Viral proteins as serological antigens

Development and clinical applications

Linn Persson Berg

Department of Infectious Diseases
Institute of Biomedicine
Sahlgrenska Academy, University of Gothenburg

UNIVERSITY OF GOTHENBURG

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Linn.persson.berg@gu.se

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To my family with all my love

Lycka är att få vara med er!

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ABSTRACT

Serological methods are based on the detection of antibodies and antigens in mainly serum but also in other body fluids such as cerebrospinal fluid (CSF). Conventional whole virus antigens are widely used in viral serological assays. These antigens usually contain a mixture of proteins from the virus of interest together with residual cell components from antigen production, which can cause diagnostic problems with cross-reactive antibodies between closely related viruses and antibodies that bind to cellular components. The methods can become more specific by using antigens based on recombinant single viral proteins that differ between closely related viruses but to which the immune system reacts strongly (immunodominant proteins).

The aim of the research has been to develop specific serological assays to detect antibodies to varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and measles virus (MeV). This has been accomplished by recombinantly producing single, specific, immunodominant viral proteins, VZV glycoprotein E (gE), EBV glycoprotein 350 (gp350) and the core part of the MeV nucleocapsid protein (N_{CORE}), for use as serological antigens in enzyme-linked immunosorbent assay (ELISA).

In **Paper I**, we show that VZVgE functions well as ELISA antigen to detect anti-VZVgE IgG antibodies. The antigen has thereafter been used in the routine diagnostics at the Department of Clinical Microbiology, Sahlgrenska University Hospital. In **Paper II**, we demonstrate that EBVgp350 performs well as serological antigen in ELISA for the detection of anti-EBVgp350 IgG. In **Paper III**, we found that patients with multiple sclerosis (MS) and their clinically healthy siblings with similar MS findings in CSF, i.e. a suspect hyperimmune phenotype, still show an increased IgG response to MeV in both serum and CSF compared with healthy controls when the previously used complex MeV whole virus antigen was replaced with MeV N_{CORE}. Our results

indicate that the reactivity is indeed specific and not caused by cross-reacting autoantibodies to cellular proteins. In **Paper IV**, patients with MS show higher IgG levels in both serum and CSF to MeV and EBVgp350 compared with healthy controls. In addition, we observed that patients with serologically verified acute infectious mononucleosis have higher serum IgG levels to EBVgp350 at follow-up after 10 years compared with healthy controls, suggesting that EBV-induced mononucleosis affects the immune system in a powerful and long-lasting way.

In **Paper V**, patients with MS treated with interferon beta (IFNβ) had higher anti-EBVgp350 and anti-MeV N_{CORE} IgG levels in serum compared with healthy blood donors. Following initiation of treatment with the monoclonal antibody natalizumab, patients' serum IgG levels decreased against both antigens, whereas levels were relatively stable during previous IFNβ treatment. Another finding was that all 728 patients with MS in the study were EBV IgG seropositive while 10 of the 144 blood donors in the control group were EBV IgG seronegative. This finding further strengthens the potential role of EBV in the pathogenesis of MS.

The developed ELISA methods can, through increased specificity, offer new diagnostic possibilities for detecting antibodies to EBV, VZV and MeV in viral infections, for control of immunity after infection/vaccination, in epidemiological investigations and in autoimmune diseases such as MS.

Keywords: Serology, ELISA, IgG, varicella-zoster virus, Epstein-Barr virus, measles virus, viral glycoproteins, multiple sclerosis

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SAMMANFATTNING PÅ SVENSKA

Serologiska metoder baseras på påvisning av antikroppar och antigen i främst serum men även i cerebrospinalvätska (CSV). Många virusserologiska metoder använder helvirusantigen som består av flera olika proteiner från viruset samt kvarvarande cellulära komponenter från antigenproduktionen. Helvirusantigener kan ge upphov till diagnostiska problem med korsreaktiva antikroppar mellan närbesläktade virus och antikroppar som binder till cellulära komponenter. Metoderna kan bli mer specifika genom att använda antigen baserade på enstaka virala proteiner som skiljer sig från närbesläktade virus men som immunförsvaret reagerar starkt emot (immundominanta). Syftet med vår forskning har varit att utveckla specifika serologiska analyser som kan användas för att påvisa antikroppar mot varicella-zoster virus (VZV), Epstein-Barr virus (EBV) och mässlingsvirus (MeV). Sådan förbättrad antigenproduktion har utförts genom att rekombinant framställa enstaka, specifika, immundominanta virusproteiner, VZV glykoprotein E (gE), EBV glykoprotein 350 (gp350) och MeV nukleokapsid protein (N_{CORE}), som serologiska antigen i enzymbunden immunosorbentanalys (ELISA). **Delarbete I** visar att VZVgE fungerar bra som ELISA antigen. VZVgE har därefter kunnat användas i rutindiagnostiken på Mikrobiologen, Sahlgrenska universitetssjukhuset. I **Delarbete II** så talar resultaten för att EBVgp350 fungerar bra som serologiskt antigen i ELISA. **Delarbete III** fastslår att patienter med multipel skleros (MS) och deras kliniskt friska syskon med MS-liknande fynd i CSV, dvs en misstänkt hyperimmun fenotyp, har ett ökat IgG-svar i både serum och CSV mot MeV jämfört med friska kontroller även när det tidigare använda komplexa MeV helvirusantigenet bytts ut mot MeV N_{CORE}. Resultaten indikerar att reaktiviteten är specifik och inte kan förklaras av korsreagerande autoantikroppar. I **Delarbete IV** uppvisar patienter med MS högre IgG-nivåer i serum och CSV mot MeV och EBVgp350 jämfört med friska kontroller. Resultaten visar även att patienter som haft mononukleos (körtelfeber) har högre IgG-nivåer mot EBVgp350 i serum vid uppföljning efter tio år jämfört med friska kontroller, vilket indikerar att EBV orsakad mononukleos påverkar immunförsvaret på ett kraftfullt och långvarigt sätt. I **Delarbete V** uppvisar patienter med MS under interferon beta (IFNβ) behandling högre IgG-nivåer i serum mot EBVgp350 och MeV N_{CORE} jämfört med friska blodgivare. Efter behandlingsstart med den monoklonala antikroppen natalizumab sjönk patienternas IgG-nivåer mot bägge dessa antigen, medan nivåerna var relativt stabila under den föregående IFNβ-behandlingen. Alla 728 patienter med MS i studien var EBV IgG-seropositiva medan 10 av de 144 blodgivarna i kontrollgruppen var EBV IgG-seronegativa. Detta fynd stärker ytterligare EBV:s potentiella roll i patogenesen bakom MS. De här utvecklade ELISA-metoderna kan genom ökad specificitet erbjuda nya diagnostiska möjligheter för att påvisa antikroppar mot EBV, VZV och MeV vid virusinfektioner, vid kontroll av immunitet efter infektion/vaccination, vid epidemiologiska undersökningar och även vid autoimmuna sjukdomar såsom MS.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Thomsson E, **Persson L**, Grahn A, Snäll J, Ekblad M, Brunhage E, Svensson F, Jern C, Hansson G.C, Bäckström M, Bergström T. *Recombinant glycoprotein E produced in mammalian cells in large-scale as an antigen for varicella-zoster-virus serology.* Journal of Virological Methods. 2011;175(1):53-9.

II. **Persson Berg L,** Thomsson E, Hasi G, Bäckström M, Bergström T. *Recombinant Epstein-Barr virus glycoprotein 350 as a serological antigen.* Journal of Virological Methods. 2020;284:113927.

III. **Persson L**, Longhi S, Enarsson J, Andersen O, Haghigi S, Nilsson S, Lagging M, Johansson M, Bergström T. *Elevated antibody reactivity to measles virus NCORE protein among patients with multiple sclerosis and their healthy siblings with intrathecal oligoclonal immunoglobulin G production.* Journal of Clinical Virology. 2014;61(1):107-12.

IV. Jons D, **Persson Berg L**, Sundström P, Haghighi S, Axelsson M, Thulin M, Bergström T, Andersen O. *Follow-up after infectious mononucleosis in search of serological similarities with presymptomatic multiple sclerosis.* Multiple Sclerosis and Related Disorders. 2021;56:103288.

V. **Persson Berg L**, Eriksson M, Longhi S, Kockum I, Warnke C, Thomsson E, Bäckström M, Olsson T, Fogdell-Hahn A, Bergström T. *Serum IgG levels to Epstein-Barr and measles viruses in patients with multiple sclerosis during natalizumab and interferon beta treatment.* Submitted manuscript.

Scientific papers not included in the thesis:

i. Lind L, Studahl M, **Persson Berg L**, Eriksson K. *CXCL11 production in cerebrospinal fluid distinguishes herpes simplex meningitis from herpes simplex encephalitis.* Journal of Neuroinflammation. 2017;14(1):134

ii. Widgren K, **Persson Berg L**, Mörner A, Lindquist L, Tegnell A, Giesecke J, Studahl M. *Severe chickenpox disease and seroprevalence in Sweden - implications for general vaccination.* International Journal of Infectious Diseases. 2021;111:92

CONTENT

ABBREVIATIONS

a.a.	Amino acid
APC	Antigen-presenting cell
BBB	Blood-brain barrier
B cell	B lymphocyte
bp	Base pair
CHO	Chinese hamster ovary
C_H	Constant heavy chain
C_L	Constant light chain
CLIA	Chemiluminescence immunoassay
CMIA	Chemiluminescence microparticle immunoassay
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CSR	Class swish recombination
DAMP	Damage-associated molecular pattern
DMT	Disease modifying therapy
DNA	Deoxyribonucleic acid
ds	Double-stranded
EBNA1/2	Epstein-Barr virus nuclear antigen 1/2
EBNA1+/-	EBNA1 seropositive/seronegative
EBV	Epstein-Barr virus
EBVgp350	EBV glycoprotein 350
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding

FAMA	Fluorescent antibody to membrane antigen
Fc	Fragment crystallizable
GC	Germinal center
Gp	Glycoprotein
HHV6A/6B/7/8	Human herpesvirus 6A/6B/7/8
HIV	Human immunodeficiency virus
HSV 1/2	Herpes simplex virus type 1/2
Ig	Immunoglobulin
IM	Infectious mononucleosis
IFN	Interferon
IFNβ	Interferon beta
IQR	Interquartile range
JCV	JC polyomavirus
N_{CORE}	The core fragment (a.a. 1–392) of the measles virus nucleocapsid protein
MeSH	Medical Subject Headings
MeV	Measles virus
MHC-I/II	Major histocompatibility complex class I and class II
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NAT	Natalizumab
NCBI	National Center for Biotechnology Information
NK cells	Natural killer cells
OCB	Oligoclonal bands
OD	Optical density
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PML	Progressive multifocal leukoencephalopathy
PPMS	Primary-progressive multiple sclerosis
PRR	Pattern recognition receptor
RBC	Red blood cell
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RRMS	Relapsing-remitting multiple sclerosis
RV	Rubella virus
SHM	Somatic hypermutation
SPMS	Secondary progressive multiple sclerosis
ss	Single-stranded
TBE	Tick-borne encephalitis
T cell	T lymphocyte
TFF	Tangential flow filtration
VCA	Viral capsid antigen
VCA+/-	VCA seropositive/seronegative
VCAM-1	Vascular cell adhesion molecule 1
V_H	Variable heavy chain
V_L	Variable light chain
VZV	Varicella-zoster virus
VZVgB	VZV glycoprotein B
VZVgE	VZV glycoprotein E
VZVgE-ag	VZVgE antigen
VZVwhole-ag	VZV whole virus antigen
WHO	World Health Organization

DEFINITIONS IN SHORT

Antibodies	Immunoglobulin molecules having a specific amino acid sequence by virtue of which they interact only with the antigen (or a very similar shape) that induced their synthesis in cells of the lymphoid series, especially plasma cells (Medical Subject Headings MeSH, National Center for Biotechnology Information NCBI).
Antigens	Substances that are recognized by the immune system and induce an immune reaction (MeSH, NCBI).
Antigen-antibody reactions	The processes triggered by interactions of antibodies with their antigens (MeSH, NCBI).
Antibody affinity	A measure of the binding strength between antibody and a simple hapten or antigen determinant. It depends on the closeness of stereochemical fit between antibody combining sites and antigen determinants, on the size of the area of contact between them, and on the distribution of charged and hydrophobic groups. It includes the concept of "avidity," which refers to the strength of the antigen-antibody bond after formation of reversible complexes (MeSH, NCBI).
Epitope	Site on an antigen that interact with specific antibodies (MeSH, NCBI).
MS trait	A suspect hyperimmune phenotype in clinically healthy siblings of patients with MS.

Immunodominant	Antigens that are easily recognized by the immune system and thus are of most importance for the specificity of the induced antibody response.
Intrathecal antibody production	Antibodies produced in the central nervous system and secreted to the cerebrospinal fluid.
Paratope	Local surface sites on antibodies which react with antigen determinant sites on antigens (epitopes) (MeSH, NCBI).
Serology	Diagnostic identification of antibodies and antigens in serum and other body fluids including cerebrospinal fluid.

1 INTRODUCTION

The term serology comes from studies of blood serum, where immunological reactions are investigated, particularly in vitro, with a focus on antigen-antibody reactions. Immunoglobulins (Ig), also called antibodies, are produced by B lymphocytes and are the humoral component in the adaptive immune system. Antibodies can be produced in response to various antigens. Medical Subject Headings (MeSH) by National Center for Biotechnology Information (NCBI) defines antibodies as: “Immunoglobulin molecules having a specific amino acid sequence by virtue of which they interact only with the antigen (or a very similar shape) that induced their synthesis in cells of the lymphoid series (especially plasma cells)”. MeSH defines antigens as: “Substances that are recognized by the immune system and induce an immune reaction”. Antigens can be part of microorganisms, other foreign substances, or, in the case of autoimmune diseases, the body's own molecules.

In medical terminology, the term serology often refers to the diagnostic identification of antibodies and antigens in both serum and other body fluids including cerebrospinal fluid (CSF). Serological methods are important for diagnosing infections and autoimmune diseases, to control immunity after infection/vaccination, and in many other situations, such as for epidemiological purposes to determine the prevalence of a particular infection. Virological diagnostic research has recently focused strongly on molecular biological methods, but serological methods remain important. It is therefore warranted to continue to improve these methods.

1.1 HUMAN IMMUNE SYSTEM

Pathogenic microorganisms such as bacteria, fungi and viruses are constantly present in our environment, but the human body has ways to protect us against disease-causing pathogens. An individual with a well-functioning immune system is termed immunocompetent. Some individuals are immunocompromised i.e. have a weakened immune system due to either primary immune deficiencies or secondary/acquired immune deficiencies due to acquired conditions e.g. immunosuppressive medications or malnutrition. In the classical partition of the human body's defenses against infections, the defense has been divided into three different levels (1):

- Barrier protection
- Innate immune system
- Adaptive immune system

1.1.1 BARRIER PROTECTION

The human body's first defenses against disease-causing pathogens are physical, chemical, and biological barriers. The skin, which display dense connections of epithelial cells, provides mechanical protection against pathogens entering the body. We are also protected from unwanted invaders by our mucous membranes that line body cavities and canals in contact with the outside world, such as the gastrointestinal, respiratory, and urogenital tracts.

The tough structure of mucous membranes can prevent pathogens from invading. The structures vary, but they all have a surface layer of tightly connected epithelial cells and a deeper layer of connective tissue. The mucus membranes also protect us by secreting mucus, a viscous fluid with inhibitory substances including bactericidal and antiviral substances, where pathogens may be entrapped and killed. There are also dynamic forces that can transport away intruders, e.g. intestinal motility and cilia in the airways. The normal microbial flora does not normally cause disease and can prevent other more pathogenic microbes from gaining a foothold by competing for microenvironments more effectively. The role of bacteria in the normal flora is more established than the role of viruses (human virome) (2, 3).

1.1.2 INNATE IMMUNE SYSTEM

The development of the innate immune system occurred earlier in evolution compared with the adaptive immune system, and it is found in both invertebrates and vertebrates. Innate immunity is important for the survival of organisms and the basic mechanisms are thus conserved among animals (4). The immune cells that are part of the innate response are neutrophils, monocytes, macrophages, dendritic cells, eosinophils, basophils, mast cells and natural killer cells (NK cells). The complement system, cytokines and acute phase proteins are also important parts of the system. Some cytokines such as interferons (IFN) have a direct antiviral effect. IFN can directly interfere with viral replication and stimulate cell-mediated immunity to respond to viral infection (5). The immune cells and molecules are present on the surfaces as well as inside the body's organs and tissues and are immediately ready to act to fight invasive pathogens.

The response occurs after recognition of pathogen-derived structures and endogenous danger signals. The pattern recognition receptors (PRRs) on the immune cells i.e. C-type lectin receptors, Toll-like receptors, NOD-like receptors, and RIG-I-like receptors recognize pathogen-associated molecular patterns (PAMPs) on pathogens and damage-associated molecular patterns (DAMPs) (6). The innate immune response has been considered to lack immunological memory (1), but this view has been questioned in recent years and there are now arguments that the concept of immunological memory must be expanded, as also the innate immune system can mount resistance to reinfection (6-9).

1.1.3 ADAPTIVE IMMUNE SYSTEM

Only vertebrates have an adaptive immune system with special molecular characteristics, including B and T lymphocytes (B and T cells) and lymphoid organs such as the spleen and thymus (10). This branch of the immune system evolved in jawed fish about 500 million years ago (10). The adaptive immune response is highly specific, and B and T cells are activated only when they encounter their cognate antigen. The adaptive immune system has an immunological memory which means that reinfection with a previously encountered pathogen induces a more vigorous and rapid response (1, 4, 10).

The innate and adaptive immune systems cooperate to fight invasive pathogens. For example, antigen-presenting cells (APCs) from the innate immune system can capture antigens and present peptides from these antigens

to T cells to activate them. The adaptive immune response can in turn both regulate and stimulate the innate immune system.

Cytotoxic T cells that express CD8 on the cell surface identify and kill cells that synthesize aberrant peptides that are presented on major histocompatibility complex class I (MHC-I) molecules, which occur in virus-infected cells. As a token of the importance of this defense mechanism, certain viruses can prevent MHC-I molecules from reaching the surface of virus-infected cells and thus hide from the cytotoxic T cells (i.e. immune evasion). However, when NK cells detect cells with fewer MHC-I molecules than normal, they will kill the cell. This is an example of how the innate and adaptive immune response interacts to eliminate pathogens. The main function of CD4+ T helper cells is to stimulate and regulate other immune cells. T helper cells can for instance increase the functional capacity of phagocytic cells and stimulate B cells to become more efficient at producing immunoglobulins (antibodies).

The humoral immunity is one of the main components of the adaptive immunity and immunoglobulins are important for providing immunity after infection/vaccination. Secreted immunoglobulins can neutralize pathogens and act as effector molecules to stimulate other parts of the immune system to fight pathogens. The human body can produce antibodies with almost infinite specificity.

After antigen recognition and activation of various B cells in the body, these cells will produce antibodies that can recognize and bind to different antigens. The antibody response in the human body is thus polyclonal because different antibodies recognize different antigens. Monoclonal antibodies are generated by a single B-cell clone, and they all recognize the same antigen. Antibody-secreting cells have been utilized to produce highly specific monoclonal antibodies that can be used for both therapeutic and diagnostic purposes (11).

Antibody-secreting cells do have a downside, which includes involvement in the pathogenesis of certain autoimmune diseases, including antinuclear/anti-DNA antibodies in systemic lupus erythematous and anti-citrullinated protein (i.e. proteins carrying the rare amino acid citrulline) antibodies in rheumatoid arthritis. Patients with severe rheumatoid arthritis who have insufficient effect on other disease-modifying antirheumatic drugs may be treated with anti-CD20 monoclonal antibodies that depletes B cells, such as rituximab (Mabthera®) (12). Antibody-secreting cells are also involved in multiple myeloma (13) and IgG4-related disease (14-17).

1.2 IMMUNOGLOBULINS

1.2.1 PRODUCTION

Hematopoietic stem cells in the bone marrow can develop into B cells in an antigen-independent process. After leaving the bone marrow, B cells will colonize secondary lymphoid organs and there, in an antigen-dependent process, they can differentiate into memory B cells and antibody-producing plasma cells. The process is initiated after a mature B cell encounters and recognizes its cognate antigen. There are T cell-independent antigens, often polysaccharide antigens, that directly activate B cells, but most antigens are T cell-dependent, which means that after antigen recognition, the B cell requires cooperation with a T helper cell that recognizes the same antigen for final activation (13, 18). After B cell activation, cell division and differentiation into memory B cells and plasma cells can occur.

B cells can produce both soluble and membrane-bound immunoglobulins simultaneously. This is done by splicing at the mRNA level, so that the same main structure can be combined with a hydrophilic short tail that provides a soluble molecule, or with a partially hydrophobic longer tail that gives a membrane-bound molecule. B cells have mainly membrane-bound immunoglobulins (the main part of the B cell receptor) while plasma cells are specialized in producing and secreting immunoglobulins (19). Plasma cells are larger than B cells and the morphology is characteristic with a small, asymmetrically placed nucleus, large cytoplasm with abundant rough endoplasmic reticulum and Golgi apparatus. The morphology of the plasma cell reflects its main function of producing and secreting Ig molecules (19).

Plasma cells can be divided into two main groups, plasmablasts and long-lived (memory) plasma cells. Plasmablasts are short-lived (20) while long-lived plasma cells can have a lifespan similar to long-lived memory B cells (21). In this context, it has been previously demonstrated that long-lived plasma cells can maintain antibody titers for long periods and in some instances throughout life (22). These long-lived plasma cells are found in the bone marrow but also in the human intestine (23-25).

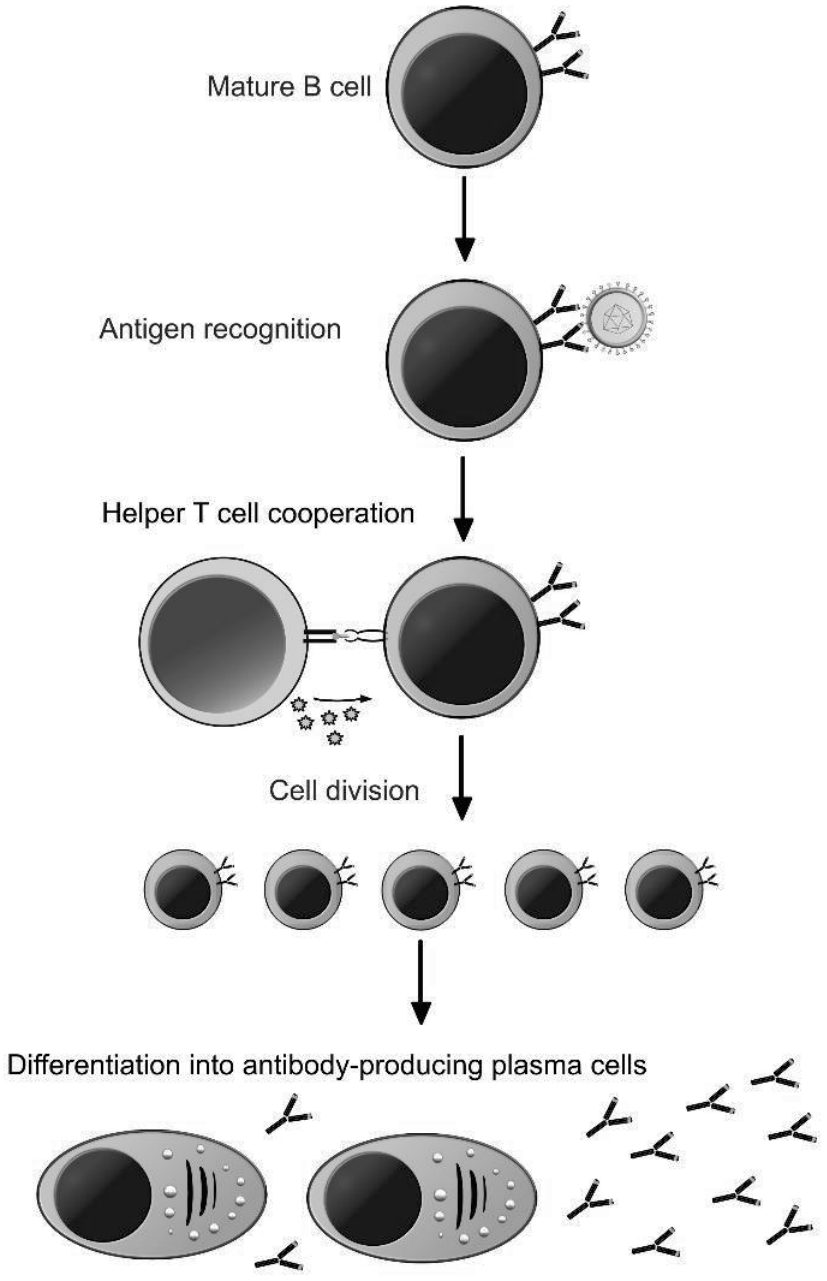

Figure 1. B cell activation and differentiation into antibody-producing plasma cells.

1.2.2 BASIC STRUCTURE

Immunoglobulins (Ig) are large molecules with an approximate molecular weight of 150kD. These molecules have a basic Y-shaped structure consisting of two heavy and two light polypeptide chains. There are two types of light chains, lambda (λ) and kappa (κ), based on small polypeptide differences. An Ig molecule has identical light chains, meaning that each individual antibody has either lambda or kappa light chains (26).

The heavy chains of an individual Ig molecule are also identical, and the chains are joined by two disulfide bonds. Each of the light chains is joined to one of the heavy chains by a single disulfide bond. Each light and heavy chain can be divided into a constant and a variable part. The variable heavy chain is often abbreviated V_H, where V stands for variable and H for heavy chain. Correspondingly, the abbreviation for the variable part of the light chain is V_L where L stands for light. The constant part of the heavy and light chains is abbreviated C_H and C_L respectively, where C stands for constant. The heavy and light chains are composed of a special type of protein domains that have a similar globular structure termed Ig fold or Ig domain (27). The structure is the result of a special folding of the protein in the tertiary structure. These Ig domains exist in several other molecules, the Ig superfamily, suggesting that such motifs have been useful protein modules during evolution (28).

The basic structure of immunoglobulins is similar, but there is a large variation in the composition of antigen-binding sites, also termed paratopes, in order to enable binding to the large numbers of different antigens that the body encounters (26). Each B cell can only express a certain type of immunoglobulin and these identical immunoglobulins all have the same specificity for binding antigen. All daughter cells of the original B cell will inherit the same antibody specificity, which means that all plasma cells from the original B cell will secrete antibodies that target the inducing antigen.

The paratope consists of the outer part of the variable heavy and light chains ($V_H + V_L$). Each individual Ig molecule has two identical paratopes because the two arms are composed of identical chains (26). The two arms of the Y-shaped Ig molecule are joined to the trunk by a flexible polypeptide chain termed the hinge region. The flexible hinge region allows the two antigen-binding arms to form different angles relative to each other, allowing binding of two different antigens if they are not too far apart. The maximum angle of the antibody arms must be able to cover the distance between the two antigens.

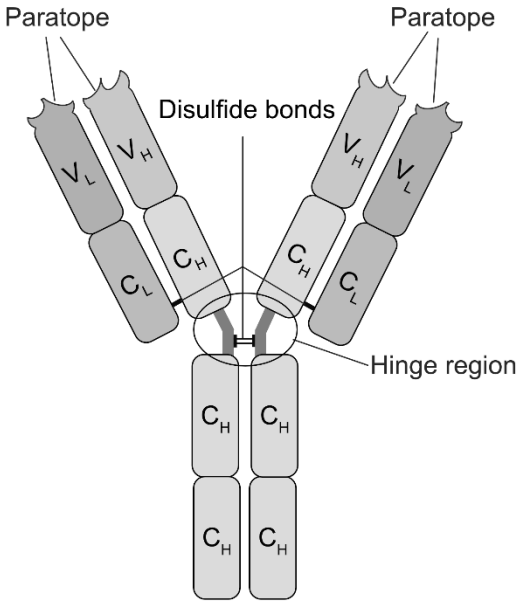

Figure 2. Immunoglobulins are composed of two light (L) chains and two heavy (H) chains. The variable parts of the light and heavy chains are abbreviated V and the constant parts C. The paratopes (i.e. antigen-binding sites) are binding to specific epitopes on antigens (i.e. antigenic determinants).

1.2.3 CLEAVAGE WITH PROTEASES

Immunoglobulins can be cleaved by proteolytic enzymes (proteases) into functionally distinct fragments. This process has been used to determine which part of the Ig molecule that is responsible for different functions. Cleavage of Ig with papain results in three fragments of the molecule. Papain cleaves the molecule above the disulfide bonds that bind the two arms that contain the paratopes. The two separated identical arms are named Fab fragments, which stands for fragment antigen binding. These fragments have monovalent antigen binding. The trunk of the Y-structured molecule has no antigen binding activity but is in intact Ig molecules important for interactions with cells and effector molecules. This part of the molecule is called Fc fragment, which stands for fragment crystallizable, because it has been observed that it easily forms crystalline structures.

Pepsin cleaves the Ig molecule below the disulfide bonds that hold the two arms of the Y-shaped Ig molecule together. The two antigen-binding arms will thus be linked in a fragment and can bind antigens with both arms (divalent antigen binding). This fragment is called $F(ab')_2$ fragment and has the same antigen-binding properties as the original antibody, but since it does not contain the Fc fragment, it cannot interact with cells and/or effector molecules. The Fc fragment will be cleaved in several smaller fragments by pepsin.

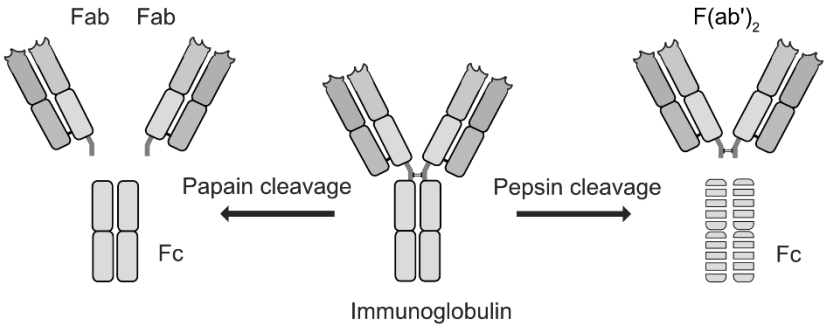

Figure 3. Cleavage of an immunoglobulin molecule with papain or pepsin protease. Fragment crystallizable (Fc). Fragment antigen binding (Fab).

1.2.4 ISOTYPES

Immunoglobulins are divided into different isotypes (antibody classes) based on differences in the constant region of the heavy chain (C_H). There are five basic structures of C_H named alpha (α), delta (δ), epsilon (ε), mu (μ) and gamma (γ). These five different C_H correspond to the five antibody classes IgA (α), IgD (δ), IgE (ε), IgM (μ) and IgG (γ) (26). The variation between isotypes include differences in the amino acid sequence of the Ig domains and the number of Ig domains in the C_H. IgA and IgG can be further divided into subclasses based on differences in the properties of the alpha and gamma chains (29, 30). Alpha is further divided into the two subclasses IgA1 and IgA2, while gamma contains the four subclasses, IgG1, IgG2, IgG3 and IgG4 (30). The different structures of the antibody classes reflect their diverse biological functions with various effector mechanisms, antigenic determinants, and biological half-life (26). The different antibody classes bind to their respective Fc receptor on immune cells.

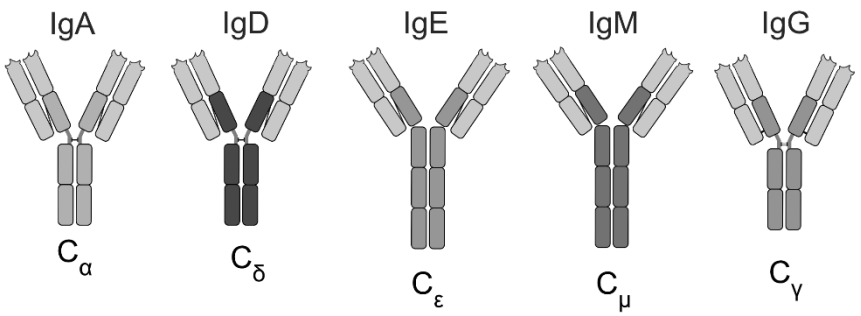

Figure 4. The five antibody classes are based on differences in the constant region of the heavy chain (C_H). The five basic structures of C_H are named alpha (α), delta (δ), epsilon (ε), mu (μ) and gamma (γ) and these five different C_Hs correspond to the five antibody classes IgA (α), IgD (δ), IgE (ε), IgM (μ) and IgG (γ).

IgA

IgA is the predominant antibody class on mucous membranes, the second most common antibody class in serum and has the highest production of all isotypes in humans (31). IgA is the most important isotype for our mucosal defense and can neutralize pathogens and microbial toxins in the gastrointestinal tract (32). In serum, IgA appears predominately in monomeric form but occurs on mucosal surfaces mainly as a dimer (26, 31, 33). Secretory IgA in dimeric form is composed of a secretory component and two Y-shaped basic IgA immunoglobulin structures that are linked by a joint chain (31). IgA is transmitted via breast milk and can therefore contribute to the defense against infections in breastfeeding infants.

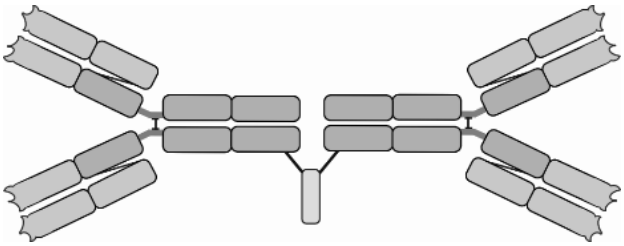

Figure 5. A secretory IgA molecule.

IgD

As with IgM, IgD is expressed early on mature B cells as a major part of the B cell receptor (34). IgD is mainly found in membrane-bound form, although small amounts of IgD can be detected in serum. The secreted IgD molecule has a monomeric form. IgD can activate basophils and thereby induce the production of antimicrobial peptides, inflammatory cytokines and B-cell activating factors (34).

IgE

Secreted IgE has a monomeric form. Through its Fc moiety, IgE can bind to Fc receptors on mast cells and basophilic granulocytes with very high affinity. This high affinity causes IgE molecules to be bound to Fc receptors of basophilic granulocytes even before any antigen is recognized by the cells. When an antigen then binds to IgE, the cell can immediately secrete various mediators. IgE is often associated with allergies and hypersensitivity, but IgE is also involved in the immune protection against parasitic helminths (35).

IgM

Secreted IgM is pentameric or hexameric (36). The pentameric form consists of five Y-shaped basic Ig structures and the hexameric form of six (36-38). IgM is expressed early during the maturation of B cells and early in an immunological response to a pathogen. IgM can activate the complement cascade through the classical pathway and thus help to clear infections by antibody-dependent cellular phagocytosis and antibody-dependent cell cytotoxicity (38).

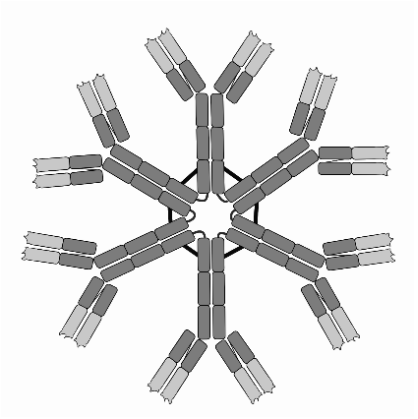

Figure 6. A secreted hexameric IgM molecule.

IgG

Secreted IgG is monomeric and the major antibody class in serum followed by IgA and IgM (26). Many cells in the immune system have Fc-gamma receptors on their surfaces and IgG is the predominant antibody class in secondary immune responses (26). IgG has many effector mechanisms including neutralization, opsonization and activation of the complement system. IgG can cross the placenta to the fetus and can thereby give the newborn a passively transferred immunity from the mother. The half-life of most IgG subtypes is approximately 3 weeks (39, 40). The neonatal Fc receptor can prolong the half-life of IgG molecules (35).

1.2.5 EFFECTOR MECHANISMS

Antibodies have effector mechanisms to protect the body from being harmed by foreign substances such as microorganisms (41). The main effector mechanisms of antibodies are:

Neutralization

Direct antibody neutralization is the process by which antibodies bind to antigens, thereby reducing or inhibiting the biological activity of pathogens/microbial components (41). Antibody neutralization can thus prevent harmful effects of pathogens/microbes in the body. As an example,

antibodies can bind to envelope proteins on viruses, thereby preventing viral binding and penetration into host cells. The antibodies will thus inhibit viral replication, further dissemination and progression of the viral disease (41). Antibodies can also neutralize toxins by binding to them, thereby inhibiting the toxins from binding to cellular receptors. Many vaccines on the market stimulate the production of neutralizing antibodies and thus protect against disease (42).

Opsonization

Opsonization is the process by which immune cells can recognize and target foreign substances by binding to opsonins (e.g. IgM, IgG and complement component C1) that cover the substances, for antibody-dependent cell cytotoxicity and/or phagocytosis.

Antibody-dependent cellular phagocytosis is the process by which pathogens are coated with antibodies to promote phagocytosis. Fc receptors on phagocytic cells can recognize and attach to antibody coated pathogens, enabling phagocytosis of the pathogen.

Antibody-dependent cell cytotoxicity is the immune process in which certain immune cells with Fc receptors can recognize and kill cells that have pathogenic antigens on their surface and are coated with antibodies. Antibody-dependent cellular phagocytosis and antibody-dependent cell cytotoxicity are effective mechanisms for the clearance of encapsulated bacteria, viruses, and virus-infected cells (43).

Complement activation

IgM and IgG can both initiate a cascade of enzymatic reactions to activate the complement system (antibody-mediated complement activation) (38, 44) where the ultimate consequences are:

- Lysis of pathogenic microorganisms. Complement factors can create a pore in the membrane of the microorganism that disturbs the osmotic balance, leading to the death of the microorganism.
- Coating of pathogens by complement factors for opsonization.
- Stimulation of inflammation by affecting the permeability of blood vessels. This leads to a greater influx of inflammatory cells and various immune defense molecules.

Antibody-dependent enhancement

There are microorganisms that can exploit the Fc receptor and complement pathways to enhance the disease through antibody-mediated responses (45, 46). This is termed antibody-dependent enhancement (41, 45, 46). An example of this phenomenon is when antibody opsonization of dengue viruses enhances viral infection. This process can occur in patients who are reinfected with another serotype of dengue virus than the one responsible for the previous infection (47). The antibodies to the dengue serotype from the previous infection may enhance the entry of the current dengue virus into monocytes/macrophages, which can lead to increased viral replication, an increased inflammatory response and aggravation of the disease (41, 45, 47).

The problem of antibody-dependent enhancement is also reflected in the difficulty of producing an effective and safe vaccine against dengue virus (48). The first licensed dengue vaccine, CYD-TDV or Dengvaxia® was in 2016 recommended by the World Health Organization (WHO) for considered use in highly endemic regions (49).

The vaccine was later found to have different effects depending on the dengue serostatus of the vaccinated individual (50). For individuals with a previous dengue infection (seropositive individuals), the vaccine is safe and effective against symptomatic dengue disease (50, 51). In contrast, seronegative individuals without a previous dengue infection, three years after the first vaccination and onwards, have an increased risk of developing severe dengue fever if they become infected with the dengue virus (50). These findings have led to the suspension of the CYD-TDV vaccination program in the Philippines, and the WHO stated in its 2018 recommendation that: “Countries should consider introduction of the dengue vaccine CYD-TDV only if the minimization of risk among seronegative individuals can be assured” (52).

1.2.6 ANTIBODY-ANTIGEN INTERACTION

The antigen-binding parts of the Ig molecule consist of amino acids (a.a.) in groups, termed complementarity-determining regions (35). These a.a. are not in line in the primary sequence of the polypeptide chain but are joined together by the folding of the protein in the tertiary structure. The paratopes (antigen-binding sites), are located in the outermost part of the variable Ig domains. Paratopes differ in their three-dimensional structure, charge, and hydrophobic properties. Which means that different antibodies can bind various structures on antigens.

Antigens can consist of many different types of molecules, such as polysaccharides, lipids, nucleic acids, small molecules (e.g. trinitrophenol) although proteins are probably the most biologically important antigens. The part of the antigen that the paratope binds to is termed antigenic determinant or epitope. A polypeptide chain can contain various linear epitopes, and, after folding of the protein, several new non-linear or discontinuous epitopes (further explained below).

The bonds between antibodies and antigens are non-covalent and reversible, formed by a combination of hydrogen bonds, hydrophobic interactions, electrostatic and van der Waals forces. That the antibody-antigen bonds are reversible means that they can alternate between binding and detaching. Antibodies recognizes epitopes in their native form. Epitopes can be classified into two main groups, continuous epitopes (often called linear epitopes), and discontinuous epitopes (often referred to as conformational epitopes).

Continuous epitopes consist of any sequential (linear) a.a. residues that can bind to a paratope. In contrast, discontinuous epitopes are composed of a.a. residues which are separated in the primary sequence, but which come in proximity in the three-dimensional folded shape. Discontinuous epitopes are more common than continuous epitopes, and it is estimated that more than 90% of antibodies recognize such targets (53).

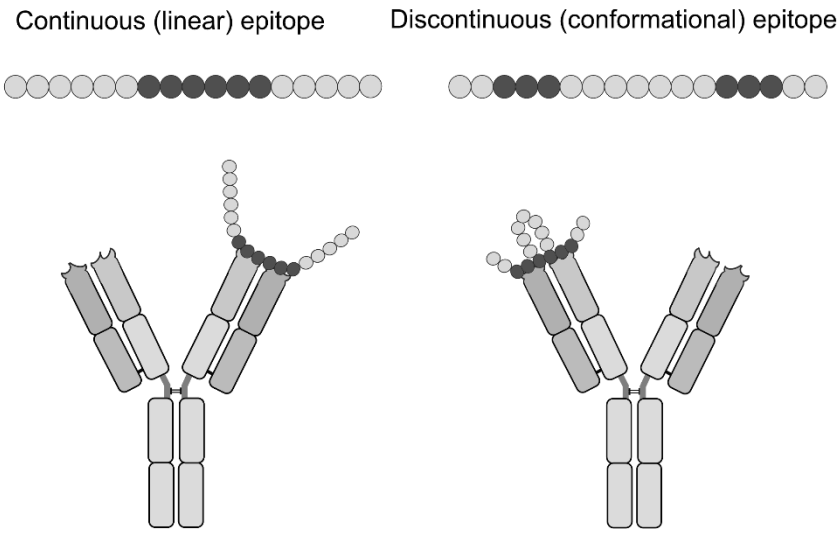

Figure 7. Figure showing a continuous (linear) epitope consisting of sequential amino acid residues in the primary sequence and a discontinuous (conformational) epitope where the amino acid residues are apart in the primary sequence but comes in proximity in the three-dimensional form.

Epitopes can either be located on the surface of the protein, facing the liquid phase, or be located inside the protein in hydrophobic parts. In the latter case, the epitope is termed cryptic because it is not exposed unless the protein is denatured. Cryptotopic epitopes in viruses are hidden from the immune response until the virion dissociates. Viral cryptotopes can be relatively conserved in genetically related viruses because such antigenic structures are part of the virus' internal structures. Consequently, such domains may share essential functions such as viral assembly and thus tend to vary less compared with proteins on the surface of viruses.

Affinity

The individual binding strength between a paratope and an epitope is termed affinity. The binding strength depends on the proximity of stereochemical fit between the paratope and epitope, the size of the contact surface between them and on the distribution of charged and hydrophobic groups. The affinity constant K_a can be affected by temperature, pH, and solvent. K_a can range from below 10^5 mol^{-1} to above 10^{12} mol^{-1} (41). The equilibrium dissociation constant (K_d) and the affinity are inversely related, which means the higher the affinity, the lower the K_d.

It is possible to determine the affinity of monoclonal antibodies since these antibodies are homogeneous and only bind to a single epitope. In contrast, polyclonal antibodies are heterogeneous and will thus contain a mixture of antibodies that will bind to various epitopes with different affinity. Thus, it is only possible to measure an average affinity for polyclonal antibodies.

Avidity

Avidity is the measure of the total binding strength of the antibody-antigen binding after formation of reversible complexes. The avidity depends on the number of interacting paratopes and epitopes, the affinity of each paratope-epitope binding and the structural arrangement of the interacting parts. IgM molecules that are pentamers have ten paratopes and hexameric IgM has twelve paratopes (36, 37). This gives IgM relatively high avidity to bind antigens even though the affinity between each paratope and epitope is not so high.

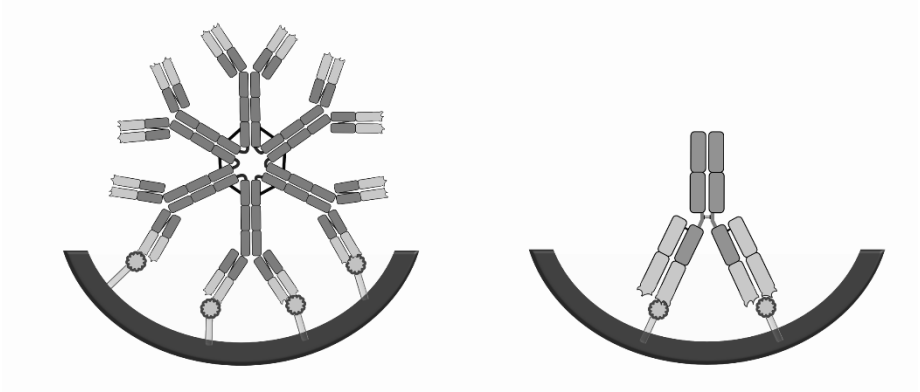

Figure 8. Hexameric IgM molecules have twelve paratopes compared with IgG molecules that have two paratopes. This gives IgM molecules relatively high avidity to bind antigens even though the affinity of each paratope-epitope binding generally is lower compared with IgG molecules.

1.2.7 ANTIBODY DIVERSITY

It is possible to produce antibodies against virtually all foreign substances provided that the substance is at least as large as required to form an epitope. The number of different antibody specificities appears almost infinite. During the development of B cells in the bone marrow, the genes encoding the variable parts of the Ig molecule are built up randomly by somatic recombination from hereditary DNA segments (54-56).

The three gene segments that encode the variable part of the heavy chain (V_H) are named variable (V), diverse (D) and joining (J). The variable part of the light chain is constructed by two gene segments V and J. The final V_H and V_L genes are constructed in a process termed the V(D)J recombination, which greatly contributes to Ig diversity (54-56). Each B cell produces only a specific type of Ig molecule. A certain B cell will survive if it recognizes a foreign substance, while B cells that bind too strongly to the body's own molecules or do not recognize foreign substances at all, will die.

A mature B cell expresses IgM or IgD as its membrane-bound B cell receptor. B cells in secondary lymphoid tissue are activated by encountering their cognate antigen, but most require stimuli from an activated T helper cell that recognizes the same antigen to begin proliferating in a process termed clonal expansion. When B cells in B-cell follicles begin to proliferate, the follicle can be transformed into a germinal center (GC). These GCs are distinct microanatomical parts in B-cell follicles where B cells during an immune

response are stimulated by special follicular dendritic cells (which present intact antigens in immune complexes) and T follicular helper cells to generate memory B cells and plasma cells (57-61). B cells will within the GCs proliferate extensively. Mutations will occur in the variable genes that will lead to diversified B-cells receptors. This process is termed somatic hypermutation (SHM) (13). B cells with receptors that have higher affinity for the antigen will be selected for survival and further proliferation. This process of generating B cells and plasma cells that produce high affinity antibodies is termed affinity maturation (13, 62).

During proliferation, some of the daughter cells can undergo a class swish recombination (CSR). It is the process by which B cells rearrange their DNA in the constant part of the heavy chain (C_H) to change it to another C_H, thereby producing another antibody isotype (e.g. from IgM to IgA, IgE or IgG) with retained antigen specificity but with other effector functions (13, 63, 64).

1.2.8 ANTIBODY RESPONSE TO VIRAL INFECTIONS

Many viruses can induce a potent and long-lasting antibody response (65, 66). To generate long-lived plasma cells that produce high affinity antibodies, B cells require cooperation with their cognate T helper cells. Activation of T helper cells is therefore essential for the development of a long-lasting and effective antibody response. The procedure in which professional antigen-presenting cells (APCs) take up antigen, process antigen into peptides and present these antigenic peptides on MHC class II molecules to T helper cells together with co-stimulatory signals to activate the helper T cells is therefore important.

The surface of many viruses consists of one or a few proteins and is highly organized with a repetitive structure. Such structures are unusual in the human body and thus both the innate and the adaptive immune system have evolved to recognize these structures as antigenic “danger signals” (67). These structures can thus be considered as pathogen-associated molecular patterns (PAMPs) (67). These repetitive motifs and other danger signals, including viral nucleic acids such as dsRNA, U-rich ssRNA and hypomethylated DNA can be detected by pattern-recognition receptors (PRRs) on cells in the innate immune system. This will lead to the induction of immune signals in the form of inflammatory cytokines (including interferons), the recruitment of neutrophiles and the activation of professional APCs, in particular dendritic cells in viral infections (65). The uptake and transport of viruses by dendritic

cells to lymph nodes are important for the activation of the adaptive immune system.

Dendritic cells are potent inducers of T cell activation where naïve T cells can differentiate into cytotoxic T cells and T helper cells. Effective priming of T cells is further facilitated by the prolonged stimulation of virus-derived antigens, which often occurs during viral infections due to viral replication in cells of the infected host (65, 66). Most B cells require the help of cognate T helper cells for final activation. The B cell will present antigenic peptides on MHC class II molecules for activated helper T cells. In response, the T helper cell will synthesize effector molecules, both cell-bound (mainly CD40L which will bind to CD40 on the B-cell) and secreted molecules to activate the B-cell.

Previous research suggests that many human viruses have a suitable size for passive transport through the lymphatic system without the need for cell-mediated transport to reach lymph nodes where the virus in its native form can interact with B cells (65). The highly repetitive surfaces of many viruses facilitate the cross-linking of B-cell receptors, which is a strong B-cell activation signal. Cross-linking of B-cell receptors can also stimulate a T-cell-independent antigenic response with IgM production (65, 66). IgM can activate the complement cascade through the classical pathway.

Other effector molecules (pentraxins) produced in response to the viral infection can also activate the complement system. Complement factor C2 can bind to B-cell receptors and facilitate the stimulation of these receptors. IgM antibodies, together with complement, can, by binding to viruses, increase the uptake of these immune complexes by APCs, which is an important step in the priming process of T helper cells and facilitates the capture of virus particles in lymphoid organs (68). The increased uptake of these immune complexes by APCs can also lead to optimal display of viral antigens by specialized follicular dendritic cells in lymph nodes, which is important for the optimal development of the antibody response.

Prior to infection/vaccination with a particular virus, there are no detectable antibodies against that virus. An exception is newborns who through the placenta may have received a passive transmission of the mother's IgG antibodies without being virus-infected themselves. An individual who lacks antibodies to a particular virus is called seronegative while an individual who has antibodies is called seropositive. The term seroconversion is used when a seronegative individual begins to produce detectable antibodies to a certain virus due to infection/vaccination and thus becomes seropositive.

In primary viral infections, IgM is often generated first and can sometimes already be detected within one to two weeks after the onset of symptoms (69, 70). The maximum IgM response occurs approximately three to six weeks after the onset of symptoms and a decline is then seen over a period of a couple of months. Sometimes, however, the IgM response may persist longer.

IgG production usually begins a little later than IgM production. The IgG response is more long-lasting compared with the IgM response and generally reaches its peak within four to twelve weeks after the onset of symptoms and persist for months or years (70). Some viruses such as herpesviruses, which remain latent in the body but also measles virus, can induce a lifelong IgG response (22). Other viruses e.g. SARS-CoV-2 has a much faster decline in IgG levels after infection (71).

In connection with a reinfection/reactivation with the same virus, memory B cells and long-lived plasma cells will be activated, leading to an early secretion of high IgG levels. Upon reinfection/reactivation, IgM will in some cases be produced at detectable levels and in other cases not. Reactivation of VZV often elicits a detectable anti-VZV IgM response (72, 73). The antibody response described above is a generalized description and it will differ depending on the pathogen and the individual's immunocompetence.

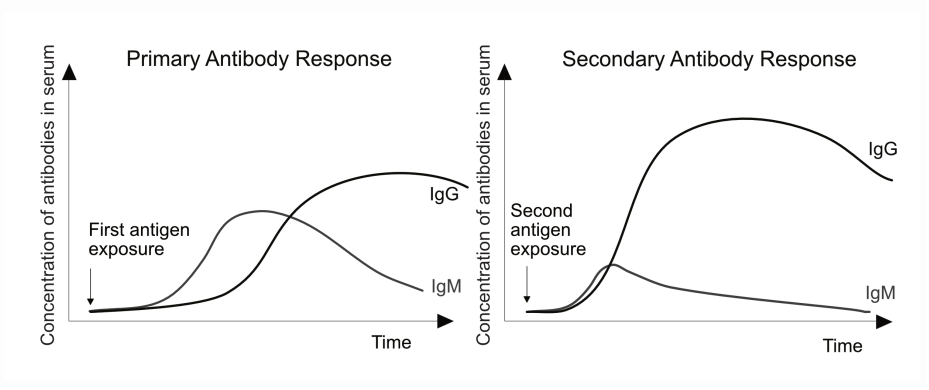

Figure 9. A generalized depiction of the IgM and IgG antibody response during a primary and a secondary infection.

1.3 VIRUSES

Viruses exist everywhere where there is biological life, and viruses, especially bacteriophages that infect bacteria, are the most abundant biological entities on the planet (74). Viruses can infect all types of living organisms, e.g. plants, algae, fungi, archaea, bacteria, and all animals including humans. Viruses require living cells to produce new viruses and they lack a complete machinery for energy production and protein synthesis. Viruses must therefore use cellular systems to produce their components and for energy production. The whole virus particle, called virion, can thus be seen as a transport container for transferring the viral genome to host cells where the virus can induce changes in the cell so that the cell will start to produce new viruses.

Viral genome

Viruses can have DNA or RNA as genetic material. The genome can be double stranded (ds) or single stranded (ss). The viral nucleic acid encodes all virus-specific proteins, both structural proteins, enzymes, regulatory elements, and factors necessary for propagation. There are some general differences between DNA and RNA viruses. DNA viruses usually have longer genomes because these viruses are genetically more stable compared with RNA viruses. RNA viruses are more prone to mutate, giving them a more genetically unstable genome compared with DNA viruses. Most DNA viruses replicate their genome in the cell nucleus where the virus can use the cell machinery. In contrast, most RNA viruses replicate in the cytoplasm.

In the human genome, some viral genetic material remains from previous retroviruses (a group of viruses that can copy their RNA to DNA using a viral reverse transcriptase) that during evolution have managed to integrate their genetic material into our genome. Viruses' ability to introduce new genetic information into cells has made them useful tools in genetic engineering.

Viral capsid

The viral genome is surrounded by a protective protein shell termed capsid. The capsid has a rigid and durable structure, which protects the nucleic acid from external influences such as changes in pH, temperature, and chemical composition. Inside the capsid, there is room for the viral genome and some viral proteins. The capsid consists of many copies of a small number of viral proteins, in a few cases only one type of protein. The structural forms of viral capsids are termed helical, icosahedral, and complex. The helical symmetry consists of repeating protein subunits that coat the nucleic acid in a helix along

its entire length. The icosahedral symmetry consists of 20 identical triangular structures, which form an almost spherical structure. Complex capsids are capsids with structures other than helical or icosahedral, as seen for some viruses, e.g. poxviruses.

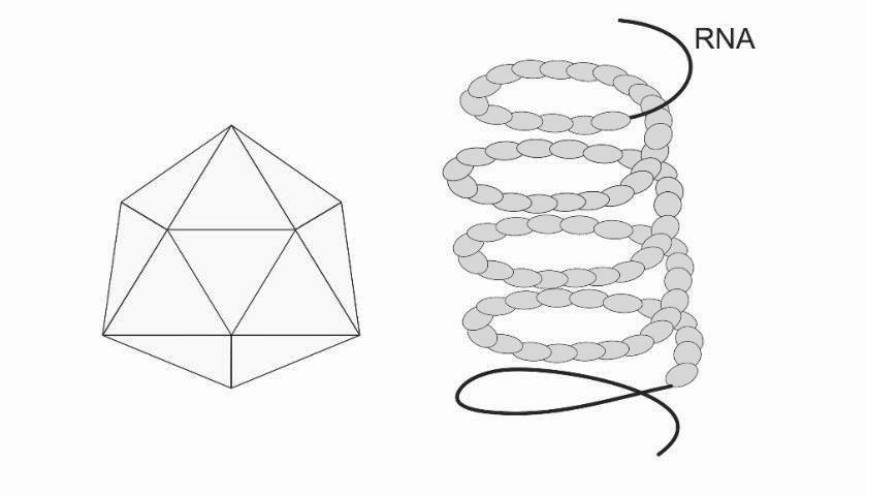

Figure 10. The viral genome is surrounded by a protective protein shell called capsid. This figure shows the two common capsid symmetries in viruses, icosahedral and helical.

Viral envelope

Some viruses also have a lipid envelope surrounding the capsid. The substructure with the core and capsid in these viruses is termed nucleocapsid. The viral genome does not encode for the lipids in the envelope, instead the virus will acquire them from host cell membranes. The viral envelope will thus consist of a bilayer of phospholipids just like our cell membranes, but the integrated proteins are primarily encoded by the viral genome. Viruses will acquire envelopes from different host cell membranes, but it is virus-specific exactly from where the acquisition will take place. Two examples of where the envelope is retrieved are the Golgi apparatus and the outer cell membrane.

The viral envelope is protective and blocks water, chemicals, and enzymes from entering the virus, but most envelopes are quite sensitive to detergents, drying and heat. An intact envelope is important for virus-cell interactions, e.g. for viral attachment and entry into host cells. Enveloped viruses are thus generally easier to inactivate compared with naked viruses, which lack envelope. Few enveloped viruses can cope with the environment in the gastrointestinal tract. The viral capsid is generally quite resistant to variations in pH, temperature, and chemical composition in the environment.

Viral glycoproteins

Integrated in the envelope are viral proteins. Many of these envelope proteins are glycoproteins, which means that the proteins have carbohydrate side chains. These viral glycoproteins are sometimes called spike proteins. The protein moiety is encoded by the virus but the glycans reflect the host cell machinery of the infected cell. Viral glycoproteins are synthesized in the same way as cellular glycoproteins. The glycoproteins are transmembrane, and the outer part (the ectodomain) is often involved in attachment and viral entry into host cells. The internal domain is often important for viral assembly.

These types of viral glycoproteins are important targets for antibodies because they are often present in relatively large amounts on the surface of the virus and often also on virus-infected cells. In naked viruses, the capsid proteins have similar functions to the envelope proteins for attachment and viral entry into host cells. Some naked viruses may have protruding proteins that originate from the capsid.

1.3.1 *HERPESVIRIDAE*

The *Herpesviridae* family consists of more than 100 identified viruses that are known to infect a diverse range of species, e.g. birds, reptiles, and mammals including humans (75). Herpesviruses have a linear dsDNA genome of 125–241 kilobase pairs (75). The genome contains 70–170 genes that encode viral proteins (76). The genome is genetically stable compared with the genome of many RNA viruses. The genome is densely packed within an icosahedral capsid. Surrounding the nucleocapsid is the tegument, which is an amorphous layer containing viral proteins. Herpesviruses are enveloped viruses with viral glycoproteins in the lipid bilayer. They have a spherical shape and measure around 150–200 nm.

After primary infection, herpesviruses will establish a lifelong latent/persistent infection with a subdued gene expression. Herpesviruses can later reactive and start the production of new viruses, which can enable the spread to new hosts. It has been shown that certain herpesviruses such as herpes simplex virus (HSV) and varicella-zoster virus (VZV) can retain their latent genomes as closed circular molecules (episomes) in the host cell nucleus (77). Studies have demonstrated that human herpesviruses 6A and 6B (HHV-6A and HHV-6B) and certain other herpesviruses, including Marek's disease virus, can integrate their viral genome into the genome of the infected host cell (78, 79). However,

much is still unknown when it comes to how various herpesviruses cause latent infection in host cells, as the area has not been sufficiently studied.

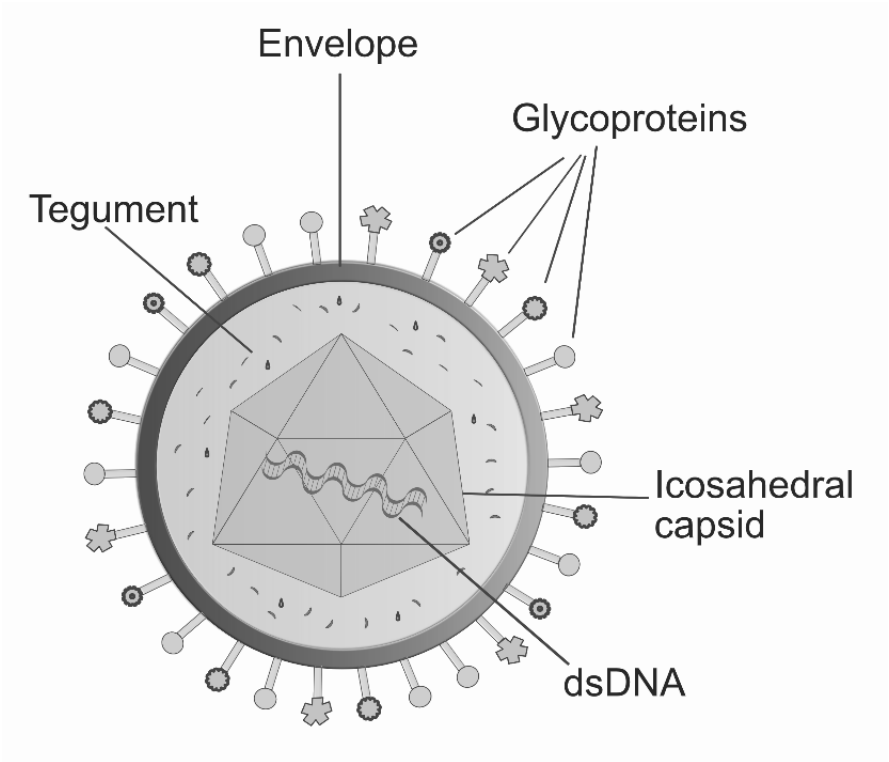

Figure 11. Schematic illustration of a herpesvirus.

Herpesviruses have evolved to adapt to their respective hosts. Herpesvirus infections are common in all animal species investigated and the viruses are spread around the globe. Many of the human herpesviruses can cause asymptomatic primary infections in early childhood, while VZV is the human herpesvirus that most often causes symptoms in primary infection, and then in the form of chickenpox (varicella). Severe disease due to human herpesviruses is seen mainly in immunocompromised individuals, fetuses, and newborns.

Members of the *Herpesviridae* family are classified into three subfamilies based on biological characteristics: *Alphaherpesvirinae (α)*, *Betaherpesvirinae (β)* and *Gammaherpesvirinae (γ)* (75, 80). All three subfamilies are represented among the nine known herpesviruses that infect humans.

1.3.2 *ALPHAHERPESVIRINAE*

- Herpes simplex virus type 1 (HSV-1, HHV-1)
- Herpes simplex virus type 2 (HSV-2, HHV-2)
- Varicella-zoster virus (VZV, HHV-3)

Herpes simplex virus type 1 and 2

HSV-1 and HSV-2 are ubiquitous around the globe but with geographic variation in seroprevalence (81). The estimated global prevalence of HSV-1 is two-thirds of all people aged 0–49 years (81). In a study regarding HSV-1 in Swedish adults, the seroprevalence was 79.4% for people who were 35–95 years old (82). The estimated global prevalence of HSV-2 is 13% for people aged 15–49 years, with the highest seroprevalence in Africa (81). In Sweden, the HSV-2 seroprevalence in adults aged 35–95 years was measured at 12.9% (82).

Both viruses infect mucoepithelial cells as their primary target and establish latency in sensory neurons. They both cause herpetic blisters; HSV-1 can induce both oral and genital lesions while HSV-2 mainly causes genital lesions. Both viruses can be reactivated after latency and cause new mucoepithelial lesions. Complications of the primary or reactivated infections include herpesvirus infections of the central nervous system (CNS), keratitis, hepatitis, pancreatitis, and pneumonitis.

1.3.3 VARICELLA-ZOSTER VIRUS

Varicella-zoster virus (VZV) is, as described above, a member of the *Herpesviridae* family and belongs to the *Alfaherpesvirinae* subfamily. The genus of the virus is *Varicellovirus* and the species is *Human alphaherpesvirus 3*. VZV is described in more detail because the virus is a central topic in the thesis.

The virus

There is one serotype of VZV, seven established clades (1–6 and 9) and two additional putative clades awaiting confirmation (83-87). VZV causes chickenpox at primary infection, which primarily affects young children. VZV can later reactivate from latency to cause herpes zoster (shingles). VZV mainly infects epithelial cells, T cells and neurons where the virus establishes latency.

VZV has the smallest genome of the known human herpesviruses (88). The linear dsDNA genome has approximately 125,000 base pairs (bp) and consists of at least 70 genes, all but 6 of which have homologues in HSV (88, 89). The genome has a unique long coding region and a unique short coding region (88, 89).

The genome is encased in an icosahedral capsid. The nucleocapsid is in turn surrounded by tegument. Outside the tegument is the lipid envelope, which contains glycoproteins encoded by the viral genome as well as cellular membrane proteins.

Glycoproteins

The VZV genome encodes the glycoproteins gB, gC, gE, gH, gK, gI, gL, gM, gN (89-91). The glycoproteins incorporated into the viral envelope are important for attachment and virus entry into host cells (90, 91). These structural components also have other important functions for the pathogenesis and replication of the virus, such as viral assembly and egress (89-93).

The glycoproteins gB, gH , gL, gM, and gN are core proteins that are conserved among the three subfamilies of the herpesviruses (94). Based on the homology with HSVgB, it is likely that VZVgB has similar functions and is critical for viral entry into host cells (89, 95). VZVgE is essential for VZV replication and infectivity in T cells and skin (93, 96-99).

VZVgE (open-reading frame 68) is the most produced glycoprotein in VZV-infected cells and the most common viral protein integrated into the viral envelope (100). Both VZVgE and VZVgB are targets for cytotoxic T cells and can induce the production of neutralizing antibodies (101, 102). VZVgE is probably the most immunogenic VZV glycoprotein (100, 102-105).

Epidemiology

VZV infections occur only naturally in humans and the virus has no animal reservoir. VZV infections are widespread around the world, especially in temperate climates where children become infected at a young age. In the absence of vaccination programs, it is estimated that over 90% of adolescents in temperate countries are infected with the virus. A recent Swedish study showed a seroprevalence of 66.7% in 5-year-old children and 91.5% in 12-year-olds (106). The seroprevalence in Swedish adults aged 35–95 years was measured at 97.9% (82).

Transmission

The infection is mainly transmitted by aerosols from vesicular fluid from the rash and is therefore highly contagious (107). The virus can also be spread from direct contact with the rash and possibly via infected respiratory tract secretions (107). The infection is transmitted mainly from individuals with chickenpox rash and to a lesser extent from individuals with zoster rash. Varicella is thought to be contagious from one to two days before the onset of the rash, but little conclusive evidence is available in the literature (108). The estimated incubation period is 14–16 days with an interval of 10–21 days. The disease is contagious until all the lesions have developed into crusts, which normally takes about 5–7 days. Immunocompromised individuals can be contagious for an extended period. If the exposure is sufficient, active herpes zoster can be transmitted to a VZV-susceptible individual and cause chickenpox.

Primary VZV infection

The virus infects via the oral cavity and upper respiratory tract where the virus will replicate in the mucosal epithelium and spread to regional lymph nodes where T cells can be infected. This is followed by a low-grade T-cell-associated viremia with the spread of the virus to the skin and possibly other organs where further replication will occur. Common symptoms in infected individuals are fever, malaise, and the characteristic, generalized rash with small itchy blisters over the body, especially over the upper part of the body and in the facial region (107). Chickenpox is in most cases a self-limiting disease, but several complications have been described (107). These manifestations include bacterial superinfection of skin, lungs, bones and blood, involvement of CNS such as cerebellar ataxia, meningitis, myelitis and encephalitis, and hemorrhagic/ischemic conditions e.g. stroke and even death (107, 109).

High-risk groups that are vulnerable for the development of severe disease and complications include immunocompromised individuals, pregnant women, and infants under one year of age (107, 110). Adults are also at greater risk of developing more severe illness compared with children (106, 107, 111). Although individuals from these high-risk groups are more likely to develop severe complications, VZV infections are so common that the incidence of these manifestations will also be substantial among non-risk groups (106). After primary infection, the virus establish latency in neurons in peripheral ganglia, i.e. dorsal root ganglia, cranial nerve ganglia and autonomic ganglia

(112-115). During latency, there is limited gene expression and replication (107).

Immunity

Primary VZV infection/vaccination will lead to the production of VZV-specific antibodies and cell-mediated immune responses that will induce immunity to chickenpox. However, both clinical and subclinical reinfection can occur after natural infection/vaccination (83, 86, 116, 117). The actual frequency of reinfections is unknown, but several studies suggest that reinfections and establishment of latency by reinfecting strains are more common than previously thought and that recombination occurs (83, 86, 116).

Reactivation

The main component of the defense against VZV is the cell-dependent immunity and when this immunity to VZV wanes, the virus can reactive and cause herpes zoster, but reactivation can also be subclinical (118). Receiving immunosuppressive drugs as well as being immunocompromised for other reasons are risk factors for VZV reactivation, along with old age as cell-mediated immunity decreases with age (119).

Herpes zoster causes a rash in the dermatome that is innervated by the ganglion from which the virus is reactivated. Reactivation of VZV will in many cases be complicated by neurological pain. Some individuals will experience persistent pain, termed postherpetic neuralgia, for months or even years after reactivation (120). Other complications that can occur are encephalitis, meningitis, VZV facial palsy (Ramsay Hunt Syndrome), myelitis, VZV vasculopathy, ocular disorders (keratitis and retinopathy), gastrointestinal disorders e.g. ulcers, hepatitis and pancreatitis and secondary bacterial infections of the skin, pneumonia and sepsis (107, 121-125). For adult patients suffering from CNS involvement following herpes zoster, sequelae with neurological complications are common (121, 126). Reactivations with complications can occur without vesicles; this is termed zoster sine herpete (127).

Treatment

Antiviral drugs that inhibit VZV replication can be used to treat VZV-infected individuals with severe disease and individuals who are considered at risk of such development. The nucleoside analogue acyclovir and its prodrug valaciclovir can be used. Another option is famiciclovir, which is a prodrug

that will be metabolized by the liver to penciclovir which has antiviral effects (128). Treatment should be started early after the first symptoms to be effective, but treatment is always recommended in Sweden for patients with severe VZV disease. Treatment can reduce virus excretion, shorten the course of the disease, and reduce the risk of complications.

Vaccines

There are effective and safe attenuated varicella vaccines, all of which are based on the VZV Oka strain (129-132). Varicella vaccines can be given in single or double dose schedules and are part of the child immunization programs of several countries, where the vaccine has significantly reduced the burden of disease (133-135). VZV vaccine is not yet part of the child immunization program in Sweden but can be obtained on the Swedish market.

The attenuated Oka strain has also been used to produce live herpes zoster vaccine (Zostavax®) but with a much higher concentration of virus compared with the chickenpox vaccines (131). The Zostavax® vaccine is given in a single dose and has been on the market in Sweden since 2013. Recently, on the market since 2020, there is an adjuvanted recombinant vaccine based on the highly immunogenic VZVgE (Shingrix®) (136-139). In contrast to the herpes zoster vaccine based on the attenuated Oka strain, this vaccine can be administered to immunocompromised individuals and induces a more robust immune response with a longer protection period, even in elderly individuals (140-142).

Viral diagnosis

Chickenpox and herpes zoster can often be diagnosed clinically except in the case of unusual disease presentations such as infection in the absence of rash and atypical rash. For example, CNS manifestations often occur in the absence of rash (122, 143-145). Laboratory tests are often necessary to diagnose atypical VZV cases and to link complications to VZV infections. Polymerase chain reaction (PCR) is the preferred diagnostic method and analyses can be performed on sample materials such as secretion from the vesicles, serum, CSF, and saliva (121, 143, 145-150).

PCR can be used to analyze and quantify VZV DNA during the acute phase but the viral load in CSF will gradually decrease during the disease. Detection of VZV DNA in CSF has in studies been mainly possible during the first seven days of disease (122, 151). Thus, the time window for detecting VZV DNA by PCR is limited. Diagnostic difficulties can arise in atypical cases when VZV

DNA cannot be detected. Serological methods for the detection of anti-VZV IgM and IgG in CSF and in acute and convalescence serum samples can be used as a complement to PCR diagnostics (152-154). Serological methods are also important for controlling immunity after infection/vaccination.

VZV serology

In one study, both serum IgM and IgG against VZV were detected within seven days after disease onset (69). A study of the antibody response in seronegative children vaccinated against VZV showed that 40% had seroconverted two weeks after vaccination and that 97% demonstrated detectable anti-VZV antibodies six weeks after vaccination (155). Another study showed that antibodies to VZV in CSF could be detected in 37% of patients with a VZV infection in the CNS after an interval of seven days from onset of symptoms (151).

VZV, HSV-1 and HSV-2 are, as previously described, genetically related viruses which all belong to the subfamily of alphaherpesviruses. They have homologous proteins and previous studies indicate that the viruses share common epitopes on gB (156, 157). These cross-reactive epitopes may give rise to cross-reactive antibodies (157-163).

The fluorescent antibody to membrane antigen (FAMA) method is considered one of the most sensitive methods for detecting anti-VZV antibodies, but the method is laborious, cannot be automated, requires experience in handling VZV, and interpretation is subjective (164, 165). The method is thus not suitable for testing large amounts of sera in routine analysis. Time-resolved fluorescence immunoassay is a sensitive method that uses purified whole cell antigen extract to detect anti-VZV antibodies, but there are enzyme immunoassay (EIA)/enzyme-linked immunosorbent assay (ELISA) methods that are more user-friendly and robust (166, 167).

VZV serological assays mainly use VZV-infected cell lysates as antigens to detect VZV-specific antibodies, but there are some methods based on glycoproteins (166, 168-171). However, whether the serological assays use VZV-infected cells, glycoproteins, or a mixture of viral proteins from VZV-infected cell lysates, most of them contain VZVgB, which may lead to cross-reactivity that can cause diagnostic problems.

1.3.4 *BETAHERPESVIRINAE*

- Cytomegalovirus (CMV, HHV-5)
- Human herpesvirus 6A (HHV-6A)
- Human herpesvirus 6B (HHV-6B)
- Human herpesvirus 7 (HHV-7)

Cytomegalovirus

The herpesviruses in the subfamily *Betaherpesvirinae* infects white blood cells, but the prototype member of this family, CMV, can also infect several other cells in the body. CMV establishes lifelong latency in monocytes/macrophages but may be reactivated periodically. It is also possible to become reinfected with another strain of the virus. CMV is spread through body fluids such as urine, saliva, blood, semen, and breast milk, but also through organ transplants.

The seroprevalence among women of reproductive age show geographic variation, from 45% to approaching 100% in different parts of the world with the highest numbers in Asia, Africa and South America and lowest in the USA and Western Europe (172). The CMV seroprevalence in Sweden is higher compared with the general figures for Western Europe (172). In Swedish adults aged 35–95 years the seroprevalence was measured at 83.2% (82).

Primary CMV infection in immunocompetent young individuals is often asymptomatic. CMV reactivations are also often asymptomatic in immunocompetent individuals. CMV infections can in immunocompetent adults occasionally cause symptoms of protracted fever, malaise, sweating, myalgia, fatigue and headache and increased transaminases (173-175).

CMV mainly causes severe disease, including hepatitis, pneumonitis, myocarditis, pancreatitis, and retinitis, in immunocompromised individuals where the immune system is unable to control the infection (176, 177). Neonatal CMV infection in premature infants and immunocompromised newborns can be aggravated by the immaturity of the immune system at this age and may include sepsis-like conditions, hepatitis, and thrombocytopenia (178-180). CMV infections in these individuals can be treated with antiviral drugs such as valganciclovir/ganciklovir and preventive measures may be indicated in individuals with high risk of severe disease (177, 178). CMV can have teratogenic effect and congenital CMV infection can cause permanent disabilities of the CNS and internal organs (181, 182). There is currently no vaccine against CMV, but research is ongoing (183).

HHV-6A, HHV-6B and HHV-7

HHV-6A and HHV-6B where previously considered to be the same virus species (184, 185). Although closely related, HHV-6A and HHV-6B have genetic, biological, and immunological characteristics that are so different that they were recently classified as two separate species by the International Committee on Taxonomy of Viruses (186, 187).

The viral genome of HHV-6 can be integrated into the genome of a small proportion of somatic cells and, in rare instances, into germ cell chromosomes, which may result in the offspring carrying a copy of the viral genome in all nucleated cells in the body (78). Inherited chromosomally integrated HHV-6 is uncommon and the frequency has been estimated at 0.2–2.9% depending on the region and population (78).

Less is known about HHV-6A and its role in human disease compared with HHV-6B. HHV-6A and B are closely related to HHV-7 (184). All three belong to the genus *Roseolovirus*, are T-lymphotropic viruses and, like other herpesviruses, establish lifelong latent infection (185). Both HHV-6B and HHV-7 are ubiquitous viruses with high seroprevalence in most countries (82, 185).

Primary infection with HHV-6B usually occur early in life after the loss of protective maternal antibodies. The infection with HHV-7 usually occurs a little later. The viruses are primarily transmitted through saliva (185). Both the primary infection and reactivations can be asymptomatic or symptomatic (185).

HHV-6B and HHV-7 (but less frequently) can cause Roseola Infantum, also known as exanthem subitum, a common childhood illness with symptoms including fever and skin rash (188, 189). Known complications of the infection are febrile seizures and status epilepticus (190). Immunocompromised individuals, especially transplant recipients, have an increased risk of severe disease in both primary infection and reactivation with the viruses (191, 192).

1.3.5 *GAMMAHERPESVIRINAE*

- Human herpesvirus 8 (HHV-8) or Kaposi's sarcoma-associated herpesvirus (KSHV)
- Epstein-Barr virus (EBV, HHV-4)

Human herpesvirus 8 (HHV-8)

The prevalence of HHV-8 varies around the world. Studies have shown seroprevalence below 10% in adult populations in North America and northern Europe but significantly higher proportion, 20–80%, in endemic areas in the Mediterranean region, the Xinjiang region of China and sub-Saharan Africa (193-196). The virus can be transmitted through several routes, including saliva, sexual, and parenteral transmission (195, 197). HHV-8, like all members of the herpesvirus family, can establish and maintain lifelong latent infection with potential later reactivation. In immunocompromised individuals, HHV-8 may cause Kaposi's sarcoma, multicentric Castelman's disease and pleural effusion lymphomas (152, 194, 198-200).

1.3.6 EPSTEIN-BARR VIRUS

Epstein-Barr virus (EBV) is a member of the *Herpesviridae* family and belongs to the *Gammaherpesvirinae* subfamily. *Lymphocryptovirus* is the virus genus, and the species is *Human gammaherpesvirus 4*. EBV is described more extensively because the virus is a central topic in the thesis.

The virus

EBV was discovered in 1964 by the two researchers Epstein and Barr (201). The virus was identified from Burkitt's lymphoma tumor cells and was the first virus to be associated with human cancer (201, 202). EBV soon proved to be a ubiquitous virus spread throughout the world (203-205). EBV has a linear dsDNA genome with approximately 172,000 bp (206), which encodes more than 85 genes (207).

EBV is currently divided into type 1 and type 2 based on the differences in the gene for Epstein-Barr virus nuclear antigen 2 (EBNA-2) (207). The dsDNA genome is encased in an icosahedral capsid. The nucleocapsid is in turn surrounded by tegument. Outside the tegument is the lipid envelope, which contains glycoproteins encoded by the viral genome.

Glycoproteins

The glycoproteins encoded by the EBV genome include gB (gp110), gH (gp85), gL (gp25), gM, gN, gp42, gp78, gp150, gp220, gp350 and BMRF2 (208). The viral glycoproteins in the envelope are essential for infecting host cells. The two glycoproteins gp220 and gp330 are encoded by the same gene (BLLF1) but gp220 will be shorter due to internal splicing of mRNA (209, 210). EBVgp350 consist of 907 a.a., with an extracellular N-terminal segment, a transmembrane segment and a small portion located on the inside of the viral membrane (211). The protein has extensive glycosylation with N- and O-linked oligosaccharide chains (209, 212). EBVgp350 is the major viral envelope protein and can be found on the plasma membrane of virus-replicating cells (208, 210, 213). The initial attachment between the virus and the B cells is between EBVgp350 and the CD21 receptor (211, 214-219). The primary target of EBV-neutralizing antibodies is EBVgp350 (220-222).

Seroprevalence and Transmission

EBV is one of the most widespread human viruses and the seroprevalence in adults is usually over 90% (204, 205). EBV is easily transmitted from one individual to another, primarily through saliva (204, 223, 224). Excretion of EBV has also been observed in saliva from asymptomatic individuals (223). In addition, the virus can be transmitted through blood, solid organ transplants and hematopoietic cell transplants (204).

EBV infection

It is common to be infected with the virus early in life. When the primary infection occurs in young, immunocompetent children it is often asymptomatic or causes symptoms that are indistinguishable from other mild childhood infections (225, 226). Infectious mononucleosis (IM) caused by EBV occurs mainly when adolescents and young adults undergo a primary infection (204, 227). Symptoms of IM includes sore throat, cervical lymphadenopathy, fever, upper respiratory symptoms, fatigue, headache, myalgia, subclinical hepatitis with increased levels of alanine aminotransferase, enlarged liver and/or spleen and skin rash (204, 227). For some individuals, fatigue will continue for several weeks to months (204). EBV mainly infects and transforms B cells but can infect other cells e.g. epithelial cells, T cells and NK cells (228, 229). The virus has a latent and a productive (lytic) cycle and it establishes a lifelong persistent infection in B cells after primary infection (230). EBV can be reactivated intermittently and can then be spread to other hosts (231).

EBV complications

EBV infections are primarily kept under control by the cell-mediated immune system and are therefore particular a problem in individuals with this type of immune deficiency (232-234). Immunocompromised individuals are at risk of developing smooth muscle sarcoma and EBV-driven B lymphoproliferative diseases, which, in immunosuppressed patients who have undergone solid organ transplantation or hematopoietic stem cell transplantation, are referred to as posttransplant lymphoproliferative disease (233, 235-237). EBV-associated malignancies such as Burkitt's lymphoma, diffuse large B cell lymphoma, Hodgkin's lymphoma, nasopharyngeal carcinoma, T/NK cell lymphoma and gastric carcinoma may develop in seemingly immunocompetent individuals (228, 232, 238, 239).

Chronic active EBV disease is very uncommon in Europe, but such cases are slightly more common in South America and Asia (240). Another serious but rare complication associated with EBV is infection-associated hemophagocytic lymphohistiocytosis (241-244). Infection with EBV, especially a primary infection in the form of IM, has been associated with an increased risk of developing multiple sclerosis (MS) (245-247). This will be described in more detail under 1.5 Multiple sclerosis.

Treatment and vaccine

There is currently no clinically effective antiviral treatment for EBV (248-250). In patients with EBV-associated diseases who are on immunosuppressive medications, a dose reduction may be performed (251). Other options for patients with EBV-associated B lymphoproliferative diseases are treatment with anti-CD20 monoclonal antibodies, which depletes B cells, such as rituximab (Mabthera®) or ocrelizumab (Ocrevus®) and cytotoxic chemotherapy (251-257). New possibilities for the treatment of EBV-associated cancers are immune cell therapies such as adoptive T-cell therapy (258).

There is currently no available EBV vaccine to prevent infection, disease, and associated diseases with the virus, but research is ongoing (258-260). The EBV glycoprotein 350/220 has been attempted as a prophylactic vaccine candidate based on its immunological properties (258, 259, 261, 262). An EBVgp350-based vaccine has in a phase two trial shown possible protection against IM but not against infection (262).

Viral diagnosis

Detection of heterophilic antibodies may contribute to the diagnosis of individuals with compatible clinical manifestations of EBV (263, 264). The sensitivity of these tests is lower in young children due to lack of production of heterophilic antibodies (264). False positive tests are also seen in several other conditions including acute infections, cancer and autoimmune diseases (204). Serological methods are frequently used to determine the EBV infection status of patients. In immunocompromised individuals where the serological response may take unusual courses, PCR is the preferred method for diagnosing current infection and for monitoring changes in viral load (265-267).

EBV serology

The two antigens primarily used to detect EBV-specific antibodies are viral capsid antigen (VCA) and Epstein-Barr virus nuclear antigen 1 (EBNA1) (268-272). Anti-VCA IgM antibodies can often be detected when symptoms of EBV disease occur (204, 273, 274). Anti-VCA IgG can be detected as early as the onset of symptoms and is often detected during the first month of illness (204, 273, 274). Anti-VCA IgM usually begins to decline after about two to three months but may persist longer, while anti-VCA IgG will persist throughout life (22, 263, 273, 274).

The generation of antibodies to EBNA1 takes longer compared with the production of anti-VCA antibodies. Anti-EBNA1 IgG can be detected no earlier than four weeks after the onset of symptoms, but it often takes around three months before these antibodies can be detected (204, 272, 275). Thus, detection of anti-EBNA1 IgG during an acute illness can be used as a marker to rule out primary EBV infection. Overall, however, there is a large variation in the serological EBV response between different individuals (204, 272-274). When anti-EBV antibodies can be detected also depends on the type of assay platform and the specific type of antigen used (204, 272).

The combination of anti-EBNA1 IgG, anti-VCA IgM and anti-VCA IgG detection can be used to determine the stage of EBV infection (276). Detection of anti-VCA IgM with or without anti-VCA IgG but without detection of anti-EBNA1 IgG indicates acute infection while detection of anti-VCA IgG and anti-EBNA1 IgG without anti-VCA IgM is typical of previous infection.

1.3.7 MEASLES VIRUS

Measles virus (MeV) belongs to the *Paramyxoviridae* family within the *Orthoparamyxovirinae* subfamily. The viral genus is *Morbillivirus* and the species *Measles morbillivirus*.

The virus

MeV is closely related to rinderpest virus, which was a pathogen in cattle before it was eradicated by vaccination in 2011 (277, 278) and it is possible that MeV developed as a zoonotic infection when humans and cattle started to live close together (279). MeV has a single negative-stranded RNA genome. The size of the genome is relatively conserved to 15,894 nucleotides, but small discrepancies have been reported (280, 281). The genome encodes six structural proteins, nucleocapsid protein, matrix protein, phosphoprotein, large protein, hemagglutinin protein and fusion protein (282).

MeV is divided into eight clades, A to H, and 24 genotypes based on the diversity of the 450 nucleotides in the carboxyterminal of the nucleocapsid protein (283). There is only one serotype of MeV. The genome is encased within a helical capsid. Surrounding the capsid is the envelope where hemagglutinin and fusion glycoproteins are incorporated.

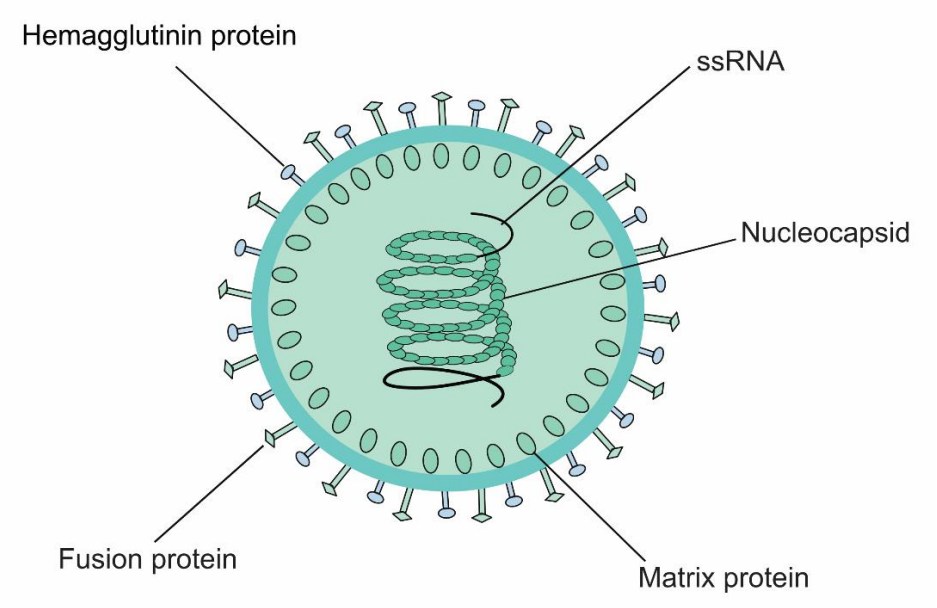

Figure 12. Schematic illustration of a measles virus.

Transmission

MeV is highly contagious and infects cells in the respiratory tract. When infected individuals cough or sneeze, the virus is transmitted via airway droplets and airborne transmission (284, 285).

Measles infection

Humans are the natural host of MeV and there is no animal reservoir although non-human primates can become infected with the virus (286-288). The incubation period is often about 10–14 days but may be as long as 23 days (289). Symptoms usually begin with fever, cough, coryza and conjunctivitis, which after around three to four days are followed by the characteristic erythematous, maculopapular rash. The small white papules on the oral mucosa, termed Koplik spots, appear a day or two before the rash and can be used clinically to diagnose the measles disease before the rash occurs.

Complications can affect many organs in the body and include pneumonia, otitis media, laryngotracheobronchitis, stomatitis, diarrhea, and neurological complications. Risk groups for complications are children under 5 years of age, adults over 20 years of age, pregnant women, and immunocompromised individuals (287). Pneumonia causes the highest MeV-related morbidity and mortality.

The neurological complications are rare but serious. MeV can cause acute encephalitis (290) and may trigger the autoimmune disease acute disseminated encephalomyelitis, which can occur during or shortly after the measles episode (291). Severely immunocompromised individuals who are unable to eliminate MeV infection due to impaired cellular immunity may develop measles inclusion body encephalitis within months after the acute infection, with progressive MeV infection in the brain causing neurological deterioration that often leads to death (291, 292). Neurological complications can also occur several years after the acute disease as in subacute sclerosing panencephalitis (293-295). The disease occurs mainly in children who are infected with MeV before the age of two. In these cases, the immune system in response to the progressive MeV infection in the brain will cause extensive brain damage that will lead to progressive loss of motor and cognitive functions, seizures, and death (293, 294). The pathogenesis behind the disease has not been fully established (295). Laboratory support for the diagnosis if often dependent on intrathecal anti-MeV IgG detection because MeV RNA is rarely detected in CSF, although MeV has been discovered in brain tissues in patients with the disease (293, 294, 296).

Immunity

Natural infection generally provides lifelong immunity with a stable and long-lasting anti-MeV IgG response (22). Immunity after vaccination is also long but the anti-MeV IgG response decreases with time (297) and breakthrough cases occur, especially after only one vaccine dose (286, 298, 299). Vaccine-modified measles usually comes with milder symptoms and may therefore be more difficult to diagnose clinically (298-300).

Treatment

There is no specific antiviral treatment for measles. The management of patients with MeV infection is therefore focused on supportive therapy with possible vitamin A supplementation, rehydration in severe diarrhea and antibiotic treatment in secondary bacterial infections.

Vaccination

MeV has been a leading cause of morbidity and mortality in the world before vaccines were developed against the disease. The control of MeV is largely due to vaccination with safe and effective attenuated live vaccines. Vaccines have been available since the 1960s and the fact that MeV has only one serotype has facilitated the use of the same type of vaccine during decades. WHO recommends two doses of MeV vaccine in all countries' childhood immunization programs and that countries striving to eliminate MeV need to achieve 95% coverage across the country (301).

Diagnosis

A clinical diagnosis can be established based on typical clinical symptoms, but it can be more difficult early in the disease when the typical rash is not present and in vaccine-modified measles (298, 299). Koplik spots have been considered a typical marker for measles, but a study demonstrated that the spots can also occur in other viral infections such as rubella (302).

PCR

MeV RNA can be detected by PCR in materials from nasopharynx and pharynx swabs, oral fluid, and urine. To increase the likelihood of detecting MeV RNA, samples should preferably be collected within seven days after the onset of the rash, but it may be possible to detect MeV RNA for months in some individuals after both natural infection and vaccination (287, 303, 304).

It is also preferable to analyze different sample materials to increase the chance of detecting MeV RNA (299, 303).

Measles serology

Although PCR is the preferred method for diagnosing acute measles (300), a measles diagnosis can also be established by serological detection of anti-MeV IgM antibodies or by a significant increase in anti-MeV IgG levels between paired sera taken in the acute and convalescent phase.

Negative measles serology in a patient with true measles infection may be due to early sampling before the patient has seroconverted. Samples taken within three days after the onset of the rash have a higher risk of becoming anti-MeV IgM-negative and sampling within 10 days have a higher risk of becoming IgG-negative (287, 299).

Cases of vaccine-modified measles, i.e. breakthrough infection, also have a higher risk of becoming anti-MeV IgM-negative (287, 299). It is well known that false positive anti-IgM MeV responses occur and may be due to nonspecific reactions such as other antibodies or rheumatoid factor (287). It can be difficult to obtain paired serum samples collected during the acute and convalescence phase and the diagnosis in these cases comes retrospectively, which is often not so useful. IgG avidity assays can be useful in identifying measles reinfections (305).

Serological analyzes are important to monitor the MeV seroprevalence in populations to gain knowledge about population immunity. Many different commercial assays are used to determine MeV seroprevalence including hemagglutination inhibition, neutralization assays, microtiter plate assays and automated immunoassays (306). The MeV nucleocapsid protein is known to induce a long-lasting antibody response and is therefore used in many serological assays (307, 308).

1.4 VIRAL SEROLOGY

Serological methods are important in the field of virology and are frequently used. The assays are used for controlling immunity after infection/vaccination, to screen individuals for potential infections and to diagnose individuals with viral disease. The methods should be sensitive, specific, reproducible, rapid, and not too expensive. Viral serological assays are based on antigen-antibody reactions. The presence of antibodies to a particular virus and/or viral antigen indicates whether the individual from whom the sample was taken has been infected with the specific virus. Most viral serological assays that detect antibodies measure the IgM and IgG responses.

Diagnostics

An infected individual's antibody isotype pattern may indicate whether the individual is undergoing a primary infection or a reinfection/reactivation. The detection of IgM antibodies to a particular virus indicates that it is a current/recent infection. If only IgM is detected but not IgG, it is likely to be a primary infection. To give an example, if anti-VZV IgM but not anti-VZV IgG is detected, it indicates that it is a primary VZV infection. If both anti-VZV IgM and anti-VZV IgG antibodies are detected, it may be a primary infection, but it may also be due to reactivation because anti-VZV IgM antibodies are often produced upon VZV reactivation (72, 73).

Antibody detection against a certain virus is performed in some cases on two occasions, first during the initial disease (acute phase) and then during the convalescent phase, after about 2–4 weeks. It is then possible to decide whether an individual seroconverts. Seroconversion means that an individual who does not have detectable antibodies to the examined virus, i.e. is seronegative, has detectable antibodies in the follow-up sample and thus becomes seropositive. Seroconversion thus indicates that the individual has a primary infection. It is also possible to examine whether an individual has undergone a reactivation/reinfection by comparing antibody levels in samples taken during the acute and convalescence phase, where a significantly increased level between the samples indicates a current/recent reactivation or reinfection with the virus.

If only IgG antibodies can be detected in the samples and not IgM, this indicates that the person has been infected or immunized with the virus before. Whether IgM is detectable or not in reactivations/reinfections varies between different viral infections and is also affected by the host's immune response.

Seroprevalence

Serological methods are important for epidemiological investigations. Seroprevalence is the number of individuals who have antibodies, i.e. are seropositive, against the virus of interest in the studied population.

Sensitivity and specificity

The reliability of diagnostic assays is often described by the specificity and sensitivity of the test. Sensitivity is a measure of the analysis' ability to detect all true positive cases of a particular disease. If the sensitivity is low, there will be individuals with the disease who will not be detected, these are called false negatives.

Specificity is a measure of the analysis' ability to distinguish between individuals with disease and individuals without disease. The analysis should become negative when the sample is derived from a healthy uninfected individual. Samples from healthy individuals who become positive are termed false positives. If many individuals are false positive, the assay has low specificity.

Antibody levels

The antibody level is the amount of antibodies produced against a particular antigen. Antibody levels are often described as antibody titers. The word titer comes from titrate which means dilute. Classically, samples have been diluted in dilution series, e.g. 1/2, 1/4, 1/8 and so on. The antibody titer is the highest dilution that still gives a positive result in the analysis, and it is expressed as the inverse of that dilution level. Titers are thus not an absolute measure but a relative concept.

The word titer is often used synonymously with antibody level regardless of whether the level of antibodies is determined using dilution series or not. Newer methods often do not use dilution series to determine antibody levels. In ELISA, the color intensity can be measured in a spectrophotometer as optical density (OD). It provides a relative measurement of the antibody level in the sample. There are also methods that provide even more quantifiable results where there are international standards to follow.

Intrathecal antibody production

The CNS is protected by the blood-brain barrier (BBB), which consists of the endothelial barrier between the CNS and cerebral capillaries, as well as the blood-CSF barrier, where CSF is produced as an ultrafiltrate of the blood mainly by the choroid plexus. (309, 310). Most proteins in CSF are blood-derived but some proteins are produced in the CNS, which is called intrathecal production. Some proteins such as albumin are derived only from the blood, while other proteins such as neuron-specific enolase are produced intrathecally (311, 312). Antibodies in CSF are both derived from blood and can be produced intrathecally. In healthy individuals, CSF antibody levels are low compared with blood levels. Proteins including our antibodies are transferred from blood to CSF primarily through diffusion across the blood-CSF barrier (309).

The transport rate depends mainly on the molecular radius of the proteins and the protein concentration in the blood (309, 313). Smaller proteins have a lower concentration gradient between blood and CSF compared with larger proteins. This is reflected by the concentration gradient for different subclasses of antibodies where IgG has a lower gradient compared with IgM, which is larger in size compared with IgG. The concentration gradient between CSF and serum for IgG and IgM is estimated to be 1:429 and 1:3300, respectively (314).

The concentration of blood-derived proteins in CSF is also dependent on the CSF flow rate, which varies between individuals and can be affected by factors such as age and various diseases (312, 315, 316). One theory is that decreased CSF flow causes a reduction in CSF exchange which leads to an increased blood-derived protein concentration in CSF (312, 314, 315). In diseases such as Guillain-Barré syndrome, brain tumors and chronic inflammatory demyelinating polyneuropathy, there is an increase of blood-derived proteins in CSF, which is probably due to a decrease in CSF outflow leading to reduced CSF turnover (314-316).

In a viral infection of the CNS, antibodies can be produced against the virus by antibody-secreting cells. A significant antibody response in CSF indicates an intrathecal antibody production, but the response in CSF must be compared with serum antibody levels because, as previously explained, antibodies in CSF can be derived from blood and the antibody levels in CSF are thus partially dependent on the protein level in the blood. Various diseases may increase the concentration of antibodies in the CNS, which may be due to decreased CSF flow and/or alterations of the barriers (309, 314-319). The decrease in CSF flow could be due to decreased circulation in the subarachnoid

space, lumbar blockage by a local stenosis/tumor, impaired drainage by arachnoid villi, or reduced CSF production (315). CNS infections such as meningitis can lead to decreased CSF flow due to increased CSF viscosity, meningeal adhesions, and deposition of cells and protein complexes in the arachnoid villi that will cause flow obstruction (315).

To investigate whether an increased levels of antibodies in CSF are due to intrathecal production or to an increased amount of blood-derived antibodies, both the antibody levels and a reference protein must be measured and compared in CSF and blood. Albumin is often used as a reference protein because it is only produced by the liver and is thus a useful indicator of the proportion in CSF that are derived from blood (320, 321). Other choices may be to measure a reference antibody response to another common virus or to measure the total IgG levels in CSF and blood (322, 323). Many formulae have been used to distinguish between an intrathecal antibody response and blood-derived antibodies including Ig synthesis rate, Ig index, Ig_{loc}, CSF/serum quotient diagrams (Reibergram) and isoelectric focusing for detection of oligoclonal bands (OCB) (314, 321, 324-327). When analyzing intrathecal antibody production, any erythrocytes in the CSF must be considered as it may be a sign of barrier damage that can occur due to traumatic lumbar puncture, neurosurgical surgery, or other causes.

1.4.1 SEROLOGICAL METHODS IN VIROLOGY

There are several different serological methods for detecting viral antigens and antibodies against viruses. Many are heterogeneous assays, also called separation assays, because these assays must separate the product to be measured from unreacted materials before detection. Although there are many different methods, many are based on the same principles. The principles include a capture system (antigen or capture antibody), addition of the analyte (the substance i.e. antigen/antibody, that the assay is designed to measure) and a detection system. Immunoassays are based on the specific binding that occurs between an antibody and the specific antigen that it recognizes. Solid-phase immunoassays are methods that use a solid phase in their capture system. Heterogeneous assays have separation steps where a common separation strategy is to wash away unbound material. The basic principles of heterogeneous solid-phase immunoassays for the detection of antibodies/antigens including the enzyme-linked immunosorbent assay (ELISA) are described below (328-330). Examples of other viral serological assays will also be presented.

Indirect detection

Indirect detection is a useful principle for detecting human antibodies in various sample materials including serum and CSF. The antigen is coated on a solid phase, which may be a glass slide, polystyrene microtiter plate, nitrocellulose membrane, nylon membrane, polyvinylidene difluoride membrane, polystyrene beads, or magnetic beads. During the incubation period, the antigen will adhere to the solid phase by passive adsorption (329, 331, 332). Unbound antigen is then washed away. The remaining areas of the solid phase where the antigen has not been bound can be blocked to avoid residual binding capacity and thus to avoid non-specific binding. This can be performed by using blocking agents e.g. bovine serum albumin or non-fat dry milk. The sample is then added and if there are complementary antibodies to the antigen, these so-called primary antibodies will bind to the antigen. In serum and CSF samples, there are polyclonal antibodies that will bind to different epitopes on the antigen.

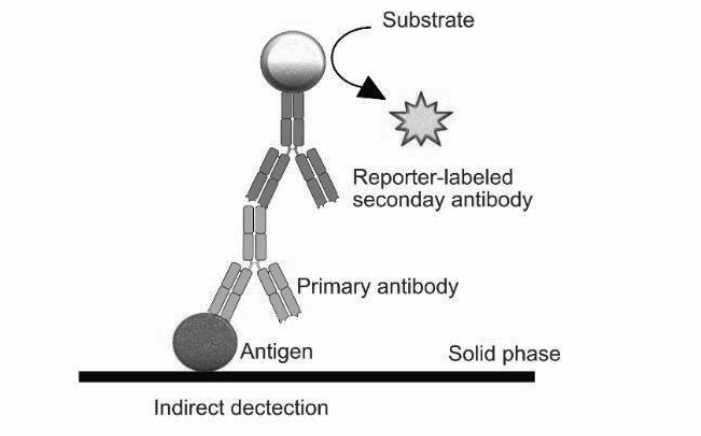

Figure 13. Basic principle of indirect detection of antibodies in a sample.

After incubation, all unbound antibodies and other unbound components in the sample will be washed away. A conjugate will then be added. The conjugate consists of a reporter-labeled, secondary antibody from another animal species that targets the Fc portion of the primary antibody. These secondary antibodies can be directed against different antibody isotypes, e.g. anti-IgM, anti-IgG, or against more than one isotype, depending on which isotype the assay is to detect. These secondary antibodies will bind if there are primary antibodies to bind to. After incubation, the unbound secondary antibodies will be washed away. For detection, a substrate is added which, together with the reporter, gives rise to fluorescence, color, or luminescence to measure the analytes in the sample. Many reporter-labeled secondary antibodies are polyclonal and

will therefore bind to several different epitopes on the primary antibody. This allows amplification of the signal because several antibodies can bind to the same primary antibody. This can amplify the signal of the assay, but it can also lead to a higher background and reduce the overall signal due to the increased risk of non-specific binding.

Direct detection

The sample with the antigen of interest is coated on a solid surface e.g. a glass slide or a microtiter plate. After incubation, unbound material from the sample is washed away. Blocking can be performed to avoid non-specific binding. A complementary reporter-labeled antibody is then added to the solid-bound antigen. After another round of incubation, unbound reporter-labeled antibodies are washed away. The next step is to add a substrate that will react with the reporter. Depending on the reporter and substrate, the detection may be based on fluorescence, color, or luminescence.

A direct detection method is faster than the other methods because it involves fewer steps. One limitation with this method is that the complementary antibody must be conjugated to a reporter, which can be quite costly and time consuming. There is also a risk that the immune reactivity of the antibody is negatively affected by the labeling with the reporter. The risk of cross-reactivity and non-specific binding is reduced in this method by not using a secondary antibody, but this also removes the possibility of amplifying the assay signal. Complex samples with many proteins can be a problem because it is possible that many of the proteins are not of interest but that they take up space on the solid surface and thereby reduce the sensitivity of the assay.

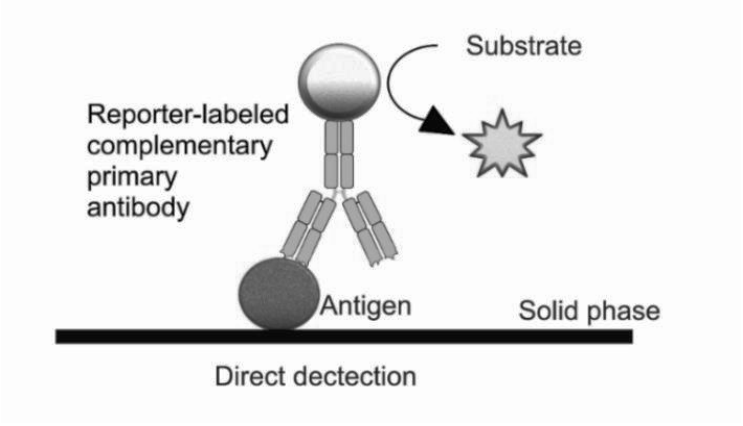

Figure 14. Basic principle for direct antigen detection.

Sandwich/capture assays

In a sandwich assay, also called capture assay, a monoclonal antibody (capture antibody) is immobilized on the solid phase to capture soluble antigen. After incubation, washing and blocking, the patient sample is added. If the complementary antigen is present in the patient's sample, it will bind to the capture antibody. The same incubation, washing, conjugate and substrate steps as described above for direct or indirect detection can then be performed. For direct detection in the sandwich assay, a secondary reporter-labeled antibody directed against the antigen will be added. For indirect detection in the sandwich assay, the secondary antibody that attaches to the antigen will be unlabeled. This secondary antibody will in turn be detected by a reporter-labeled antibody from another animal species. It is crucial that the reporter-labeled antibody does not bind to the capture antibody. It is also important that the capture antibody and the secondary antibody bind to different epitopes that are located far enough apart on the antigen.

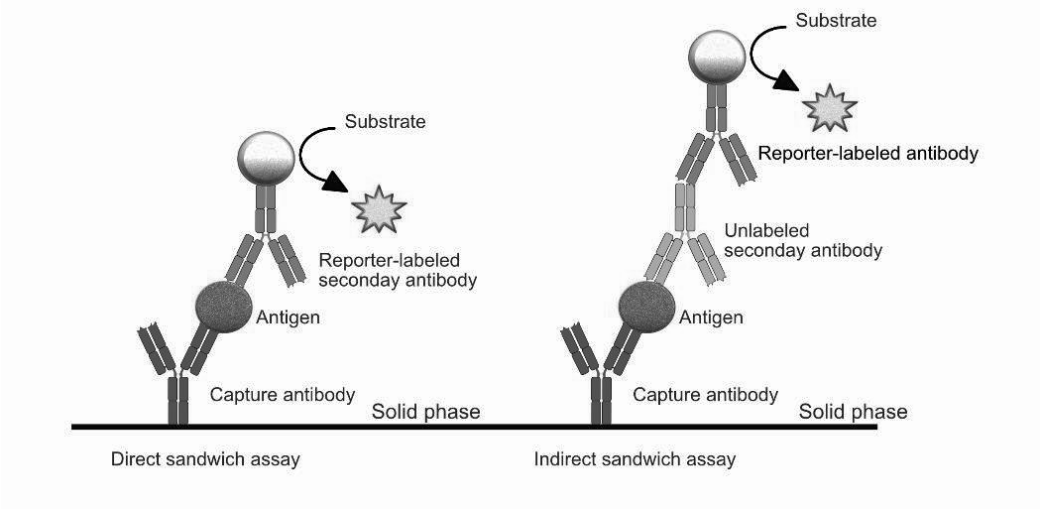

Figure 15. Basic principle for a direct and indirect sandwich assay.

Competitive/inhibition assays

The basic principles described above with direct and indirect detection, and sandwich assays can all be adapted to competitive/competition assays, also called inhibition assays. The distinguishing feature of these assays is that the sample with an unknown amount of antigen/antibodies of interest will compete for binding with a reporter-labeled standard analyte for attachment to a limited number of binding sites on capture antibodies/antigen. The higher the concentration of the antibodies/antigens of interest in the patient's sample, the

less reporter-labeled standard analyte will bind to the capture antibodies/antigen. Unbound reporter-labeled standard analyte will be washed away in following steps, and this will weaken the signal in the assay when the substrate is added. In a competitive assay, the generated signal is thus inversely proportional to the amount of bound antigens/antibodies in the patient´s sample.

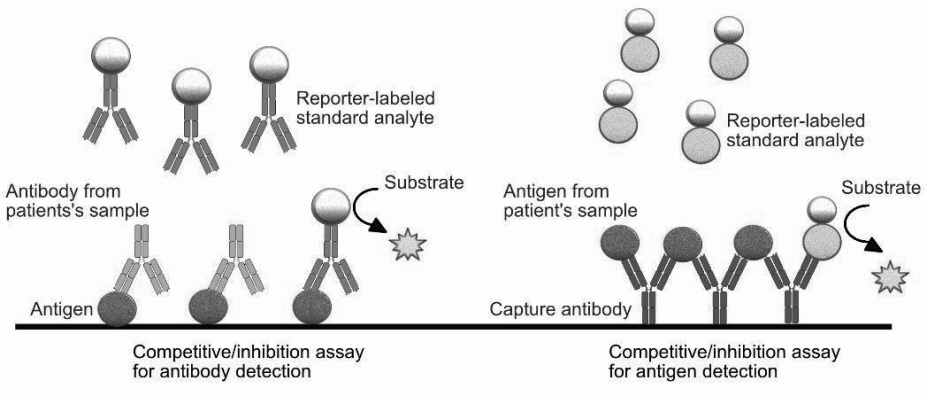

Figure 16. Basic principles for competitive/inhibition assays for antibody or antigen detection.

Structural arrangement

Solid-phase immunoassays have immobilized antigen/capture antibodies on a solid surface that provide a high concentration of epitopes located to a specific area. The advantage is that this gives a higher chance of forming antibody-antigen complexes over a certain time frame compared with if the antigen/capture antibodies were free in solution. When a paratope from an IgG molecule has bound to an epitope, the other paratope is more likely to bind an identical epitope if the epitope is close, which is the case when the antigen is immobilized on a solid surface compared to if it is free in solution.

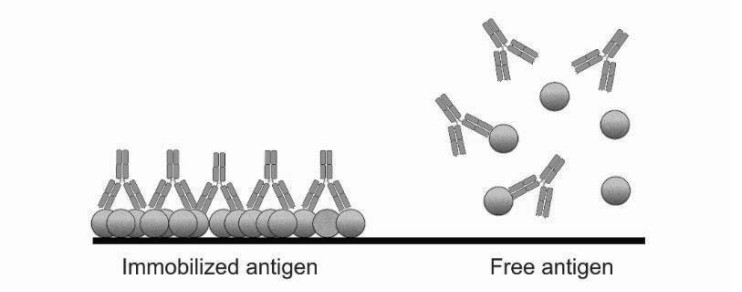

Figure 17. A higher quantity of antibody-antigen complexes is formed during a certain time frame if the antigen is immobilized on a solid phase compared to complexes formed when the antigen is free in solution.

However, it is important to consider how the three-dimensional structure of the antigen can change when used in serological assays. Antigens and antibodies can be conformationally altered by immobilization to solid phases (329). Some assays, including Western blot, use denatured proteins, which limit the three-dimensional structure of the proteins and the presentation of conformational epitopes. Other assays, such as immunofluorescence with virus-infected cells, will to a greater extent retain the natural forms of the proteins.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA) were developed at the same time by different research groups in Sweden and the Netherlands (333, 334). The methods are based on the same principles for detection of antibodies/antigens as described above (328, 335). The methods can detect small quantities of antigens and antibodies in fluid samples such as serum and CSF. In both ELISA and EIA, an enzyme is used as the reporter to detect biological molecules. The most used enzymes are probably alkaline phosphatase and horseradish peroxidase. The enzyme signal will increase over time by continuing to turnover more substrate unless a stop solution is added to the assay. Microtiter plates are often used as a solid phase in these assays. ELISA/EIA methods are widespread throughout the world with many adaptations (336).

Radioimmunoassay

The radioimmunoassay (RIA) was first described in 1960 by Rosalyn Yalow and Solomon Berson for measuring endogenous plasma insulin (337). The development of the RIA was awarded the Nobel Prize in 1977, but only Yalow was able to receive the prize since Berson passed away in 1972 (337). The basic principles of solid-phase immunoassays apply to RIA. Radioisotopes are used as reporters for the reaction. Although the methods are sensitive and specific, the problems with radioisotopes when it comes to laboratory safety and the aspect of disposal of radioactive waste have prompted the development of other techniques that use other reporters for the reactions.

Chemiluminescence immunoassays

Chemiluminescence immunoassays (CLIA) determine the concentration of antigen/antibodies by the intensity of the luminescence that is emitted from the chemical reaction in the assay (338). The basic principles of solid-phase immunoassays also apply to CLIA. These methods can have direct or indirect detection, be sandwich assays and be competitive or non-competitive. The

reporter in these assays such as acridinium, together with the substrate, can create a chemiluminescent reaction, which emits light and is measured as relative light units. The signal is directly proportional to the concentration of antigen/antibodies in the sample. Assays using magnetic microparticles as solid phase are often termed chemiluminescence microparticle immunoassays (CMIA). CLIA methods have been widely used in virology laboratories due to the ability to analyze many samples in a relatively short time, the high degree of automation and most importantly, the fact that the methods are sensitive and specific (271, 339-341).

Line immunoassay

The principle of line immunoassay is that antigens to be tested are adsorbed in separate bands on a membrane strip of e.g. nylon. The patient sample is added and if it contains complementary antibodies to any of the antigens, these antibodies will bind to the respective antigen during the incubation period. Unbound antibodies are then washed away. To detect the bound antibodies, a secondary antibody conjugated to an enzyme is added. After incubation, washing is performed to remove unbound secondary antibodies. The substrate is then added. Positive reactions are seen as bands on the strip. Each test strip also contains control bands to determine that sufficient sample material has been added to the strip.

Western blot

Western blot is an assay technique where sample identification occurs in part through molecular weight and specific antibody binding. After denaturation of the proteins in the antigen, they are separated by electrophoresis, usually in polyacrylamide gel. The proteins migrate in the gel and the final position depends on the size, conformation, and charge of the protein. Small proteins travel longer compared with large proteins. The separated proteins can then be stained directly on the gel with e.g. Coomassie blue or transferred with electrical voltage to a membrane, e.g. nitrocellulose membrane. If the proteins are transferred to a membrane, the same overarching principles as previously presented can be used for detection of antigen/antibodies.

Direct detection to determine if the protein of interest is present can be done by adding reporter-labeled antibodies and substrate. Indirect detection can be performed by adding a patient sample or a monoclonal antibody along with a secondary reporter-labeled antibody (conjugate). The reporter can for example be an enzyme which, together with the substrate, creates a color change on the membrane. The thickness of the band gives an indication of the amount of

antigen. Western blot provides good control of the specificity of antibody binding and is often used as a confirmatory test.

Immunofluorescence

Immunofluorescence methods have been widely used in virology laboratories. The methods can detect antigen/antibodies by using a fluorochrome as a reporter which together with added substrate can emit fluorescent color. Common fluorochromes are fluorescein which gives a yellow-green color and rhodamine which gives a red color (70). A fluorescence microscope is used to observe the reaction. Virus-infected cells that attach to glass slides are often used in these assays. In direct immunofluorescence, the primary antibody labeled with a fluorochrome together with the substrate can directly detect the viral protein. This can be used to detect virus-infected cells.

In indirect immunofluorescence, antibodies to a particular virus can be detected. The patient's sample is added to virus-infected cells. If the sample contains antibodies to the virus, the antibodies will attach to the virus-infected cells. These antibodies can then be detected using a secondary antibody labeled with a fluorochrome. Immunofluorescence is a rather labor-intensive method, which cannot be automated, and which takes a long time to master. An advantage of the method is that it is possible to locate where on the virus-infected cell the antibodies bind.

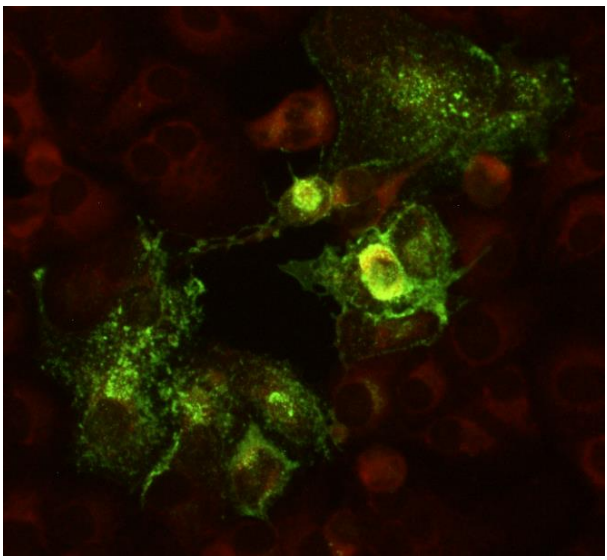

Figure 18. An immunofluorescence photograph showing fluorescent stained anti-varicella-zoster virus glycoprotein E (VZVgE) monoclonal IgG antibodies that have attached to VZV-infected cells.

Hemagglutination inhibition assay

The hemagglutination inhibition assay was among the first serological assays in diagnostic virology. The method is based on the hemagglutination process where viral hemagglutinin glycoproteins on the surface of certain viruses e.g. influenza virus, binds to the sialic acid receptors on the surface of erythrocytes and cause the red blood cells (RBCs) to agglutinate together in complexes. The presence of specific antibodies will inhibit this process and thus the viruses will not agglutinate the erythrocytes. The method is limited for viruses with hemagglutination glycoproteins e.g. influenza virus, MeV, rubella virus (RV) and dengue virus (342-344).

Latex agglutination test

In a latex agglutination test, latex beads are coated with the virus of interest, e.g. RV. These beads are added to the patient's serum sample. If the serum sample contains antibodies to the virus, the antibodies will agglutinate with the beads. The agglutination titer is the highest serum dilution where there are enough antibodies for agglutination to occur (345). Most patients, with a symptomatic primary EBV infection, will develop heterophilic antibodies i.e. IgM antibodies produced due to the polyclonal response of EBV-infected B cells. These antibodies can agglutinate mammalian erythrocytes. Detection of heterophilic antibodies is a rapid, simple, and widely used method for diagnosing EBV-induced mononucleosis (264). Monospot, a commonly used test, is a latex agglutination assay that uses equine erythrocytes, and if the patient's sample contains heterophilic antibodies, the antibodies will agglutinate the erythrocytes (204, 264).

Complement fixation test

The complement fixation test is another example of an early serological method. The serum sample from the patient is heated so that the complement proteins but not the antibodies in the sample are destroyed. Subsequently, a standardized amount of complement proteins and the antigen of interest are added to the sample. If the serum sample contains antibodies to the added antigen, the formation of antibody-antigen complexes will occur. If complexes are formed, the complement proteins will bind to the complexes. In the next step, animal RBCs carrying a complement-binding antibody are added. If large amounts of antigen-antibody complexes have been formed, there will be no free complement proteins in the solution as they will have bound to the complexes, but if there are not enough antigen-antibody complexes to bind all the complement proteins, the remaining ones will bind and lyse the RBCs (70).

Neutralization assay

Neutralization assay is a clinically relevant method for identifying the presence and magnitude of functional antibodies that can prevent viral infection (346). The patient's serum in different dilutions and a defined amount of the infectious virus will be added to cell cultures. The cells will be infected by the virus if there are not enough neutralizing antibodies that will bind to the surface of the virions and prevent them from entering the cells. Whether the virus has infected the cell culture can be determined by the presence or absence of cytopathic cell effects or other evidence of ongoing infection using immunoreactive techniques. The neutralization titer is the highest serum dilution that prevents infection of the cells. Neutralization assays are labor intensive but may be warranted to use in some cases to examine immunity including after vaccination.

1.4.2 ADVANTAGES OF SEROLOGICAL METHODS

Many of the serological methods currently in use are sensitive, specific, reproducible, fast, and easy to perform. Some of the methods can be automated, which minimizes the risk of human error and increases the ability to analyze many samples in a short time. Serological analyzes are often inexpensive, making them cost-effective. Some serological assays are suitable as screening methods for infectious diseases in blood donors, during pregnancy, before transplantations and before surgeries. In addition, serological methods are important for viral epidemiological studies in the population and to increase our knowledge of various viral diseases.

Serological assays can help determine different phases of an infection, for instance to define different stages of hepatitis B. Serological tests for hepatitis B include analyzes of several hepatitis B-specific antibodies and antigens. The different combinations of serological responses to these antibodies and antigens can be used to determine if a patient has acute or chronic hepatitis B. Hepatitis B serology can also demonstrate if an individual is immune to hepatitis B due to previous infection/vaccination or is susceptible to the disease.

Serology can be used in later stages of a disease and after a disease outbreak when it is no longer possible to detect viral RNA/DNA by PCR. To give an example, serological methods are the primary diagnostic method for diagnosing tick-borne encephalitis (TBE) because there is little chance of detecting viral RNA during the second phase of the disease when the patient

has begun to experience neurological symptoms, which is when most patients seek medical attention. Serological assays do not require active parts of the virus for analysis, which may facilitate sampling.

1.4.3 LIMITATIONS OF SEROLOGICAL METHODS

It is not feasible to perform serological analyzes without having an aim for the investigation. It is thus important to decide on the basis of the patient's history and symptoms which antigens/antibodies are to be analyzed.

Serological interpretation

The interpretation of serological results can be complicated. The serological response is affected by the type of virus, whether it is a primary infection, a chronic infection, a reactivation or a reinfection, the length of time that has elapsed since the time of infection and the immune system of the infected individual. The test result can also be affected by the method used to detect antigen/antibodies. Viruses have different abilities to induce antibody production and individuals have different abilities to generate antibodies. The normal level of antibodies in healthy people also varies depending on, among other things, the patient's age and immunocompetence. Serological results should be interpreted in a clinical context where the patient's disease panorama and disease course are considered. It is also important to consider whether it is a common or rare viral disease and whether the individual has received blood products as this may result in a passive transmission of antibodies that can be detected in assays and cause confusion.

Antibody dynamics

A serological analysis provides a snapshot of a dynamic situation and repeated sampling may therefore be necessary for conclusions to be drawn. When antibodies cannot be detected against a particular virus, it may be because the individual is uninfected by the virus, but it may also be because antibodies have not yet been produced. For some viral infections it can take quite some time from the time of infection until seroconversion occurs e.g. in infections with hepatitis C virus. The patient may thus have symptoms and be contagious, but the diagnosis cannot be based on antibody detection, this situation is called the serological window phase of infection (347-349). This is an important reason why many western countries use PCR for screening blood donors (350). The window phase for serological diagnosis of some infections e.g. human

immunodeficiency virus (HIV) has been shortened by introducing combination tests that detect both HIV antigen and anti-HIV antibodies (351, 352).

Some individuals produce such low levels of antibodies that they may never reach the established detection limit and these individuals may therefore be seronegative in the serological analysis even though they have been infected/vaccinated. This may be the case for some immunocompromised individuals who are unable to generate an adequate antibody response to infections (353). There are also rare cases of individuals with hepatitis C virus infection who will not generate antibodies to the virus (354). Another example is that approximately 5–10% of healthy individuals do not generate anti-EBNA1 IgG after an EBV infection (204, 272). Antibodies are also not generated at an exact time after a certain infection because there are differences in the kinetics of individuals' antibody production. For instance, it has been shown that most individuals generate anti-EBVgp350 IgG before the onset of clinical symptoms (274, 355) but this is not true for everyone (274, 356). The variations between different individuals' antibody responses can be a challenge when developing and interpreting serological analyzes.

If the serological diagnosis needs to be determined by taking acute and convalescence samples, the long time between samples will relatively often allow the patient to recover before the diagnosis is determined. As an example, a primary VZV infection or reactivation can be correctly diagnosed by analyzing acute and convalescence samples, to detect seroconversion or significant titer increase, but it will probably not be so useful as it takes a long time for the antibodies to be generated and PCR can provide a faster diagnosis in atypical cases (107).

Sensitivity and specificity

In the perfect serological assay, all individuals with the viral antigens or antibodies of interest would be positive and all individuals lacking the antigens/antibodies would be negative. Unfortunately, it is impossible to develop these perfect assays. Serological analyzes have different established limits for what counts as positive or negative results. The test may also have a gray area where it is uncertain whether the detected reactivity is due to true antibody/antigen reactivity or if it is false/non-specific. The limits are established after analysis of many samples that have been determined to be true positive or negative. For serological analyzes, it is practically impossible to set limits that provide both 100% sensitivity and specificity. Some true negative samples may show non-specific reactivity and some true positive samples may

have such low levels of antibodies that they fall below the established detection limit for the assay.

Depending on how the analysis is to be used, it may be more important to prioritize sensitivity or specificity. When screening for transmissible infections such as HIV and hepatitis C, it is important to use very sensitive assays so as not to miss any true positive cases. The lack of specificity can be supplemented by other more specific methods. However, in rare cases, even with further analyzes, it can be difficult to determine with certainty whether the reactivity is due to a true antibody response or if it is non-specific (357). The disadvantage of the lack of specificity includes not being able to use donated blood products due to false positive or indeterminate test results and possible negative effects of notifying individuals of deviating test results (358).

Predictive values

The positive predictive value is the proportion of patients with a positive test result who truly have the disease, while the negative predictive value is the proportion of patients with a negative test who truly do not have the disease. These predictive values depend on the sensitivity and specificity of the assay but also on the prevalence of the disease in the population. When implementing a diagnostic test for a particular disease in a clinical laboratory, it is therefore important to consider how the prevalence of that disease will affect the performance of the assay.

Cross-reactivity

The sensitivity and specificity of all viral serological methods depend on the binding between antibodies and antigen. Whole virus antigens are often used as serological antigens. These antigens are produced by infecting cell cultures with the virus of interest, allowing virus replication to take place, and finally extracting the virus proteins. Whole virus antigens contain various proteins from the virus but also residues from the infected cells. Thus, antibodies with different specificity may attach to many different epitopes on the viral proteins, which may increase the sensitivity of the assay, but there is a risk that the remaining cell components give rise to unwanted responses.

The disadvantage of whole virus antigens is also that closely related viruses may have similar epitopes and if many proteins are included in the antigen, the risk increases that these similar epitopes, called cross-reactive epitopes, are included in the antigen. Cross-reactive antibodies that target an epitope on a particular virus can then instead bind to a cross-reactive epitope on a closely

related virus. This type of cross-reactivity can, for instance, cause diagnostic problems in analyzes of antibodies against flaviviruses (359-361) and alphaherpesviruses (157-159, 162, 323). Narrowing down the number of viral proteins in the antigen to a single protein or to peptides can lead to higher specificity but also loss of sensitivity. This is especially important to keep in mind when using peptide antigens as they only consist of short stretches of amino acids with few epitopes and probably no conformational epitopes.

Non-specific binding

It is important to consider the risk of non-specific binding in the serological assays and how this may interfere with the performance of the analyzes. A disadvantage of the passive adhesion of antigen to a solid phase is the risk of attachment of unwanted proteins remaining from antigen production. The antigen may have cell residues or other components left over from production that may adhere to the solid phase and cause unwanted binding to antibodies in the patient's sample. Antibodies in human samples can also directly attach to parts of the solid phase where antigen has not bound and create false positive reactions. The extent to which this occurs depends on the components of the individual sample and how well the blockage of the wells prevents this non-specific binding. False positive reactions can also occur due to nonspecific binding of antibodies to antigens through protein-to-protein interactions. Nonspecific reactions due to unwanted binding to the secondary antibody are also well known (362). Another established factor for interference in serological analyzes is the rheumatoid factor (287, 363-365). In addition, both false negative and false positive reactions can occur due to unwanted interactions between reactants and buffer components (362, 366).

IgM assays

Diagnostic problems with cross-reactivity and non-specific binding that cause false positive results are usually a larger problem in IgM assays compared with IgG assays and it has therefore generally been more difficult to develop specific IgM assays (163, 365, 367-369). This may be because IgM molecules generally have lower affinity compared with IgG molecules, but still have relatively high avidity due to the many paratopes present on an IgM molecule. Other diagnostic problems with IgM assays includes polyclonal antibody responses. In response to certain viral infections, such as a primary infection with EBV, B cells can produce IgM against several different viruses, which is called polyclonal B-cell activation (370). In some infections, the IgM antibody response remains for a long time even if the infection is no longer in an active phase. It can then be difficult to determine the stage of infection because IgM

in these cases is not a marker for acute/recent infection. Diagnosing breakthrough infections of TBE virus and MeV in vaccinated individuals can be complicated by a later or absent IgM response (287, 371).

Immune complexes

In some viral infections such as hepatitis B, immune complexes can cause diagnostic problems. Antibodies to hepatitis B bound to viral antigen in complexes can prevent antibody detection in the serological assays and the patient becomes false negative (372).

Technical difficulties

As with all diagnostic methods, technical problems can arise when performing serological analyzes. Pipetting errors can occur both when the assays are performed manually and mechanically, even if the risk of manual errors is higher. Self-thawing freezers should not be used to store reagents as the freeze-thaw cycles can destroy protein activity, causing the proteins to be denatured or inactivated. The buffer system used must be compatible with other reagents. Buffers containing phosphate can interfere with the commonly used enzyme alkaline phosphatase and reduce enzyme activity. Samples with endogenous phosphates can also interfere with the enzyme. When the plates are washed, too high a vacuum can dry out the coated protein and too low a vacuum can leave residual liquid in the wells. It is also important not to let the wells dry out between different steps as drying can mean loss of protein activity.

A well-known problem with ELISA is the "edge effect" which is a phenomenon where the optical density (OD) values of the peripheral wells compared with the central wells of the microtiter plate have higher or lower values than expected. This is often due to differences in temperature or lighting between the central and peripheral wells. The microplates are often incubated at 37 °C instead of room temperature to shorten the incubation time. The problem may be that the peripheral wells reach higher temperatures faster compared with the central wells. If the substrate is light-sensitive and the plates are developed in a room with strong light, the wells closest to the light source may have a higher development effect compared with the other wells.

1.5 MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic inflammatory disease in the CNS. The etiology and pathogenesis are not established, but most hypotheses suggest an interaction between genes, especially those that control the immune system, and environmental risk factors such as low vitamin D levels, low sun exposure and smoking (373, 374). These risk factors are believed to trigger autoimmune reactions to myelin, which involves both T and B cells.

The damage mechanisms involve both demyelinating and axonal damage, the latter of which leads to irreversible disability. Demyelination and axonal damage reduce or inhibit the impulse transmission along the axons. The name multiple sclerosis comes from multiple scarring that occurs due to the inflammation-causing lesions in the CNS. They are visible as hyperintense T2-weighted lesions on magnetic resonance imaging (MRI) and active lesions may be detected as contrast-enhancing lesions on T1-weighted MRI scans, indicating damage of the blood-brain barrier (BBB).

Phenotypes of MS

Based on clinical features, MS is traditionally divided into three different phenotypes, relapsing-remitting MS (RRMS), primary-progressive MS (PPMS) and secondary progressive MS (SPMS). Approximately 90% of patients initially follow the RRMS course with acute exacerbations (also termed relapses or attacks) from which they fully or partially recover (375). Although patients with RRMS may experience clinical stability between relapses, MRI reveals that lesion formation occurs mostly without new or aggravated symptoms.

The neurological symptoms vary depending on where the lesion (s) occur in the CNS. Common symptoms associated with onset of MS are regional motor and/or sensory impairment and impaired vision due to optic neuritis. After 20–30 years of RRMS course, the disease will subsequently convert into SPMS. During this stage, there is a slow progression of disability with or without superimposed relapses. Patients with PPMS have a similar progressive decline in their neurological function from the onset of the disease.

Immunopathology of MS

Traditionally, MS has been conceived as a two-stage disease, where the first stage is dominated by recurrent inflammation and the second stage is dominated by a neurodegenerative phase (376, 377). Contrasting this view are

autopsy studies showing active inflammation and demyelination in the CNS also during end stages of MS and obvious degeneration of both white and grey matter of the brain in early stages of MS (378). A new theory of the course of the disease is that there is a diffuse smouldering pathological process were the disease affects the entire CNS (376). The theory behind smouldering MS is that MS is a single-stage disease and that even early in the disease, not only relapses occur, which cause acute focal damage with axonal damage but also slower processes such as demyelination and energy deficits that eventually lead to neurological deterioration with atrophy of the brain and the spinal cord (376). It is unclear to what extent these delayed processes are dependent on the focal inflammation. Later, post-neurodegenerative processes occur and with increasing age, age-related neurodegenerative processes also contribute to patients losing neurological function (376). The finding that accumulation of disability in patients with RRMS is not associated with relapses supports this theory of smouldering MS (379).

Epidemiology

The onset of MS occurs mainly in young adults, between 20–40 years of age, mean at 32 years (380). In Europe, the disease is a major cause of non-traumatic neurological impairment (381). Twice as many women as men suffer from the disease (380). The total prevalence of MS is estimated at 2.8 million patients: 35.9 per 100,000 population (380). Sweden is one of the countries with the highest incidence and prevalence of MS in the world with almost 200 per 100,000 persons with MS and 900–1000 new cases annually (382, 383). The prevalence of MS is uneven worldwide with higher prevalence in temperate climates in both the southern and northern hemispheres, but the increasing incidence of MS with latitude gradient is not as evident in more recent studies in the northern hemisphere (384, 385). The overall prevalence of the disease is increasing globally and especially in females (380, 384-386).

Diagnosis

Diagnosing MS is performed by weighing the patient's history, clinical symptoms, disease course, detection of CSF-specific oligoclonal IgG bands (OCB), findings on MRI and fulfilling the criteria of dissemination in time and space according to the revised McDonald's criteria from 2017 (387). The improved criteria enable earlier diagnosis and thereby earlier treatment of the disease, which can improve the course of the disease. When an MS diagnosis cannot be established after the first exacerbation, the patient is diagnosed with clinically isolated syndrome.

Treatment

There is currently no curative treatment for the disease, but for patients with RRMS, there are several disease-modifying treatments that reduce the relapse rate, number of lesion formation and disability development. There is also evidence that the degeneration is mitigated with lower rate of brain and cervical cord atrophy development. Treatment should be started in close proximity to the onset of the disease. The treatment is usually highly effective early in the course of the disease and may reduce the disease's impact on the long-term health-related quality of life. The goal of treatment is to reduce the inflammatory activity and thereby improve prognosis.

Interferon beta (IFNß) was the first disease modifying therapy (DMT) approved for RRMS and has been used since the mid-1990s (375). IFNß are antiviral cytokines that have a moderate effect on inflammation in MS. The side effects are usually quite mild and the most common are skin irritation at the injection site and influenza-like symptoms. Other treatments for MS include glatiramer acetate, dimethyl fumarate, diroximel fumarate, teriflunomide, fingolimod, ponesimod, ozanimod and the monoclonal antibodies natalizumab (NAT) (Tysabri®), alemtuzumab (Lemtrada®) and the anti-CD20-directed monoclonal antibodies rituximab, ocrelizumab and ofatumumab that depletes circulating B-cells. All of these medications reduce the inflammatory component of the disease through various mechanisms of action. The anti-CD20 B-cell depleting monoclonal antibody rituximab has shown a high treatment effect and the off-label use of this drug has increased in Sweden (388-390).

The choice of therapy is personalized and based on clinical factors such as number and severity of exacerbations, early disability development, the lesion load and rate of new lesions on MRI, the course of the disease, age, comorbidities, and several other factors that include tolerability and safety. Treatment with the monoclonal antibodies NAT, alemtuzumab and those targeting anti-CD20 may be more effective but there is a higher risk of severe side effects. MS exacerbations can be treated with high doses of corticosteroids or by plasmapheresis when steroids seem insufficient to halt relapse progression. Unfortunately, it has been more difficult to develop effective therapies for progressive multiple sclerosis. The explanation may be that the pathogenic mechanisms are not as well understood, which causes problems in developing efficient medications (391). However, ocrelizumab has recently been approved for inflammatory active PPMS and siponimod for inflammatory active SPMS.

The patients in Paper V were treated with NAT and the mechanisms of action of that therapy will therefore be explained in more detail. NAT, is a recombinant, humanized monoclonal IgG4 antibody that binds to the α4 subunit of α4β1 integrins (also known as very late antigen-4) and α4β7 integrins (392, 393). The α4β1 integrin, which is an adhesion molecule found at high levels on the surface of all leukocytes except neutrophils, binds to vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells (394-396). The interaction between VCAM-1 and α4β1 integrins is important for leukocyte adhesion and transmigration of cells across the BBB to the CNS (395-398).

The binding of NAT to α4β1 integrins therefore inhibits leukocyte migration into brain tissue, which reduces inflammation and prevents the formation of lesions (392, 396, 399). The treatment effect of NAT is high, but the treatment may give rise to serious side effects such as progressive multifocal leukoencephalopathy (PML) (393, 400). PML is caused by infection of the CNS by JC polyomavirus (JCV). Treatment with NAT has also been associated with herpesvirus infections of the CNS and primary central nervous system lymphoma (401-403).

Etiology

The etiology behind MS is not yet fully understood but is thought to be due to complex interactions between genes and environmental factors. EBV, vitamin D, and smoking are among the most well-established environmental risk factors for developing the disease (245, 373, 374, 404-409). IM, which is caused by a delayed primary EBV infection, has been shown to particularly increase the risk of MS development (246, 247, 410-412). Whether EBV is a prerequisite for developing MS is still unknown, and some researchers question whether there are any true EBV seronegative patients with MS (413-415). A recent study suggesting EBV as a cause of MS showed that the risk of developing MS increased 32-fold after EBV infection and that increased serum levels of neurofilament light chain (a marker of neuroaxonal degeneration) occurred only after EBV seroconversion (245).

Antibody response

Most patients with MS show an intrathecal antibody production that can be detected as OCB by electrophoresis of CSF (416, 417). OCB have previously been considered stable and represent a signature pattern for individual patients, but it has now been shown that these bands can change over a longer period of time but that the change in most cases probably is not affected by disease

progression or immunosuppressive therapy (418). Patients with other autoimmune or infectious diseases may also present OCB in CSF, why it cannot be regarded as a specific marker for MS (419, 420). A small proportion of the IgG antibodies in OCB are directed against viral antigens and myelin proteins, but the vast proportion of these proteins have unknown targets (421).

Patients with MS demonstrate higher EBV seroprevalence (245, 405, 413, 414, 422, 423) and increased serum levels of anti-EBV antibodies (404, 423-426) compared with healthy controls, but the intrathecal antibody response to EBV is relatively low compared with the intrathecal response to certain other neurotropic viruses such as MeV, RV and VZV (427-431). The intrathecal production of anti-MeV, anti-RV and anti-VZV IgG, termed the MRZ reaction, is a common finding in patients with MS and can be used as a complement to other diagnostics (429-431). A few studies have also shown an increase in serum anti-MeV IgG levels in patients with MS compared with healthy controls (432-434). Not all neurotropic viruses are positively associated with MS; for example, not CMV, and individuals who are CMV seropositive have shown a reduced risk of developing MS (245, 435-437).

Why patients with MS have this increased IgG antibody response to certain neurotropic viruses compared with healthy controls is unknown and therefore requires further research. Due to the association between MS and EBV, the idea has arisen to use the antibody response to EBV and/or the EBV viral load as surrogate markers for disease activity and treatment effect in MS (438-443). Some studies support that disease activity is correlated with the antibody response to EBV (440, 441) while other studies do not (442-444). Further studies on the association are therefore warranted. Most serological studies on the antibody response to EBV in patients with MS have used EBNA (especially EBNA1) and VCA as antigens (404, 407, 408, 414, 424, 425, 428, 440-444). Analysis of antibodies to EBVgp350 may therefore, as a sensitive and specific antigen, provide new information on the humoral immune response to EBV in patients with MS.

Some healthy siblings of patients with MS demonstrate a suspect hyperimmune phenotype with OCB in CSF and increased IgG antibody levels against certain neurotropic viruses in both CSF and serum compared with healthy controls (445-447). These siblings have been termed siblings with MS trait (446, 447).

In this thesis, improved serological methods are used to examine antibody responses to certain viral antigens in patients with MS.

2 AIM

The overall aim was to develop specific serological assays to detect IgG antibodies to varicella-zoster virus (VZV), Epstein-Barr virus (EBV) and measles virus (MeV) and to use these assays for clinical applications.

The specific aims were:

- To produce and evaluate recombinant, single immunodominant viral proteins, VZV glycoprotein E, EBV glycoprotein 350 and the core part of the MeV nucleocapsid protein as serological antigens in enzyme linked immunosorbent assay.

- To use the newly developed serological assays to examine antibody responses to VZV, EBV and MeV in patients with multiple sclerosis.

- To study the role of antibody response to EBV as a potential part of the pathogenesis of multiple sclerosis.

3 PATIENTS AND METHODS

3.1 PATIENTS

3.1.1 PAPER 1

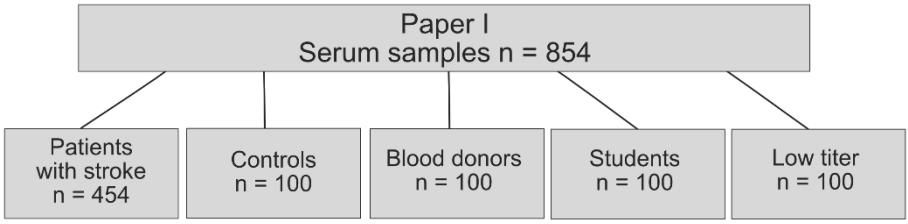

Figure 19. Figure depicting the analyzed serum samples in Paper I.

Serum samples from five different groups were analyzed in Paper I. A total of 454 samples from patients with ischemic stroke were analyzed from four stroke units in western Sweden together with 100 population-based controls matched for age and sex. The other three groups consisted of blood donors (n = 100), students (n = 100) and serum samples with low anti-VZV IgG titers (n = 100). A total of 854 samples were analyzed in the study. All samples were coded before analysis. A schematic illustration of the patient material can be seen in Figure 19 and the characteristic of the five groups are presented in Table 1.

Table 1. Age and sex of the participants in the five different groups from whom serum samples were analyzed in Paper I. Abbreviation: Not determined (n.d.).

	Total N	Mean age (years)	Female N (%)
Patients with stroke	454	57.4	159 (35%)
Controls	100	56.4	36 (36%)
Blood donors	100	n.d.	n.d.
Students	100	26.9	51 (51%)
Low titer	100	37.9	n.d.

3.1.2 PAPER II

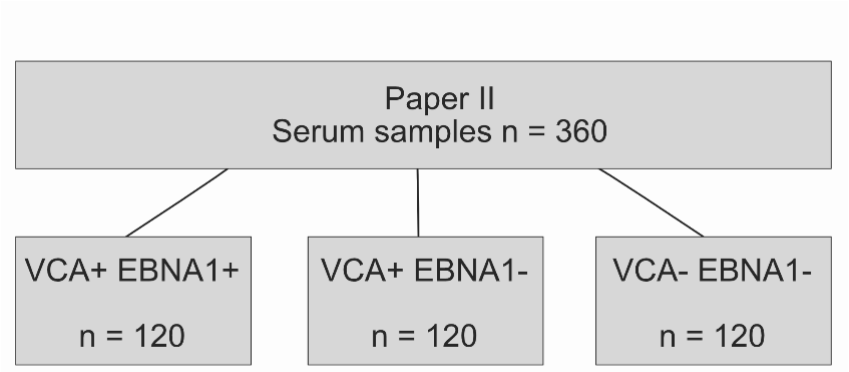

Figure 20. A schematic figure of the analyzed serum material in Paper II. A total of 360 serum samples were analyzed for detection of IgG antibodies to Epstein-Barr virus glycoprotein 350. Abbreviations: Viral capsid antigen (VCA), Epstein-Barr virus nuclear antigen 1 (EBNA1), seropositive (+), seronegative (-).

The analyzed serum samples in Paper II were sent to the Department of Clinical Microbiology, Sahlgrenska University Hospital in Gothenburg, Sweden in 2014 and 2015 for clinical analysis of anti-EBNA1 and anti-VCA antibodies. The samples were analyzed using ARCHITECT i4000SR immunoassay analyzer (Abbott).

The individuals agreed to store the samples in Biobank Väst after analysis for possible later use. For the study, a total of 360 serum samples from Biobank Väst were collected and anonymized for analysis of anti-EBVgp350 IgG antibodies. Of these 360 samples, 120 were VCA and EBNA1 IgG seropositive (VCA+EBNA1+), 120 were VCA and EBNA1 IgG seronegative (VCA-EBNA-), and 120 were VCA IgG seropositive but EBNA1 IgG seronegative (VCA+ EBNA1-). The patient material is illustrated in Figure 20.

3.1.3 PAPER III

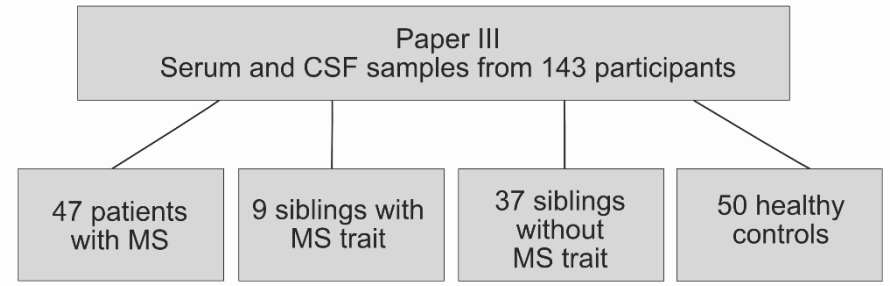

Figure 21. A schematic figure of the analyzed patient material in Paper III. Abbreviations: Multiple sclerosis (MS). A suspect hyperimmune phenotype seen in clinically healthy siblings of patients with MS (MS trait).

The patient material was the same as in a previous study except for one male sibling without MS trait where sample material was lacking (445). There were nine clinically healthy siblings of patients with MS who in the previous study presented two or more OCB in CSF and several showed increased anti-MeV IgG levels in CSF and/or serum compared with healthy controls (445). These siblings were categorized as having MS trait (a suspect hyperimmune phenotype) (445). In total, the patient material consisted of paired serum and CSF samples from 47 patients with MS, 9 siblings with MS trait, 37 siblings without MS trait and 50 healthy controls. Of the patients with MS, 22 had RRMS, 22 SPMS and 3 PPMS. The control group had fewer women and a lower median age compared with the groups with patients and siblings. The patient material is displayed in Figure 21. The age and sex distribution between the groups is displayed in Table 2.

Table 2. Age and sex of participants in Paper III.

	Total N	Median age (range years)	Female N (%)
Patients with MS	47	46 (22–63)	31 (66%)
Siblings with MS trait	9	43 (34–58)	5 (56%)
Siblings without MS trait	37	46 (22–66)	24 (65%)
Healthy controls	50	32 (18–57)	15 (30%)

3.1.4 PAPER IV

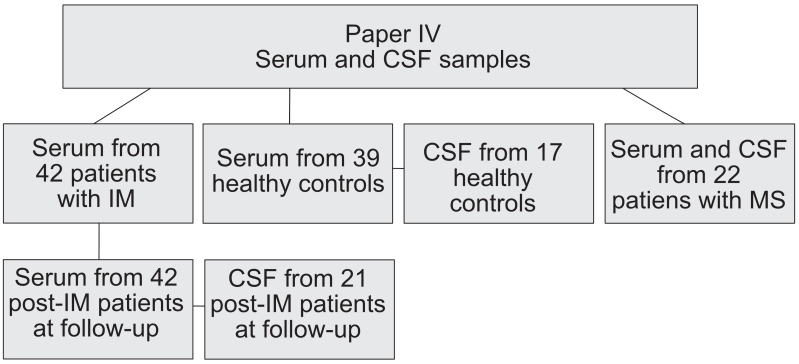

Figure 22. The patient material in Paper IV. Abbreviations: Infectious mononucleosis (IM). Cerebrospinal fluid (CSF).

The patient material in Paper IV consisted of serum samples collected from 42 individuals with serologically verified acute IM sometime between 2003 and 2007. Serum samples from the same individuals (post-IM patients) were obtained at follow-up approximately 10 years after their IM episode. A serum sample was obtained from all 42 individuals and 21 also contributed with CSF. As a control group, serum and CSF samples were obtained from 17 healthy controls that denied a history of IM. Of these healthy controls, 15 were EBV seropositive. In addition to these healthy controls, serum samples from 24 EBV seropositive blood donors were included. In total, the sample material in the EBV seropositive control group consisted of 39 serum samples and 15 CSF samples. The patients with MS included in the study were sampled between 1996 and 1997 and both serum and CSF samples from these individuals were analyzed. Patients with MS were age and sex matched with the post-IM patients at follow-up. Of the patients with MS, 13 had RRMS and 9 had SPMS. The patient material is presented in Figure 22 and Table 3.

Table 3. Age, sex and analyzed sample material from the participants in Paper IV. Of the 17 healthy controls that contributed with cerebrospinal fluid (CSF), 15 were Epstein-Barr virus seropositive.

	Total N	Median age (range years)	Female N (%)
Infectious mononucleosis (serum)	42	18 (11–34)	24 (57%)
Follow-up (serum)	42	28 (22–43)	24 (57%)
Follow-up (CSF)	21	27 (22–40)	13 (62%)
Healthy controls (serum)	39	23 (18–34)	21 (54%)
Healthy controls (CSF)	17	25 (20–46)	11 (65%)
Multiple sclerosis (serum and CSF)	22	35 (18–45)	16 (73%)

3.1.5 PAPER V

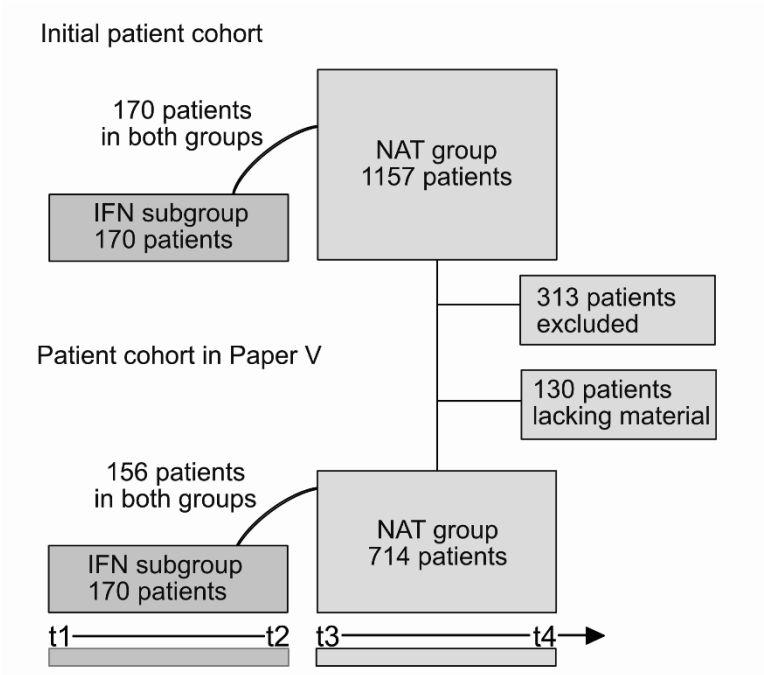

Figure 23. Schematic figure showing the patient material in Paper V. Serum samples were collected on two occasions from 170 patients with multiple sclerosis during treatment with interferon beta (IFNβ) at time point 1 (t1) and t2. The 170 patients in the IFN subgroup were then treated with natalizumab (NAT). The paired serum samples from the patients in the NAT group were obtained immediately prior to the first infusion of NAT, at time point 3 (t3) and the last available sample during NAT therapy, at t4. The initial NAT group consisted of 1157 patients. Of these, 313 were excluded in a previous study due to insufficient quantification of antibodies to JC polyomavirus or previous treatment with intravenous immunoglobulin. Serum material was also lacking from 130 patients for the antiviral IgG assays in the current study. The NAT group analyzed in this study consisted of 714 patients, of whom 156 were also part of the IFN subgroup.

The patient material in Paper V was obtained from patients with MS enrolled in the Swedish pharmacovigilance study for NAT (IMSE), where post marketing surveillance of NAT was performed (448, 449). All 1157 patients in the initial NAT group were treated with NAT before March 2010 (448-450). In a previous study, 313 patients were excluded due to insufficient quantification of anti-JCV antibodies or previous treatment with intravenous immunoglobulins, while samples from the remaining 844 patients were analyzed (450). The antiviral analyzes in the present study could be performed for 714 patients who had enough serum left for the analyzes, while serum

material was lacking from 130 patients. The paired serum samples from the patients in the NAT group were obtained by sampling immediately prior to the first infusion of NAT, at time point 3 (t3) and the last available sample during NAT therapy, at time point 4 (t4). The median time between the samples was 12 months with an interquartile range (IQR) of 7–24 months.

For 170 patients in the initial NAT group, serum samples had been taken during previous IFNβ treatment at t1 and t2 (before the start of NAT therapy). The median time between samples was 13 months with an IQR of 7–25 months. In the current study, 14 of the 170 patients in the IFN subgroup lacked material for the samples taken before (t3) and during (t4) NAT treatment why only 156 patients had paired samples taken both during IFN treatment and before and during NAT treatment. A total of 728 individual patients with MS were included in the present study. A sex- and age-matched control group with 144 blood donors was also included in the study. The characteristics of these three groups are presented in Table 4 and a schematic illustration of the patient material is presented in Figure 23.

Table 4. Characteristics of the participants in the natalizumab (NAT) group, the interferon (IFN) subgroup and the healthy controls. Δt is duration of time in months between samples taken at t1–t2 and t3–t4, with interquartile range (IQR).

	Total N	Median age (range)	Female	Median Δt months (IQR)
IFN subgroup t1–t2	170	37 (16–58)	106 (62%)	13 (7–25)
NAT group t3–t4	714	37 (12–63)	500 (70%)	12 (7–24)
Blood donors	144	35 (18–63)	100 (69%)	

3.2 METHODS

3.2.1 ANTIGEN PRODUCTION

The quality of viral serological methods has improved over time, but further improvements are warranted. We decided to use single, immunodominant viral proteins to develop new antigens with both high specificity and sensitivity. The expression system for antigen production is important to consider as residues from the antigen production can cause non-specific reactions.

We have focused on expression systems without human or primate components to avoid the risk of autoantibodies to these components. In addition, it is important to consider which cells should be used to recombinantly generate proteins, since post-translational modifications, such as glycosylation of viral proteins, depend on the host cell (451, 452). We have used Chinese hamster ovary (CHO) cells to express the two viral glycoproteins (VZVgE and EBVgp350) used as serological antigens in our studies because these cells are well established, including their pattern of glycosylation, for mammalian recombinant protein production (453).

The VZVgE and EBVgp350 antigens were developed in collaboration with the core facility Mammalian Protein Expression at the University of Gothenburg, Sweden. The MeV nucleocapsid antigen was produced by our collaborators at Centre National de la Recherche Scientifique, Université Aix-Marseille, Marseille, France.

3.2.2 VARICELLA-ZOSTER VIRUS GLYCOPROTEIN E

The candidate protein for developing a VZV specific antigen was the immunodominant gE. A BLAST analysis did not show extensive homologies between VZVgE and HSVgE (data not shown). An expression vector containing the gene segment encoding a.a. 1–539, corresponding to the signal sequence a.a. 1–24 and the extracellular domain a.a. 25–539 of VZVgE was generated. This was done by PCR amplification of VZV DNA encoding a.a. 1–539 from VZV strain Dumas. The amplified PCR fragment was cut with restriction enzymes and cloned into pcDNA6/myc-His A vectors.

The vector was transfected into CHO K1 cells. The cells were cultured and cloned in several cycles to generate a viable production of VZVgE. Several stable clones that secreted high levels of VZVgE were produced and one clone was adapted to growth in serum-free suspension culture. The cells were then

cultured in a perfusion bioreactor where VZVgE was secreted into the medium. This was done to yield larger quantities of VZVgE. The perfusion culture was set up in a 3 L Biobundle bioreactor and 12.5 L of cell-free harvest could later be collected. The harvest was centrifuged, pre-filtered and then concentrated by tangential flow filtration (TFF). The protein was purified from the concentrate by using 1 mL HiTrap chelating columns loaded with Co2+. Imidazole was used to elute the bound protein. Western blot was used to analyze the fractions.

3.2.3 EPSTEIN-BARR VIRUS GLYCOPROTEIN 350

The EBV transmembrane envelope protein EBVgp350 consists of 907 a.a. with an 860 a.a. long extracellular N-terminal segment. EBV DNA constructs encoding different parts of EBVgp350, a.a. 1–506, 751–860 and 502–860, were derived from EBV strain B95-8 (GenBank accession number M10593). The three EBV DNA constructs were synthesized and ligated into pcDNA6myc-His vectors and adapted for expression in CHO cells. The His6 tag from the vector was left on the C-terminal end of the protein for expression.

The two DNA constructs a.a. 1–506 and 502–860 were combined to generate a construct encoding the whole extracellular domain a.a. 1–860 of EBVgp350. The cloning steps were performed using *Escherichia coli* XL-1 Blue. The recombinant EBVgp350 constructs were produced by transient transfection of FreeStyle™ CHO-suspension cells. The NovaCHOice® Transfection kit was used according to the manufacturer's instructions to perform the transfections. Small-scale 5 ml transfections were performed in 50 ml TubeSpin™ tubes and large-scale transfections with EBVgp350 a.a. 1–506 and 1–860 were performed in 3 L Biobundle bioreactors.

The supernatants from the cell cultures were harvested, centrifuged, and pre-filtered followed by TFF concentration. Protein was purified from the concentrate using a 1 mL Hitrap™ Chelating HP column loaded with 0.1 M CoCl2, at a flow rate of 1 mL/min. Imidazole was used to elute bound protein. The collected fractions were analyzed by Western blot. Then, the 20 mM imidazole fractions were pooled and dialyzed against 3 × 3 L phosphate-buffered saline (PBS) using Spectra/Por® 4 dialysis membrane tubing. The final protein concentrations were measured with Thermo Scientific™ NanoDrop 2000 to 0.7 mg/ml for EBVgp350 a.a. 1–860 and 0.6 mg/ml for a.a. 1–506.

3.2.4 MEASLES VIRUS NUCLEOCAPSID ANTIGEN

To produce the MeV antigen the immunodominant MeV nucleocapsid protein was used. The plasmid pet21a/NFlag-H6, which encodes MeV nucleocapsid protein (strain Edmonston B) with an N-terminal flag sequence (454) and a C-terminal hexahistidine tag (455) was inserted in *Escherichia coli*, strain Rosetta [DE3] pLysS (Novagen) for antigen expression.

The MeV nucleocapsid protein, was detected in the soluble fraction of the bacterial lysate and the protein was purified from the bacterial lysate by immobilized metal affinity chromatography using Chelating Sepharose Fast Flow Resin preloaded with Ni^{2+} ions (Amersham Pharmacia Biotech) as previously described (455, 456). The N_{CORE} fragment (a.a. 1–392) was obtained from the nucleocapsid protein by limited proteolysis using trypsin (455). The core part of the nucleocapsid protein was chosen as serological antigen because it is conserved and structurally ordered while the diversity of the carboxyterminal is the basis for the division of MeV into clades and genotypes (283). The N_{CORE} antigen was purified to homogeneity (>95%) in two steps: immobilized metal affinity chromatography and gel filtration.

3.2.5 WESTERN BLOT

After antigen production, gel staining and Western blot were performed in Paper I, II and III to identify VZVgE, EBVgp350 and MeV N_{CORE}. Western blot was also used in Paper I to analyze serum samples that showed discordant results between the two VZV ELISA methods used in the study. The antigens VZVgE, EBVgp350 and MeV N_{CORE} were denatured and then separated by gel electrophoresis. In Paper I, VZVgE was stained directly on the gel with silver, or the Novex Colloidal Blue Staining Kit. In Paper III, MeV N_{CORE} was stained directly on the gel with Coomassie blue. VZVgE, EBVgp350 and MeV N_{CORE} were also transferred with electrical voltage to nitrocellulose membranes or Immobilon-P membranes.

To detect VZVgE, either the mouse monoclonal antibody VZVgE sc-56994 or serum samples from patients were used. For the mouse monoclonal, the used conjugates were alkaline phosphatase goat anti-mouse Ig or horseradish peroxidase goat anti-mouse Ig. For the human antibodies, the used conjugate was horseradish peroxidase polyclonal rabbit anti-human IgG. The substrates used to detect the mouse monoclonal were BCIP/NBT developing solution or chemiluminescent substrate Immobilon™ Western. To detect human anti-VZV IgG antibodies, 4-chloro-1-naphtol was used as the substrate. EBVgp350

was identified by using Penta-His™ Antibody as the primary antibody and alkaline phosphatase goat anti-mouse IgG as secondary antibody. Detection was performed by using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as substrate. MeV N_{CORE} was identified by anti-measles IgG antibodies in human serum or mouse monoclonal antibody 5.227 against the nucleocapsid protein. The conjugates used were either horseradish peroxidase polyclonal rabbit anti-human IgG or anti-mouse IgG. The substrate used was 4-chloro-1-naphtol.

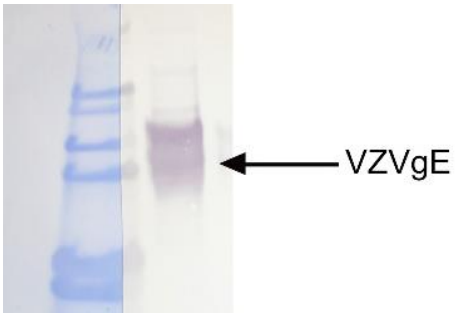

Figure 24. Western blot using varicella-zoster virus glycoprotein E (VZVgE) as antigen and a human serum sample containing anti-VZVgE IgG antibodies. Molecular marker was Mark 12 Unstained standard.

3.2.6 IMMUNOFLUORESCENCE

Indirect immunofluorescence was used in Paper I to analyze the samples that showed discordant results between the VZV ELISA methods. Green monkey kidney cells AH1 cell line (SBL/83) were infected with VZV according to internal diagnostic routine at the Department of Clinical Microbiology, Sahlgrenska University Hospital in Gothenburg, Sweden. The cells were fixed with acetone to glass slips and the serum samples were then added. If the sample contained anti-VZV IgG, the antibodies attached to the virus-infected cells. Fluorescein-labeled goat anti-human IgG was used as conjugate.

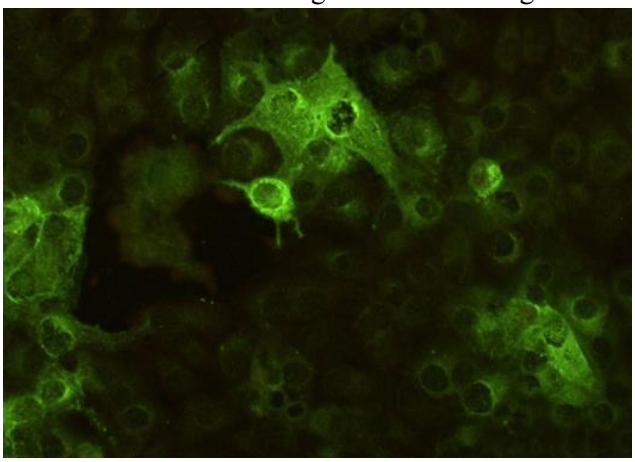

Figure 25. An immunofluorescence photograph showing fluorescent stained anti-varicella-zoster virus (VZV) IgG antibodies that have attached to VZV-infected cells.

3.2.7 ELISA

Indirect ELISA methods were used in the studies to analyze IgG antibodies to VZVgE, EBVgp350 and MeV N_{CORE}. The following were analyzed:

Paper I: Anti-VZVgE IgG in serum samples.
Paper II: Anti-EBVgp350 IgG in serum samples.
Paper III: Anti-MeV N_{CORE} IgG in serum and CSF samples.
Paper IV: Anti-VZVgE, anti-EBVgp350 and anti-MeV N_{CORE} IgG in serum and CSF samples.
Paper V: Anti-EBVgp350 and anti-MeV N_{CORE} IgG in serum samples.

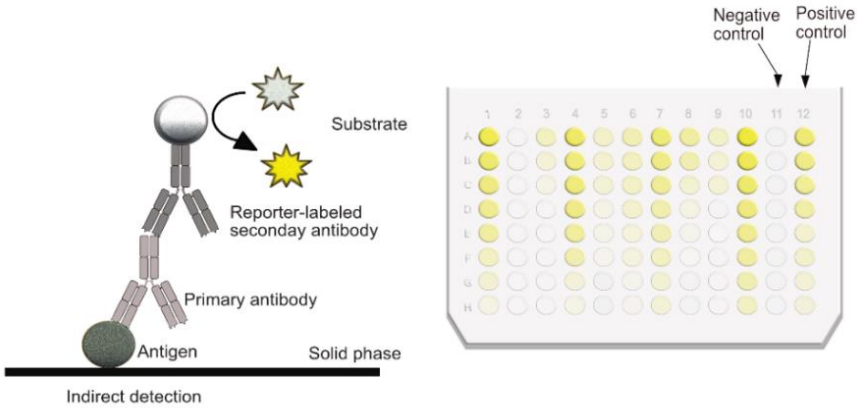

Figure 26. The figure illustrates the indirect ELISA method and an example of an ELISA plate ready for measurement in a spectrophotometer. In the example, the plate is coated with measles virus N_{CORE} antigen. The serum samples are in two-fold dilutions from 1/200 to 1/25600. The negative control is in row 11 and the positive control is in row 12. Samples from patients with MS, their siblings or healthy controls are in row 1–10.

The antigens were diluted with carbonate buffer and then coated on Nunc MaxiSorp™ 96-well high protein binding ELISA plates. After incubation and washing, the plates were blocked with non-fat dry milk diluted in PBS to avoid nonspecific binding. After blocking, patients' serum and CSF samples were added to the plates. If the samples contained complementary IgG to the viral antigens on the plates, the antibodies attached to the antigen. After incubation, the plates were washed to remove unbound antibodies and other components. A diluted conjugate, Alkaline Phosphatase AffiniPure $F(ab')_2$ Fragment Goat Anti-Human IgG, was then added to all wells. After incubation, all unbound conjugate was washed away. Substrate solution, phosphatase substrate dissolved and diluted in diethanolamine buffer, was added to the wells and if secondary antibodies conjugated to enzyme were present, the enzyme induced a color change of the substrate to yellow. Positive and negative control samples were added to all assays in order to control the quality of each analyzed ELISA plate. The optical density (OD) values were measured in a spectrophotometer.

3.3 STATISTICAL METHODS

In Paper II, a receiver operating characteristic (ROC) curve was performed in GraphPad Prism 7.3 to evaluate the diagnostic accuracy of the indirect ELISA method for detecting anti-EBVgp350 IgG. A two-sided statistical test was performed for the ROC curve in which a p-value smaller than 0.05 was considered significant.

In Paper III, the independent groups were compared using the Mann-Whitney U test. Patients with MS and their siblings were compared using Wilcoxon signed-rank test. IBM SPSS statistics 20 was used for the statistical analyzes. The statistical tests were two-sided and p-values smaller than 0.05 were considered significant.

In Paper IV, the Mann-Whitney U test was used to compare patients with IM with the control group. Where applicable, non-parametric ANOVA was used for comparisons. Wilcoxon signed-rank test was used for pairwise comparison between patients with IM and the same patients later at follow-up. To model age-adjusted group differences, quantile regression was used (457). P-values smaller than 0.05 were considered significant.

In Paper V, the statistical analyzes were performed using SPSS Statistics 27. The Mann-Whitney U test was used to compare anti-EBVgp350 and anti-MeV N_{CORE} IgG levels in patients with MS during IFNβ treatment at t1 and blood donors. Wilcoxon signed-rank test was used to compare the anti-EBVgp350 and anti-MeV N_{CORE} IgG levels, between the samples collected during IFNβ treatment at t1 and t2 and before and during NAT treatment at t3 and t4. The statistical tests were two-sided and p-values <0.008 were considered significant due to Bonferroni correction for multiple tests.

3.4 ETHICS

The samples from patients and controls were approved for use in Paper I by the Research Ethics Committee at the University of Gothenburg with ref. no. Ö 469-99.

The samples in Paper II were collected for clinical purposes and sent to the Department of Clinical Microbiology, Sahlgrenska University Hospital, Gothenburg, Sweden for analysis of anti-EBV antibodies. The serum samples used in the study were deidentified prior to analysis of anti-EBVgp350 IgG antibodies and the results cannot be traced back to individuals.

Ethical approval to use the samples in Paper III was given by the Research Ethics Committee in Gothenburg, ref. no. 361-96.

The material from post-IM patients was approved for use in Paper IV by the Research Ethics Committee in Umeå, ref. no. 2017-484-32M, which is an updated version of the prior application ref. no. 2011-198-31M with previous update ref. no. 2013-226-32M. The material from patients with MS was approved for use in the study by the Research Ethics Committee in Gothenburg, ref. no. 361-96 with updates S8-97 and R584-98.

In Paper V, ethical approval was obtained from the Stockholm Regional Ethical Committee and the Swedish Ethical Review Authority ref. no. 2006/845-31/1, ref. no. 2005/535-31/1, ref. no. 2009/1977-32 (updated 2010-08-06) and ref. no. 2019-04420.

Some sample materials included in the studies, such as the serum samples in Paper II and serum samples from blood donors in Paper I and V, are not covered by the Swedish act (2003:460) on ethical review of research involving humans. The samples were not collected for use in the studies and the samples were deidentified before analysis so the results cannot be traced back to individuals. Ethical permission is therefore not required in this context.

4 RESULTS AND DISCUSSION

4.1 PAPER I

There is a need for rapid, simple, and reliable serological assays for the determination of anti-VZV IgG responses, both for the diagnosis of VZV infections and for the analysis of immunity to the virus. In Paper I, immunodominant VZVgE was recombinantly expressed in CHO cells and used as a serological antigen in indirect ELISA to develop a sensitive and specific assay for the detection of anti-VZV IgG antibodies.

VZVgE production

VZVwhole-ag contains, in addition to many of the proteins in VZV, also cellular components. When narrowing down the number of proteins used in an antigen, the sensitivity may decrease and if only a single protein is to be used, it must be immunodominant for the serological analysis to achieve sufficient sensitivity. Glycoproteins are major components of the virus' outer surfaces where they are exposed on the viral envelope. Thus, these glycoproteins are accessible to the body's immune system and are common targets for human antibodies. VZVgE has been found to be the most immunogenic glycoprotein on VZV (100, 102-105) and was also an appropriate candidate antigen in the development of a new, more specific serological VZV assay due to the lack of extensive homologies with HSVgE.

Large-scale production and purification of recombinant VZVgE expressed in CHO K1 cells was performed successfully. CHO cells were used because they are well established for recombinant mammalian protein production (453). CHO cells are also known to produce proteins with complex N-glycans and short core O-glycans. This is important to consider because VZVgE contain carbohydrates with N- or O-linked glycosylation and the glycosylation pattern depends on the host cell. Recombinant VZVgE was analyzed by liquid chromatography electrospray mass spectrometry and two sialylated O-linked glycans were confirmed. N-glycans were not analyzed but are likely to be present because CHO cells, as previously mentioned, are known to generate complex N-glycans. VZVgE had previously been produced in CHO cells but only on a small scale (100). In Paper I, VZVgE was generated under well-monitored conditions and therefore protein production should be reproducible. Recombinant VZVgE was generated in CHO cells adapted to growth in suspension and the protein production could be performed on a large scale in perfusion bioreactor culture.

Comparison between VZVgE and VZVwhole-ag

The new serological VZVgE antigen (VZVgE-ag) was compared with a conventional whole virus antigen (VZVwhole-ag) in indirect ELISA. Of the 854 serum samples analyzed, 846 (99.1%) showed consistent results. A total of 830 samples were VZV IgG seropositive and 16 were seronegative with both methods. Only eight samples showed discrepant results between the analyzes. These samples were all VZV IgG seropositive with VZVwhole-ag but seronegative with VZVgE-ag. The discordant samples were further analyzed by Western blot using VZVgE as antigen and by indirect immunofluorescence using VZV-infected cells. From these further analyzes, it was determined that one sample was VZV IgG seropositive, two seronegative and five remained indeterminate. A flow chart of these results is presented in Figure 27.

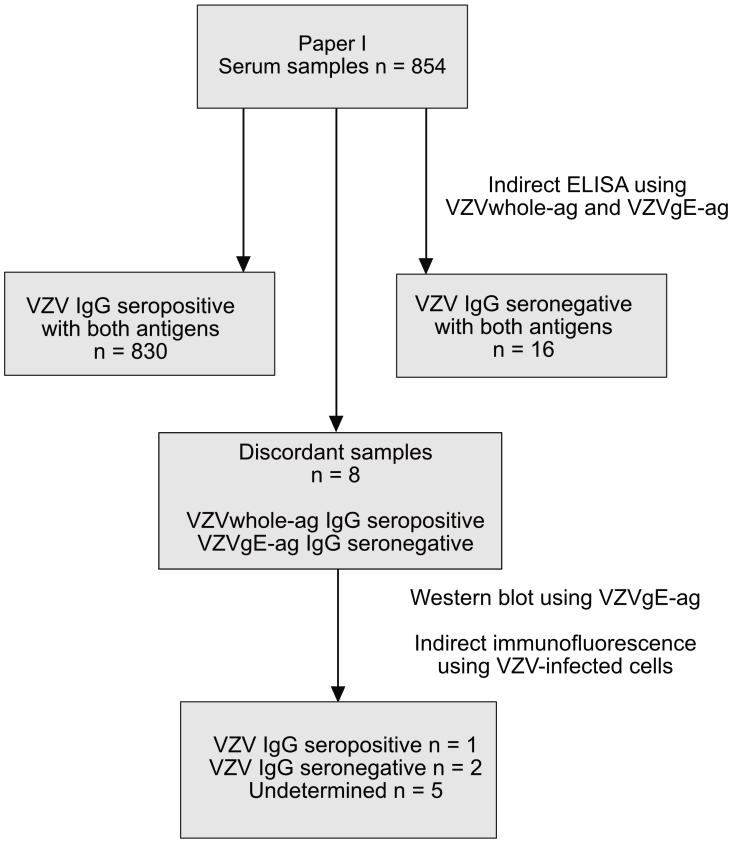

Figure 27. Flowchart showing the results of the 854 serum samples analyzed by indirect ELISA using varicella-zoster virus (VZV) whole antigen (VZVwhole-ag) and VZVgE antigen (VZVgE-ag).

The ELISA analyses using VZVwhole-ag generally yielded higher anti-VZV IgG levels in serum compared with VZVgE. This was not as pronounced for patients with stroke and the age-matched elderly controls. The older subjects generally showed higher anti-VZVgE IgG levels compared with the younger participants i.e. blood donors and students (Figure 28). Overall, the results showed that VZVgE had high specificity and sensitivity as a serological antigen in ELISA.

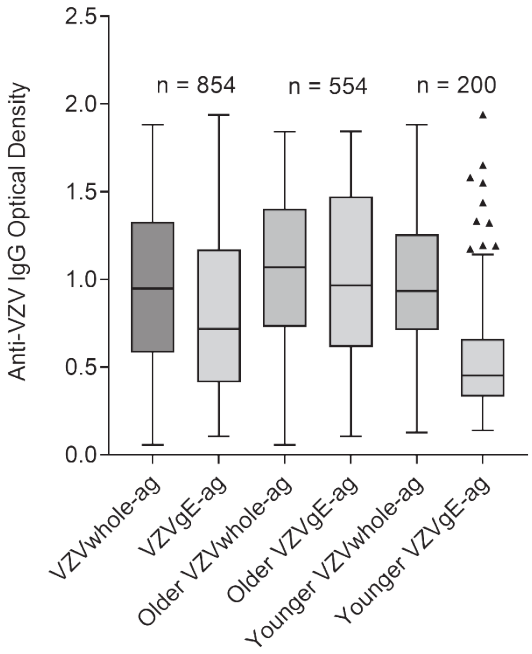

Figure 28. Tukey box plot displaying the optical density values for the 854 serum samples diluted 1/800 and analyzed by indirect ELISA using varicella-zoster virus (VZV) whole virus antigen (VZVwhole-ag) and VZVgE antigen (VZVgE-ag). The figure also shows the results for the combined group with older participants (n = 554), i.e. stroke patients (n = 454) and age-matched elderly controls (n = 100) and the group with younger participants (n = 200), i.e. blood donors (n = 100) and students (n = 100).

Importance of VZV serology methods

VZV serology methods are not normally of great value in diagnosing acute VZV infections, i.e. chickenpox and herpes zoster. These diseases can usually be diagnosed clinically, but in case of uncertainty, PCR is the preferred laboratory analysis (72). However, VZV is one of the most common viruses that causes infections in the CNS and the infections give rise to a wide range of CNS manifestations, often without rash (122, 126, 143-145). Serological assays of anti-VZV antibodies are in these cases an important complement to PCR analyzes (126, 458). The time window for detecting VZV DNA is limited and when VZV DNA cannot be found, detection of intrathecally produced anti-VZV antibodies can lead to the diagnosis (122, 126, 151).

There may exist several VZV CNS infections that remain undiagnosed due to the lack of clinical awareness in the absence of the characteristic rash. Once the diagnostic question has emerged, the time window for detection of VZV DNA may already be closed. This may well be the case in some patients with stroke, even though the link between stroke and VZV is now established (123, 459). One study showed that in patients with VZV vasculopathy, analysis of intrathecal anti-VZV antibodies was a more sensitive method compared with detection of VZV DNA to diagnose the disease (154).

Patients' history of VZV infection can in rare cases be unknown and/or unreliable as several other viruses can cause viral rashes with reddish or pink spots, which for individuals without medical training can lead to misdiagnosis of the disease. Laboratory determination of VZV immunity is therefore necessary in some cases. The following are examples of when determination of VZV immunity may need to be performed (i) immunocompromised individuals exposed to VZV (ii) before the introduction of immunosuppressive medications in patients to determine the need for VZV vaccination and after vaccination to control the immune response (iii) healthcare professionals with unknown VZV serostatus exposed to VZV or considering vaccination (iiii) pregnant women exposed to VZV and with uncertain history of previous infection (iiiii) women with unknown serostatus considering vaccination before attempting pregnancy and also other adults with unknown serostatus considering vaccination.

Although immunity to VZV is mainly due to the cell-dependent immune response and this immune response is the best indicator of immunity, it is a more labor-intensive and complicated procedure to analyze this cell-dependent response. Thus, for routine laboratory analysis of VZV immunity, the detection of anti-VZV IgG is the primary method (460). Sensitive and specific serological methods for anti-VZV IgG detection are also important for evaluating VZV seroprevalence in the population. One limitation when VZV seroprevalence in Sweden was estimated in a recent study was the risk of false-positive results due to cross-reactive anti-HSV IgG antibodies (106).

Cross-reactivity

VZV serology methods are widely used and of high value, but it is important that they are specific in the detection of anti-VZV IgG as the results are used to diagnose VZV CNS infections and to guide vaccination decisions and therapeutic interventions for VZV-exposed pregnant women and immunocompromised individuals. There are many different VZV serology methods used in laboratories around the world. Common to most methods is

that they are at risk of detecting cross-reactive HSV antibodies because they contain VZVgB, which is likely to have cross-reactive epitopes with HSVgB (156, 157). Detection of cross-reactive antibodies can cause false positive results that lead to misdiagnosis or incorrect assumptions about immunity (157-161).

The indirect ELISA method developed in Paper I with VZVgE as antigen was shown in a later study to reduce the risk of detecting cross-reactive anti-HSV-1 IgG antibodies in patients with PCR confirmed CNS infections with HSV or VZV, compared with the use of VZVwhole-ag (323). The increased specificity of the indirect VZVgE ELISA method is an improvement in VZV serology diagnostics to prevent false positive results due to cross-reactive antibodies. VZVgE has been used as serological antigen in indirect ELISA for the detection of anti-VZV IgG in the Department of Clinical Microbiology, Sahlgrenska University Hospital. The laboratory is the national reference laboratory for herpesviruses.

The VZVgE ELISA method for the analysis of anti-VZV IgG antibodies was compared with the routine clinical assays, VZVwhole-ag ELISA and indirect immunofluorescence using VZV-infected cells, to evaluate the performance of the VZVgE ELISA. The results showed that the VZVgE ELISA had high sensitivity and higher specificity compared with the routinely used methods and these serological assays were then removed from the routine diagnostics (data not shown). Moreover, the serological VZVgE antigen developed by us has been successfully used in studies in Finland for the detection of anti-VZV IgG antibodies (461, 462). In addition, a recent study has successfully reported the use of another VZVgE antigen in a diagnostic CLIA method (463).

VZVgE vaccine

The Shingrix® vaccine, which is based on VZVgE, has proved to induce a strong immune response (136-139) and even give rise to a more robust immunological memory with a longer protection period compared with the Zostavax® vaccine, which is based on a higher dose of the attenuated Oka strain (140-142). The higher protective effect of the Shingrix® vaccine may be due in part to different glycosylation patterns caused by the generation of the vaccines in different cell types (464). Recombinant VZVgE in the Shingrix® vaccine is expressed in CHO cells while the attenuated Oka strain in the Zostavax® vaccine is expressed in human fibroblasts. Thus, VZVgE glycan occupancy and glycan structures may show differences between the two vaccines, which may affect the induction of the immune response (464). The strong immune response to VZVgE expressed in CHO cells in the Shingrix®

vaccine strengthens the argument for also expressing the serological VZVgE antigen in CHO cells.

The anti-VZV IgG response was higher in serum samples from older individuals, i.e. ischemic stroke patients and their age-matched controls compared with younger individuals, i.e. blood donors and students. This suggests that reactivity to this protein may increase with age and possible reactivation. The high antibody levels against VZVgE suggest that the glycoprotein is a strong inducer of the immune response even in older individuals, which may be a contributing factor to the Shingrix vaccine's success in protecting against herpes zoster.

Anti-VZV IgG antibody levels induced after vaccination are lower compared to the levels obtained after natural infection, which is why anti-VZV IgG levels after vaccination are more difficult to detect in serological assays (465). It may therefore be beneficial to have a serological assay based on the same glycoprotein as that used in the Shingrix® vaccine. A chickenpox vaccine using VZVgE is not currently on the market, but it would be beneficial as it is likely that immunocompromised individuals who cannot be vaccinated with the live attenuated chickenpox vaccine can tolerate a subunit vaccine.

4.2 PAPER II

In Paper II, a sensitive and specific serological assay was developed using EBVgp350 as antigen. Serum samples with previously known serostatus against VCA and EBNA1 were analyzed with the new EBVgp350 ELISA method for the detection of anti-EBVgp350 IgG antibodies.

Comparison between longer and shorter EBVgp350 constructs

EBVgp350 is extensively glycosylated (209, 218) and glycosylation is important for the antigenicity of the protein (451, 466). As with VZVgE, the glycosylation of EBVgp350 depends on the host cell used to express the protein (451). CHO cells, which are mammalian cells, are known to generate less numerous but still quite similar glycosylation to that expressed in virus-infected human host cells (467). CHO cells have also been successfully used in the past to express EBVgp350 (468, 469) and these cells were therefore selected to generate the recombinant EBVgp350. In paper II, two protein constructs consisting of a.a. 1–860 or a.a. 1–506 were successfully produced in CHO cells.

The two protein constructs were then used as serological antigens in indirect ELISA. A total of 21 VCA and EBNA1 IgG seropositive (VCA+EBNA1+) samples and 21 VCA and EBNA1 seronegative (VCA-EBNA1-) samples were analyzed by indirect ELISA using the two antigens. The seroreactivity against the longer protein construct, i.e. a.a. 1–860, was found to be higher compared with that against the shorter protein segment, i.e. a.a. 1–506.

The antibody responses to different parts of EBVgp350 show great variation between individuals (466). This may partly explain why the analyzed serum samples in Paper II show higher anti-EBV IgG reactivity against the longer protein, i.e. the a.a. 1–860 construct, which contains more epitopes compared with the 1–506 protein. The higher IgG reactivity detected against the longer protein may be due to reactive sequences located between a.a. 741 and a.a. 860 (466) which thus only the longer construct contains. Previous studies analyzing anti-EBVgp350 antibody responses have either used a long protein such as a.a. 1–860, or a short construct, i.e. a.a. 4–450, why it has not previously been possible to compare the reactivity of antigens at different protein lengths of EBVgp350 (274, 356).

Analysis of samples

The longer protein construct with higher reactivity was then used for indirect ELISA analysis of the entire clinical material with 360 serum samples diluted 1/400 and with known IgG serostatus against VCA and EBNA1. The samples that were VCA+EBNA1+ and many of the VCA+ EBNA1- samples showed reactivity against EBVgp350. The samples that were both VCA and EBNA1 IgG seronegative generally showed no anti-EBVgp350 IgG reactivity. The results are presented in Figure 29.

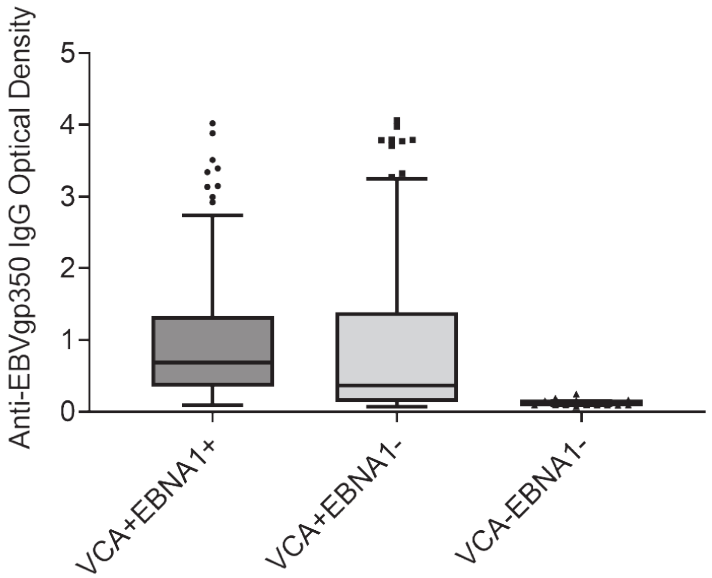

Figure 29. Serum samples (n = 360) with known serostatus to viral capsid antigen (VCA) and Epstein-Barr virus nuclear antigen 1 (EBNA1) were analyzed by indirect ELISA in dilution 1/400 for detection of IgG antibodies to Epstein-Barr virus glycoprotein 350 (EBVgp350). In total there were 120 VCA and EBNA1 seropositive samples (VCA+EBNA1+), 120 VCA seropositive but EBNA1 IgG seronegative samples (VCA+EBNA1-) and 120 VCA and EBNA1 IgG seronegative samples (VCA-EBNA1-). The Tukey box plot show the anti-EBVgp350 IgG reactivity in these samples measured as optical density.

A receiver operating characteristic (ROC) curve was generated to analyze the diagnostic performance of the EBVgp350 ELISA method. The VCA+EBNA1+ samples (n = 120) were defined as true positive samples and the VCA-EBNA1- samples (n = 120) were defined as true negative samples in the analysis. The ROC curve showed that the EBVgp350 ELISA method had high sensitivity and specificity. The OD value for cut-off between EBVgp350 IgG seropositive and seronegative samples was set at 0.162, based on the mean

of the VCA-EBNA1- IgG seronegative samples with the addition of two standard deviations. According to the ROC curve, the cut-off point had a sensitivity of 90% and a specificity of 98%.

Anti-EBVgp350 IgG was detected in 108/120 (90%) of the VCA+EBNA1+ serum samples. Of the VCA IgG seropositive but EBNA1 IgG seronegative (VCA+ EBNA1-) samples, 79/120 (66%) were EBVgp350 IgG seropositive. Only 2/120 (2%) of the VCA-EBNA1- IgG seronegative samples had detectable anti-EBVgp350 IgG. Thus, a total of 118/120 (98%) of these samples were anti-EBVgp350 IgG seronegative. The results are illustrated in Figure 30.

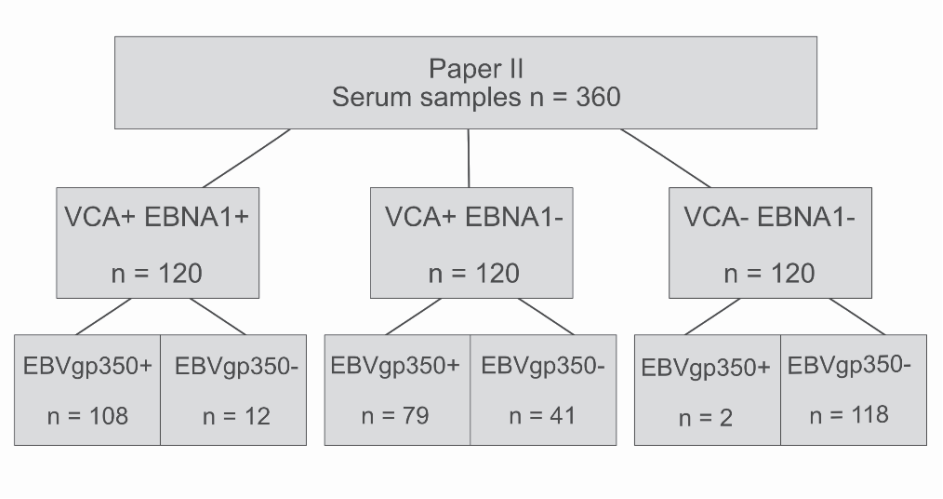

Figure 30. Schematic figure showing how many of the serum samples (n = 360) with known serostatus against viral capsid antigen (VCA) and Epstein-Barr virus nuclear antigen 1 (EBNA1) were Epstein-Barr virus gp350 (EBVgp350) IgG seropositive (+) and seronegative (-).

Analysis of anti-EBNA1 IgG and anti-VCA IgM/IgG can often identify the stage of an EBV infection, but in some special situations, it can be difficult. In these cases, it may be beneficial to perform an additional EBV serological analysis to increase the chance of correctly determining the patient's history of EBV infection. The antibody response to EBV varies between individuals. It is known that some individuals do not produce detectable anti-EBNA1 IgG levels even though it has been long enough since they became infected with the virus (204, 272). The kinetics of generating anti-EBV antibodies to various EBV antigens also differ between individuals (204, 272-274, 355, 356). This may partly explain why some VCA+EBNA1+ IgG seropositive serum samples did not show detectable anti-EBVgp350 IgG and why two VCA-EBNA1-negative samples were EBVgp350 IgG seropositive.

Anti-EBVgp350 IgG has been studied as a potential reverse marker for the severity of EBV-induced disease (274, 355). The results from a study showed that patients with higher anti-EBVgp350 IgG levels had milder IM disease compared with patients with lower IgG levels (274). Another study found no correlation between peak levels of various EBV antibodies, including anti-EBVgp350, and the severity of IM symptoms (355). A third study on the subject demonstrated that the generation of EBVgp350-neutralizing antibodies was associated with the EBV viral load in blood during IM (356).

Furthermore, our study presented in Paper IV, showed that IM induces a strong antibody response to EBV and that individuals with IM even after 10 years have increased anti-EBVgp350 IgG levels compared with healthy controls (470). Further research with sensitive and specific EBVgp350 serological assays may clarify whether anti-EBVgp350 IgG is a marker for milder EBV disease, which may strengthen the logic for the development of an EBVgp350 vaccine.

EBVgp350 vaccine

There is currently no EBV vaccine available on the market, but research is ongoing (258-260). A vaccine based on EBVgp350 produced in CHO cells showed in a phase two trial, a possible effect to prevent IM but not against asymptomatic EBV infection (262). EBVgp350 is still an important candidate protein for a subunit vaccine against EBV and serological methods that analyze the IgG response to this protein may therefore be needed in the future to measure antibody responses after vaccination (258-260, 471).

4.3 PAPER III

Patients with MS show increased IgG antibody reactivity to certain neurotropic viruses including MeV compared with healthy controls. The mechanisms behind the increased response to these viruses are unknown and therefore require further investigation. The MeV reactivity detected in serological assays may be specific to MeV antigens or nonspecific against other components of the viral antigen. In Paper III, we examined the anti-MeV IgG specificity in patients with MS, their clinically healthy siblings and healthy controls using a more specific serological assay with the conserved core portion of the MeV nucleocapsid protein (MeV N_{CORE}) as the serological antigen.

Patients with MS

Serum samples diluted 1/400 and CSF samples diluted 1/40 from the subjects were analyzed by indirect ELISA using MeV N_{CORE} as antigen. Patients with MS showed higher levels of anti-MeV N_{CORE} IgG measured as OD in both serum ($p < 0.001$) and CSF ($p < 0.001$) compared with healthy controls. Moreover, patients with MS had higher IgG levels in CSF ($p < 0.001$) but not in serum ($p = 0.2$) compared with the whole group of 46 healthy siblings. These results are presented in Figure 31.

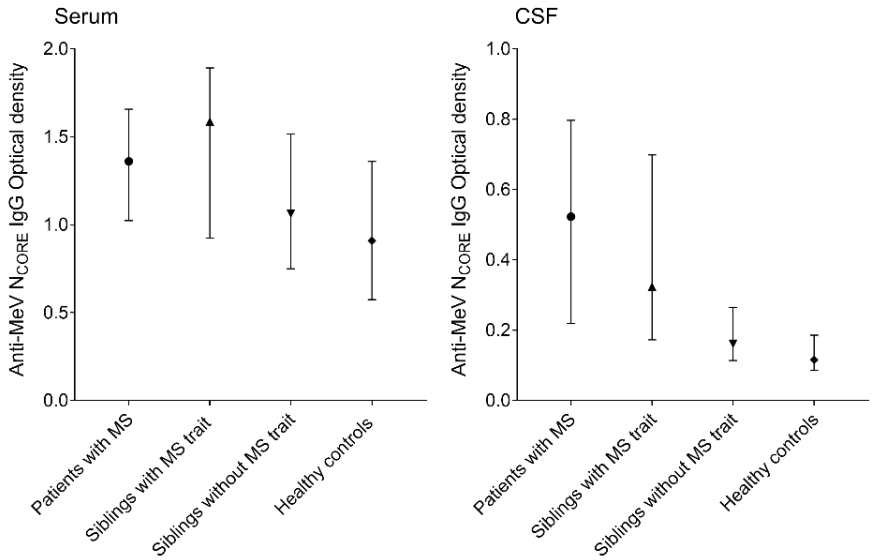

Figure 31. IgG antibody reactivity to measles virus nucleocapsid antigen (MeV N_{CORE}) in serum samples diluted 1/400 and cerebrospinal fluid (CSF) samples diluted 1/40, from patients with MS (n = 47), siblings with MS trait (n = 9), siblings without MS trait (n = 37) and healthy controls (n = 50). Reactivity is measured as optical density and median values with interquartile range are illustrated in the figure.

The OD_{CSF}/OD_{serum} values were calculated for each patient. When comparing the OD ratios between different groups, the patients with MS had a higher ratio compared with both healthy controls ($p < 0.001$) and clinically healthy siblings ($p < 0.001$).

The previous study that analyzed the identical patient material (except for a male sibling without trait) showed similar results but used MeV whole virus antigen generated in human fibroblasts in indirect ELISA (445). The antibody response to MeV is part of the common and characteristic MRZ reaction seen in patients with MS where intrathecally produced antibodies to MeV, RV and VZV are detected (431, 472-475). The antibody response in serum to MeV is not as closely investigated but a few studies, including the precursor study to the present one, have shown that serum anti-MeV IgG reactivity is also increased in patients with MS compared with healthy controls (432, 433, 445).

The new and advantageous in the present study is the specific MeV N_{CORE} antigen that has been used in the serological assay. MS is considered an autoimmune disease and MeV whole virus antigens commonly used in serological assays may increase the risk of autoantibody detection as there may be residual human/primate cellular components in the antigen remaining after antigen production. The risk of autoantibody detection decreases when the recombinantly produced MeV N_{CORE} antigen lacking human/primate components is used. Thus, the diagnostic specificity for detecting anti-MeV-specific IgG increases when this ELISA method is used. Although we cannot completely rule out the risk of non-specific binding or cross-reactive antibodies, it is more likely that the increased anti-MeV N_{CORE} IgG levels detected in serum and CSF samples from patients with MS in Paper III are due to MeV-specific antibodies.

Intrathecal antibody production in the form of OCB in CSF is not a specific marker for MS but may in some clinical contexts support an MS diagnosis (320, 387, 445, 476). This has been considered in the 2017 revision of the McDonald's criteria for diagnosing MS (387). MS can sometimes be difficult to diagnose and the MRZ response can in these situations be used to support the MS diagnosis (430, 477-480). Even in patients who do not present OCB in CSF, there may be a positive MRZ reaction that can support an MS diagnosis (474, 480, 481). In addition, the MRZ reaction may be a useful marker for distinguishing MS from other autoimmune diseases with similar symptoms (477-480).

Siblings with MS trait

Siblings with MS trait showed higher anti-MeV N_{CORE} IgG levels compared with healthy controls in serum ($p = 0.01$) and CSF ($p = 0.002$). In addition, the siblings with MS trait showed higher anti-MeV N_{CORE} IgG levels in CSF ($p = 0.04$) but not in serum ($p = 0.08$) compared with siblings without MS trait. The results are presented in Figure 31. Siblings with MS trait demonstrated higher OD_{CSF}/OD_{serum} values compared with healthy controls ($p = 0.008$), while siblings without trait did not ($p = 0.5$).

Approximately 16 years after the first sampling, a follow-up was performed regarding the siblings with MS trait, where it was found that none of these siblings had developed MS. The underlying pathophysiology to why some clinically healthy siblings of patients with MS demonstrate this immunological reaction is unknown. One hypothesis is that these siblings are genetically susceptible, but that additional environmental triggers are required to develop the disease which may not occur (446, 447).

4.4 PAPER IV

Several studies have shown that the risk of developing MS increases after IM (246, 247, 373). However, this relationship has not been prospectively investigated from the time of IM but mainly retrospectively from when the MS disease was diagnosed. In Paper IV, the antibody response to EBVgp350, VZVgE and MeV N_{CORE} was examined in sera from patients with acute IM and 10 years later at follow-up. The antibody responses in the patients with acute IM was compared with the seroreactivity to these viruses, both in healthy controls and in patients with MS.

Serum antibodies to EBV in patients with IM

The levels of anti-EBNA1 and anti-EBVgp350 IgG in the serum samples diluted 1/1000, from the 42 subjects with acute IM, were generally low and significantly lower compared with the 39 EBV seropositive healthy controls. Most patients with acute IM had lower levels of anti-VCA IgG than the healthy controls, but many were, unlike the healthy controls, anti-VCA IgM seropositive.

At follow-up, anti-VCA, anti-EBNA1 and anti-EBVgp350 IgG levels had increased in patients with previous IM. The post-IM patients' anti-EBVgp350 IgG levels were higher at follow-up compared with the 39 healthy controls ($p = 0.007$). However, there was no significant difference in anti-EBNA1 or anti-VCA IgG levels between patients at follow-up and controls, although the post-IM patients showed a tendency of having higher levels against the latter antigen (Table 5).

The finding that individuals with a history of IM showed higher levels of anti-EBVgp350 IgG compared with healthy controls at follow-up after 10 years suggests that a primary EBV infection in the form of IM affects the immune system in a different and more powerful way compared with an asymptomatic primary EBV infection. EBV establishes latency after the primary infection to later reactivate. The virus will remain in the host for life and the virus will induce a lifelong antibody response. Anti-EBV IgG antibody levels have been shown to be stable with no apparent decrease over time (22). Thus, even if the EBV seropositive healthy controls acquired their EBV infection earlier compared with the participants with IM, the levels of anti-EBV IgG antibodies are not likely to be lower due to the difference in the duration of the EBV infection. One hypothesis is that primary EBV infection in the form of IM, with a higher viral set-point, induces a stronger response from the immune system that give rise to higher levels of anti-EBVgp350 antibodies compared

with an asymptomatic/mild infection and that this is the reason behind the increased anti-EBVgp350 IgG levels that are detected at 10-year follow-up in post-IM patients. It has previously been shown that the amount of EBV virus in blood and the amount of CD8+ T cells correlate with the severity of the disease (482) and that the amount of EBV virus in blood is associated with the production of EBVgp350 neutralizing antibodies (356).

The increased serum levels of anti-EBVgp350 in post-IM patients compared with healthy controls were not reflected in increased levels of anti-EBNA1 and/or anti-VCA IgG antibodies. There are biological differences in the antibody response to various viral antigens from the same virus both in terms of kinetics and general antibody production. It is known that not all individuals infected with EBV show a detectable IgG response to EBNA1 (272).

EBVgp350 is an immunodominant envelope glycoprotein and the primary target for EBV-neutralizing antibodies (220-222). Therefore, it is likely that acute IM may induce a higher production of anti-EBVgp350 IgG compared with asymptomatic/mild EBV infections. Another reason for the higher anti-EBVgp350 IgG levels in post-IM patients compared with healthy controls may be more prevalent recurrent EBV reactivations, but this question has not been studied. The exact composition of the complex VCA antigen is unknown to the general research community, but it is likely that EBVgp350 is part of the content. It is possible that our immunodominant EBVgp350 antigen may detect higher levels of EBVgp350-specific IgG antibodies compared with the VCA antigen which also consists of many other components to which the EBVgp350-specific antibodies are less likely to attach.

Serum antibodies to VZV and MeV in patients with IM

Serum levels of both anti-MeV N_{CORE} and anti-VZVgE IgG were higher during acute IM compared with the levels in the same subjects at follow-up (Table 5). The increased antibody reactivity to both VZV and MeV in the patients with acute IM may be due to EBV-induced polyclonal B-cell activation.

There was no significant difference in serum anti-VZVgE IgG levels in post-IM patients at follow-up compared with healthy controls. IgG antibody levels to VZV normally show a moderate decrease over time (22). Post-IM patients at follow-up and healthy controls showed similar anti-MeV N_{CORE} IgG levels in serum. In an age-adjusted analysis, the post-IM patients showed a moderate increase compared with healthy controls ($p = 0.014$). IgG antibody levels to MeV are generally stable over time after natural infection (22) but declines with time after vaccination (297).

Table 5. IgG antibody reactivity to Epstein-Barr virus gp350 (EBVgp350), Epstein-Barr virus nuclear antigen 1 (EBNA1), viral capsid antigen (VCA), measles virus nucleocapsid antigen (MeV N_{CORE}) and varicella-zoster virus glycoprotein E (VZVgE). The analyzed serum samples were diluted 1/1000 and the cerebrospinal fluid (CSF) samples diluted 1/10. The sample material was collected from patients with acute infectious mononucleosis (IM), these patients at follow-up (FU), healthy controls (HC) and patients with MS (MS). The reactivity is presented as optical density (OD) or sample to cut-off (S/CO). Median values with interquartile range (IQR) are presented. EBV IgG seropositive (EBV +).

Median (IQR)	EBVgp350 IgG OD	EBNA1 IgG S/CO	VCA IgG S/CO	VCA IgM S/CO	MeV N_{CORE} IgG OD	VZVgE IgG OD
Serum samples						
IM (n=42)	0.1 (0.062)	0.38 (0.27)	7.0 (8.04)	10.3 (13.3)	0.86 (0.89)	1.19 (1.02)
FU (n=42)	1.16 (1.85)	15.4 (13.5)	56.1 (30.4)	0.24 (0.35)	0.53 (0.59)	0.59 (0.53)
HC EBV+ (n=39)	0.68 (0.84)	17.0 (14.0)	41.8 (34.9)	0.13 (0.56)	0.48 (0.56)	0.53 (0.40)
MS (n=22)	1.98 (1.24)				2.5 (1.2)	0.75 (1.0)
CSF samples						
FU (n=21)	0.31 (0.42)				0.21 (0.17)	0.15 (0.05)
HC (n=17)	0.18 (0.24)				0.17 (0.17)	0.15 (0.13)
HC EBV+ (n=15)	0.18 (0.22)				0.17 (0.17)	0.15 (0.13)
MS (n=22)	0.31 (0.41)				1.78 (1.41)	0.46 (0.81)

CSF antibodies to EBV, VZV and MeV in patients with IM

The total IgG levels in CSF were within the normal range in the healthy controls and in the post-IM patients at follow-up. None of these individuals showed OCB in CSF. The CSF samples were diluted 1/10 in the serological analysis. Anti-EBVgp350 IgG levels were significantly higher in post-IM patients at follow-up compared with the whole group with 17 healthy controls ($p = 0.01$) but not compared with only the 15 EBV IgG seropositive controls ($p = 0.08$). The CSF/serum ratio was determined based on the results of paired samples with CSF (diluted 1/10) and serum (diluted 1/1000) giving the CSF/serum dilution ratio of 1/100. The CSF/serum ratio for EBVgp350 IgG was lower among post-IM patients at follow-up compared with the healthy controls while no differences between the groups were seen for MeV N_{CORE} and VZVgE. That the CSF/serum ratio for EBVgp350 was lower for the post-IM patients at follow-up compared with healthy controls may be due to the increased serum levels of anti-EBVgp350 IgG in the patients.

Analysis of serum and CSF samples from patients with MS

The 22 patients with MS showed higher serum levels of anti-EBVgp350 IgG ($p = 0.006$) and anti-MeV N_{CORE} IgG ($p < 0.0001$) compared with the 39 healthy controls. However, serum levels of anti-VZVgE IgG were not higher in patients with MS compared with controls. In CSF, patients with MS had slightly higher IgG antibody levels against both EBVgp350 ($p = 0.048$) and VZVgE ($p = 0.01$) compared with controls. More pronounced were the increased CSF levels of anti-MeV N_{CORE} IgG in the patients compared with the controls ($p < 0.0001$) (Table 5). The CSF/serum ratio for MeV N_{CORE} and VZVgE but not for EBVgp350 was higher in patients with MS compared with healthy controls.

It is well known that patients with MS show increased IgG responses to all three viruses, EBV, VZV and MeV, compared with healthy controls, but that the increased antibody response to VZV and MeV have mainly been reported in CSF while the increased antibody response to EBV has mainly been seen in sera (428, 472-475, 483). Previous studies of antibody responses to different viruses in patients with MS show no direct correlation between serum and CSF IgG levels (431, 484). In this study, patients with MS showed a moderate increase in anti-EBVgp350 IgG levels in CSF compared with healthy controls. In previous studies, anti-EBV IgG levels in CSF have often been relatively low compared with serum levels (428, 483). The antibody response to EBVgp350 in patients with MS has not been extensively investigated in contrast to the carefully studied antibody responses to EBNA1 and VCA. Further studies are therefore needed to conclude whether patients with MS have increased levels of anti-EBVgp350 IgG in CSF compared with healthy controls.

An established risk factor for developing MS is primary EBV infection in the form of IM (246, 247, 373) and a recent study provides serological support that late EBV infections increases the risk of developing MS (485). A feature in individuals with presymtomatic MS is higher antibody reactivity to EBV compared with healthy controls, especially to EBNA1 (424, 425, 486, 487). However, the antibody reactivity to EBVgp350 has not been studied in this context. In conclusion, our results show that patients with acute IM display increased levels of anti-EBVgp350 IgG in serum at follow-up after 10 years. One hypothesis, that may relate to this finding, is that primary EBV infection in the form of IM can affect the immune system in a powerful and unusual way, which can be a triggering factor for continued biological events that after many years can lead to autoimmune diseases such as MS. Our present results support the argument for continuing to investigate the role of EBV in the pathogenesis of MS.

4.5 PAPER V

In Paper V, we wanted to identify whether the IgG reactivity to EBV and/or MeV is altered in patients with MS during interferon beta (IFNβ) treatment and/or after initiation of Natalizumab (NAT) treatment. A secondary goal was to identify if any patient with MS was EBV IgG seronegative.

Patients with MS vs healthy controls

When comparing serum IgG levels between patients with MS and the 144 healthy blood donors, samples taken at t1 from the subgroup of 170 patients who had samples collected during previous IFNβ treatment were used (see Figure 23 for definition of the different time points). The reason why these samples were used in the comparison was that the patients were most treatment naïve at this time before starting treatment with NAT. Patients with MS had higher levels of both anti-EBVgp350 ($p = 0.0006$), and anti-MeV N_{CORE} IgG ($p < 0.0001$) compared with the healthy blood donors.

Why patients with MS have increased levels of anti-EBV and anti-MeV IgG in sera compared with healthy controls is unknown. By using our recombinantly produced antigens EBVgp350 and MeV N_{CORE} which are based on single, immunodominant viral proteins lacking human/primate cellular residues, we reduced the risk of detecting autoantibodies to cellular components and/or cross-reactive antibodies. We have thus enhanced the specificity for detecting EBV- and MeV-specific antibodies. Our results suggest that patients with MS truly have an increased reactivity to these viruses. The increased anti-EBV and anti-MeV IgG levels in patients with MS have led to suggestions for the use of these antibody responses, primarily EBV, as surrogate markers for MS disease activity and/or treatment efficacy (440-444, 488, 489).

Changes in antibody levels during treatment

The changes in IgG levels between the 170 paired samples collected during IFNβ treatment at t1 and t2 and the 714 paired samples collected before at t3 and during NAT treatment at t4 are shown in Figure 32. The IgG levels against EBVgp350 did not change in the IFN subgroup between t1 and t2 and only 93 of 170 patients (55%) had lower IgG levels in the t2 follow-up sample compared with the first sample obtained at t1. For the 714 patients who started NAT treatment, IgG levels against EBVgp350 decreased between the samples collected before starting treatment at t3 and during treatment at t4 ($p < 0.0001$).

IgG levels against EBVgp350 decreased during NAT treatment in 509 of 714 patients (71%).

There was a slight decrease in IgG levels to MeV N_{CORE} between the samples collected at t1 and t2 from the subgroup of patients with MS during IFNβ therapy ($p = 0.006$), but only 95 of 170 patients (56%) demonstrated an antibody decline, which makes this observation of reduction somewhat less convincing (Figure 32). The patients in the NAT group showed a more pronounced decline in IgG antibodies to MeV N_{CORE} between the samples obtained at t3 and t4 ($p < 0.0001$). In total, 538 of 714 patients (75%) demonstrated a decline in anti-MeV N_{CORE} IgG levels after initiating NAT therapy.

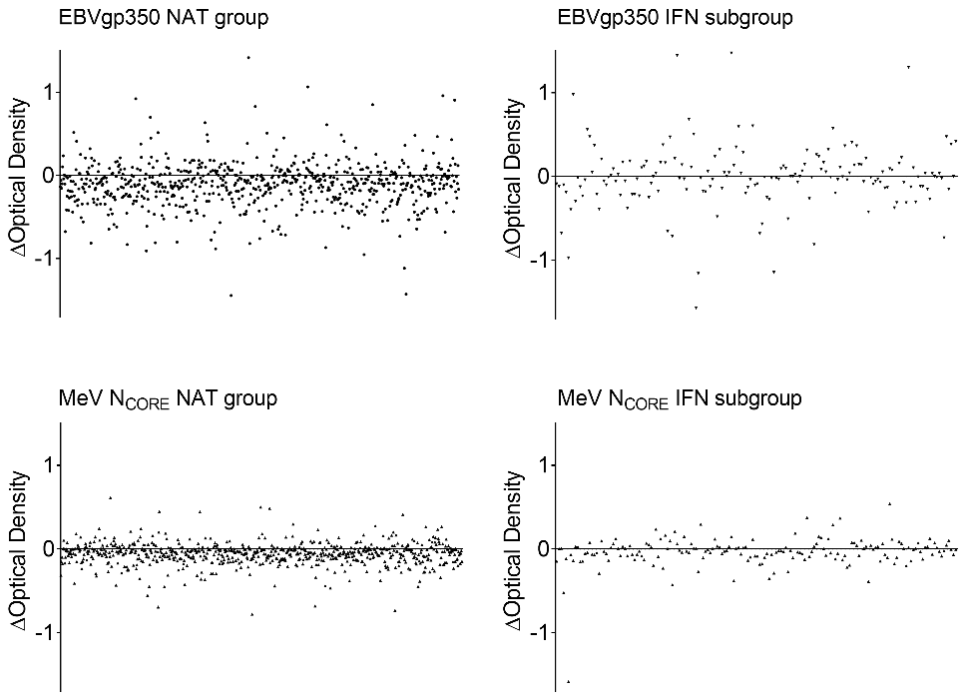

Figure 32. Delta (Δ) optical density (OD) values are shown as dots in the figure to illustrate the difference between the IgG antibody levels for anti-EBVgp350 and anti-MeV N_{CORE} IgG, respectively, for each paired serum sample obtained during IFNβ treatment (n = 170) at t1 and t2 and before and during NAT treatment (n = 714) at t3 and t4. ΔOD values were obtained by subtracting the OD value of the first sample taken at t1 or t3 from the OD value of the second sample taken at t2 or t4 (i.e. t2 minus t1; t4 minus t3). ΔOD values above zero indicate an increased antibody level and values below zero indicate a decreased antibody level.

The time span was short and similar, approximately one year, between the serum samples collected during IFN treatment at t1 – t2 and before (t3) and during (t4) NAT treatment. The results showing that anti-EBVgp350 and anti-MeV N_{CORE} IgG decline after initiating NAT therapy but are relatively stable during IFNβ treatment suggest that the decrease is not a general decrease due to time and increased age in the sampled individuals.

The anti-EBV IgG antibody response usually lasts for life without any significant reduction (22). Anti-MeV IgG antibody levels are generally stable and persist throughout life after natural infection in healthy individuals (22) but decline with age after vaccination (297). In contrast, the anti-MeV IgG response in patients with MS tends to increase over time both after natural infection and vaccination (433). Our results thus suggest that the decrease in anti-EBVgp350 and anti-MeV N_{CORE} IgG is associated to the initiation of NAT therapy.

There are studies that show a mild decrease in total serum IgG levels during NAT treatment (490-492). One of these studies could only demonstrate a mild IgG decline in the longitudinal part of the study (491). In another study, no decrease could be observed (493). In the study prior to the current one, on the same material, it was shown that the IgG antibody response to JCV and two herpesviruses, CMV and VZV, differed in patients with MS after starting NAT therapy. Anti-JCV and anti-VZV IgG declined but, in contrast, anti-CMV IgG increased slightly (450). (Our VZVgE antigen was used for anti-VZV IgG detection.) This shows that NAT does not suppress IgG reactivity against all viruses, which may suggest that the reduction of anti-EBVgp350 and anti-MeV N_{CORE} IgG is not part of a general IgG decline.

Previous studies have not found any change in anti-EBNA1 IgG levels during NAT treatment (443, 444, 489). One study found an increase in anti-VCA IgG during NAT treatment (444), while another was unable to detect a change (489). That these antibody responses did not decrease after initiating NAT therapy as we show with decreased anti-EBVgp350 IgG levels may be due to different biological purposes with the antibodies, different sensitivity of the viral antigens and/or the small samples sizes in some of these studies. No decrease in serum anti-MeV IgG levels during NAT treatment has been observed in previous studies with relatively small sample sizes (491, 493).

It is possible that NAT treatment causes changes in patients' immune responses that affect the production of anti-EBV and anti-MeV IgG antibodies. Affinity maturation of B cells requires support from T helper cells and insufficient help can lead to impaired plasma cell production which can affect the antibody

production. It is also possible that NAT has more direct effects on B-cell function (492, 494).

Patients with MS have an increased risk of developing certain infectious diseases in comparison with the general population (495, 496). The use of disease-modifying treatments may increase this risk. It has been established that NAT therapy increases the risk of PML and that the decrease in antibodies to JCV may be linked to this increased risk (400, 450, 497). Primary central nervous system lymphoma and herpesvirus infections of the CNS are associated with NAT therapy but less is known about these associations and whether lower antibody levels to certain herpesviruses may increase the risk of developing the diseases (401-403).

Seroprevalence

An antibody response to EBVgp350 could be detected in 718 patients with MS. Only 10 patients did not show detectable antibody levels. All samples where an antibody response to EBVgp350 could not be detected were also analyzed using EBNA1 and VCA as antigens. The 10 EBVgp350 seronegative MS patients showed detectable levels of anti-EBNA1 and/or anti-VCA IgG, indicating that all patients with MS were EBV IgG seropositive. Among the control group with blood donors, 14 of 144 (9.5%) were EBVgp350 IgG seronegative. Further analysis of these EBVgp350 seronegative patients showed that four blood donors had detectable antibodies to EBNA1 and/or VCA but 10 (6.9%) were both anti-EBNA1 and anti-VCA IgG seronegative.

In the IFNβ subgroup, 15 of the 170 samples (8.8%) collected at t1 had undetectable IgG antibody levels against MeV N_{CORE} and at t2, 18 of 170 samples (10.5%) were seronegative. In the NAT group, 80 of 714 samples (11.2%) were seronegative at t3 and 108 of 714 samples (15.1%) were seronegative at t4. In total, 41 of 144 blood donors (28.5%) were IgG MeV N_{CORE} seronegative.

Patients with MS have a high EBV seroprevalence and it is unclear if there are any patients with MS who are not infected with the virus (413, 414, 424). The few EBVgp350 IgG seronegative samples in this study were also analyzed with the antigens EBNA1 and VCA and the results showed that all patients with MS were EBV IgG seropositive. The different assays for the detection of anti-EBV IgG have different sensitivities and specificities and it is unlikely that any assay has both 100% sensitivity and specificity (276). So, it is more robust to use several different assays to determine EBV seroprevalence, but a secure definition of seronegativity remains however elusive. There are few EBV

seronegative patients with suspected clinically isolated syndrome or MS but in these cases, EBV seronegativity may be a useful biomarker to identify patients where a different diagnosis is more likely and further investigations are warranted.

Table 6. Epstein-Barr virus glycoprotein 350 (EBVgp350) IgG seronegative samples from patients with multiple sclerosis during treatment with interferon (IFN) (3/170) and before and during treatment with natalizumab (NAT) at time point 3 (t3) (11/714) and t4 (14/714). In addition, the EBVgp350 IgG seronegative samples were assayed with the EBNA1 and VCA antigens. Abbreviations: seronegative (-), seropositive (+), gray zone (GRZ).

Patient	EBVgp350	EBNA1	VCA
1	–	+	+
2	–	GRZ	+
3	–	+	+
4	–	+	+
5	–	+	+
6	–	+	+
7	–	+	+
8	–	–	+
9	t3+ t4–	+	+
10	t3+ t4–	+	+
11	t3+ t4–	+	+
12	IFN+ NAT–	+	+
13	IFN– NAT–	+	+
14	IFN– NAT–	+	+
15	IFN– NAT+	+	+

5 CONCLUSION

In **Paper I**, VZVgE was shown to work well as serological antigen in indirect ELISA. The new method had high sensitivity and specificity compared with an ELISA method using VZVwhole-ag. The use of VZVgE reduces the risk of detecting cross-reactive anti-HSV IgG antibodies compared with using VZV antigens that contain many VZV proteins including VZVgB, as is the case in most commercial VZV assays. At the Department of Clinical Microbiology, Sahlgrenska University Hospital, the VZVgE antigen has been successfully used as an ELISA antigen in the routine diagnostics for detection of anti-VZV IgG antibodies for approximately a decade.

A sensitive and specific ELISA method using EBVgp350 as serological antigen was developed in **Paper II** for the detection of anti-EBVgp350 IgG antibodies. Two protein constructs of different lengths were produced and tested as antigens in indirect ELISA. The longer protein construct containing a.a. 1–860 gave higher reactivity compared with the shorter construct containing a.a. 1–560 thus suggesting that longer EBVgp350 constructs should be used as serological antigens and in possible vaccine development.

In **Paper III**, **Paper IV**, and **Paper V**, the recombinant, immunodominant MeV N_{CORE} antigen devoid of human/primate components was used as serological antigen in indirect ELISA for detection of anti-MeV IgG antibodies. Our results indicate that the increased anti-MeV IgG antibody levels detected in serum and CSF in patients with MS and their siblings with MS trait compared with healthy controls are indeed due to MeV-specific antibodies and not caused by cross-reacting autoantibodies.

Previously, mainly the antibody responses to EBV VCA and EBNA1 have been analyzed in patients with MS. We now add knowledge by analyzing anti-EBVgp350 IgG, which can provide new pieces of the puzzle to understand the possible role of EBV in the pathogenesis of MS. Our finding in **Paper IV,** that patients with mononucleosis have increased levels of anti-EBVgp350 IgG at follow-up after 10 years, supports the hypothesis that a primary EBV infection in the form of mononucleosis can affect the immune system in a powerful and unusual way that can be a trigger for continued biological events that after many years may possibly result in MS disease.

In **Paper V**, we observed that natalizumab treatment of patients with MS was associated with a moderate decrease in serum anti-EBVgp350 and anti-MeV N_{CORE} IgG antibodies. In contrast, in a previous study based on the same

sample material, anti-CMV IgG levels were slightly increased by this treatment, which argues against a general IgG decline as an explanation (450). We can also conclude that all 728 patients with MS in the study were EBV IgG seropositive while 10 of the 144 blood donors in the control group were seronegative. This finding further strengthens the potential role of EBV in the pathogenesis of MS.

Our specific ELISA methods using VZVgE, EBVgp350 and MeV N_{CORE} as serological antigens can, through increased specificity offer new diagnostic possibilities for detecting antibodies to these antigens in viral infections, in controlling immunity after infection/vaccination, in epidemiological investigations and in autoimmune diseases such as MS.

6 FUTURE PERSPECTIVES

This thesis aims at improving serodiagnosis of viruses, including EBV, VZV and MeV, through methodological development. Below are some clinical applications of such refined serology.

VZVgE-based serological methods including our VZVgE ELISA may be important in immunization control after vaccination with VZVgE subunit vaccines. Specific and sensitive VZV serological assays such as our VZVgE ELISA can also be used to diagnose VZV infections of the CNS when the time window for detection of VZV DNA in CSF is closed. VZV infections such as VZV vasculopathy can be difficult to diagnose in the absence of the characteristic rash and further research in this area may establish the incidence of VZV infections in the CNS. For example, future studies may identify the incidence of VZV-induced stroke by analyzing anti-VZV antibodies in acute and convalescent serum samples from stroke patients and/or by detecting intrathecally produced anti-VZV antibodies in CSF from these patients one to two weeks after the stroke episode.

Moreover, specific serological assays can in some clinical contexts be used to support an MS diagnosis. The intrathecal MRZ reaction can be employed as a diagnostic complement to other laboratory investigations and EBV seronegativity may be used as a marker for patients who warrant further investigation.

EBV infection increases the risk of developing MS, but it has not been determined whether this virus is a prerequisite for developing MS. Additional large-scale seroprevalence studies of patients with accurately diagnosed MS can identify whether there are EBV seronegative patients with MS or whether all patients are seropositive. In these seroprevalence studies, it is important to use several specific assays in order not to miss any true EBV seropositive cases. If all patients with MS are EBV seropositive, it would further strengthen the role of EBV in the pathogenesis of MS.

In addition, our EBVgp350 ELISA may be used to further investigate the serological relationship between EBV and MS. Our next step will be to analyze the anti-EBVgp350 IgG reactivity in CSF samples from more patients with MS, including the reactivity in CSF samples collected before and after patients start treatment with natalizumab.

Immunosuppressive treatments are becoming more common, but a major disadvantage is severe side effects such as the higher risk of developing severe

infections. As an example, rituximab, which depletes B cells, can greatly reduce the antibody response to pathogens, thereby increasing the risk of infection, including a propensity for viral infections in the CNS. Further research on the monitoring of antibody responses to certain viruses could show whether there are critically low antibody levels where the risk of active infections increases sharply. This can lead to preventive measures such as vaccination, antiviral therapy, and/or reduction of immunosuppressive therapy.

EBVgp350 has been one of the top EBV vaccine candidates and if such a vaccine were to come, our novel EBVgp350 ELISA may be used to analyze the antibody response after vaccination. The ELISA method may also be used to investigate whether there is an association between antibody reactivity and the severity and/or treatment effect for various EBV disease manifestations such as IM and EBV-induced tumors.

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