surface area of the epidermis. Activated DCs are short lived. Rapid turn over of DCs ensures that the antigens they bring to the lymph node are regularly updated. The knowledge of DCs is developing fast and many new kinds of DCs with various functions have been described lately. Recent studies suggest that DCs may be specialized to accomplish distinct functions in the course of the immune response and multiple DC subtypes are engaged in the response to a single infection (Iwasaki 2004).

Natural killer (NK) cells develop from lymphoid progenitors in the bone marrow, and constitute 5-15% of circulating lymphocytes. NK cell activation is regulated by a combination of cell surface stimulatory and inhibitory receptors, many recognizing MHC class I molecules. The "missing self" hypothesis depending on the presence of inhibitory receptors on NK cells interacting with MHC class I was first presented 1981 (Klas Kärre, PhD thesis 1981). According to the "missing self" model one of the NK cells special functions is to react specifically against cells that lack completely or express low levels of MHC class I molecules, such as tumor cells or cells infected by certain viruses. Like macrophages NK cells contain granules that contain destructive enzymes and chemicals. Release of these granules can result in killing of tumor cells and virus-infected cells, by activating their inherent apoptosis program. An additional mechanism of killing is the interaction of Fas ligand expressed on the NK cell surface, with Fas on the surface of the target, signaling the target to go into apoptosis. NK cells themselves are activated by cytokines, mainly produced by myeloid cells, such as interferons and IL-12 which are secreted e.g. by virally infected cells. NK cells also

produce large amounts of cytokines such as IFN-γ and TNF α. Two major functional groups of NK cells have been described according to their CD16 and CD56 high or low expression on the cell surface: One group is called CD56 bright NK cells, specialized in cytokine release and the second group is called CD56 dim NK cells, specialized in cytotoxicity. This subset of NK cells is also capable of antibody dependent cellular cytotoxicity (ADCC) by binding of their surface CD16 receptor to antibody-coated target cells.

ADAPTIVE IMMUNITY

The defining characteristics of adaptive immunity are exquisite specificity for distinct molecules and an ability to remember and respond more vigorously to repeated exposures to the same microbe. It is also sometimes called acquired immunity to emphasize that potent protective responses are acquired by experiences. The components of adaptive immunity are lymphocytes and their products. Key cells are T and B-lymphocytes expressing antigen specific receptors on their cell surface. There are two types of adaptive immune responses called humoral immunity and cell-mediated immunity, mediated by different components of the immune system and function to eliminate different types of microbes.

B-cells

Humoral immunity is mediated by B-lymphocyte-produced antibodies in the blood and mucosal secretions. B-cells are the only cells capable of producing antibody molecules and therefore the central cellular component of humoral immune response. B cells develop in the bone marrow and mature B-cells are found mainly in lymphoid follicles, secondary lymphoid tissues, bone marrow, and in low numbers in the circulatory system. The basic structural unit of an antibody is composed of two identical heavy chains and two identical light chains. N-terminal variable regions of the heavy and light chains form the antigen-binding sites, whereas the C-terminal constant regions of the heavy chains functionally interact with other molecules in the immune system. Secreted antibodies perform various effectors' functions, including neutralizing antigens, activating complement and promoting leukocyte-dependent destruction of microbes.

Neutralizing antibodies (NT) Antibodies can actually bind to a virus while it is still outside a cell and can keep the virus either from entering or from replicating once it has entered. Some neutralizing antibodies can bind to the part of the virus that would plug into the cellular receptor, preventing the virus from docking on the surface of the cell. The virus is instead hung out to dry opsonised and ready to be eaten by phagocytes.

Antibodies against bacterial toxins. Antibodies can bind to invading bacteria and opsonise them for destruction. When antibodies opsonise bacteria or viruses they bind to the invader with their Fab regions, leaving their Fc tails available to bind to Fc receptors on the surface of cells like macrophages. In this way, antibodies form a bridge between invader and the phagocyte (macrophage), bringing the invader close, preparing for phagocytosis. In fact when a phagocyte's Fc-receptors bind to antibodies that are opsonising an invader, the phagocytic ability increases.

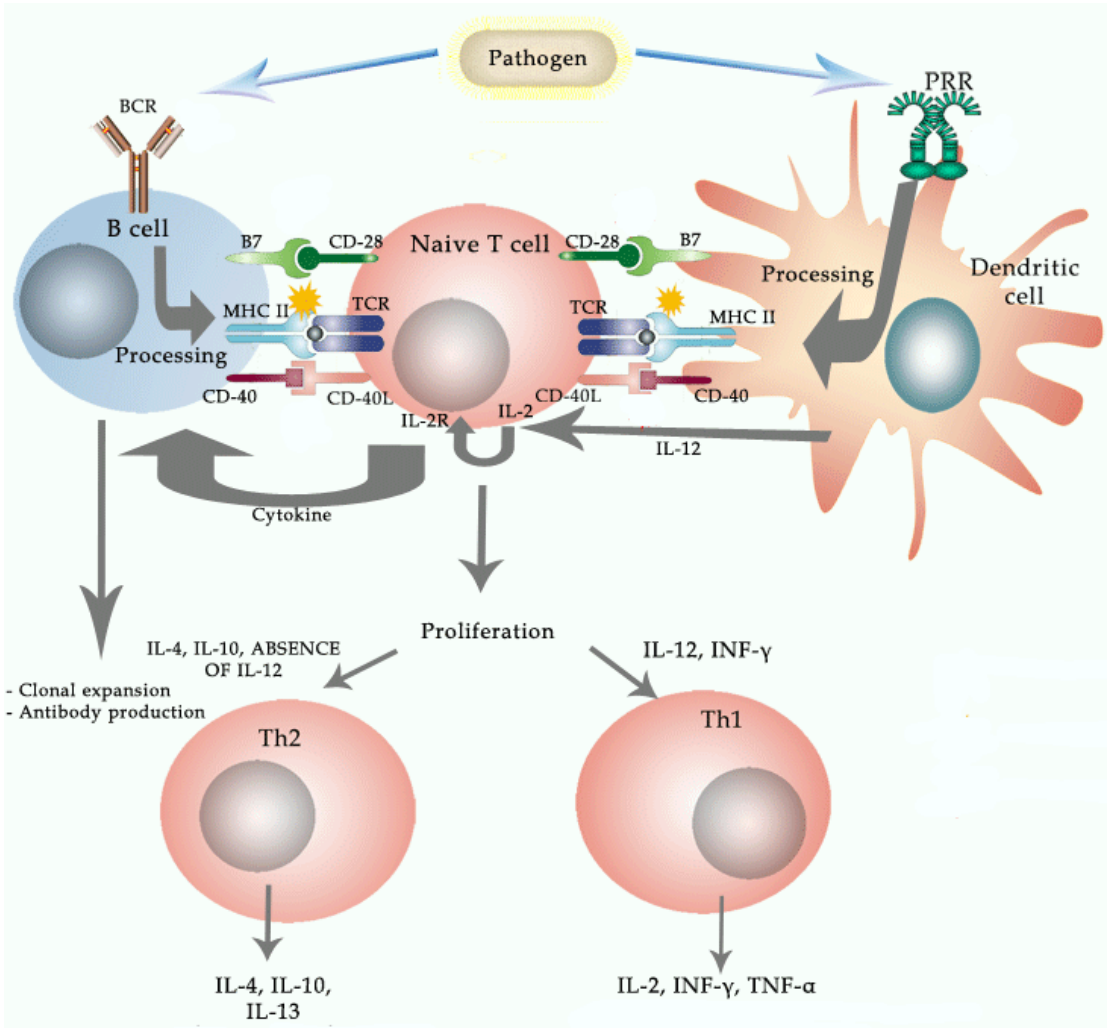
T-cells

Intracellular microbes such as viruses and some bacteria survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Defence against such infections is a function of cell-mediated immunity which promotes the destruction of microbes residing in phagocytes or killing of infected cells to eliminate reservoirs of infection. Cell-mediated immunity is mediated by T-lymphocytes (T-cells) representing the majority of circulating lymphocytes. T-lymphocytes mature in the thymus, circulate in the blood, populate secondary lymphoid tissues, and are recruited to peripheral sites of antigen exposure. They express antigen receptors (TCRs) that recognize foreign peptide fragments bound to self-MHC molecules. By the structure of the TCR, T-cells can be classified as Tgd cells or Tab-cells, and most Tab-cells in peripheral blood and inflammatory tissues express either CD4 or CD8 molecules on their cell surfaces. Functional subsets of T-lymphocytes include $CD4^+$ helper T-cells and $CD8^+$ CTLs. All T-cells express the CD3 complex, a T-cell specific receptor on the cell surface. APCs are responsible to present antigens to T-cells, when they meet in the lymphoid organ or in infected tissues. For naïve T-cells, recognition and activation requires the presence of co-stimulatory signals. Once T-cells have been activated they start to produce IL-2 whose main effect is to stimulate T cell proliferation and clonal expansion.

Cytotoxic T-lymphocyte (CTL, $CD8^+$). A virus-infected cell can be eliminated through the killing of the host cell by the immune system. Cytotoxic T-cells ($CD8^+$) that recognizes and kills host cells infected with viruses or other intracellular microbes usually executes this. CTLs usually express $CD8^+$ and recognize microbial peptides displayed by class I MHC molecules. The release of cytokines by CTL facilitates and strengthens the immune defence. Cytokines attracts other T-cells to the site, and then activation leads to the production of IL-2, IFN-γ, TNF-α, and the chemokine MIP-1ß and MIP-1α and RANTES. The elimination of target cells involves the release of granules containing membrane pore-forming proteins and enzymes (perforin, granzymes or lysine); or the interaction of Fas and Fas ligand or TNF-α and TNF-receptor *(*Janeway 2004*)*. All cells, except the erythrocytes have MHC class I molecules on the cell surfaces and T-cells can attack and kill all types of infected cells. Certain pathogens may inhibit the host's survival mechanisms by suppressing the MHC class I presentation thereby avoiding T-cell attacks (which then instead can be performed by NK cells detecting the pathogen's effect on the MHCs).

Helper T cells ($CD4^+$): The functional subsets of T-lymphocytes whose main effector functions are to activate macrophages in cell-mediated immune responses are also needed for the activation of B-cells to mature into IgG producing plasma cells. $CD4^+$ T-cells can also mediate cytotoxic activity e.g. by using Fas-Fas-ligand interactions. Another important effector function for $CD4^+$ T-cells is employed for the killing of microorganisms with the ability to survive intracellular inside macrophages: the macrophage inform the $CD4^+$ T-cell of the presence of the hidden pathogen by MHC class II associated presentation of pathogen-derived antigens and the $CD4^+$ T-cell respond by activating and amplifying the macrophage's killing mechanisms. The T-cell branch of the immune system can respond to a virtually infinite variety of exogenous antigens, thus including the possibility of self-antigen recognition and dangerous

autoimmune reactions. Regulation is required to control excessive effector T-cell responses against exogenous antigens. Several different types of T-cells with different functions have now been recognized.



Th1: A subset of helper T-cells that secrete a particular set of cytokines, including IFN-γ and whose principal functions are to activate $CD8^+$ T-cell CTLs and stimulate phagocyte-mediated defense against infections.

Th2: Another subset of helper T-cells, whose principal function, is to stimulate IgE and eosinophil activity/mast cell mediated immune reactions and to down-regulate Th1 responses. Th2 may secrete IL-4, Il-5 and IL-10 and is important for humoral and anti-parasitic defense.

T regulatory cells: Ten years ago a suppressor T-cell subtype able to suppress effector T-cell proliferation and cytokine production either through direct cell-to-cell contact or by the secretion of the anti-inflammatory cytokine IL-10 or TGF-ß was reported and these cells were called T regulatory cells (Treg) (Sakaguchi 2000). Today, it is believed that suppressor function can be mediated also by other T-cells. Increasing evidence suggest that T reg cells generates during human viral infections such as HIV, HCV and herpes virus infections and may contribute to the suppression of virus specific immune responses. This immune suppression may favor viral persistence, but it may also protect the host from pathogenic tissue damage at the site of infection. Treg cells play an important role in the maintenance of tolerance by regulating immune responses to self and foreign antigens (Sakaguchi S. 2005).

Th17: A newly discovered subset of T-cells that do not produce the classical Th1/ Th2 cytokines but are instead capable of secreting large amounts of IL-17. They play a critical function for protection against certain microbial challenges, particularly against extracellular bacteria and fungi. In humans, the 3 major inducers and/or sustainers of Th 17 differentiation are IL-6, IL-21 and IL-23. The Il-21 cytokine produced by Th17 is a stimulatory factor for Th17 differentiation and serves as the positive feedback amplifier, as does IFN γ for Th1 and Il-4 for Th2 cells. Recently, Th17 cells have been reported to be associated with organ-specific autoimmune diseases, such as psoriasis, asthmatic bronchitis and inflammatory bowel disease (Tesmer LA 2008).

Cytokines are involved in virtually every aspect of immunity and inflammation. Understanding the function of individual cytokines is complicated because their role can vary depending on the cellular source, target and phase of the immune response. Numerous cytokines have both pro-inflammatory and anti-inflammatory potential, with the contrasting outcome observed being determined by the immune cells present and their state of responsiveness to the cytokine. Cytokines are small structural proteins or glycoproteins that have multiple sources, target and functions. Over 40 cytokines are now recognized and many have been discovered during the last years. They regulate host defenses against infection and injury in a paracrine and autocrine fashion. Activated T-cells and macrophages are major cytokine producers. Immune regulatory cytokines can be subdivided into two different groups; Th1 cytokines (IFN-γ, IL-2, TNF-α, and IL-12) involved in cellular immune responses and Th2 cytokines (IL-4, IL-5, and IL-13) in particular responsible for induction of certain humoral immune responses. Cytokines produced by Th1 and Th2 cells can regulate the production of each other. Il-10 has emerged as a key immune-regulator during infection with viruses, bacteria, fungi, protozoa and helminthes. IL-10 may inhibit the activity of Th1 cells, NK cells and macrophages even though macrophages are the major source of IL-10 during infection. IL-10 can thus impede pathogen clearance if needed to reduce immune pathology.

Chemokines are polypeptides involved in cellular migration and cell-to-cell communication that selectively and sometimes specifically controls adhesion, chemotaxis and activation thus playing an important role for the trafficking and migration of leukocytes to inflammatory and lymphoid tissues. The concentration of IP-10 and MIP-1ß in cerebrospinal fluid during viral meningitis has been shown to correlate to the degree of mononuclear infiltration in the cerebrospinal fluid. The endogenous system of chemokines in the brain acts in concert with neurotransmitter and neuro-peptide systems to govern brain function (Adler 2006).

Cytokine	Source	Function
IL-2	Antigen activated T-cells	Stimulates T-cell proliferation and apoptosis and effector functions in NK cells and B-cells
TNF- α	Monocytes, macrophages, and many other cells	Stimulates the recruitment of neutrophils and macrophages to the infection site
IL-4	T-helper cells	Stimulates the development of Th2 cells and prevent macrophage activation. Stimulate production of IgE antibodies
IL-5	T-helper cells	Stimulates the production of IgA-antibodies, B-cell proliferation and maturation of eosinophils
IL-6	Fibroblasts and endothelial cells	Stimulates the production of acute phase proteins and the maturation of B-cells and antibody production
IL-10	Activated macrophages and T-helper cells	Inhibition of macrophage activation and an important link between specific and nonspecific immune responses.
IL-12	APCs	Induce IFN-γ production by Th1 and NK cells.
IL-13	Th2 cells, epithelial cells	Stim. B-cells to produce IgE antibodies. Inhibition of macrophages and antagonist for IFN-γ.
IFN-γ	NK cells, Th1 cells	Activates proliferation of T, B and NK cells. Upregulates the expression of MHC class I and II molecules.
MIP-1ß	T-cells, B-cells and macrophages	Chemoattractant for memory Th1 cells, NK and dendritic cells; activation and proliferation of T-cells, Il-2 secretion and Il-2R expression.
IP10 (CXCL-10)	Monocytes, T- cells. Induced by IFN-γ stimulation	Selective Th1 lymphocyte and monocyte chemo attractant, promoting lymphocyte adhesion to activated endothelial cells.
IFN-α	Lymphocytes, monocytes, and macrophages	Confer resistance to viruses on target cells, inhibits proliferation.

GENERAL INFECTION IMMUNOLOGY

When acute, chronic or persistent viral infections trigger the immune system the infected host will respond. The nature of the response will depend on virus type, infectious dose, and the site of infection, route of transmission, genetic background and immune competence of the host. Viruses typically infect a wide variety of cell populations by using normal cell surface molecules as receptors to enter the cell. After entering the cell, viruses can cause tissue injury and disease by any of several mechanisms. Viral replication interferes with normal cellular protein synthesis and function and leads to injury and ultimately death to the infected cell. Innate and adaptive immune responses to viruses are aimed for blocking infection and eliminating infected cells.

The principal mechanisms of innate immunity against viruses are inhibition of infection by type I IFNs and NK cell- mediated killing of infected cells. Type I IFNs inhibit viral infection of uninfected cells and viral replication in infected cells by inducing an anti-viral-state. NK cells may kill cells infected with viruses and mediate an important mechanism of immunity against viruses in the early course of infections, before an adaptive (primary) immune response have been developed. NK cells also recognize infected cells in which the virus has managed to shut off class I MHC expression by identifying them as non-self and target them for destruction. Cells like macrophages and dendritic cells of the innate immune system also have the important role of activating the $CD4^+$ T-cell and thus the efficient forces of the adaptive immune response.

$CD4^+$ T-helper cells will usually not itself execute effector mechanisms in virus infection. Nevertheless, the helper lymphocyte is crucial for the defense against viruses, since it is the key regulator of the adaptive immune response, directing the activation and maturation of different effector arms by the production of cytokines and through direct cell-to-cell communication, such as humoral responses and CTLs.

Humoral immunity induced by previous infection or vaccination, is able to protect individuals from certain viral s such as hepatitis B virus or morbilli virus infection. Antibodies are effective against viruses only at the extra-cellular stage. Antiviral antibodies may function as neutralizing antibodies by binding to viruses blocking the binding by the virus to cell-surface molecules to prevent virus entry into host cell.

Elimination of viruses that reside within cells by the adaptive immune response is mediated by $CD8^+$ T-cells which kill infected cells. $CD8^+$ T-cells can undergo massive proliferation during viral infection and most of the proliferating cells are specific for a few viral peptides. Some of the activated T-cells differentiate into effector $CD8^+$, which can provide cytotoxicity and lysing of infected cells, and also secrete cytokines such as IFNγ, which possesses some antiviral activity.

Memory T cells can be divided into central memory and effector peripheral (activated) memory subsets, which are endowed with different capacities to home to lymphoid or non-lymphoid tissues, to proliferate in response to antigen or cytokines and to perform effector functions. Sallusto et al demonstrated that central memory cells and peripheral

memory cells can be distinguished as different phenotypes since the former does not, but the latter do express the chemokine resptor CCR7 (Sallusto f 1999). Peripheral memory cells represent a population of T cells that secrete large amount of cytokines, may execute cytotoxicity and reacts rapidly following pathogen challenge; whereas central memory cells may be more often localized to lymphoid tissues, conferring long-term immunity, and in particular respond to pathogen challenge by primarily proliferative responses (Lanzavecchia 2005).

Some agent strategies to avoid host immune defense and consequences

Antigenic variation: Parasites evade protective immunity by reducing their immunogenicity and by inhibiting host immune responses. Parasites have developed remarkably effective ways of resisting immunity such as changing their surface antigens during the lifecycle in host, so called antigenic variation. Two forms of antigenic variation have been well defined. One is when the mature tissue stages of parasite produce different antigens than the infective stages do. For example the infective sporozoit stage of malaria differs from the merozoites that reside in the host responsible for chronic infection. The second example of antigenic variation is the continuous variation of major surface antigens seen in African trypanosomes such as Trypanosoma brucei and Trypanosoma rhodesiense. Infected individuals experience waves of blood parasitemia and each wave represents an antigen expression that differs from the previous wave. By the time the host produce antibodies against the parasite, an antigeni-cally different organism has grown. More than hundred such antigenic variations can occur during one infection. One consequence of antigenic variation is that it is difficult to effectively vaccinate and sometimes treat individuals against these parasites.

Parasitic granulomas: Some parasites are too big to be killed of by the immune system but Th2 cells solves this by producing granulomas around the parasites or by inducing plenty of antibodies that can cover the parasites surface. Some parasites and their products induce granulomatous responses with concomitant fibrosis. In Schistosoma mansoni eggs deposited in the liver stimulate $CD4^+$ cells that in turn activate macrophages and induce DTH reactions. DTH reactions result in the formation of granulomas around the eggs. The granulomas serve to contain the eggs deposited in the liver but severe fibrosis associated with this chronic cell-mediated response leads to disruption of venous blood flow in the liver, portal hypertension and cirrhosis.

Bacterial granulomas: Certain intracellular living bacteria like Mycobacterium tuberculosis may resist killing within phagocytes, persist for long periods causing chronic antigenic stimulation and T- cell and macrophage activation which may result in the formation of granulomas surrounding the microbes. This type of inflammatory reaction may serve to localize and prevent spread of the microbe but may also be associated with severe functional impairment due to tissue necrosis and fibrosis.

Inhibition of antigen processing and class-1 presentation
Some viral genes encode proteins that modulate host immune responses. The human cytomegalovirus produces two proteins, US2 and US11, which bind to class I molecules in the endoplasmatic reticulum and actively carry or dislocate these

molecules into the cytosol where they cannot be loaded with antigenic peptides and are degraded in the proteasome. The consequence of blocking class 1-peptide is that the infected cells show reduced expression of stable class 1 molecules on the surface and do not display viral peptides for T-cell recognition.

IMMUNOLOGICAL METHODS

For many years, the dominating assays for measuring specific CMI was the two conventional assays for proliferative and for cytotoxicity. These are labor-intensive, in particularly the cytotoxicity assays which is extremely so. These assays are associated with great variations both in terms of intra- and inter-assays, and provide measurements expressed as stimulation indices and % cytotoxicity, respectively, and can only demonstrate the presence and the magnitude of a response to a pathogen (-antigen) but no information whatsoever on the nature of such responses. Over the last decade, a number of novel techniques for characterizing CMI have been developed and become widely employed. New assays may be used for measuring proliferative responses but usually they focus on detection and characterization of cytokine responses or/and characterization of the expression of cell surface molecules of responding cells. The older methods were regularly utilizing peripheral blood mononuclear cells (PBMC) which demanded time-consuming isolation of cells with risk of introducing several possible errors, whereas many new assays is based on whole-blood which is much more convenient. A number of new and old methods will be presented.

Proliferative response by the H^3-tymidine uptake assay
T-cell proliferation has traditionally been measured by incorporation of a radioactive tracer, incorporated in newly synthesized DNA of dividing cells. The assay provides information of the total amount of DNA-synthesis in a culture but do not provide information on the phenotype or proliferating cells.

Proliferative response by flow cytometry.
A flow cytometric analogue to the thymidin uptake assay, assessing DNA synthesis, is the Brdu-assay. Another flow cytometric assay is the CFSE assay, by which cells are stained with CFSE and proliferative responses are measured as declining CFSE content for each generation of daughter cells developed during proliferation. By the FASCIA, proliferative responses are recorded as the development of lymphoblasts.

Cytotoxicity assay
The cytolytic activity has been a traditional measurement of $CD8^+$ cytotoxic T-cell function, as well as NK cell function. For assessment of MHC class I restricted antigen-specific lyses, infected or antigen-loaded autologous B-cell lines or similar are used as target cells and fresh or expanded antigen-specific CTLs, as effector cells. The cytolytic process consists of a number of steps, including 1) recognition and binding of the cytolytic cell target cells 2) delivery of lethal hit to the target cells and 3) target cell lyses and recycling of the effector cell.

Cytotoxicity measured by flow cytometry.
A flow cytometric CTL assay has been presented, using the release of CFSE and PI as markers, and providing possible characterization of targets and effectors by immunophenotyping (Godoy et al, 2005).

Cytokine responses by Elispot
The enzyme-linked immunospot, Elispot detects cells that secrete cytokines following antigen stimulation. PBMCs are cultured with antigen in plates that have been coated with anti-cytokine antibodies. The secreted cytokine is captured by the antibody and revealed with a second enzyme-conjugated antibody. The secreted cytokine molecules form spots, each one corresponding to a cytokine secreting cell. This assay is a robust and highly sensitive method but cannot, without particular isolations steps, provide any information on the nature of the cytokine-producing cells. By Elispot, the production of only one cytokine – usually IFN-γ – is measured and for detection of more, additional Elispots must be simultaneously performed.

Cytokine responses by Intracellular Cytokine Staining, (ICS)
ICS is a relatively new flow cytometry-based method that requires culture in the presence of a protein transport inhibitor to retain and accumulate the produced cytokines inside the cells; fixation, to maintain structure of the cells and permeabilitsation to allow the entry of cytokine-antibodies into the cells. This method allows the simultaneously determination of several cytokines in combination with enumeration and phenotypic identification of the responding lymphocyte population. One drawback of the ICS assay is that a large number of cytokines cannot yet be detected by the method, usually because these cytokines is not present in concentrations enough for reaching above the detection limit. ICS can be performed with whole blood as well as with PBMC. This method is become more and more popular as a valuable tool in clinical studies and vaccine trials lately.
Detection of cytokine responses by other methods
ICS is a relatively new flow cytometry-based method that requires culture in the
Prior to the development of convenient Elispot and ICS assays, other techniques were employed. One highly sensitive but labor-intensive assays was the demonstration of intra-cellular cytokines by immunofluorescence microscopy (Andersson U 1988). Furthermore, cultures of PBMCs have been employed for the measurement of secreted cytokines into the cultured supernatants, examined by ELISAs or during recent years by Luminex. Lately, a very commercially available convenient assay for the detection of IFN-γ in supernatants of whole blood culture (Quantiferon) has become available.

Tetramer-based techniques:
Tetramer assays are based on the visualization of antigen-specific T-cells by the use of fluorescently labeled peptide-MHC complexes that bind to specific T-cell receptors. Tetramer staining has been used to enumerate, characterize and purify peptide specific $CD8^+$ cells (Murali-Krishna 1998) and reagents for similar detection of specific $CD4^+$ T-cells is becoming available**.** The primary advantage of tetramer staining is it doesn't require in vitro antigen stimulation or culture. The disadvantages include the lack of functional correlate, except for what is provided by simultaneously performed immunophenotyping, and the technical need for very sophisticated and complex reagents.

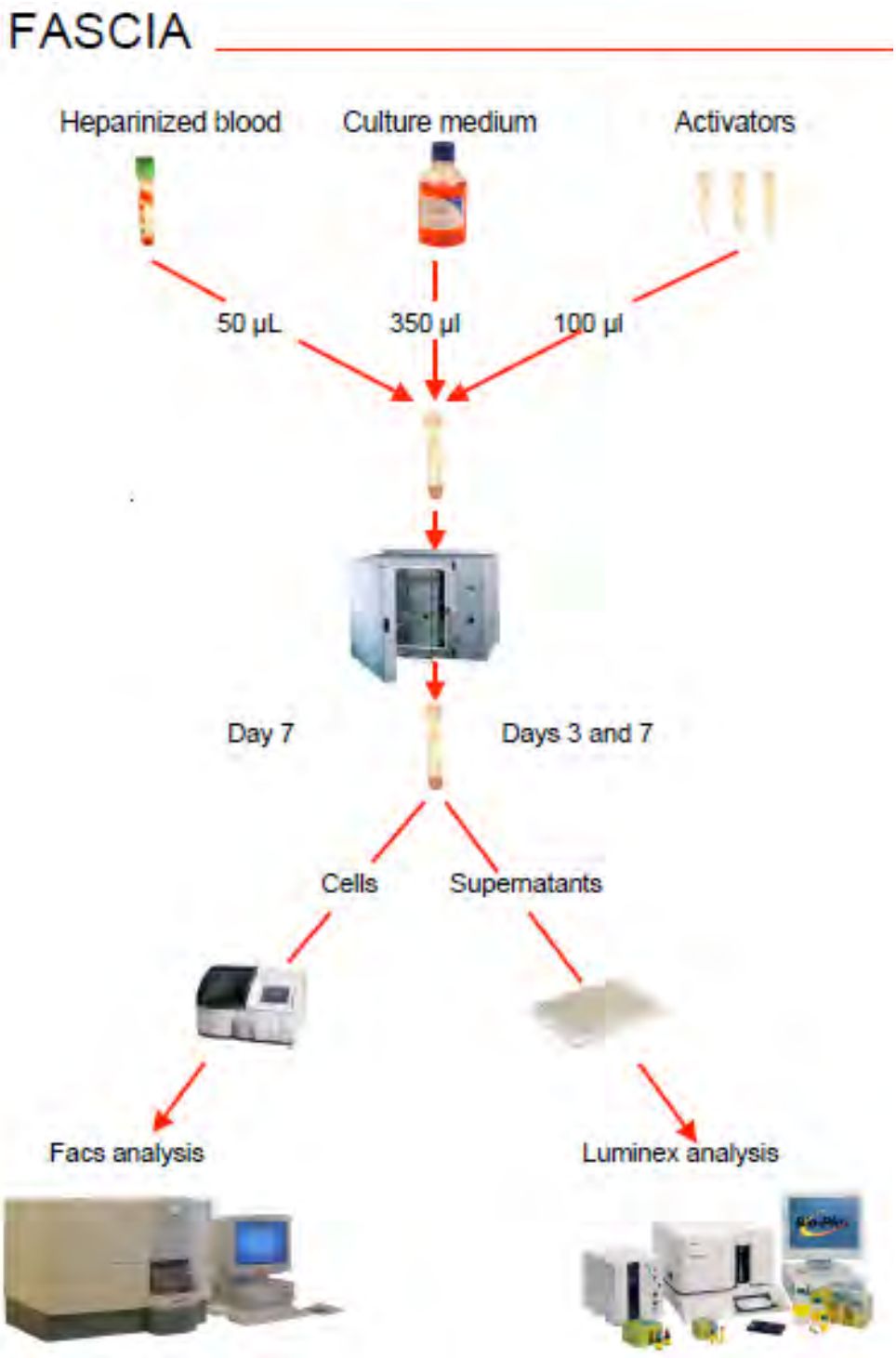
Flow-cytometric Assay of Specific Cell-mediated Immune-response in Activated whole-blood (FASCIA)

FASCIA is an in-house accredited method developed at SMI for the analysis of whole blood (but can also be employed for PBMC) which is very convenient and FASCIA can as easily be done for single samples as it can be scaled up for large studies. This assay uses FACS as well as Luminex analysis and can thus provide cell-mediated immune responses as proliferative responses (by FACS blast detection), characterization of responding cells (by FACS immunophenotyping) and characterization of large cytokine profiles of responding cells (by Luminex assessment).

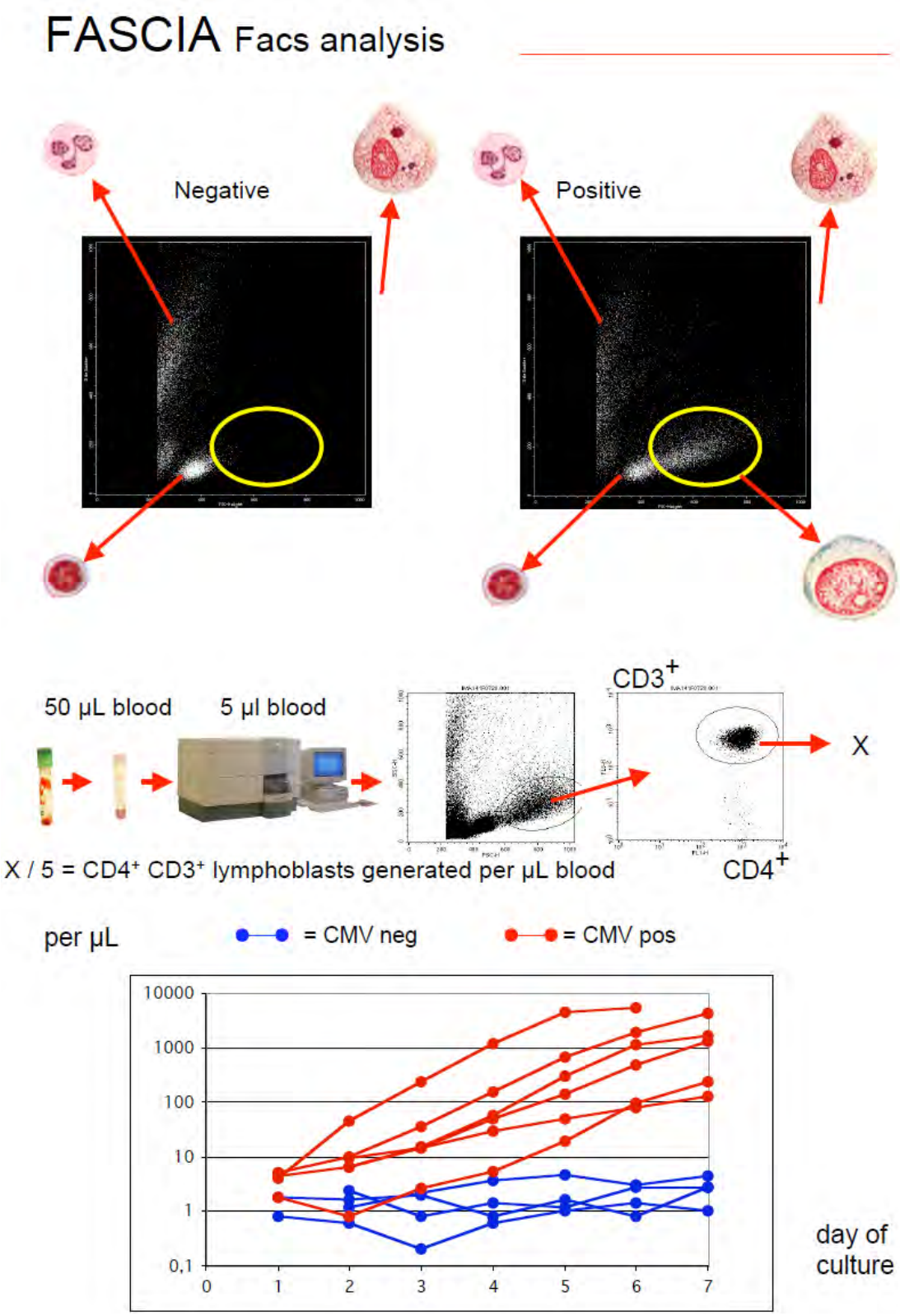
Cultures are usually set up using whole blood samples, cultivated for 7 days in the presence or absence of stimulators and results are assessed as the number of antigen-specific lymphoblast that are generated (Gaines 1996, Svahn 2003). Only small amount of blood is needed, which is especially useful for studies of children. The exclusion of extensive separation and preparation procedures saves time and ensures that cells are kept in an environment more similar to in vivo. Possible enrichment or depletion of cell subsets and pre-activation of cells during preparation, resulting in an increased background and a decreased sensitivity are avoided.

By FACS analysis, responding lymphoblast are readily detected by their scatter profiles and combined with staining, the advantages of multi colour flow cytometry can be employed for the extensive characterization of responding cells by immunophenotyping and also following permeabilisation for the demonstration of intracellular cytokines.

Culture supernatants are removed from parallel cultures day 3 and from the original culture, prior to cell analysis, day 7 (or other time-points if needed) and tested for concentrations of different cytokines using multiplex immunoassays, such as the Luminex. This analysis can also be performed using the Cytometric Beads Assay (CBA). The Luminex xMAP technology uses distinct colour-coded polystyrene beads conjugated with capture antibodies and biotinylated detection antibodies, enabling the simultaneous measurement of numerous different cytokines at wide ranges of concentrations. Compared to the ICS assay, the FASCIA with Luminex analysis cannot identify the exact cells, which produce the cytokines, but a large number of cytokines and chemokine cannot be detected by ICS assays whereas the FASCIA Luminex analysis is very sensitive and can detect very small amounts of cytokines such as Th2 cytokines.

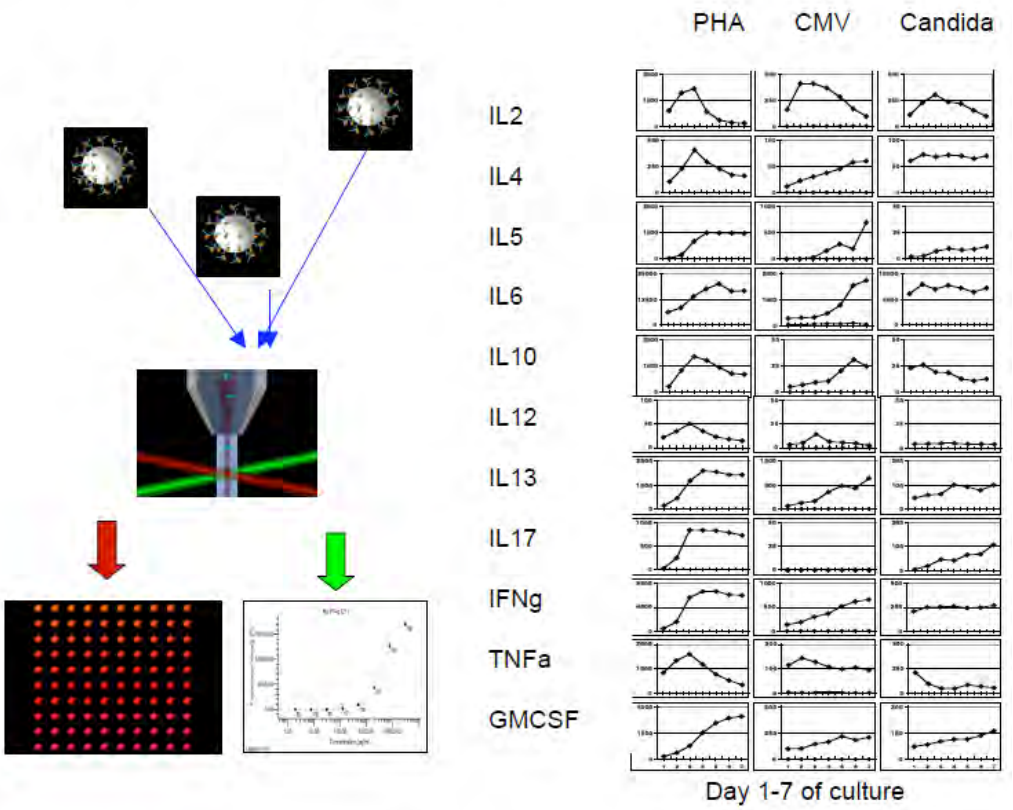


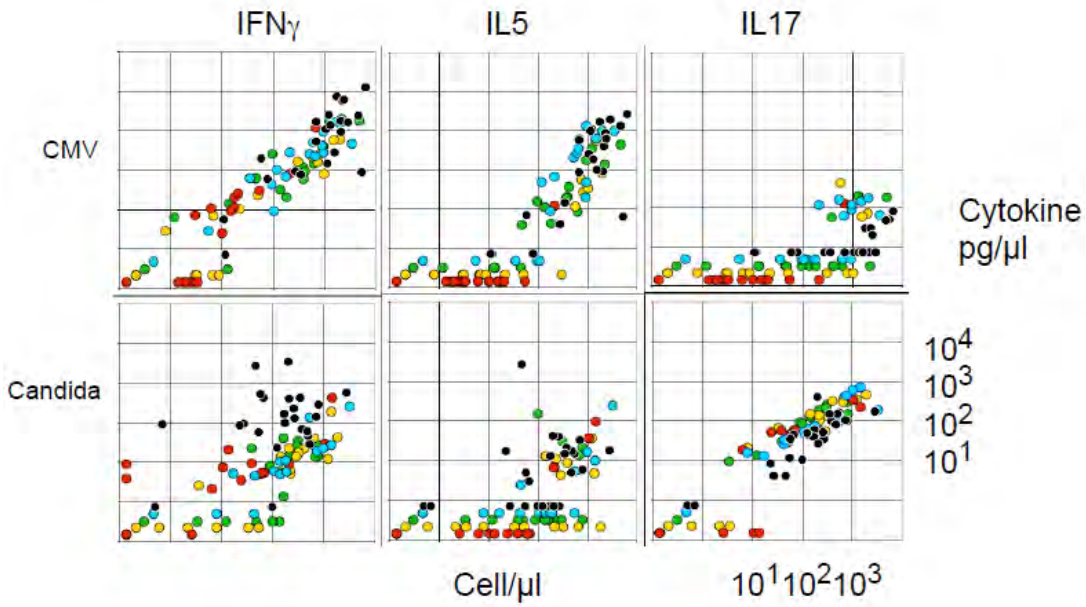
FASCIA (Flow-cytometric Assay for Specific Cell-mediated Immune-response in Activated whole-blood) set up, culture, harvest and analysis by Facs and Luminex, illustrated on the following pages.



Top: Scatter profile for neutrophils, macrophages, small lymphocytes and lymphoblasts (yellow region) for whole-blood samples cultured with only medium (Negative) and with influenza antigens (Positive).
Middle: Following immunophenotyping, 1/10 of sample is acquired and results are expressed as the number of $CD3^+$ $CD4^+$ lymphoblast generated per µl peripheral blood cultured.
Bottom: Proliferative responses and increasing number of $CD3^+$ $CD4^+$ lymphoblast in cultures with CMV nucleocapsids and whole blood from 6 CMV-antibody positive (red) and 4 CMV-antibody negative (blue) subjects.

FASCIA Luminex analysis





Left: General principle for Luminex detecting read beads corresponding to the different cytokines tested and green fluorescence correlated with cytokine concentrations determined by comparison with standards.
Right: The concentrations of cytokines and chemokines in FASCIA cultures with specific antigens can usually be measured day 7 but some analyses need to be measured at other time-points e.g. day 3.
Bottom: Cytokine profile (IFNg, IL5, IL17) in relation to proliferative responses to CMV and Candida measured in HIV-negative healthy controls (black) and in HIV-positive patients:

without anti-HIV treatment and with decreasing numbers of CD4 counts (green, yellow, red); or on effective anti-HIV and not detectable HIV-RNA (blue).

HERPES SIMPLEX AND THE IMMUNE SYSTEM.

HUMORAL IMMUNITY AND HSV INFECTION

A humoral immune response rapidly develops during primary genital HSV infection. The early HSV-specific mucosal antibodies are of the IgA isotype and these antibodies can be detected as early as day 4-5 post infection, followed by IgG1 and IgG3 subclass antibodies appearing day 11-16 (Milligan 1995). There is no evidence that antibodies may protect against clinical manifestations of HSV and several vaccine studies have demonstrated that neutralizing antibodies induced against HSV glycoproteins does not provide protection against HSV infection or disease.

ADAPTIVE IMMUNITY AND HSV INFECTION

Many studies have shown that cell-mediated immune responses are important for the resolution of HSV disease (Bernstein 1999). However, HSV has evolved mechanisms to evade the host immune responses at several levels. It can interfere with innate immunity by blocking interferon type 1 signaling and it can dampen adaptive immune responses by suppressing T cell recognition of infected cells by inhibiting antigen presentation. The acquired immune response to herpes simplex viruses includes both $CD4^+$ and $CD8^+$ T-cells activation. In a recent paper early appearing $CD4^+$ T-regulatory cells were reported to facilitate migration and other responses by allowing a timely entry of immune cells into lesion tissues (Lund 2008). In the initial phase of infection $CD4^+$ T-cells play a crucial role for coordination of the immune response: in the absence of $CD4^+$ T cells, the $CD8^+$ T-cells develop poorly. HSV-specific $CD8^+$ T-cells are essential for control and resolution of primary HSV infection, as well as for control of reactivation in latent infection. They have been found in trigeminal ganglia of herpes simplex type 1 infected patient where they suppress viral reactivation by production of IFN-γ and non-cytotoxic granules (LIU 2001, Knickelbein 2008). Under stress, $CD8^+$ T-cell secretion of IFN-γ and release of lytic granules is reduced and then, latently established HSV may be activated and clinical illness developed. Moreover, accumulating evidence indicates that latency involves chronic low-level immune activation including secretion of IFN-γ and TNF-α in response to frequent but subclinical viral reactivation (Dunn 2002, Theil 2003).

Immune deficiencies and HSV infection

Immune compromised patients with defects in cell-mediated immunity experience more severe and more extensive HSV infections than those with deficits in humoral immunity, such as X-linked agammaglobulinemia (Pass 1979, Conley 2005). Patients with HIV-induced cellular immunodeficiency are known to frequently suffer from prolonged reactivated HSV infections with severe and complicated mucocutaneous lesions (Maier JA 1986, Stewart JA 1995, Orange 2002). From previous studies, we also know that congenital deficiencies of NK and CD4+ T- cells can lead to disseminate HSV infections (Orange 2002, Gupta 2007). Biron and Byron described a child who could not express CD16 and CD56 molecules and thus were NK deficient, which experienced a severe varicella infection, followed by CMV pneumonitis, and then disseminated HSV infection (Biron 1989). UNC-93B and TLR 3 deficiency resulting in impaired IFNα production have been found in some cases of HSV-1 encephalitis in children (Casrouge 2006, Zhang 2007).

Immunity and genital HSV infection

In HSV-2 as well as HSV-1 genital infection, the frequency of recurrences decline by time and the clinical manifestations of recurrences are less severe than the illness experienced at primary infection, indicating that adaptive (CMI) immunity may develop into at least some control of HSV. The efficiency of this control appear to be higher for HSV-1 than HSV-2 genital infection since the frequencies of patients experiencing recurrence after first episode genital infection of HSV-1 and HSV-2 were found to be 14% and 60%, respectively in a follow-up study (Corey 1982). The likelihood of an asymptomatic seroconversion to HSV-2 instead of developing an associated illness was increased by a factor of 3 if the patient was already carrying HSV-1 and in case of a symptomatic HSV-2 infection, those already carrying HSV-1 experienced a less severe and shorter illness than previously HSV-naïve individuals (Langenberg 1999) demonstrating that HSV-1 specific adaptive immunity is cross-reactive and may execute certain control of subsequent HSV-2 infection. However, the recurrence rate following asymptomatic and symptomatic primary genital HSV-2 infection appeared similar (Corey 1982), suggesting that an early protection from clinical illness during the primary HSV-2 attack from the outside may perhaps be different from the control against reactivation from latent HSV-2 in ganglion.

In a study of partners to HSV-2 infected persons, it was shown that transmission of HSV-2 between partners usually takes place within the first year and seldom later (Mindel 2000, Cunningham 2001) Posavad and Corey found that 4 out of 10 HSV seronegative partners o patients with genital HSV-2 infection displayed HSV-specific proliferative responses suggesting that exposure to HSV may induce HSV-specific CMI and lead to protection against infection (unpublished observations). Already in 1978, Corey et al showed that the early appearing peak levels of HSV-specific proliferative responses correlated inversely with duration of virus shedding and clinical illness in patients with genital HSV infection (Corey 1978). Similarly, high levels of IFN-g produced by HSV-specific CD4+ T-cells from peripheral blood were associated with a lower frequency of recurrences in genital HSV infection (Cunningham 1984). A number of studies have compared HSV-specific CMI in PBMC in patients suffering from recurrent genital HSV-2 infection with asymptomatic HSV-2 infected individuals. One group reported that HSV-specific production of high levels of IFN-γ and low levels of IL-10 correlated with asymptomatic HSV-2 infection, and another group found that patients with recurrent infections produced significantly lower levels of Th1 cytokine responses to HSV-2 specific sGg-2 and mGg-2 but no difference in proliferative responses tan patients with recurrent HSV-2 infection (Eriksson 2004).

In another study, lymphocytes were daily recovered from genital tissue HSV lesions and tested for the presence of HSV-specific $CD4^+$ T-cell proliferative response, $CD8^+$ T-cell cytolytic response, and NK cell cytotoxicity. The $CD4^+$ T-cell and NK-cell mediated reactions did not change over time, but cytotoxic activity of $CD8^+$ T-cells increased in subsequent cultures and was also associated with viral clearance in lesions demonstrating the important role of $CD8^+$ T-cells for HSV control and recovery from HSV-2 illness (Koelle 1998).

Immunity and HSV meningitis

HSV-2 meningitis is usually a complication of primary genital HSV-2 infection generally only occurring in HSV-naive patients without prior HSV-1 infection, suggesting a protective role against the spread to CNS by the cross-reactivity provided by HSV-1 specific immune responses. Once established, HSV-2 can be reactivated leading to recurrent meningitis and suggestive to be the major cause of Mollaret's syndrome of benign recurrent lymphocytic meningitis (Bergström 1990, Tedder 1994). There is yet no information available in the medical literature on HSV-specific CMI in human patients with HSV-2 meningitis but experiments using murine models have suggested that HSV-binding to Toll like receptors TLR2 and TLR9 may be important for the resistance against HSV-2 meningitis in mice (Sörensen 2008).

AIMS

To improve diagnostics of acute aseptic meningitis in general and of herpes simplex meningitis in particular.
To increase the understanding of why some, otherwise immune competent individuals suffer from severe and frequent expressions of herpes simplex virus infections while others control the infection. To characterize the nature of the immune response in herpes simplex virus infection in order to identify individuals in whom frequent and/or severe manifestations may be expected.

Specific aims:

Paper I to evaluate a quantitative PCR for detection of HSV-DNA and VZV-DNA in CSF on a well-characterized material of patients with herpes simplex type-2 meningitis (HSM) and acute aseptic meningitis (AAM) of unknown origin.

Paper II to evaluate the etiological outcome and clinical usefulness of routinely performed TBEV serology and CSF-PCR for entero and herpes simplex viruses in patients with AAM and /or mild meningoencephalitis.

Paper III to investigate the immune responses of primary HSV-1 and HSV-2 genital infections and to analyze if these could be associated with frequency of relapses during a 12 months follow-up period.

Paper IV to investigate the adaptive immune responses in recurrent meningitis and recurrent genital herpes simplex virus infected and to study factors of importance in the constitutive immune defense in a selection of individuals with different expression of herpes simplex virus infections.

MATERIAL AND METHODS

For detailed information about material and methods used in this thesis, see respective paper.

Ethical clearance
The studies included in thesis were performed after approval from the – institutional Review Boards and Ethical Committees at each participating site. All subjects gave their signed informed consent prior to study enrollment.

Statistics
For details about statistical analysis used in this thesis, see respective study. Comparisons were made using the *t* test and the X^2 test (Paper I)**.** Kruskal-Wallis test was used to compare cell-mediated immune responses between groups and time-points (Paper III), Spearman test was employed for calculations of correlations (Paper III) and the Mann-Whitney *U*-test (Paper II, III and IV) was used when appropriate. A p-value<0.05 was considered statistically significant.

RESULTS AND DISCUSSION

PAPER I

Efficient and sensitive diagnostic methods are needed in the management of viral infections in the central nervous system. An evaluation of the sensitivity of PCR in early diagnosis by using CSF from patients with primary and recurrent HSV-2 meningitis is hitherto lacking. Quantitative PCR methods are developed and advantageous to previously used qualitative nested PCR methods in many ways.

In this study we evaluated a new real-time PCR method for the detection of HSV-2 and VZV DNA in CSF from clinically well-characterized patients. The assay was applied on CSF samples collected consecutively and analyzed retrospectively from 110 patients during 1988-2004. All patients > 15 years of age with clinical signs of meningitis and CSF pleocytosis and culture negative for bacteria and fungi belonging to two main groups were included. One group (n=65) consisted of patients with HSV-2 meningitis (HSM) or with strongly suspected HSV-2 etiology meeting one or more of the inclusion criteria (HSV-2 DNA in CSF detected with nested PCR, present seroconversion to HSV-2, concurrent herpetic genital lesions and negative differential diagnoses, recurrent meningitis and, earlier, verified HSV-2 meningitis, and recurrent meningitis and a history of meningitis of unknown origin and HSV-2 antibodies in acute phase serum and negative differential diagnosis). This group was further subdivided into those with primary clinical infection and those with recurrent infection. The other group consisted of patients with acute aseptic meningitis (n=45) of unknown origin (AAM), negative in tests for enteroviruses, *Borrelia burgdorferi*, TBE and HSV-1 and not meeting the criteria for HSM.

Nested PCR was performed routinely for HSV-1 and -2 DNA using previously described, qualitative PCR assays. The design of the new real-time PCR assay was based on a previously published method designed for the detection of HSV-DNA and VZV-DNA in skin lesions (Schmutzhard 2004), with some modifications. Primers were selected from Glycoprotein D for HSV-1, glycoprotein G for HSV-2 and open reading frame (ORF) 29 for VZV. Commercial DNA standards were used for quantification, in which serial dilutions were run in parallel with the clinical samples. Detection sensitivities of nested and real-time PCR were compared and the methods detected DNA equally well down to 55 to 60 copies per reaction. Below this level real-time PCR gave more positive reactions.

With RT-PCR HSV-2 DNA was found in 80% (52/65) of patients with clinical HSV-2 meningitis compared to 72% (42/65) found by nested PCR. Additional HSM diagnoses were identified in the recurrent meningitis group and one in the AAM group, The detection rate of HSV-2 by real-time PCR was increased in the group of patients with recurrent HSM, (70%) compared with nested PCR (52%), although not statistically significant

The sensitivity of real-time PCR for the diagnosis of HSV-2 meningitis was found to be high, though somewhat lower in recurrent infection (70%, 19/27) than primary (87% 33/38) infection

Ten samples were positive in real-time PCR only. Five of them had copies <1000 copies/ml and 5 had 1,000- 10,000 copies/ml. Four samples were found positive in nested PCR only. Among them 2 were found positive in re-analysis having viral loads <1,000 copies/mL.

VZV was detected in 2 of 45 samples (4.4%) from AAM patients.

Viral meningitis remains an important cause of morbidity and financial burden and merits efforts to improve diagnostic, treatment and prevention options. The etiological diagnosis of HSV-2 meningitis is of particular importance as this condition carries a considerable risk of future recurrent neurological symptoms (Aurelius 2002, Tedder 1994). With HSV-2 diagnosis, accurate information about prognosis and treatment options is possible.

In our study, real-time PCR verified a larger number of cases of HSV-2 meningitis than the previously used nested PCR. In the absence of a golden standard, using the above stated criterias; the sensitivities for the detection of HSV-2 DNA by real-time PCR could be estimated to 87 % and 70% in primary and recurrent meningitis, respectively. The missing cases as well as the discrepancy between the PCR methods may be explained by minute DNA concentrations around the level of detection, and maybe unevenly distributed, in the samples.

Mucocutaneous lesions in association with the meningitis were noted in 23 out of 38 (61%) patients with primary and 16 out of 27 (59%) with recurrent HSM in line with previous publications. Asymptomatic genital recurrences with shedding may occur in spite of the lack of visible lesions and may also have preceded the meningitis by one or two weeks thereby avoiding an obvious linkage

VZV was found in 4.4% (2/45) of the patients. There was no previous history of vesicular rash in childhood. Both experienced their primary meningitis in their thirties (31 and 35 years) with 5-6 days of neurological symptoms before admission. Analysis of acute phase serum of the patients showed high levels of VZV IgG antibodies, indicating reactivated infection. None of them presented a clinical picture of vesicular rash. These facts underline the fact that VZV should be considered in the etiological diagnosis in adults with or without the zoster rash.

Significant differences were shown with larger numbers of leukocytes and monocytes and higher levels of lactate and albumin in the primary HSM group. This is in line with HSV-2 viral load being significantly higher in primary than in recurrent meningitis ($p<0.05$). Viral loads of >10,000 copies were found exclusively in patients with primary HSV-2 meningitis, while viral loads of < 1000 or between 1000 and 10,000 copies were found in all of the patients with recurrent HSV-2 meningitis Correlation was found between viral load and the amount of leucocytes, monocytes, protein and albumin found in CSF. This correlates well with the observation that primary meningitis clinically tends to be more intense, and with prolonged course of infection. One may speculate that a primary infection will cause the greatest amount of inflammation in the meninges and thus presents highest amount of pathological cerebrospinal fluids findings due to the host immune response.

The patients with first-time meningitis came to the clinic on an average one-day later, proposing that patients with recurrent infections have been encouraged to seek medical attention early for the benefit of antiviral treatment. Accordingly, the mean time with

neurological symptoms before lumbar puncture was significantly shorter in patients with recurrent HSM. This could be of importance for the amount of DNA detected in CSF as in previous investigations of series of CSF samples the viral load has shown to decrease over time (unpublished data). This suggests even greater differences in viral loads in primary versus recurrent meningitis than presented by our data.

Summarized: Real-time PCR for the diagnosis of HSV-2 meningitis was evaluated in a large clinically well characterized material and found to verify a larger number of cases of HSV-2 meningitis than the previously nested PCR in the group of recurrent meningitis. Quantitation of DNA enables further research of disease prognosis and treatment.

PAPER II

Our aim of this second paper was to investigate the etiology of acute aseptic meningitis (AAM) in an adult population in Stockholm, and the diagnostic efficiency using a limited first-line combination of microbiological assays. A consistent use of PCR analyses for herpes-simplex virus (HSV) DNA and enterovirus (EV) RNA in CSF as well as ELISA for IgM in serum to tick-borne-encephalitis virus (TBEV) during season established a diagnosis in the majority, 62%, of 419 patients with acute aseptic meningitis.

The three agents, EV, HSV and TBEV, represent the most common and important AAM agents in our region. EV is the major cause of meningitis in adults in Scandinavia. Although EV meningitis usually is a mild and self-limiting disease poliomyelitis is caused by EV and outbreaks of other neuropathogenic EV strains occur. HSV-2, which may occur without blisters, carries a considerable risk of future intermittent neurological morbidity and is the major cause of recurrent AAM. Specific antiviral therapy is available for treatment and/or prophylaxis. TBEV occurs with seasonal variation in Stockholm. It usually causes AAM or mild meningoencephalitis but severe encephalitis and myelitis may occur and a protracted post TBEV-syndrome with mainly cognitive CNS dysfunctions may follow in a substantial percentage. During the last 10-year period the number of TBEV cases has increased and an extended geographical spread of infected ticks have emerged in Sweden.

The inclusion criterias in the study were strictly clinical. Patients presenting signs indicative of AAM such as fever, headache and neck stiffness or mild meningoencephalitis such as fever, headache and slightly altered consciousness in combination with pleocytosis (>5 mononuclear leucocytes x 10 6/L) in CSF were included. Cases with clinical AAM and a clear-cut meningitis or meningoencephalitis-associated virological finding were also included even though the CSF assay was normal or missing. A total of 419 adults with AAM or mild meningoencephalitis treated at Karolinska or Danderyd hospital during a 6-year period 1999-2004 were included. AAM and mild meningoencephalitis was considered as a single clinical entity and designed as AAM. The serum samples were drawn on admission with a median of 2 days after onset of meningitis symptoms. If a positive result was obtained with one of the tests other etiological analyses were not performed. If the tests yielded negative results further diagnostic measures were taken based on clinical and epidemiological

observations. Additional microbiological assays were performed in some patient's serology for EV and for HSV in 123 and 130 patients (about 30 % each), and CSF-PCR for VZV in 42 (10 %).

The EV diagnosis was based on detection of EV RNA in CSF and/or isolation of EV in CSF or detection of EV IgM in serum combined with clinical findings indicative of current EV infection. TBEV diagnosis was based on TBEV IgM activity in serum. The diagnosis of HSV was based on 1) detection of HSV-1 or -2 DNA in CSF 2) recurrence of meningitis without etiological findings and previous meningitis of HSV-2 aetiology verified by CSF-PCR and/or HSV-2 IgG-seropositivity and findings of HSV-2 DNA in current genital blisters and/or 3) current seroconversion of HSV-2 specific IgG. The VZV diagnosis was based on detection of VZV DNA in CSF or current blisters or seroconversion of VZV IgG or IgM as a sign of current infection.

Using the first-line combination of assays an etiological diagnosis was made in 255/419 patients (62 %) Using the extended criteria for EV and HSV diagnosis was verified in additionally 13 + 13 patients (67%). Other viral diagnoses of aseptic meningitis were obtained in additional 15 cases. Overall an etiology was established in 302 (72%) of the 419 patients with aseptic meningitis.

EV was found to be the major causative agent (27%) followed by TBEV (21%) and HSV-2 (19%) and VZV (2%). AAM due to HSV-1 was diagnosed in only on patient based on AAM and HSV-1 DNA detected in concurrent blisters. An etiological diagnosis was not established in 117 cases (28 %). All of them had pleocytosis in the CSF but due to a lack of sample material, adequate testing according to the protocol was not performed in some of them. .

The overall median age was 36 years (range 18 – 88 years). There was a significant difference in age between the EV (32 years) and the TBEV meningitis patients (48 years). The overall male/female percentages were 44 and 56 %, respectively. Females dominated in all groups except in the TBEV group. A seasonal variation was observed in the cases with EV, peaking in autumn, and in the TBEV group with a peak in August. The cases of meningitis of unknown etiologies also showed a seasonal variation with most cases occurring during late summer and autumn while the HSV-2 cases were distributed equally around the years.

In this study the initial intention was to investigate all cases with the proposed analyses. However, since all patients did not follow this protocol initially, some samples had to be analyzed additionally, retrospectively. Furthermore, some samples were missing, especially in TBEV cases where the diagnosis was established by clinical findings and serology. This may have confounded the results but on the other hand the study probably reflects the clinical situation in a relatively proper manner.

The relatively long duration of the study period decreases the impact of temporal variations in the epidemiological spectrum. The incidence of AAM of 7 .4 on average is lower than earlier reports of 10-20 per 100,000 adults. This may partly be explained by the fact that TBEV cases and probably also AAM of other etiologies may have been

treated in other regional hospitals without being referred to our hospitals and the true incidence being higher than we noted.

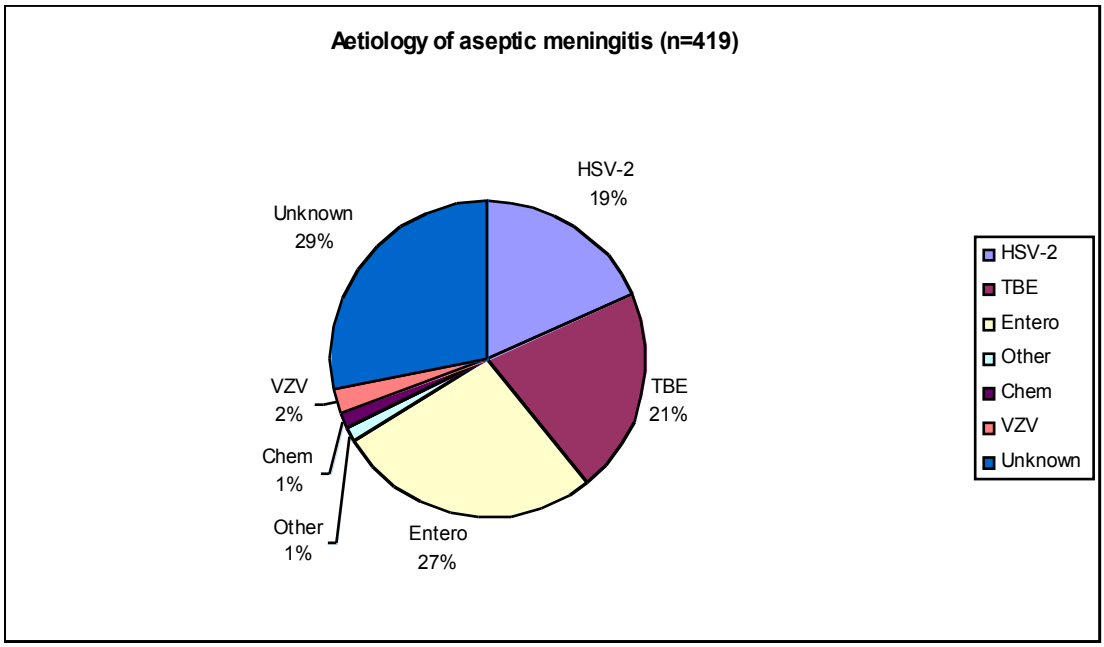


Figure 1. Distribution of aetiology in 419 patients with acute aseptic meningitis and meningoencephalitis 1999-2004 in Stockholm Sweden

The proportion of EV infections is considerably lower than in many other studies. During our study period only a single outbreak of enterovirus occurred (coxsackie B5 in 2000), which may explain the low incidence of EV cases and also contribute to the lower overall incidence. Another explanation to the relatively low EV meningitis incidence could possibly be the fact that most of the individuals in this group may have been attending day-care centers in childhood and thereby been previously exposed to EV infections. This may have resulted in a greater heard immunity against EV among them is in line with earlier publications with a notable lower incidence due to acquired immunity among elderly.
In the figure 2, the age distribution in relation to different etiologies, during 6 years period is presented.The EV diagnosis showed the highest peak in the age group 25-34 years old.

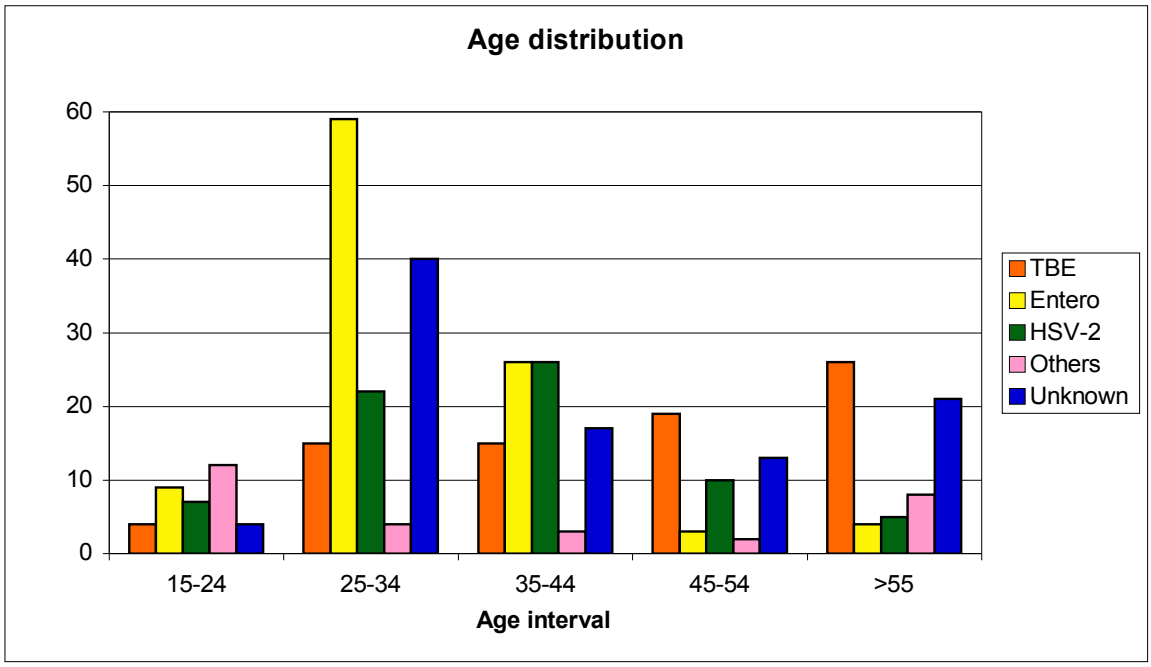


Figure 2. The age distribution during 1999-2004, of 419 patients with acute aseptic meningitis in relation to age distribution.

A higher proportion of females were found in our study compared to earlier studies. His may be partly due to our finding of a relatively large proportion of HSV-2 cases among which a female predominance is well known.
In a large proportion, almost one-third, the cause of meningitis remained unknown.

A relatively high diagnostic yield was found in our study. This was mainly obtained by the limited combination of assays for EV, HSV and TBEV diagnosis. This rendered the diagnosis in the vast majority of the etiologically diagnosed cases (255/302, 84.4%). The relatively high prevalence of TBEV in the Stockholm region and the high sensitivity of the TBEV IgM test and an acceptably high sensitivity of CSF-PCR for EV as well as awareness of HSV-2 as a cause of meningitis also without blisters and better HSV diagnostic assays could all have contributed to this outcome.

Still, the cause remained unknown in a large proportion, almost one third, of the cases. This may in some cases be due to lacking CSF and/or serum samples. However, adequate sampling and assays were performed in about three-quarters of the patients with an unknown diagnosis. EV diagnosis in many of the undiagnosed cases could possibly be indicated by age, and seasonal variation. Enteroviral RNA seems to be cleared from CSF relatively quickly after infection. If the patient presents late in the course of disease an EV diagnosis may be obtained by analyzing fecal samples by virus isolation or PCR, which was not performed in this study. It is possible, or likely, that the EV yield and total etiological outcome would have been greater if virus isolation and EV PCR on fecal samples, and EV IgM tests, had been performed routinely. Although HSV-2 diagnosis was found in a substantial percentage of cases in the present study, by means of clinical findings combined with serology, some cases may have

remained undiagnosed. This may be indicated by the female predominance in the undiagnosed group where 61 % were women and 39 % men, as HSV-2 meningitis normally exhibits a strong female predominance. Detection of TBEV-specific IgM antibodies in serum is a sensitive tool in the meningoencephalitis phase and it is unlikely that many TBEV cases that were investigated remained undiagnosed in our study. As only a limited number of samples were investigated by CSF-PCR for VZV, some VZV cases may be hidden in the group with unknown aetiology.

The priority of extensive and expensive laboratory investigations may be questioned with the limited availability of antiviral therapies. The clinical picture of AAM may initially imitate that of acute bacterial meningitis, neuroborreliosis, tuberculosis meningitis or severe HSV encephalitis, which all requires prompt and adequate treatment. Thus it is important to distinguish these severe but treatable diseases from AAM and mild meningoencefalitis. In the clinical situation the etiological diagnosis may help in the exclusion of a severe and treatable cause of CNS infection. This may contribute to minimizing unnecessary examinations, antibiotic treatments and days of hospitalization. Furthermore etiological diagnosis of AAM is of benefit per se, especially in cases with prolonged recovery or complicating events. The patient may be informed about the prognosis and uncertainty avoided.
With our proposed first-line assays the cost is within an economically justifiable range in view of the high diagnostic yield. We propose that CSF-PCR for EV and HSV-1 and -2 and TBEV serology should be performed in all cases of AAM if a diagnosis is not pinpointed by the clinical findings. HSV-1 should be included although it rarely causes meningitis because it may cause severe encephalitis that requires rapid antiviral treatment. Further development of cost-effective diagnostic methods to explore the causes of the still vexingly large proportion of cases with an unknown aetiology remains a challenge.

PAPER III

The aim of this study was to investigate and characterize the immune response of patients presenting with their first episode HSV genital infection and to distinguish whether immune markers correlation with frequency of recurrences during a follow up period could be found.

A change in the epidemiological pattern of primary genital herpes simplex infection has been reported in United Kingdom and Scandinavia with HSV-1 being increasingly recognized as a cause of genital herpes (Ross JDC 1993, Tayal SC 1994, Löwhagen 2002). Traditionally HSV-2 accounted for the majority of genital HSV cases with a prevalence of 20-30% in adult population (Forsgren 1994). However, the vast majority of both orolabial and genital HSV infections are asymptomatic (Spruance 1984, Wald 1995). For those who experience symptoms at the first episode, the clinical picture ranges from a severe infection characterized by general symptoms and extensive, painful, genital and perigenital ulcerations to a mild illness with minimal localized vesiculation and ulceration. The severity of symptoms associated with genital herpes is dependent on several factors including viral load, breach of genital mucosa, previous exposure to HSV and genetic factors determining epidermal cell and immune restriction of viral replication. Severe first episodes are more likely to occur when the

individual has never previously been exposed to either virus. Individuals who have previously been exposed to and acquired the other HSV-type tend to have less severe first episode (non-primary episode).

The first challenge for this study was to properly classify the patients. Although none of these patients had experienced previous clinical symptoms suggesting genital HSV infection (and they thus had "clinically primary infections"), it became evident that some had acquired a sub clinical infection prior to this occasion. The patients were divided in three groups after a thorough characterization based on virus detection and type-specific serological status: primary HSV-1 infection (n=8, primary HSV-2 infection (n=9) and non-primary HSV infection (n=12). The non-primary HSV-2 infection group included patients with prior HSV-1 infection as well as patients already carrying HSV-2, which had previously been latent but now reactivated causing first recurrence and first episode symptoms. Four patients were excluded from the study since they could not be classified generally since virus detection had not been performed. One patient, with HSV-1 virus detected in genital blisters and medical records of a clinically well defined infection, was excluded since he did never develop HSV-antibodies or specific cell-mediated immune response during twelve months follow up period. This patient even experienced a recurrence of symptoms (or a new infection?) 2 months later according to medical records. This patient was treated at two occasions with early initiated antiviral therapy according to standard procedures at the clinic.

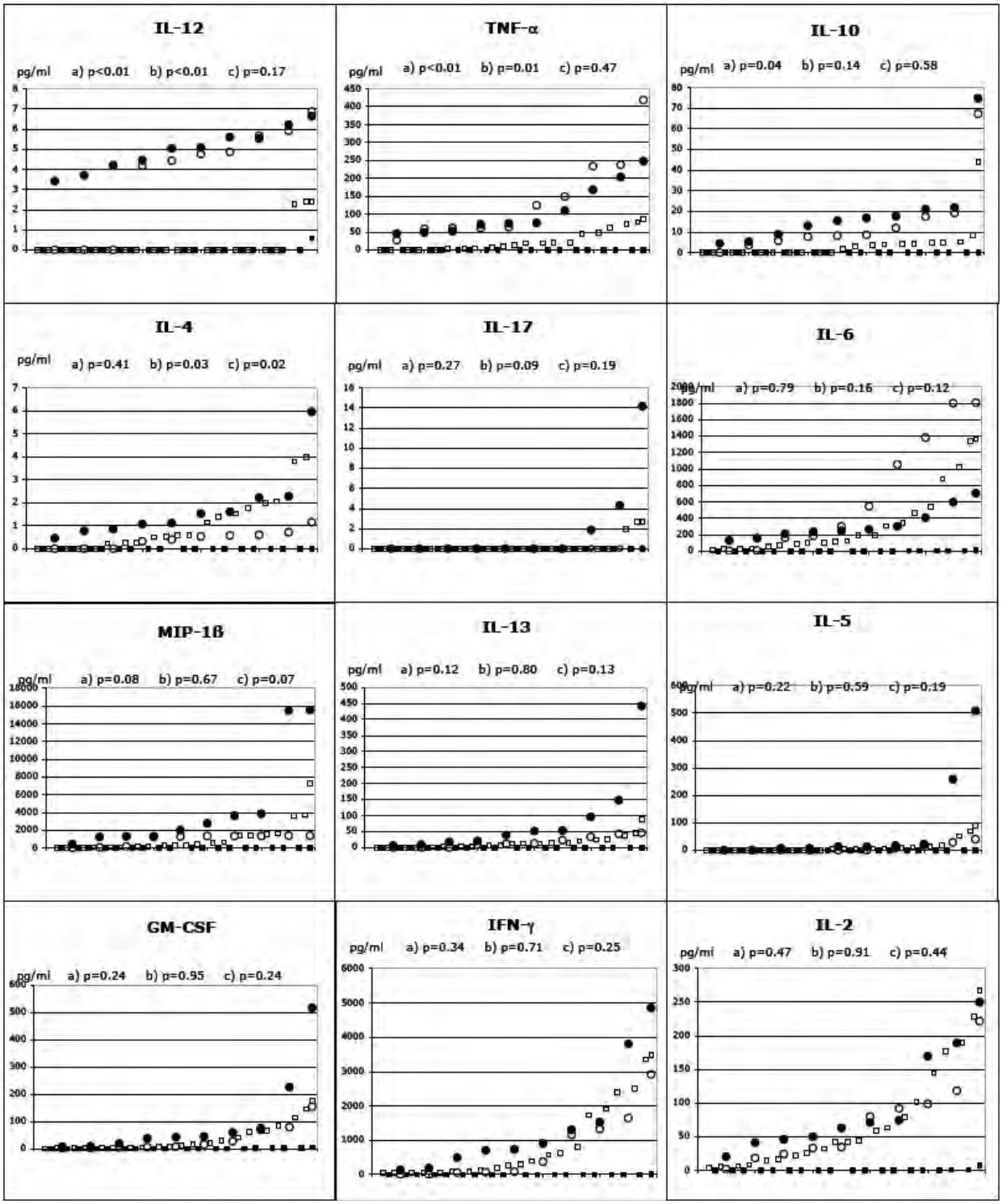
The time-points for follow-up visits with sampling were chosen with regard to a previously published article on proliferative answer in HSV infections (Corey 1983). Our results showed that a strong HSV-specific cell mediated response (CMI) measured as $CD4^+$ blasting and secretion of cytokines developed early in the course and often before appearance of detectable HSV-antibodies in primary HSV infection. Peak responses were detected already during symptoms of acute illness and then declined over time. This was particularly evident in patients with HSV-1 infection probably due to low frequency of recurrences and fewer episodes of antigenic re-stimulations. Patients with non-primary HSV tended to display lower CMI during acute illness but response was maintained and did dot significantly change during the subsequent year We also studied and characterized the correlation between specific T-cell responses and the frequency of reactivation following a primary genital HSV infection. The frequency of recurrences during the follow-up year was clearly lower for patients with primary HSV-1 infection ($p<0.02$) whereas the other two groups recurrence rate were not significantly different. Among patients with non-primary infection, the recurrence rates appeared not to differ between those previously carrying HSV-1, HSV-2, or both, but too few subjects were studied to detect significant differences.

We employed Luminex analysis by which we were able to simultaneously detect levels of several cytokines. Patients with primary infections generally display a vigorous immune cytokine response already at first visit. A clear correlation between level of cytokine produced at first visit and number of recurrences experienced over the year was observed: the more cytokine produced, the less recurrences in particular for IL4, IL10 and MIP1β. The responses declined with lower cytokine levels and fewer $CD4^+$

T-cell blasts in responses recorded during the follow-up period. This was particular evident for many cytokines in patients with primary HSV-1 infection. This may be due to fewer recurrences and thus leading to less antigenic stimulations for the immune response. By contrast, the HSV-specific cytokine and proliferative responses did not decline with time in patients with non-primary HSV-infections.
Il10 has been described as a regulatory cytokine able to suppress immune responses to reduce immune-pathological effects and tissue damage caused by excessive immune activation (Suvas S 2004). Il4 is another anti-inflammatory cytokine which is able to induce differentiation of naïve T-cells into Th2 cells and is secreted in large amounts in patients suffering from allergic diseases. Eriksson et al reported that patients with recurrent HSV-2 infection responded to HSV-2 with lower levels of IL4 and IL10 than subjects with asymptomatic HSV-2 infection (Eriksson 2004). Their and our results could indicate that HSV-2 specific memory cells producing high levels of IL4 and IL10 can mediate better protection against symptomatic recurrence than memory cells with different cytokine profiles.

The HSV-specific IP10 responses recorded for patients with non-primary infection were significantly higher at all three time-points compared to patients with primary HSV infection. However, the IP10 response increased for patients with primary HSV-1 infection over the year in contrast to the decline recorded for other chemokine and cytokines. IP10 can be induced by many different stimuli such as IFNγ, IFNα and certain viruses. MIP1β is a chemokine secreted by both T and NK cells, and has been shown to posse antiviral capacity by binding to a protein on visions and generating pores in the viral envelope (Nakayama 2006). MIP1β may also be important for viral clearance by recruiting leucocytes and by killing of virus.

The frequency of recurrences following primary HSV-2 genital infection may at least partly be predicted by an early HSV-specific IL-4 and IL-10 response. Similar but not as strong, correlations were also found for patients with primary HSV-1 infection, which could be due to the limited numbers of recurrences in this group. In conclusion, the early appearing HSV-specific CMI in primary genital HSV infection may determine the clinical future and the particular host-agent relationship between HSV and man. However, our results are only valid for clinical recurrences. It is possible that all our patients sub clinically did shed virus similarly, or even that our patients with less clinical recurrences in fact had more biological recurrences and shed more virus. Such a relation could be advantageous for the host, as he or she would be free of symptoms. On the other hand, the infected person would probably then more easily transmit the virus to other persons.



Cytokine levels in cultures with HSV-1 nucleo capsids and whole blood from HSV-antibody negative (n=19, closed squares) and HSV-antibody positive (n=21, open squares) healthy blood donators; and from patients with recurrent genital HSV infections (n=10, open circles) and recurrent HSV meningitis (n=10, closed circles). Results for each group were sorted ascending anddisplayed with distances inversely correlated to the number in each group to facilitate comparison over the whole range and Student's t-test was employed to compare HSV-antibody positive donators with a) recurrent HSV meningitis, and b) recurrent genital HSV; and for comparison of c) the two groups of patients.

PAPER IV

In this paper we focused on the HSV-specific immune responses in patients with recurrent HSV-2 meningitis, a clinical syndrome that has become more frequently recognized during the last decade, but with yet no studies reported and information available on the immunologic aspects. We included 10 such patients and compared them with 10 patients with recurrent genital HSV-2 infection and also 21 HSV seropositive and 19 HSV seronegative healthy blood donors.

Meningitis is usually presenting as a complication of primary genital infection generally thus appearing only in those previously seronegative for HSV-1. The first episode of HSV-2 meningitis usually appears within 2 weeks from a primary genital infection. Corey at al found that symptoms suggestive of aseptic meningitis were observed in 36% of the women and 13% of the men in 268 studied patients with primary episode of genital herpes type 2 infections. A female predominance is striking in HSV-2 meningitis with a female-to-male ratio of about 2:1-6:1 reported (Corey 1983, Read 1999, Franzen-Röhl 2008). In recurrent meningitis infections, half of the patients experiences painful lesions prior to, or at the time of recurrent meningitis (Gonzales 2003, Franzen-Röhl 2007) suggesting that recurrences occur at both sites simultaneously.

In our study we found that patients with recurrent HSV-2 meningitis had elevated T-cell blasting and cytokine response against HSV-antigens during clinical remission. Perhaps this higher HSV-CMI, as compared with patients with recurrent genital or asymptomatic infection is due to a low grade but persisting activation and “boosting” presentation of HSV-2 virus from sacral ganglion or perhaps this site provides stronger stimulation to the immune system than do HSV-2 localized elsewhere.

In our search for suitable candidates for our study we also came across a small group of asymptomatic seronegative partners (4), to our frequent recurrent genital HSV patients. Why did these individuals remained seronegative, after a medium of eight years of matrimonial experiences? Did they possess something extra or something less? What is the secret for their protection against herpes simplex virus infections? Could possibly HSV neutralizing IgA in the genital tract (Hirbod 2006), equivalent with the findings of protective mucosal immune responses against sexual HIV acquisition, be applicable also to HSV infections? We tested these four seronegative partners but could not detect any HSV-specific CMI in contrast to Corey et al, who recorded such responses in 4 of 10 seronegative partners.

In a recent study it was showed that HSV-2 infected persons were found to genitally shed HSV-2 during frequent and short periods of reactivations but the majority of these periods of activation were subclinical (Mark KE 2008). A critical role for the local mucosal immune system was suggested in prevention of clinical symptoms (Zhu 2007). Do patients with recurrent HSV-2 meningitis also present intermittent genital shedding from reactivation in the sacral ganglia followed by neuronal spreading? This has not yet been investigated in HSV-2 patients.

Our patients with recurrent HSV-2 meningitis displayed, during remission, elevated HSV-specific proliferative responses and cytokine production (see figure), in particular regarding Th2 cytokines in combination with increased TLR3 and TLR9 expression on dendritic cells as well as increased IFN-a production by TLR3 agonist compared to asymptomatic and healthy HSV-2 carriers. Why? Are the elevated immune responses a consequence of strong and repeated stimulation and presentation of the enemy for the immune system over and over again? And is the possible deviation of the immune response into a Th2 profile there from the beginning, and in part responsible for the clinical result and recurrent meningitis, or is that deviation a later phenomenon reflecting an immune response which is maturing into a profile that will be more suitable for the infected person? Or – could the strong responses be, not the effect, but in part also the cause of the clinical consequence? Is it possible that the meningitis is less the effect of the pathogen virulence and more the result of a too active immune response, which has not been, regulated enough by the system?

These questions may also be relevant for another clinical situation regarding treatment of patient with recurrent meningitis. Continuous prophylactic antiviral treatment to these patients is overall beneficial by reducing numbers of recurrences and improving quality of life. But when such treatment is discontinued, the patients often immediately relapse and then experience more frequent recurrences and more severe symptoms than before the treatment was initiated. This is of course probably due to viral reactivation because of loss of therapy-mediated viral control but another explanation could be that the activated enemy in turn activates the virus-specific immune forces, and that it is the forces more than the enemy that is causing the clinical illness? Could hyper immune reactions be involved in the pathogenesis of recurrent HSV-2 meningitis?

To the best of our knowledge, this study was the first on HSV-specific immune responses in human recurrent HSV-2 meningitis. More studies are clearly needed of both primary HSV-2 meningitis and recurrent HSV-2 meningitis in greater detail. As the incidence of primary HSV-2 infection in previously HSV-naïve persons appear to be increasing, the incidence of meningitis and the prevalence of recurrent meningitis will probably also increase and new and more strategies for clinical management will be needed.

CONCLUSIONS

Paper I

1) The sensitivity of real-time PCR for the diagnosis of HSV-2 meningitis was found to be high, though somewhat lower in recurrent infection than primary infection.
2) The viral load of HSV-2 DNA in CSF was significantly larger and the inflammatory changes in the CSF more pronounced in primary meningitis than in recurrent.

Paper II

1) A consistent use of CSF-PCR for EV and HSV, and TBEV serology established a diagnosis in the majority of 419 acute aseptic meningitis patients (62%).
2) Enteroviruses (27%) were the most prevalent etiological agent of aseptic meningitis during a 6-years period in Stockholm, followed by TBE (21%), HSV-2 (19%).

3) Using extended sets of assays for viral detection, established a diagnosis of acute aseptic meningitis in 302 patients (72%).

Paper III

1) We found an inverse correlation between numbers of recurrences and cytokine response at day of inclusion in patients with primary HSV-1 and HSV-1 infection.
2) In patients with primary HSV infection CMI responses declined over time, whereas patients with non-primary infection displayed stable CMI during follow-up year.
3) The frequency of recurrences following primary HSV-2 genital infection may partly be predicted by an early HSV-specific IL-4 and IL-10 response.

Paper IV

1) Patients with recurrent HSV-2 meningitis had elevated T-cell blasting and cytokine response against HSV-antigens during clinical remission.
2) Patients with recurrent HSV-2 meningitis had elevated Th2 cytokine production, somewhat increased NK-cell response and increased TLR 3 and 9 as well as TLR induced interferon response during clinical remission.

CONCLUDING REMARKS:

Thanks to the fact that my PhD studies have involved clinical, virological and immunological aspects of the herpes simplex virus, I've been introduced to a broad palette of the research field.

Herpes virus infections usually don't manifest themselves as severe clinical problems only in rare situations and could therefore be considered of peripheral interest to us compared to the" big" diseases like tuberculosis, HIV or malaria.

In order to be able to understand the nature of the HSV virus as well as given the excellent opportunity to study the host response to viral infections, HSV creates a wonderful research model. In my research I had the privilege to take part of frontier research on the adaptive and the innate immune response to viral persistent infections in humans, a truly dynamic, stimulating and expanding field.

Herpes viruses definitely serve as an endless source of sophisticated proteins that interact with cellular counterparts and teach us about the virus as well as their host. In the process of an invading persistent viral infection, various mechanisms are involved to shape the immune system in favor of the virus. Some of these pathways overlap and one immunological pathway may not be enough to control virus.

During viral persistence, the balance between the virus and host immune response is crucial. The immune system keeps the virus in check and the virus counters by evading the immune response to avoid clearance, sometimes by forcing the balance in favor of the virus thereby causing disease in majority of cases. In most persistent infections like the herpes family the virus creates latency and hid in host for later reactivation, the continuous presence of the viral antigen renders virus-specific T cells to become somewhat dysfunctional. These differences can range from severe functional impairments, to more subtle changes in the light of infections with a lower virus burden. Recent work in various animal models has shed light on immune regulatory molecules or cytokine that affect viral persistence and/or T-cell function and interventions that modulate these factors.

Little has yet been evaluated on humans in the field of HSV-2 infection in peripheral blood cells. In our study of the proliferative cell-mediated immune response and cytokine profile of patients with first period genital herpes infections we have been able to distinguish specific patterns. Such research will open up for new possible avenues for immunotherapeutic interventions though this must be applied with care and great understanding, because they may present unintended side effects.

In our studies of recurrent meningitis an increased level of T-cell response was found along with increased expression of TLRs. Such disturbances are perhaps understood in terms of imbalance of T-cells that leads to predominance in the relative contribution of either Th1 or Th2 cytokines. This may lead to organ-specific autoimmunity or allergies and certain other diseases with Th2 predominance.

Further research need to focus on the nature of the immune response against persistent viral infections to improve our understanding of viral control and pathways that can be targeted for possible intervention. Possibly by developing immune modulators, new antiviral drugs, protective antiviral antibodies and/or therapeutic vaccines. This will lead to future advancement in the field of immune regulatory treatments beneficial to all patients suffering from persistent virus infections.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Diagnos av akut viral hjärnhinneinflammation och immunförsvaret vid herpes simplexvirus infektioner

Bakgrund: Infektioner med herpes simplexvirus (HSV) är utbredda i samhället. Herpes simplex Typ 1 (HSV-1) och Herpes simplex Typ 2 (HSV-2) kan båda infektera mun eller genitala slemhinnor. Viruset stannar kvar i kroppen efter en första infektion och kan sedan återkomma med skov av smärtsam sårbildning. Efter symptomgivande genital herpes får hela 90 % (recidiv) återfall under första året och >40 % får >6 nya skov. Det är oklart varför en del individer blir recidivfria och tycks ”kontrollera” infektionen medan de flesta drabbas av upprepade attacker. Sannolikt behövs att ett effektivt immunsvar utvecklats och detta kan i sin tur bero på vilket stöd T-lymfocyterna får från det medfödda immunsvaret. HSV-2 kan också orsaka neurologiska symptom, framför allt hjärnhinneinflammation. Idag är 30 % av alla icke-bakteriella hjärnhinneinflammationer av okänd natur. Det finns ett behov av effektiva och känsliga diagnostiska metoder i handläggningen av akuta virusinfektioner i centrala nervsystemet.

Delarbete I Vi har utvärderat en ny diagnostisk metod och har funnit en ökad känslighet vid analys av HSV-2 orsakad hjärnhinneinflammation. En s.k. realtids-PCR (RT-PCR) för detektion av HSV DNA och Varicella-Zoster virus (VZV) DNA i ryggmärgsvätska och som nu utvärderats på ett stort kliniskt välkarakteriserat patientmaterial. Känsligheten befanns vara 87 % vid första gångs infektion och 70 % vid återfall av HSV-2 hjärnhinneinflammation. Virus mängden av HSV-2 DNA i CSF befanns vara signifikant högre hos patienter med primära hjärnhinneinflammationer jämfört med sekundära. VZV detekterades i 4,4 % av fallen.
(Franzen-Röhl E, Tiveljung-Lindell A, Grillner L, Aurelius E, J Clin Microbiol 2007).

Delarbete II På Danderyds sjukhus och Karolinska Universitetssjukhuset Solna samlades prov in på alla vuxna patienter som sökte med kliniska symptom på akut icke bakteriell hjärnhinneinflammation under åren 1999-2004. Proven analyserades med PCR på CSF för herpes simplex virus (HSV) DNA och enterovirus (EV) RNA samt ELISA IgM i serum för påvisande av tick-borne-encephalitis virus. Utvärderingen av den effektiviserade analys kombinationen verifierade agens hos 255 av 419 (62 %) patienter med icke-bakteriell hjärnhinneinflammation. EV(27 %) utgjorde den största etiologiska gruppen av hjärnhinneinflammation, följd av TBEV (21 %) och HSV-2 (19 %). Med hjälp av utvidgade analyser diagnostiserades sammanlagt 302 patienter (72 %) med hjärnhinneinflammation. (*Elisabeth Franzen-Röhl, Kenny Larsson, Eva Skoog, Annika Tiveljung-Lindell, Lena Grillner, Elisabeth Aurelius, Martin Glimåker; Scandinavian Journal of Infectious Disease, 2008; 914-921)*

Delarbete III Den kliniska bilden av HSV-2 infektion innefattar vanligen återkommande genitala blåsor och ibland även symptom på hjärnhinneinflammation och dessa individer lider av återkommande episoder av dessa manifestationer. I Sverige finns 200-400 individer med diagnosen ”återkommande HSV-2 hjärnhinneinflammation”. Immunologiska brister kan vara inblandade i dessa sjukdomsuttryck. Patientproven analyseras med FASCIA, en ny metod för storskalig lymfocytstimulering och bestämning görs av det specifika proliferativa T-cellssvaret med FACS. Naturen av detta svar karakteriseras genom analys av cytokin profilen. Vi studerade också vilka receptorer som uttrycks och aktiveras på Natural killer - cellens yta. Patienter med återkommande HSV-2 hjärnhinneinflammation hade förhöjt T-cells produktion samt förhöjda cytokin nivåer vid en HSV-antigen stimulering. T-cells respons är ökat hos patienter med hjärnhinneinflammation med ett samtidigt ökat uttryck av Toll-like receptors 3 och 9 (TLR). Således bedömer vi att HSV-2 hjärnhinneinflammation inte är orsakad av immunologiska funktionella brister i det förvärvade eller medfödda immunförvaret.

Delarbete IV Under det sista årtiondet har ett ökat antal rapporter från USA och Europeiska länder visat på ett ändrat epidemiologiskt mönster hos genital herpes. Kliniska och immunologiska data insamlades från 29 patienter som följdes under ett år efter sin första episod av genital herpes eller hjärnhinneinflammation. Det kliniska förloppet följdes och blodprov togs under akutskedet, efter 8 veckor och efter 12 månader. Svaren jämfördes med svaret hos individer med icke symptomgivande infektion (anti-kroppspositiva individer). Antalet minnesceller som svarar på HSV-specifikt stimulus (CMI) detekteras med FACS. Dessutom gavs cytokinprofil på svarande celler genom mätning av olika cytokiner. Hos patienter med första gångs infektion av herpes simplex virus infektion sjönk cellförmedlade immunsvaret över tid, emedan de med ”återkommande” infektioner uppvisade ett stationärt cellmedierat svar. För patienter med första gångs HSV-2 infektion, var nivåer av HSV-specifikt IL-10 och IL-4 svar vid första besöket signifikant invers korrelerat med antal återfall under det följande 12 månaderna. Frekvensen av antalet återfall efter första episoden av genital HSV-2 infektion kan delvis förutsägas genom ett tidigt IL-4 och IL-10 svar.

Betydelse: Våra studier innebär förbättrad kunskap om handläggning patienter som söker med kliniska symptom på en akut icke-bakteriell hjärnhinneinflammation. Det är viktigt att förstå att individer inte behöver uppvisa svåra defekter med total avsaknad av en immunologisk celltyp för att ha nedsatt försvar mot en viss virusinfektion. Våra immunologiska studier ger möjlighet att arbeta vidare med att identifiera individer med benägenhet för svår sjukdom. Därigenom får vi underlag för rådgivning och ställningstagande till antiviral behandling och i förlängningen, möjlighet till framtagandet och användning av immunmodulerande behandling och ev. ett vaccin.

ACKNOWLEDGEMENTS

My wish to express my sincere gratitude to all who helped me during these studies:

My dear husband **Hannes** and to my children **Daniel, Samuel, and Gabriel, Maria** and **Isac**, for your love and tremendous joy and simply by being the greatest gift in life. You remind me of the eternal questions and I am truly blessed. Thank you for being supportive and patient during my research especially these last months.

None of these studies would have been possible without the participation of all the patients. So I am sincerely grateful for everyone's trust and endurance. Without your history and willingness to share, this work would not been possible. Also a special thank you to those of you that willingly and enthusiastic volunteered as control group.

My sincere gratitude to:
Elisabeth Aurelius, my main supervisor, the head of the CNS section at the infectious diseases clinic at Karolinska University hospital, Solna, who encouraged and introduced me to this work. She also shared her devotion for the patients and wide knowledge of viral infections in the central nervous system. Thank you also for fruitful comments on my manuscripts and sharing your experience in both science and practical work. It has indeed been adventurous!

Hans Gaines, my co-supervisor, head of the section for clinical immunology at SMI for your unique scientific competence and for your untiring encouragement and patience during the latter part of the studies. For sharing your "addiction" to research and science and for creating the best working atmosphere, always having time for stimulating discussions. Also, immensely grateful for the inspiring conversations at lunch and generous practical support.

The co-authors and collaborators at MTC: Klas Kärre, Louise Berg and Danika Schepis **(**MTC), for sharing their expert knowledge, especially on the innate immunity field and overall scientific guidance. For valuable discussions and good spirit during our work. (Also, wonderful box plots).

Friends and collaborators at the dept. of immunology and vaccinology (IVA), Smittskyddsinstitutet. Kristina Franck and Fredrik Atterfelt, Lena Andersson, Mehrdad Monsavi-Jazi, Teghestie Tecleab, Johan Brännström, Maria Widfeldt, Petra Jones and Maria Lagrelius for outstanding technical skills, working capacity and invaluable help. The head of IVA, SMI, Rigmor Thorstensson, for providing outstanding laboratory resources and overall support and enthusiasm for research.

Special thanks to the head Harry Beitner and all personal at the Dept of Dermatology and Venerology, SESAM, Solna, Huddinge and City for enthusiastic work in the inclusion of patients. Especially thanks to three fantastic nurses: Mona Enander, Elisabeth Klingsell and Elisabet Wennberg at SESAM, Solna. This study could not have been accomplished without your devotion and personal efforts.

The co-authors for excellent scientific knowledge and comments on the manuscript along with the encouragement for this project, especially to Martin Glimåker, Annika Tieveljung-Lindell, Arne Vikström and Lena Grillner and Jan-Åke Liljeqvist.

Markus Maeurer for sharing his tremendous knowledge in the clinical and immunologic field but also in a spiritual way.

Kristina Broliden, my mentor, you always managed to find solutions to every problem along the road like a guarding angel.
The FoUU group at my clinic for supporting me during my research years.
Kjerstin Björkholm for kindly helping me when needed.

Anna Törner for being able to explain complicated statistical methods in an easy comprehensive way. Cécile Everett for excellent layout and computer management.

The present and former heads of the Unit of Infectious Diseases at Karolinska University hospital Solna for support and providing necessary working conditions. Especially Åke Åkesson who granted me the necessary time to start my PhD training in 2006.

Friends and colleagues at the Department of Infectious Diseases at Karolinska University Hospital Solna, for the friendly, open, positive atmosphere and for sharing ups and downs of the everyday work at the hospital with me. Thank your for supportive laughs and open minded, constructive analysis. We still have something special….

Ann Atlas, my colleague and roommate for your loyal friendship, encouragement and endless discussions, sharing with me this new world of wonders and possibilities. Lennart Östlund for wonderful chats and excellent time-management and support of my research! Ami Lexmark, the anchor of the Infectious Diseases clinic.

Mrs. Leslie Ducey, Richmond Indiana, my American sister, for excellent linguistic revision on my thesis. You are the best!

Eva Noren, my oldest friend and personal coach. For creative, inspiring discussions on a professional level but also for practical solutions to big and small problems.

Frida & Benjamin and the ”Waljansons” for being free spirits and also my extended family along with excellent Mahjong partners, thus being important distractions from research during late nights.

For all the relatives and friends of my family including for being inspiring and wonderful people and together you are the essence of my own Universe.
The End is definitely the Beginning!

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